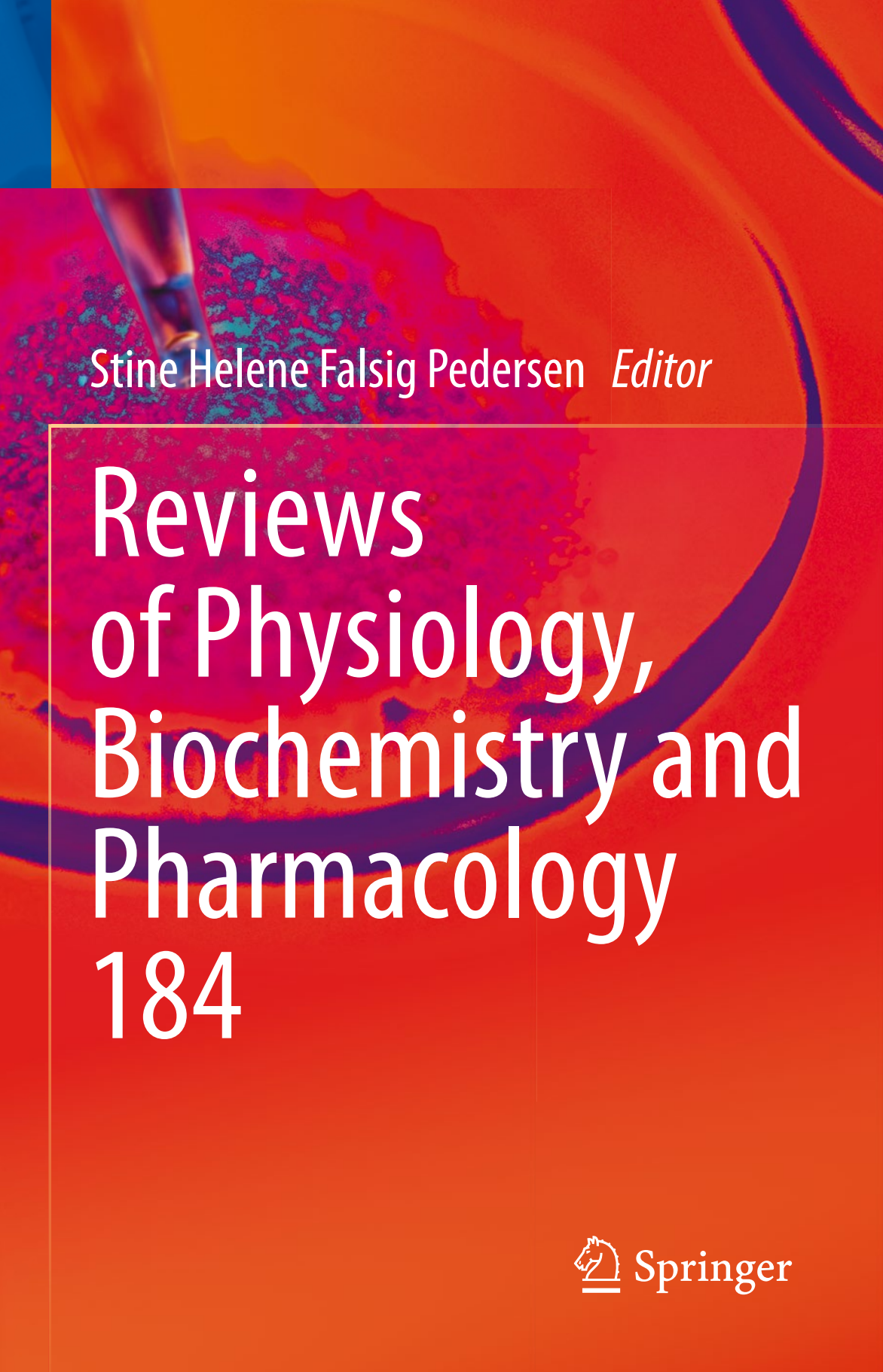
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Editor
Stine Helene Falsig Pedersen
Department of Biology
University of Copenhagen
Copenhagen, Denmark

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The Endocannabinoid System in *Caenorhabditis elegans*

Rubén Estrada-Valencia, María Eduarda de Lima, Aline Colonnello, Edgar Rangel-López, Nariani Rocha Saraiva, Daiana Silva de Ávila, Michael Aschner, and Abel Santamaría

Contents

R. Estrada-Valencia
Laboratorio de Aminoácidos Excitadores, Instituto Nacional de Neurología y Neurocirugía, Mexico City, Mexico

Facultad de Ciencias, Universidad Nacional Autónoma de México, Mexico City, Mexico

M. E. de Lima, A. Colonnello, E. Rangel-López, and A. Santamaría (✉)
Laboratorio de Aminoácidos Excitadores, Instituto Nacional de Neurología y Neurocirugía, Mexico City, Mexico

N. R. Saraiva and D. S. de Ávila
Laboratório de Bioquímica e Toxicologia em Caenorhabditis elegans, UNIPAMPA, Uruguaiana, Brazil

M. Aschner
Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY, USA

Abstract The existence of a formal Endocannabinoid System in *C. elegans* has been questioned due to data showing the absence of typical cannabinoid receptors in the worm; however, the presence of a full metabolism for endocannabinoids, alternative ligands, and receptors for these agents and a considerable number of orthologous and homologous genes regulating physiological cannabinoid-like signals and responses – several of which are similar to those of mammals – demonstrates a well-structured and functional complex system in nematodes. In this review, we describe and compare similarities and differences between the Endocannabinoid System in mammals and nematodes, highlighting the basis for the integral study of this novel system in the worm.

Keywords *Caenorhabditis elegans* · Cannabinoid receptors · Endocannabinoid system · Endocannabinoids · *N*-acylphosphatidylethanolamine · Nematodes

Abbreviations

2-AG	2-Arachidonoylglycerol
5-HT	Serotonin
AA	Arachidonic acid
AC	Adenylate cyclase
ACEA	Arachidonyl-2′-chloroethylamide
ACh	Acetylcholine
AEA	Anandamide
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BLAST	Basic local alignment search tool
CAPS	Calcium dependent secretion activator
CB1R	Cannabinoid receptor 1
CB2R	Cannabinoid receptor 2
CBD	Cannabidiol
CBDV	Cannabidivarin
CBR	Cannabinoid receptor
CBRs	Cannabinoid receptors
cGMP	Cyclic GMP
CNS	Central nervous system
DA	Dopamine
DAGLα/β	Diacylglycerol lipase α/β
DAS	Dafachronic acids
ECS	Endocannabinoid system
EPA	Eicosapentaenoic acid
EPEA	Eicosapentaenoyl ethanolamide
ESI-IT-MS	Electrospray-ionization ion-trap MS/MS
FAAH	Fatty acid amide hydrolase

FAS	Full analysis set
GABA	γ-Aminobutyric acid
GC	Growth cone
GFP	Green fluorescent protein
GLU	Glutamate
GPCR	G protein-coupled receptors
GTP	Guanosine triphosphate
IIS	Insulin/IGF-1 signaling
JNK	c-Jun N-terminal kinase
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
NAEs	*N*-acylethanolamines
NAPE	*N*-acylphosphatidylethanolamine
NAPE-PLD	*N*-acylphosphatidylethanolamine-specific phospholipase D-like hydrolase
NHR	Nuclear hormone receptor
NPC1	Niemann-Pick type C1
NSBP-1	Nematode sterol binding protein 1
PDE	Phospho-diesterases
PEGCs	Phosphoethanolamine glucosylceramides
PKA	Protein kinase A
PLC	Phospholipase C
PUFAs	Polyunsaturated fatty acids
RGC	Retinal ganglion cell
RNAi	RNA interference
R-Smads	Receptor-regulated receptors of small mothers against decapentaplegic
SID-GC-MS	Stable isotope dilution gas chromatography-mass spectrometry
STRM-1	Sterol A-ring methylase-1
Svh	Suppressors of *vhp-1*
TGF-β	Transforming growth factor beta
THC	Delta-9-tetrahidrocannabinol
TOR	Target-of-rapamycin
TRPV1	Transient receptor potential V1

1 Introduction

The endocannabinoid system (ECS), uncovered from molecules obtained from *Cannabis sativa*, mediates several effects on the nervous system. It is constituted by cannabinoid receptors (CBRs), enzymes, and endogenous cannabinoid ligands. Experimental and clinical data show that endogenous cannabinoids elicit the activation of the cannabinoid receptor 1 (CB1R) and cannabinoid receptor 2 (CB2R) and

other proteins. As a consequence, this activation triggers relevant physiological effects. It is also known that the ECS actively participates in several physiological processes in the central nervous system (CNS). In fact, its components participate in a considerable number of neuromodulatory functions such as immunomodulatory processes, neurotransmission mechanisms, and cell signaling pathways. *Caenorhabditis elegans* (*C. elegans*) is a soil-free nematode that has been widely used in the scope of scientific research. It is considered an optimal in vivo model to study aging and longevity due to the complete characterization of its genome and transcriptome. Its easy management and low maintenance cost make the nematode an ideal experimental model for pharmacological and toxicological studies. Numerous strains are available, some of which include deletion-type mutations in specific genes. Other transgenic strains express green fluorescent protein fused to promote the expression of genes that encode proteins of interest. Despite these advantages, the literature regarding the effects of the ECS on *C. elegans* is scarce due to a limited number of studies. Here, we reviewed the available on this topic with the aim at amplifying and strengthening the utility of the *C. elegans* model to elucidate the physiology of the ECS in mammals.

2 The Endocannabinoid System (ECS)

The ECS has been extensively studied since its discovery some decades ago. In the early 1990s, determining the mechanisms underlying analgesic and depressive effects on the mammalian CNS was imperative. As a first approach, it was thought that the lipophilic nature of cannabinoids had a non-specific effect on cell membranes. After the discovery of delta-9-tetrahidrocannabinol (THC), the main addictive and psychotropic cannabis-derived molecule, several mechanistic-related findings followed shortly later. For instance, THC mapping and characterization of its anchoring sites in the brain raised the concept of CBRs. Indeed, these receptors displayed a similar nature to those G protein-coupled receptors (GPCR). From here on, the discovery of the CB1R was proposed to explain the psychotropic and addictive effects of cannabis. These receptors are found throughout the brain, mainly in the olfactory bulb, hippocampus, basal ganglia, and cerebellum. However, they are also distributed in the presynaptic terminals, peripheral nervous system, gastrointestinal tract, and liver, among others (Zou and Kumar 2018).

After the discovery of CB1R, another CBR was identified, known as CB2R. Later on, its presence was demonstrated in immune cells and peripheral tissues such as the cardiovascular system, adipocytes, and the reproductive system. In recent years, some studies pointed to CB2R expression in the brain, particularly in microglia, the latter functioning as immune cells. CB2R is also expressed in neurons, although not at the same proportion as CB1R. In addition, it is proposed that these receptors play a role in nociception and neuroinflammatory processes (Ilyasov et al. 2018; Munro et al. 1993).

Nowadays, it seems clear that the ECS affects a noteworthy array of physiological processes in organisms such as embryogenesis and development (Bukiya 2019), nociception (Woodhams et al. 2015), cell growth, immune and inflammatory responses (Del Río et al. 2018), and energy balance (Gatta-Cherifi and Cota 2016). To date, several cannabinoid-based clinical studies have revealed that they mediate various effects in the pathogenesis of several nervous system disorders. Moreover, their implication in neurodegeneration processes, demyelination, epilepsy, seizures, and traumas has also gained recent attention (Ilyasov et al. 2018).

The ECS is composed of endocannabinoids, the enzymes involved in their synthesis and degradation, and the plasmatic and mitochondrial membrane receptors (CB1R and CB2R) (Bénard et al. 2012; Maya-López et al. 2020). CB1R and CB2R are associated with the Gi/0 protein subunit which suppresses adenylate cyclase (AC) upon binding with their ligands (Cristino et al. 2020; Fraguas-Sánchez et al. 2018; Zou and Kumar 2018). In regard to natural ligands, the two most studied endocannabinoids are *N*-arachidonoylethanolamine or anandamide (AEA) and 2-arachidonoylglycerol (2-AG). Their synthesis is catalyzed by *N*-acylphosphatidylethanolamine (NAPE)-specific phospholipase D-like hydrolase (NAPE-PLD) and diacylglycerol lipase α/β (DAGLα/β), respectively. In contrast, their hydrolysis is mediated by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively.

A plethora of cannabinoids includes multiple primary fatty acid amides and *N*-acylated amino acids, as well as some other molecules. In addition, each of these endocannabinoids may interact with particular molecules, resulting in a more complex mechanistic network that hinders their study. Altogether, this expanded ECS has been recently named the endocannabidiome (Veilleux et al. 2019).

The ECS participates in various physiological functions such as memory, cognition, locomotion, sleep, reproduction, and pain perception. Accordingly, the ECS represents an important field of study for the development of efficacious therapies. Such therapies aim at alleviating symptoms and control progression of CNS pathologies, including chronic pain, eating disorders, brain tumors, and neurodegeneration (Maya-López et al. 2020; Oakes et al. 2019).

Particularly, the mammalian nervous system is difficult to study due to the complexity of molecular interactions that influence individual behavior. Therefore, simpler models that can provide reliable evidence regarding the ECS effects on the modulation of different pathologies have been advanced (Oakes et al. 2019). Consequently, the ECS study could be further enhanced by approaching its effects in less complex animal models than mammals themselves.

3 *Caenorhabditis elegans* General Characteristics

Caenorhabditis elegans (*C. elegans*), a free-living nematode with a worldwide distribution across humid-temperate regions, has become a referent animal model for research in recent years. This organism represents a relevant in vivo model

because of its multiple advantages. For instance, its small size and affordable maintenance allow a simpler storage in reduced spaces at the laboratory. In addition, its short reproductive cycle (3 days), large brood size, and availability of thousands of mutant strains enable scientists to obtain results in shorter times. Moreover, *C. elegans* has a fully characterized nervous system which consists of 302 neurons in hermaphrodites. Its fully sequenced genome with more than 65% genes found in humans and multiple conserved molecular pathways make this model advantageous at several levels. Consequently, this nematode offers great potential in terms of accessibility since its experimental use does not require the approval of animal care/ethical committees (Frézal and Félix 2015; Harrington et al. 2010; Shen et al. 2018).

In regard to neurotransmitter systems, *C. elegans* possesses four major biogenic amines (octopamine, tyramine, dopamine (DA), and serotonin (5-HT)) that are responsible for modulating behavioral responses to changes in the environment (Chase and Koelle 2007). The actions of these neurotransmitters have been observed in neurons and muscles, regulating egg laying, pharyngeal pumping, locomotion, and learning, to name a few. While octopamine is not found in mammals, DA and 5-HT are known to act in the nematode's CNS through receptors and signaling mechanisms similar to those inherent to mammals (reviewed by McVey et al. 2012). DA is also responsible for habituation to touch (McDonald et al. 2006). 5-HT, commonly used by several motor neurons in the nematode, modulates motor activity and inhibits egg laying and pharyngeal pumping (reviewed by McVey et al. 2012). 5-HT signaling also stimulates the nematode to remain in food-rich areas. Another neurotransmitter shared with mammals, γ-aminobutyric acid (GABA), is responsible in *C. elegans* for muscle relaxation during locomotion, while modulating defecation. Both GABA and glutamate (GLU) may act as excitatory or inhibitory neurotransmitters in the worm. In the case of GLU, excitatory signals are mediated by ionotropic glutamate receptors, controlling the reversal following nose touch (reviewed by McVey et al. 2012). In contrast, acetylcholine (ACh), which is also present in mammals, exerts only excitatory functions in the nematode, regulating locomotor, pharyngeal pumping, egg laying defecation, and male mating (reviewed by McVey et al. 2012). In regard to purinergic transmission, although it has been reported that P2X-type ion channels activated by adenosine triphosphate (ATP) (Fountain and Burnstock 2009) might be lost in the nematode (Agboh et al. 2004; Bavan et al. 2009), more recently Lopes Machado et al. (2018) reported that the effects of *Ilex paraguariensis* extracts on lipid storage in the worm depend on the purinergic system (ADOR-1). In general terms, evidence seems to confirm that no members of P2X family nor of P2Y family have been found in the nematode. Collectively, this evidence encourages the need for more detailed characterization of the role of this system in the physiology of *C. elegans*.

Due to the characteristics previously mentioned, *C. elegans* has been widely used in a plethora of research areas. For instance, genetic studies may evaluate a gene of interest by the mechanistic study of genes using RNA interference (RNAi). They also assess the ability of mutant strains to overexpress, lack by knockout, or have a reporter gene associated with it (Shen et al. 2018). Its use in neurodegenerative disease models has been useful due to its simpler nervous system compared to the

one present in vertebrates (Harrington et al. 2010). Additionally, *C. elegans* has gathered attention in aging studies because of its reduced lifespan of around 21 days. Such a feature represents a better choice for longevity studies than other vertebrate models with extended life span. Therefore, the nematode is optimal as an in vivo model preserving multiple regulatory pathways involved in its physiology (Shen et al. 2018).

Among its conserved pathways, the insulin/IGF-1 signaling (IIS) pathway is one of the most studied due to its involvement in aging, homeostasis, lifespan, oxidative and thermal stress responses. The IIS pathway reaches these effects by the regulation of many molecules, in particular the transcription factors DAF-16 and SKN-1. These transcription factors are homologs to the mammalian FOXO family and Nrf2, respectively (Blackwell et al. 2015; Mukhopadhyay et al. 2006).

During early development, SKN-1 has a key role in the development of the entire digestive system. In addition, its expression has been associated with proteostasis, homeostasis of metabolism, immunity, and a wide range of detoxification processes. SKN-1 has been thoroughly studied for its importance as a regulator of lifespan extension and its involvement in oxidative stress response. Such modulatory activities are carried out by upregulated genes involved in the three detoxification phases (Blackwell et al. 2015). In comparison, DAF-16 participates in processes such as regulation of lifespan, dauer development, metabolism, immunity, and thermal and oxidative stress as well (Mukhopadhyay et al. 2006).

In this review we aim at strengthening the viability of *C. elegans* as a model to assess the effects linked to the ECS in the CNS. For this purpose, we highlight the genetic similarity between the nematode and mammals, focusing the homologous pathways in these two phylogenetic groups.

4 The Endocannabinoid System in *C. elegans*

In the last 12 years, research has focused on the existence of an ECS in *C. elegans*, despite the contradictory phylogenetic results. In this section we discuss the proposed molecules comprising the so-called *Caenorhabditis elegans*' endocannabinoid system.

The ability of *C. elegans* to synthesize endocannabinoids was demonstrated for the first time in 2008. For this, electrospray-ionization ion-trap MS/MS (ESI-IT-MS) was used to evaluate the presence of AEA and 2-AG in *C. elegans* Bristol N2 (wild type), AB1 (Australian worms), CB4856 (Hawaiian worms), TR403 (wild type), and *fat-3* mutants. Except for *fat-3* mutants, the presence of endocannabinoids was demonstrated in all assayed strains. It was suggested that such endocannabinoids were not detected in *fat-3* mutants due to their lack of Δ^6 desaturase activity. As a consequence, nematodes displayed inhibited ability of synthesizing long chain polyunsaturated fatty acids (PUFAs) (Lehtonen et al. 2008). Further studies have suggested that AEA is expressed abundantly in the pharynx of N2 worms. In

addition, there is an increase in its concentration during the development cycle, reaching its peak at the L2 stage and decreasing onwards (Lucanic et al. 2011).

AEA metabolism is regulated by its mammalian orthologs. During its synthesis, NAPE-PLD has an ortholog with two isoforms called NAPE-1 and NAPE-2. Transfection assays of both isoforms coupled to green fluorescent protein (GFP) showed that NAPE-1 is synthesized in interneurons proximal to sensory neurons. Moreover, NAPE-1 is synthesized in ventral nerve cords. Meanwhile, NAPE-2 is expressed abundantly at the ventral nerve cords and vulva muscles. In addition, its functional orthology was demonstrated by in vitro experiments. In these studies, *Escherichia coli* were transformed with both genes and produced recombinant proteins capable of synthesizing AEA in vitro (Lucanic et al. 2011; Harrison et al. 2014).

On the one hand, it was demonstrated that AEA degradation is regulated by FAAH-1, the ortholog enzyme of mammalian FAAH. Coupling of *faah-1* gene with a GFP reporter evinced that it is mainly expressed at the pharynx, close to the neurons expressing *nape-1* and *nape-2* genes. In addition, its functional orthology was demonstrated by the observation of an increase in *N*-acylethanolamines (NAEs). Such an increase was observed after treating worms with URB597, an inhibitor of FAAH, or by the incorporation of *faah-1* RNAi in worms (Lucanic et al. 2011).

On the other hand, 2-AG metabolism orthology does not seem as favorable as in the case of AEA; however, the nematode's sequence F42G9.6a codifies the transcript necessary for synthesizing DAGL-2 (also referred to as DAGL-1), which is considered an ortholog of DAGLα/DAGLβ (McPartland et al. 2006). In addition, *dagl-2* overexpression has been associated with an increased lifespan. In contrast, worms with diminished *dagl-2* expression exhibited reduced lifespan and oxidative stress resistance against paraquat induced-toxicity at 10 and 40 mM (Lin et al. 2014). Nonetheless, DAGL-2 ability to synthesize 2-AG has not been fully demonstrated. Moreover, the nematode lacks an ortholog for the 2-AG degrading enzyme, MAGL. Despite MAGL absence, recent studies evaluating longevity discovered that JZL184, a MAGL inhibitor, extended *C. elegans* lifespan. The observed effect was the result of inhibiting FAAH-4, which degrades monoacylglycerols such as 2-AG and AEA into smaller molecules (Chen et al. 2015). Figure 1 summarizes and compares AEA and 2-AG metabolism in *Homo sapiens* and *C. elegans*.

The presence of an ECS in *C. elegans* is further supported by the presence of receptors that bind AEA and 2-AG. The binding of the cannabinoid [^{3}H]CP55,940 had been previously reported in the nematode's neural tissues. However, the receptors to which this cannabinoid bind were unknown until recent years (McPartland et al. 2006). AEA effects observed in axon regeneration inhibition after axotomy have been shown to be dependent on the GPCRs NPR-19 and NPR-32 (Pastuhov et al. 2016). Besides, NPR-19 is responsible for AEA nociceptive effects in octanol avoidance assays, as well as the TRPN-like channel *osm-9* (Oakes et al. 2017, 2019).

There are some other processes in which ECs have been proven to participate in the worm, such as reproductive development. However, they are independent of the previously mentioned receptors, and the mechanisms involved in such processes will be mentioned later in this review.

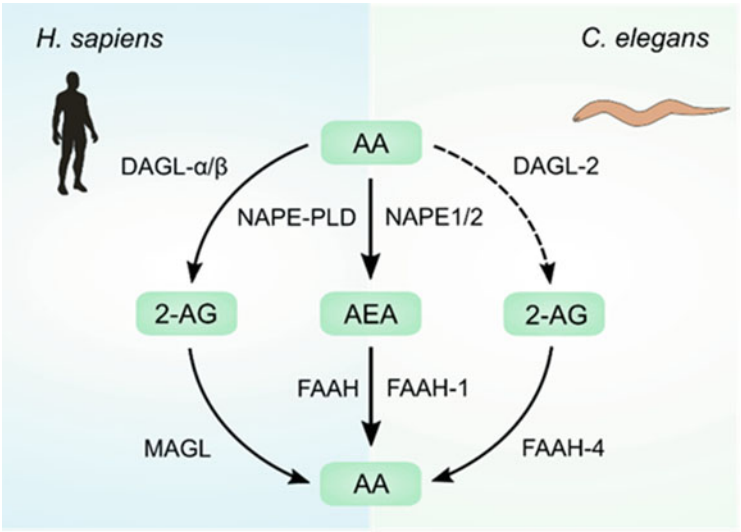

Fig. 1 AEA and 2-AG metabolism in *Homo sapiens* and *C. elegans*. In mammals (represented by humans in the figure), anandamide (AEA) is synthesized by *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) from arachidonic acid (AA), and degraded by fatty acid amide hydrolase (FAAH). In comparison, in *C. elegans*, the orthologs NAPE-1 and NAPE-2 synthesize AEA, and FAAH-1 catabolizes it. In the case of 2-arachidonoylglycerol (2-AG), this compound is synthesized in mammals from AA by diacylglycerol lipase-α (DAGL-α) or DAGL-β and catabolized by monoacylglycerol lipase (MAGL). In *C. elegans*, the ortholog DAGL-2 may be responsible for its synthesis, and the non-homolog FAAH-4 is in charge of its degradation

4.1 C. elegans Phylogenetics

During the twentieth century, several articles were published describing the toxicity effects of hemp crops. To explain these effects, during the early 2000s the first sequence alignment search was performed. The search was done with BLAST 2.0 to determine the homologous sequences for the mammalian CB1R, CB2R, and FAAH, in *C. elegans*. These studies revealed that none of the CBR candidates overcame the algorithmic threshold established to consider them as homologous to humans due to the presence of several substitutions at important amino acidic residues. As a consequence, they were considered non-homologous as well as non-functional under these criteria. The most similar sequence found in the nematode was F15A8.5, which exhibited a similarity of 24% against 64% of human CB1R sequence. The sequence C02H7.2 also showed a high degree of homology, with a similarity of 23% against 61% of human CB1R sequence (McPartland and Glass 2001). In addition, (1) the lack of evidence of specific binding of cannabinoids in the phylogenetic clade Ecdysozoa, (2) the absence of functional CBRs, and (3) the fatty acid amide hydrolase in these organisms suggested that the cannabinoid system may have never existed in this clade, or that it may have been lost during evolution (Lutz 2002). However, a new scope of sequence alignments was performed. The sequence

alignment compared CB1R and CB2R sequences of a wide variety of vertebrates, invertebrates, and that nematode sequence C02H7.2. This alignment compared 20 amino acid residues that are essential for specific ligand binding to CB1R or CB2R. From the data obtained from this new analysis, it was possible to generate a functionality matrix with binary scoring which allowed the search for putative CBR homologs. In agreement with previous studies, C02H7.2 was discarded as a putative ortholog of CB1R; this was due to the substitution in 19 of the 20 amino acid residues analyzed, which were relevant for binding of various cannabinoids. The conserved amino acid corresponded to Y5.39, reflecting affinity for the synthetic cannabinoid CP 55,940 and a proper signal transduction (McPartland and Glass 2003).

After initial Basic Local Alignment Search Tool (BLAST) and Full Analysis Set (FAS) assessments, BLAST comparisons were combined with the contained data in a phylogenetic tree. This tree was composed of 12 different organisms and 10 genes related to the endocannabinoid system. This phylogenetic tree was built using the best registered hits in the genome and aligning them with ClustalX Multiple Sequence Alignment software. After this initial analysis, the graphic outputs were generated using the TreeView family tree builder tool. For this analysis, the included genes were *Homo sapiens* CB1R, CB2R, Transient Receptor Potential V1 (TRPV1), GPR55, FAAH, MAGL, COX2, NAPE-PLD, DAGLα, and DAGLβ (McPartland et al. 2006). Under this new approach, the previously analyzed sequence C02H7.2, that codifies the GPCR NPR-19, was considered a strong candidate as a CB1R homolog due to the FAS scores obtained, as well as the high consistency observed with the species' phylogenetic tree. By using this combined analysis, it was also determined that the sequence B0218.1a is a homolog for human FAAH. The sequence Y37E11AR.4 for NAPE-PLD and the sequence F42G9.6a were also found homologs for DAGLα and DAGLβ. The results concerning the presence of TRPV1 were not so evident; however, the sequence *osm-9* seemed to have a phylogenetic similarity with TRPV1, as well as TRPV4. The lack of synapomorphy is noteworthy given their placement between the TRPV4 clade and the outgroup *Hs*TRPA1 (McPartland et al. 2006). The TRPV1 channels are relevant for the ECS study considering their involvement in nociception inhibition upon binding of AEA (Oakes et al. 2019).

The discrepancy between BLAST scores and the phylogenetic tree in regard to a CB receptor homolog reflect the tools used for each analysis. Previous reports have stated that BLAST scores of sequence similarity do not imply phylogenetic proximity by itself as the alignment matches do not reflect the evolutionary distances (Koski and Golding 2001). On the one hand, the BLASTed sequences corresponded to only a motif of the receptor sequence. On the other hand, a phylogenetic analysis usually consists of analyzing a wider region of the sequence. For this reason, a proper recreation of the receptor phylogenetic tree could give a better outlook in the search of ortholog candidates.

4.2 Role of Cannabinoids in the Establishment of Reproductive Development in C. elegans

During development, *C. elegans* undergoes different larval stages (L1, L2, L3, L4) and the adult, culminating in its reproductive development. However, exposure to unfavorable conditions during L1 stage may condition the worm undergoing an alternate larval stage (dauer or L2d) to resist those conditions (Erkut and Kurzchalia 2015; Fielenbach and Antebi 2008).

The dauer stage occurs when the worm senses different cues such as cold, heat, food scarcity, high salinity, and high population density. Such stressors will activate different signaling pathways that regulate this process. The four main signaling pathways involved in this process are (1) guanylyl cyclase, (2) transforming growth factor beta (TGF-β), (3) insulin/IGF-1 signaling (IIS), and (4) steroid hormones. The steroid hormone pathway includes dafachronic acids (DAS), which converge in the activation of the nuclear hormone receptor (NHR) DAF-12 (Erkut and Kurzchalia 2015; Fielenbach and Antebi 2008).

The first insight into endocannabinoids involvement in the integration of dauer or reproductive developmental signals was made while seeking for the presence of *N*-acylethanolamines (NAEs) by stable isotope dilution gas chromatography-mass spectrometry (SID-GC-MS). The ECS is involved in the regulation of nutrient intake and energy balance. Thus, it was unknown if the presence of AEA or eicosapentaenoyl ethanolamide (EPEA) might play a role in nutrient availability sensing in *C. elegans*. Surprisingly, developmental delay and increase of lifespan in *rfIs-22* and *rfIs-23* mutants were observed. In addition, a decrease in various NAEs concentrations, such as EPEA and AEA, was observed. Both mutants overexpress *faah-1* by containing extra copies of the abovementioned gene. This led to further testing of several cannabinoids such as EPEA, AEA, and 2-AG. Additionally, some synthetic cannabinoid related molecules, such as AM251, were tested. All these cannabinoids regulated reproductive development in a range of 2.5–50 μM (Galles et al. 2018; Lucanic et al. 2011; Rodrigues et al. 2016). Figure 2 depicts a schematic representation of the EPEA and AM251 signaling in *C. elegans*.

The concentration of NAEs throughout the worm's life cycle supports their involvement in the commitment to reproductive development. An increase in NAEs concentration has been observed from hatching until the L2 phase, followed by a decrease as they become adults. In contrast, worms that develop into L2d larvae exhibit a decrease in NAEs concentration (Lucanic et al. 2011). The mechanisms by which cannabinoids exert this function are not fully understood; however, in this review we mention the main proposed mechanisms studied up to date.

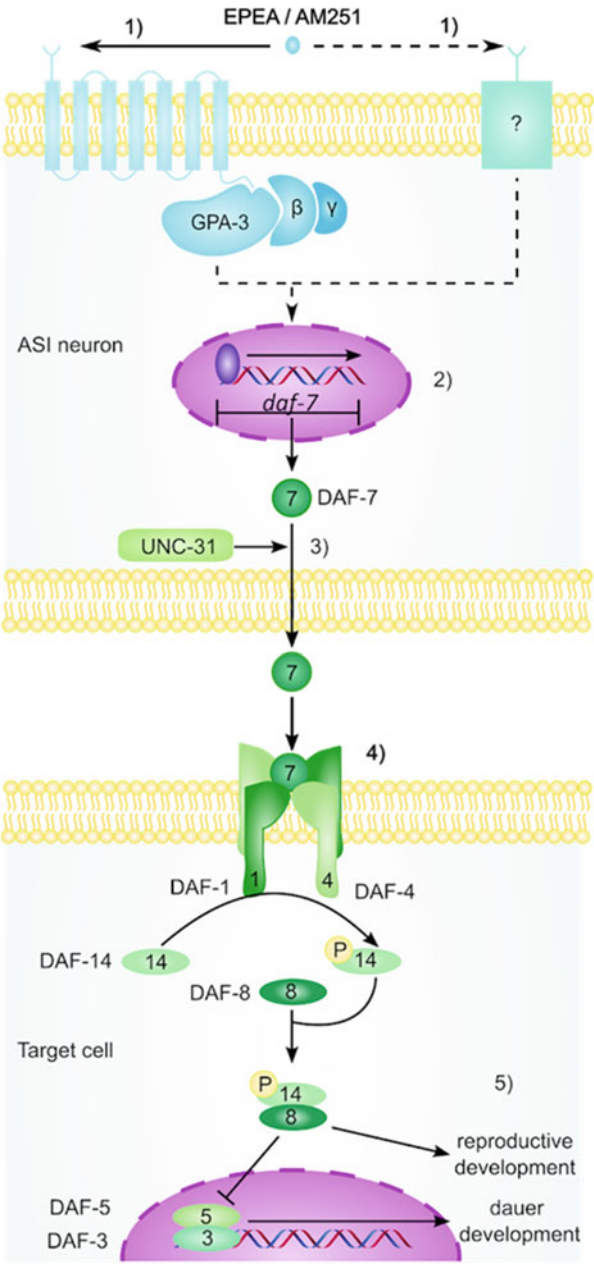

Fig. 2 Involvement of EPEA and AM251 signaling in the promotion of reproductive development. (1) AM251 and EPEA bind to a GPCR containing the subunit GPA-3, or to an unknown receptor, or to another cell that ends up communicating with the ASI neurons; (2) the signal received promotes DAF-7 (ortholog for mammalian GDF11) synthesis in ASI neurons; (3) The synthesized DAF-7 is secreted in extracellular medium by UNC-31 (ortholog for mammalian CADPS). (4) The secreted DAF-7 binds to the tetrameric receptor conformed by DAF-1 and DAF-4, triggering the TGF-β-dauer development signaling pathway. Upon binding with the receptor, the DAF-1 subunit phosphorylates DAF-14, which binds to DAF-8 and forms a complex capable of translocating to the nucleus. Once translocated, the DAF-14/DAF-8 complex inhibits the pro-dauer smads DAF-3/DAF-5 complex, resulting in reproductive development

4.3 Transforming Growth Factor Beta (TGF-β) Signaling Pathway

The Transforming Growth Factor Beta (TGF-β) pathway regulates a wide variety of developmental and homeostatic processes in *C. elegans*. These processes include dauer development, body size, lipid metabolism, innate immunity, and aging. In turn, these physiological features involve the participation of two pathways: the dauer and the Sma/Mab pathway (Savage-Dunn and Padgett 2017). The dauer pathway begins with DAF-7 binding to a tetrameric serine-threonine kinase receptor. This receptor is composed of two subunits of the receptor type I DAF-1 and the type II receptor DAF-4. The binding confers the capability of the receptor type II to phosphorylate the receptor type I, which then activates its kinase activity. The phosphorylation of the receptor type I allows the recruitment of the two Receptor-regulated receptors (R-Smads), DAF-8 and DAF-14. Once the R-Smads have been phosphorylated by DAF-1, they are capable of binding and antagonizing the co-smad DAF-3 in the nucleus. The nuclear events promoted by this signaling pathway have functions associated with the regulation of dauer development, aging, and lipid metabolism (Savage-Dunn and Padgett 2017).

The mutants *daf-7* and *daf-2* have a dauer formation constitutive (Daf-c) phenotype. This is because *daf-7(e1372)* mutants present a substitution in the *daf-7* pro-domain coding region that leads to improper splicing of *daf-7* mRNA. As a result, the mutation in *daf-7* leads to the absence of the dauer pathway signaling or TGF-β signaling pathway (Pierce et al. 2001). In comparison, *daf-2(e1368)* mutants present a substitution at the region that codifies for the ligand-binding domain. This mutation leads to reduced affinity of the ligand to this receptor which participates in the IIS pathway (Kimura et al. 1997). It should be noted that both mutants must be grown at 25°C to achieve the Daf-c phenotype throughout the population (Kimura et al. 1997; Pierce et al. 2001).

To determine the cause of developmental delay in mutants overexpressing *faah-1,* worms were exposed to various NAEs concentrations. Next, worms were tested in dauer formation assays. The assays were performed in *daf-2* mutants grown at 24°C and exposed to NAEs ranging from 2.5 to 50 μM. Then, the number of gravid adults was recorded 3 days after exposure. Results showed that EPEA and AEA were the only NAEs capable of recovering the Daf-c phenotype in a concentration-dependent manner. In this case, EPEA displayed a stronger effect than AEA. Eicosapentaenoic acid (EPA), an EPEA precursor, was also evaluated to analyze its possible involvement in the inhibition of Daf-c phenotype; however, EPA proved incapable of recovering reproductive development. The ability of EPEA to recover the Daf-c phenotype was evaluated in a wide variety of mutants with defective genes that express Daf-c phenotype. Nevertheless, none of the mutants seemed a proper candidate, including the *npr-19* and *daf-7* genes (Lucanic et al. 2011).

Further experiments attributed EPEA's mechanism in promoting reproductive development to its involvement as a nutrient signaling. This hypothesis was first proposed due to the dependence on the transcription factor *pha-4* to detect an

increase in lifespan in mutants overexpressing *faah-1*. The *pha-4* is necessary for lifespan extension under dietary restriction conditions. On the other hand, the mutant *rsks-1*, having a defective ortholog of the kinase 6 involved in target-of-rapamycin (TOR) nutrient sensing pathway, showed a significant decrease in EPEA concentration. In addition, EPEA treatment was capable of reverting the lifespan extension observed in *rsks-1* mutants (Lucanic et al. 2011).

Recent new evidence advanced the hypothesis that the EPEA-mediated reproductive development effect is dependent on *daf-7* expression level. The possible dependence in this signaling pathway was proposed by another group who reports the lack of effect in dauer formation after 72 h exposing the *daf-7; fat-3* double mutants to EPEA 50 μM at 20°C. The results obtained showed that in this double mutant, 80% of EPEA treated worms formed dauer larvae (Galles et al. 2018). This double mutant *daf-7;fat-3* harbors the previously described mutation of *daf-7*, as well as the deletion of the coding region of *fat-3* gene, which encodes for the Δ6-desaturase protein FAT-3. This protein is required for arachidonic acid (AA) and EPA synthesis.

Additional data seem to support the reliance on DAF-7, since after a screening performed in the Bioactive Lipid Library, only the CB1R inverse agonist AM251 was found to promote growth in *daf-2* mutants. This effect was only observed in worms exposed to AM251 before differentiating into L2d. This was similar to the peak concentration of NAEs observed during their development, and it was necessary at least an AM251 5 μM dose. It is noteworthy that in dauer formation assays it was observed that AM251 could not exert its effects in defective *daf-7* and *daf-4* mutants. Additionally, this ligand and receptor are influenced by the presence of the calcium dependent secretion activator (CAPS) ortholog *unc-31* (Rodrigues et al. 2016).

It remains unknown if cannabinoids exert their effects directly on ASI neurons by eliciting the expression of *daf-7* and then initiating the signaling cascade leading to reproductive development, or if they bind to another possible molecule which eventually activates this pathway. On the other hand, it has been proven that the AM251 effect is also dependent on the guanylyl cyclase activity protein DAF-11, and partially on the G protein-coupled receptor α-subunit, named GPA-3, which is expressed in ASI neurons (Rodrigues et al. 2016). However, in a *gpa-3* strain overexpressing the GPCR subunit GPA-3, there are diminished levels of daf-7 mRNA by the reduction of cGMP. These observations could be explained by an increase of phospho-diesterases (PDE) mRNA levels, particularly PDE-1 and PDE-5, from which the first one is expressed in the brain (Hahm et al. 2009). The dependence in GPA-3 and TGF-β to achieve a satisfactory reproductive development signal may seem contradictory due to the antagonizing effect of the first one in the second one. Nonetheless, further studies should help explain this complex mechanism of regulation.

4.4 Role of Cannabinoids in Cholesterol Transport

Through its evolution, *C. elegans* has lost the capacity of auto-synthesizing cholesterol. However, dietary incorporation of sterols is essential for physiological processes such as growth, locomotion, proper molting, and dauer development (Entchev and Kurzchalia 2005; Martin et al. 2010).

When worms ingest a proper amount of cholesterol, it is used as a precursor of the DAS, the main promoters of reproductive development. In contrast, insufficient concentrations of sterols promote the development of L2d in the F1. This effect may be replicated in mutants of the main cholesterol transporters, *ncr-1* and *ncr-2*, which are homologs of mammalian Niemann-Pick type C1 (NPC1). Double mutants *ncr-1;ncr-2* present a deletion of the codifying regions for both cholesterol transporters. Such deletion leads to a Daf-c phenotype due to an unsuitable intracellular transport of cholesterol (Kurzchalia and Ward 2003).

Several molecules, such as phosphoethanolamine glucosylceramides (PEGCs), can recover the reproductive development in F1 generation of *ncr-1;ncr-2* mutants through a cholesterol transport related mechanism (Boland et al. 2017). These observations, together with the finding of a modulating role from endocannabinoids in the dauer formation, led researchers to question if these lipids could also mediate this process by a cholesterol transport related mechanism. To test this hypothesis, dauer formation assays were performed in L1 worms or in embryos after exposure to 50 μM AEA or 2-AG for 72 h. Both cannabinoids recovered the reproductive phenotype not only in *ncr-1;ncr-2* double mutants, but also in *daf-7;fat-3* double mutants (Galles et al. 2018). These authors also suggested that AEA and 2-AG effect on reproductive development promotion could be an indicator of an enhancement in the transport of cholesterol reserves. Such effect is independent of *ncr-1* and *ncr-2* levels and non-dependent in *daf-7* conversely to EPEA and AM251. In addition, neither of them acted as substitutes of DAS as their reproductive development effect is nullified when exposing *daf-9* mutants to AEA or 2-AG. The gene *daf-9* codifies the cytochrome P450 enzyme DAF-9, which is necessary for DAS synthesis. Additionally, AEA and 2-AG mechanism of action is independent of PEGCs, but it works in parallel to them. This was proven by evaluating dauer formation assays upon AEA or 2-AG (50 μM) exposure. Additionally, worms were treated with RNAi or exposed to a chemical inhibitor of the enzymes that synthesize PEGCs precursors. The inhibition of PEGCs synthesis did not affect cannabinoids inhibition of dauer formation (Galles et al. 2018).

Currently, there is a lack of studies regarding the exact mechanism which is elicited by AEA and 2-AG; however, the effects of these endocannabinoids may be explored in further studies. For instance, they could be investigated by evaluating their possible participation in the downregulation of molecules involved in DAS synthesis inhibition such as the methyl transferase sterol A-ring methylase-1 (STRM-1) (Hannich et al. 2009), or their role in the upregulation of proteins that synthesize DAS as *hsd-1* (Patel et al. 2008), sterol binding proteins such as the nematode sterol binding protein 1 (NSBP-1) (Cheong et al. 2013), or even by acting

through cholesterol transporters as *lrp-1* (Entchev and Kurzchalia 2005), or among other unknown or not yet described cholesterol related proteins. Of these proteins, LRP-1 could be an interesting target for future studies since the mammalian LRP1 has proven to be upregulated in its mRNA expression in a blood-brain barrier (BBB) model after cannabinoids treatment (Bachmeier et al. 2013). Moreover, LRP1 has been associated with cholesterol accumulation in mice macrophages (Lillis et al. 2015). Forthcoming research may help in elucidating the mechanisms involved in the effects promoted by endocannabinoids so that a more supported explanation may be considered.

4.5 Involvement of Endocannabinoids in Axon Regeneration

Previous studies have shown that p38 and c-Jun N-terminal kinase (JNK) pathways have a role on axon regeneration in *C. elegans*. In addition, both of these mitogen-activated protein kinase (MAPK) cascades are inhibited by the MAPK phosphatase VHP-1. For this reason, a genome-wide RNAi screen was performed to identify the suppressors of *vhp-1* induced lethality. From this screening, 10 genes were designated as suppressors of vhp-1 (svh). Surprisingly, the *svh-3* gene corresponded to the enzyme *faah-1*, which is responsible for metabolizing molecules such as the endocannabinoid AEA (Li et al. 2012). These findings encouraged researchers to evaluate the effects of AEA and FAAH-1 in axon regeneration. The null-phenotype *faah-1(tm0511)* adult mutants had reduced frequency in axon regeneration after laser axotomy of γ-aminobutyric acid (GABA)-releasing D-type motor neurons. To evaluate if ECs accumulation was responsible for this effect, axon regeneration was tested upon exposure for 6 h of AEA or EPEA 290 μM. Both of them showed a reduction in axon regeneration, though AEA had a much more significant effect. In addition, this effect was only possible in adults, as axon regeneration in L4 worms was not affected upon AEA exposure or in *faah-1(tm5011)* mutants (Pastuhov et al. 2012).

Axon regeneration inhibition by AEA is achieved through GPCRs activation containing the GOA-1 subunit, an ortholog of mammalian Goα. This was demonstrated by the lack of inhibition on axon regeneration in the loss-of-function *goa-1 (n1134)* mutants exposed to AEA 290 μM after axotomy. The signaling of AEA through GPCRs containing GOA-1 continues with the inhibition of another GPCR subunit, EGL-30. This was shown in loss-of-function *egl-30(ad805)* and gain-of-function *egl-30(tg26)* mutants. These animals exhibited defects in axon regeneration as well as suppression of axon regeneration inhibition upon AEA exposure, respectively (Pastuhov et al. 2012). While the signaling for axon regeneration inhibition mediated by AEA has been partially elucidated, the GPCR that contains the GOA-1 subunit was unknown because of an ortholog CB receptors absence. Recently, a new analysis was performed using FASTA records by analyzing the presence of 16 similar or identical amino acids that are structurally or functionally relevant for CB1R or

CB2R. After further analysis, only four AEA receptor candidates were obtained: NPR-19, NPR-24, NPR-32, and NPR-35.

Mutants presenting a deletion for each of these candidate receptors were crossed with *nape-1* mutants. This mutant overexpresses NAPE-1 and has an axon regeneration inhibition phenotype constitutively. Only *npr-19* null mutants were capable of suppressing the *nape-1* mutant phenotype. However, the effects obtained were not statistically relevant, thus leading researchers to question if there could be two receptors acting independently on AEA-mediated axon regeneration inhibition. The double mutant *npr-19; npr-32* was shown to enhance this suppressive effect (Pastuhov et al. 2016). In agreement with these results, the double mutants *faah-1 (tm5011); npr-19(ok2068)* and *faah-1(tm5011); npr-32(ok2541)* were able to recover the defective axon regeneration observed in *faah-1* mutants. In addition, *npr-19* and *npr-32* mutants showed resistance to AEA related axon regeneration inhibition after being exposed to AEA 290 μM (Pastuhov et al. 2016). Later on, the effects of AEA in the spatial dynamics of axon regeneration were tested in terms of the angle in which they grow. Both mutants, *npr-19* and *npr-32*, were shown to have a slight decrease in axon regeneration misguidance. However, the double mutant *npr-19;npr-32* was shown to have a more significant reduction in axon regeneration misguidance. Overall, these results suggest that AEA exposure develops a misguidance in axon regeneration which is dependent on the presence of both NPR-19 and NPR-32 receptors.

In mammals, a similar effect on axon growth has been observed. Purified retinal ganglion cell (RGC) cultures exposed to arachidonyl-2′-chloroethylamide (ACEA), a selective CB1R agonist, showed a reduction in growth cone (GC) and number of filopodia. Additionally, CB2R has also been proven to be relevant on axon guidance in a similar manner. Experiments performed in RGC exposed to CB2R agonists, JWH015 and JWH133, have shown a decrease in the length of projections and growth cone surface area. However, it is important to consider that these mechanisms were caused by a reduction of cyclic adenosine monophosphate (cAMP) levels and by activating protein kinase A (PKA) (Argaw et al. 2011; Duff et al. 2013).

Corroborating these results, 2-AG has also been shown to be involved in axon guidance. A particular arrangement of three components comprising the ECS (DAGL-α, MAGL, and CB1R) is observed in the growth cone of RGC. Exposure to O-2050, an antagonist of CB1R, is associated with an increase in the median of the axon length. In contrast, treatment adding JZL184, an inhibitor of MAGL, prevents this phenotype (Stark and Caprioli 2016).

Overall, these results show that exposure to cannabinoids leads to a similar effect in axon growth; however, it should be considered that, in order to achieve this effect, the mechanisms involved act through similar receptors but may activate different downstream pathways. This may exclude the extrapolation of results between the nematode and humans, but it is something that should be further discussed when additional data on the role of the ECS in *C. elegans* become available. Figure 3 shows a schematic representation of the ECS-mediated signaling in axon regeneration.

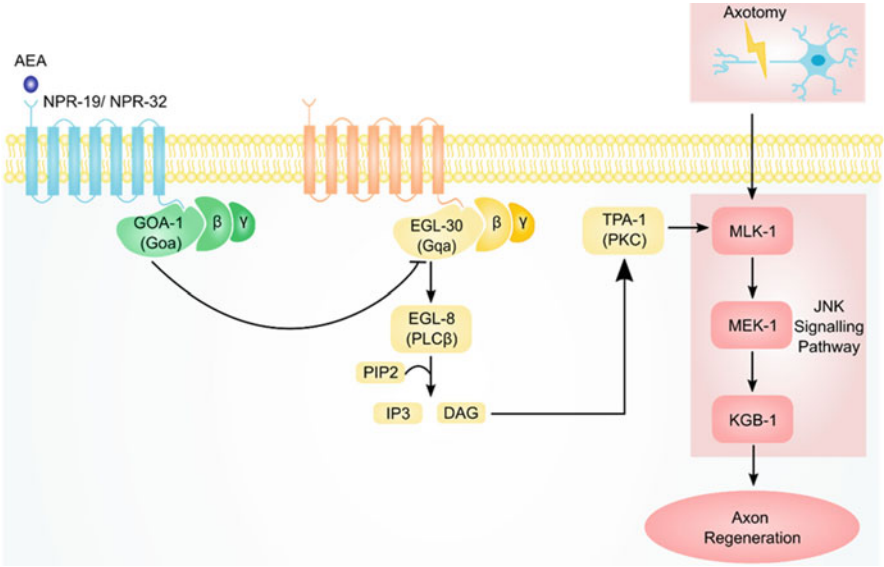

Fig. 3 Regulation of axon regeneration by the Endocannabinoid System. Upon axonal damage, the JNK signaling pathway is activated and promotes axon regeneration. One of the main activators of this pathway is the GPCR subunit EGL-30 (homolog for Gqα), which activates EGL-8 (homolog for PLCβ) and hydrolyzes phosphatidylinositol bisphosphate (PIP2) to produce inositol triphosphate (IP3) and diacylglycerol (DAG). In turn, DAG promotes TPA-1 (homolog for protein kinase C) activation, which then activates the JNK signaling pathway by phosphorylation of MLK-1. The binding of anandamide (AEA) with either receptor NPR-19 or NPR-32 activates the GOA-1 subunit (homolog for Goα) and inhibits EGL-30. This ends up promoting axon regeneration inhibition

4.6 Involvement of Endocannabinoids in Behavior

The studies performed by Oakes et al. (2019) identified that cannabinoid modulation exerts effects on forward and reversal movement exclusively by the activation of the monoaminergic system. Forward movement was evaluated by measuring the number of body bends for 20 s, after worms were exposed to 2-AG 0.4, 8, 32, and 320 μM for 15 min. The first experiments showed that worms exposed to 2-AG had a reduction in body bending ratio, and this effect was nullified when tested in knockout mutants *ser-4* and *octr-1*, but not in *npr-19* (Oakes et al. 2017, 2019). The forward movement decrease has been shown to rely on different mechanisms that involve both dopamine and serotonin. In contrast, the reversal movement is only dependent on the presence of serotonin.

In the case of dopamine, this neurotransmitter has been shown to be important for forward movement because of the lack of decrease in body bend ratio upon exposure of 2-AG 32 μM in *cat-2* and *dop-4* null mutants. These mutants have an improper function of their dopaminergic system, as *cat-2* codifies for a tyrosine hydroxylase and *dop-4* for a GPCR that binds to dopamine. In addition, this effect seems to be achieved by modulating the release, but not the reuptake of dopamine, as suggested

by the reduction of body bending in *dat-1* mutants not expressing this neurotransmitter transporter. The ADE dopaminergic neurons were identified responsible for this effect, by evaluating *cat-2::mCherry* transgenes fluorescence, and observing increased fluorescence in this pair of neurons upon exposure to 2-AG. Subsequently, the stimulation of dopamine release relies on the previous activation of the TRP channel *trp-4*. This is suggested by the slight and similar pattern of body bend ratio decrease that *trp-4*-null mutants and *trp-4* RNAi knockdown worms showed in comparison with *cat-2* mutants after 5, 10, and 15 min of exposure (Oakes et al. 2019).

In comparison with dopamine, the serotoninergic-dependent reduction of forward movement relies on the activation of *ser-4* in AIB neurons rather than *dop-4* in yet unidentified neurons. The importance of serotonin is further supported by the lack of effect on body bend ratio in *cat-1* and *cat-4* null mutants, which have a deficient transport and synthesis of dopamine and serotonin. This is due to *cat-1* which codifies for a synaptic vesicular monoamine transporter and *cat-4* for an ortholog of GTP cycle hydrolase 1. In addition, the authors discard the reliance only in dopamine by observing the lack of 2-AG effect in the *tph-1* null mutants that are unable to synthesize the tryptophan hydroxylase. Additionally, the reduction of forward movement on both of these mutants was able to be recovered by the exposure to either dopamine or serotonin. The release of serotonin mediated by 2-AG occurs through the activation of *osm-9*, a TRPV1-like channel. This was proven by obtaining similar patterns observed in the decrease of forward movement in *osm-9* and *tph-1* mutants upon exposure to 2-AG for 5, 10, and 15 min. It is noteworthy that *osm-9* activation was shown to occur at the ADF neurons, as demonstrated by the lack of effect in body bends by the selective RNAi knockdown of *tph-1* and the subsequent rescue of *tph-1* in these neurons (Oakes et al. 2019).

The mechanism of reversal movement modulation by cannabinoids in a serotonin-dependent manner is similar but slightly different to that one occurring on forward movement. The reversal movement was tested as the number of spontaneous reversals per 3 min, after 10 min of incubation with agents. This exposure to 0.4, 8, and 320 μM 2-AG increases the number of spontaneous reversals in N2 strain. The authors suggest as a first instance that this process relies entirely on the serotonergic system as was demonstrated by the lack of effect in *tph-1* null mutants but not in *cat-2* or *npr-19* mutants. In addition, the authors proposed that this process might occur by the activation of OSM-9 in ADF neurons as a consequence by the lack of effect in *osm-9* null mutants. Finally, the authors mention that it also could be associated with the further increase of serotonin release; however, the released serotonin activates different receptors, such as MOD-1 and SER-1, as there was a lack of effect in *mod-1* and *ser-1* null mutants (Oakes et al. 2019).

The evidence provided herein suggests that 2-AG interacts with the aforementioned channels OSM-9 and TRP-4 to achieve reversal and forward movement modulation (Oakes et al. 2019); however, further studies should be focused on determining if the interaction between these channels and cannabinoids is direct or indirect. In the case of OSM-9, it is evolutionarily related to human TRPV1 and TRPV4 (slightly more to the second one), and this remains relevant as both have

been proven to be activated by cannabinoids (McPartland et al. 2006). In turn, 2-AG activates directly TRPV1 but indirectly TRPV4 (Muller et al. 2019), raising the question on whether this interaction is direct or mediated by another mechanism wholly independent of NPR-19 signaling.

4.7 Exogenous Cannabinoids Effects in C. elegans

C. elegans also responds to exogenous cannabinoids, particularly because they have an ECS but also they have other neurotransmitter systems that can be affected by cannabinoids. Phytocannabinoids found in *Cannabis* spp. have been used as recreational drugs due to the sensory perception alterations they cause, although they have been most recently used for their many medicinal properties. The pharmacological effects of *Cannabis* spp. are attributed to their major components: the psychoactive phytocannabinoid THC and the non-psychoactive cannabidiol (CBD), which have been recently described by their analgesic, anti-inflammatory, anxiolytic, and anti-convulsant activities (Blessing et al. 2015; Donvito et al. 2018; Ibeas Bih et al. 2015; Rosenberg et al. 2017). Although structurally different, phytocannabinoids and endocannabinoids act by activating the same receptors and causing similar cellular responses as they possess similar molecular structures (Di Marzo and Piscitelli 2015).

Even though more than 80 phytocannabinoids have been isolated and identified in *Cannabis* species, up to date those studied in *C. elegans* are the two non-psychoactives cannabidiol (CBD) and its propyl analog cannabidivarin (CBDV) (Hunter Land et al. 2020; Shrader et al. 2020). Their safety and pharmacological assessment in *C. elegans* is advantageous since it is possible to determine the mechanism of these cannabinoids, and even unknown effects. *C. elegans* has a complete neuronal network with a well-characterized synaptic connectivity and, as already described, a conserved cannabinoid signaling system.

In this context, it has been demonstrated that 6 h exposure to high concentrations (up to 4 mM) of CBD did not cause any premature death to *C. elegans* at any of the tested concentrations, which confirms its safety use (Hunter Land et al. 2020). In fact, the study could not determine an LC_{50} since no signs of toxicity were observed. Remarkably, a long-term exposure to CBD prolonged worms' lifespan and improved health span, as indicated by increased locomotion of aged worms treated with this cannabinoid. It is noteworthy that CBD exposure at low concentrations (up to 40 μM) conferred stress resistance to worms (Hunter Land et al. 2020). These findings are valuable since they bring translatable data on the long-term safety of CBD, a major concern for its acceptance in patient treatments.

The dopaminergic system is well conserved between mammals and *C. elegans*; taking advantage of this evidence, Shrader et al. (2020) studied CBD and CBDV effects on this neurotransmitter system. As DA, CBD and CBDV exposure induced paralysis in the swimming movement. Using a *dop-3* mutant (homolog for the mammalian D2-like receptor), the authors observed that worms did not paralyze in

the swimming assay, thus suggesting that phytocannabinoids are binding to this receptor and they require the D2-like receptor to exert their effects. In the absence of tyrosine hydroxylase, the step-limiting enzyme for dopamine synthesis, *cat-2* mutant worms do not paralyze; however, when receiving exogenous DA, CBD, or CBDV they reduced swimming movements, confirming that they are binding to dopamine-receptors. The co-treatment with DA, CBD or CVDV resulted in no changes in paralysis, indicating that these cannabinoids are occupying the same receptors as dopamine (Shrader et al. 2020). These data indicate that phytocannabinoids can act as dopaminergic agonists in a mechanism supporting their use for Parkinson's disease and schizophrenia treatment.

It is noteworthy that no study on the effects following the exposure to the psychoactive THC in *C. elegans* has been published so far. The first licensed cannabis-based treatments were dependent on THC pharmacological effects; however, bioethical concerns have been an obstacle, since this cannabinoid possesses psychoactive properties. The few published studies on phytocannabinoids in *C. elegans* reveal a research gap that needs to be fulfilled.

4.8 Involvement of Endocannabinoids in Nociception

The concept that *C. elegans* can express a nociception-like response to adverse stimuli is of major relevance when considering that the nematode is commonly used as an alternative model for mammals to attend ethical issues in the later. Evidence collected so far suggests that aversive behavior in the nematode constitutes a useful tool to assess nociceptive-like responses, thus strengthening the concept that worms may resemble behaviors that are typically studied in mammals.

Oakes et al. (2017) reported that L4 wild type nematodes (N2) grown in agar plates containing 60 µL of 2-AG or AEA displayed a significantly inhibited octanol-aversive behavior. Conversely, NPR-19 receptor knockout nematodes [*npr-19 (ok2068)*] exposed to these same conditions exhibited a drastic decrease in octanol-aversive behavior inhibition. Such findings could be interpreted as an endocannabinoid-modulated response to "stressful" stimuli, such as octanol. In this case, the cannabinoid-like NPR-19 receptor seems to play a crucial role in several endocannabinoid-dependent behaviors in *C. elegans*. Supporting this data, it has been well-documented that in mammals, CB1R activation by 2-AG or AEA triggers antinociceptive effects. Additionally, *npr-19*-null mutants exposed to AEA were able to recover the antinociceptive effect mentioned upon being transfected by a vector containing the full-length of human *CNR1* cDNA, which encodes CB1R. It is well-known that CB1R modulation in humans is related to disorders such as depression and anxiety (Oakes et al. 2017). Therefore, this effect might be related to that observed in these assays using *C. elegans* mutants.

Given that NPR-19 is expressed in a small number of neurons, including glutamatergic pharyngeal motoneurons and URX sensory neurons, NPR-19 activation modulates avoidance, feeding, and pharyngeal pumping behaviors. In addition,

N2, serotonin [*ser-4 (ok510)*], and octopamine [*octr-1(ok371)*] receptor knockout nematodes were exposed to 2-AG at 3.2, 32, or 320 μM. At the higher concentrations (32 and 320 μM), the knockout mutants showed aversive behavior, suggesting that both OCTR-1 and SER-4 are necessary for the cannabinoid-mediated inhibition of nociception at higher concentrations. Altogether, these findings revealed that higher exogenous 2-AG concentrations are necessary to compensate for the OCTR-1 and the 5-HT-like SER-4 receptors absence. In normal conditions, these monoaminergic receptors are essential in inhibiting aversive, feeding, and pharyngeal pumping behaviors. Moreover, 2-AG treatment has proven to significantly increase serotonin endogenous levels in *C. elegans* by stimulating its release (Oakes et al. 2017).

Finally, since several effects induced by cannabinoids in *C. elegans* depend on their concentrations/doses, route of administration, time of exposure, larval stage at the time of administration, and the type of cannabinoid per se, we include in Table 1 a detailed description of the conditions used in several reports assessing reproductive, behavioral, motor and nociceptive responses evoked by these agents in the nematode. For all reports, consideration should be taken on the fact that the cited concentrations do not represent the intra-worm concentration ingested or absorbed; thus even the higher concentrations used by some groups might keep translational relevance for human cases.

5 Conclusion and Perspectives

In this review, we presented the most recent knowledge and evidence available supporting the existence of an ortholog ECS in the nematode, *C. elegans*. These observations are based on three main facts: (1) The presence in the worm of the two most well-known endocannabinoids, 2-AG and AEA, and other less studied cannabinoid-related molecules, such as EPEA, as it has been demonstrated by ESIT-IT-MS (Lehtonen et al. 2008) and SID-GC-MS (Lucanic et al. 2011); (2) *C. elegans* harbors proteins that are involved in the metabolism of endocannabinoids, and most of them are orthologs of their mammalian counterparts (McPartland et al. 2006). Moreover, it has been fully demonstrated that NAPE-1, NAPE-2 (Harrison et al. 2014), and FAAH-1 (Lucanic et al. 2011) are enzymes involved in the synthesis of endocannabinoids; (3) concurrently, some authors have demonstrated the presence of receptors that are activated upon exposure to cannabinoids in *C. elegans*, and they are capable of modulating different mechanisms during worm development and survival. Particularly, NPR-19 and OSM-9 are some of these ortholog candidates to cannabinoids-mammalian receptors. The presence of this kind of receptors is one of the main controversial topics, and despite initial sequencing studies on the *C. elegans* genome did not show clear homologies with the canonical CB1R and CB2R mammal receptors, the use of more complex molecular analysis platforms that involved phylogenetic data from different species and the simultaneous analysis of genes related to the endocannabinoid system made it possible to propose the existence in this nematode of homologous candidates to the

Table 1 Detailed experimental conditions used in various protocols evaluating reproductive, behavioral, motor and nociceptive responses evoked by cannabinoids in *C. elegans*

	Cannabinoid	Concentration	Administration pathway/time	Larval stage	Involved cellular components	Reference
Promotion of reproductive development	AEA	50 μM	Spotted in solid medium/72 h	Eggs	Nonspecified	Lucanic et al. (2011)
			Mixed with feeding bacteria prior to seeding/72 h	L1	Nonspecified, Independent of *daf-7*	Galles et al. (2018)
	2-AG	50 μM	Mixed with feeding bacteria prior to seeding/72 h	L1	Nonspecified Independent of *daf-7*, *ncr-1*, *ncr-2*	Galles et al. (2018)
	EPEA	50 μM	Spotted in solid medium/72 h	Eggs	Independent of *daf-7*	Lucanic et al. (2011)
			Mixed with feeding bacteria prior to seeding/72 h	L1	Dependent of *daf-7*	Galles et al. (2018)
	AM251	2.5, 5, 10, 25, 50 μM	Spotted in solid medium/72 h	Eggs	Dependent of *daf-7, daf-4, unc-31, gpa-3*	Rodrigues et al. (2016)
Axon regeneration inhibition	AEA	290 μM	Dissolved in buffer S/6 h	Young adults	Dependent of *npr-19* and *npr-32*	Pastuhov et al. (2012, 2016)
	EPEA	290 μM	Dissolved in buffer S/6 h	Young adults	Nonspecified	Pastuhov et al. (2012)
Nociception inhibition	AEA	Nonspecified	Spreading in solid medium/10 min	L4	Nonspecified	Oakes et al. (2017)
	2-AG	3.2 μM	Spreading in solid medium/10 min	L4	Dependent of *npr-19*	Oakes et al. (2017)
		32, 320 μM	Spreading in solid medium/10 min	L4	Dependent of *npr-19*, octr-1, ser-4	Oakes et al. (2017)

(continued)

Table 1 (continued)

	Cannabinoid	Concentration	Administration pathway/time	Larval stage	Involved cellular components	Reference
Decrease of forward movement (body bend ratio)	2-AG	Nonspecified	Spreading in solid medium/ 30 s to 20 min	Young adults	Dependent of *osm-9*, *ser-4, trp-4*, *dop-4*	Oakes et al. (2019)
Increase of spontaneous reversal movement	2-AG	0.4, 8, 320 μM	Spreading in solid medium/ 10 min	Young adults	Dependent of *osm-9*, *mod-1*, *ser-1*	Oakes et al. (2019)

typical CBRs present in other evolutionarily more related organisms. Particularly, the NPR-19 receptor meets most requirements as a putative candidate for the mammalian CB1R homolog. As mentioned before, this receptor is codified by the sequence C02H7.2 and shares functionality with their human counterparts (McPartland et al. 2006). Despite the presence of several substitutions in amino acid residues of importance for binding to AEA (McPartland and Glass 2003), the co-injection of *npr-19* and GIRK1/2 cRNAs in oocytes of *Xenopus laevis* enabled the analysis of changes in the current of K^+ upon exposure to 2-AG and AEA. These findings support the concept that both cannabinoids are capable of binding to this novel receptor, showing EC_{50}values of 395 nM for 2-AG and 14 nM for AEA, similar to those concentrations observed for human CB1R (Oakes et al. 2017). In addition, *npr-19*-null mutants were shown to recover their antinociceptive phenotype upon exposure of 2-AG after being injected with a vector containing the *CNR1* cDNA, which encodes for the human CB1R (Oakes et al. 2017). These data not only suggest that both of these endocannabinoids bind to NPR-19 receptor, but also support the notion that the function of this receptor can be recovered by transfecting worms with the mammalian CB1R. The observed functional similarity should be enough to dissipate the previous phylogenetic incongruences that had been registered and to consider NPR-19 as a novel CB1R-like receptor.

Some other receptors in *C. elegans* such as OSM-9 and NPR-32 have also been analyzed and it was found that they share similarity to other mammalian CBRs. OSM-9 has been most widely studied and it has been established to be phylogenetically similar to the TRPV1 and TRPV4 channels (McPartland et al. 2006). Oakes et al. (2019) have named it a $TRPV_1$-like channel based on its function and effects related to cannabinoids exposure. Additionally, OSM-9 has been involved in certain cannabinoid related mechanisms in the worm (Oakes et al. 2019). However, its direct or indirect role in the activity of the cannabinoids must be assayed in future research. In contrast, NPR-32 has a slightly more adverse outlook to be considered as a putative CBR. Initially, it was proposed as a candidate for CB1R ortholog by analyzing its structure, revealing high similarity to human GPR55, though authors did not mention anything related to the amino acid residues used for this analysis, or the criteria they followed to suggest that NPR-32 is related to GPR55 (Pastuhov et al. 2016). In addition, the direct binding of cannabinoids to this receptor has not been fully demonstrated, but the dependence of cannabinoid-mediated axon regeneration inhibition suggests that this candidate for CBR must be considered in further studies. Finally, TRP-4 is another novel component from a channel receptor that was regulated by cannabinoids, but it has not yet been characterized as an ortholog of any mammalian receptor.

The functionality of an endocannabinoid system in *C. elegans* has been experimentally demonstrated in a variety of processes and metabolic pathways, which represents a new and promising field of research to address the participation of endogenous and exogenous cannabinoids in the modulation of several dysfunctions that have been observed in mammal models. Collectively, the evidence described and discussed herein corroborates the use of the *C. elegans* model as a promising and adequate tool for studying how the nematode's ECS modulates processes inherent to

neurodegenerative diseases (such as excitotoxicity, redox imbalance, protein aggregation, disorders due to altered neurotransmitters release, or malfunctions of membrane channels, among others). Using this nematode as an in vivo model in a short time provides a plethora of novel information, bypassing the ethical limitations present for the use of more complex animal models, and with high therapeutic expectations to offer patients suffering from neurological disorders with some improvements in their early diagnosis, treatment, and quality of life. Nonetheless, the assumptions derived from the study of this system in nematodes should be cautiously considered as some of the mechanisms that have been proven to be regulated by the proposed ECS seem to have been conserved in humans, such as the axon regeneration inhibition, while some others are inherent only to the worm and are absent in humans, such as the reproductive cycle over dauer development.

Similarly, further studies should consider displaying a proper planning in the developmental stage of worms prior to the exposure to cannabinoids, the concentrations to be tested, or even the proposed cannabinoids to be used. The stage of development of *C. elegans* prior to treatments with cannabinoids or its agonists-antagonists is highly relevant for consideration, as it has been shown that during early development the synthesis of AEA and EPEA increases until the worm commits to the dauer development or reproductive development, followed by a proportional decrease (Lucanic et al. 2011). These data could suggest that cannabinoids become less relevant after this commitment, but it has been shown that in adults these molecules regulate axon regeneration inhibition (Pastuhov et al. 2016). Moreover, to define the moment when axon regeneration inhibition should be tested is also relevant, as this process occurs in adults but not in L4 stage (Pastuhov et al. 2016), showing that the stage and the process are relevant to analyze specific events. The concentration of cannabinoids is another factor which determines the activation of various components that regulate the same or similar effects, as was shown in octanol avoidance analysis that evaluated the antinociceptive effects of 2-AG. In this study, the presence of NPR-19 was required to observe the inhibition of nociception at several doses of 2-AG. Here, the authors reported that at the higher concentrations of 2-AG, SER-4 and OCTR-1 were also essential to achieve the observed effect (Oakes et al. 2017). If this dose-dependent effect is a specific process in these nematodes, it should be evaluated further when more information is available in regard to the ECS in *C. elegans*.

On the basis of the reviewed evidence, we suggest that cannabinoid related studies performed in this in vivo model should include at least one of the two most well-studied cannabinoids, AEA or 2-AG. If these considerations are followed, the obtained data might exclude the incongruences observed in the different mechanisms presented during the inhibition of dauer formation elicited by EPEA when compared with that observed for AEA and 2-AG. While that the partial dependence of EPEA (Lucanic et al. 2011) and 2-AG on the activity of the CB1R-like receptor has been described, the participation of NPR-19 remains unclear. If this receptor plays a role in dauer formation inhibition through the effects of AEA and 2-AG, then this might be explained by differences in the affinity of these molecules as EPEA has been shown to be a weaker agonist of CB1R when compared to AEA and 2-AG

(McDougle et al. 2017). Similarly, the amino acid substitutions found in NPR-19 compared to the sequence of CB1R in key residues for different ligands binding should not be underestimated.

Finally, non-mammalian models have also contributed immensely to the understanding of a great variety of processes in much more complex organisms, leading to great advances in biomedical research. However, the presence of this novel ECS in *C. elegans* renders the nematode as an adequate model for studying processes and diseases where cannabinoids play significant roles, in contrast to other models lacking altogether this system, such as *Drosophila melanogaster*. Considering the reviewed data, we encourage to continue the future research of this endocannabinoid system as an appropriate approach to demonstrate the importance that this system might have not only in this nematode, but also to reveal the potential utility of this model in biomedical research in order to improve health conditions in neurological patients in whom the involvement of the endocannabinoid system dysfunction has been ascribed.

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Inherited Ventricular Arrhythmia in Zebrafish: Genetic Models and Phenotyping Tools

Ewa Sieliwonczyk, Vladimir V. Matchkov, Bert Vandendriessche, Maaike Alaerts, Jeroen Bakkers, Bart Loeys, and Dorien Schepers

Contents

Abstract In the last years, the field of inheritable ventricular arrhythmia disease modelling has changed significantly with a push towards the use of novel cellular cardiomyocyte based models. However, there is a growing need for new in vivo

E. Sieliwonczyk (✉), B. Vandendriessche, M. Alaerts, and B. Loeys
Center of Medical Genetics, Faculty of Medicine and Health Sciences, University of Antwerp and Antwerp University Hospital, Antwerp, Belgium
e-mail: ewa.sieliwonczyk@uantwerpen.be

V. V. Matchkov
Department of Biomedicine, Pulmonary and Cardiovascular Pharmacology, Aarhus University, Aarhus, Denmark

J. Bakkers
Hubrecht Institute for Developmental and Stem Cell Biology, Utrecht, The Netherlands

D. Schepers
Center of Medical Genetics, Faculty of Medicine and Health Sciences, University of Antwerp and Antwerp University Hospital, Antwerp, Belgium

Laboratory for Molecular, Cellular and Network Excitability, Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium

models to study the disease pathology at the tissue and organ level. Zebrafish provide an excellent opportunity for in vivo modelling of inheritable ventricular arrhythmia syndromes due to the remarkable similarity between their cardiac electrophysiology and that of humans. Additionally, many state-of-the-art methods in gene editing and electrophysiological phenotyping are available for zebrafish research. In this review, we give a comprehensive overview of the published zebrafish genetic models for primary electrical disorders and arrhythmogenic cardiomyopathy. We summarise and discuss the strengths and weaknesses of the different technical approaches for the generation of genetically modified zebrafish disease models, as well as the electrophysiological approaches in zebrafish phenotyping. By providing this detailed overview, we aim to draw attention to the potential of the zebrafish model for studying arrhythmia syndromes at the organ level and as a platform for personalised medicine and drug testing.

Keywords Cardiac electrophysiology · Gene editing · Inheritable ventricular arrhythmia · Zebrafish

1 Introduction

The exploration of the genetic aetiology of inherited cardiac arrhythmia has recently encountered significant challenges, which require a revision of its concept. Many genes previously associated with inherited arrhythmia are currently under dispute (Adler et al. 2020; Hosseini et al. 2018), and there is increasing evidence that complex inheritance patterns might underlie its pathogenesis (Bezzina et al. 2013). Nevertheless, much remains to be discovered, as evidenced by the incomplete yield of genetic analyses in inherited arrhythmia syndromes, although this varies by condition with the highest yield observed for long QT syndrome (LQTS) (60–80%) (Ingles et al. 2020) and the lowest for Brugada syndrome (BrS) and short QT syndrome (SQTS) (20–30%) (Campuzano et al. 2018; Ingles et al. 2020).

The assignment of pathogenicity to genetic variants identified in established or candidate disease genes for inherited arrhythmia is particularly challenging. This process can be facilitated by functional characterisation in model systems which are able to recapitulate the complex genetic architecture of these disorders, and, at the same time, remain suitable for phenotyping the cardiac electrical and mechanical activity and morphology.

Non-cardiac cellular models (e.g. HEK-293 cells) are too limited to provide this complex paradigm. The recent utilisation of induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs) was considered a major breakthrough, as it enabled the study of cardiomyocyte function within the patient's own genomic background (Shaheen et al. 2017). However, the generation of iPSC-CMs is expensive and time-consuming, and the cells often show a high degree of variability and immaturity (Shaheen et al. 2017). Importantly, iPSC-CMs remain an in vitro model, and can

therefore not be used for the assessment of cardiac pathophysiology in the body, where the heart is exposed to mechanical, hormonal and neuronal regulation.

Murine models have been implemented in the experimental study of several inherited arrhythmias (Huang 2017). However, the translation of findings from mouse to human is not always straightforward, as the cardiac electrophysiology substantially differs between these species. A murine heart is characterised by a faster heart rate, a shorter action potential (AP) duration, a lack of a distinct plateau phase (Kaese and Verheule 2012) and distinct ion channel compositions (Nerbonne et al. 2001).

The zebrafish (*Danio rerio*) is a widespread laboratory animal, which is frequently used for the assessment of functional and structural consequences of genetic variants. Despite a greater evolutionary distance, the zebrafish heart is a surprisingly suitable model for human cardiac electrophysiology. Although the zebrafish cardiac anatomy differs from humans because of a single atrium and ventricle, the zebrafish cardiac AP and electrocardiogram (ECG) resemble the human to a larger extent than the mouse heart does (Nemtsas et al. 2010). Zebrafish are also less expensive to house, produce a large number of offspring and are easier and quicker to genetically modify (Rafferty and Quinn 2018). These advantages make this experimental animal model a particularly attractive tool for high-throughput assays (e.g. phenotype based drug discovery) (MacRae and Peterson 2015) and personalised medicine research.

Numerous studies have examined the zebrafish cardiac electrophysiology (Abramochkin et al. 2018; Bovo et al. 2013; Brette et al. 2008; Nemtsas et al. 2010; Ravens 2018), as well as the potential of zebrafish for inherited cardiac arrhythmia modelling (Poon and Brand 2013; Verkerk and Remme 2012; Vornanen and Hassinen 2016). In our review, we provide a comprehensive overview of previously developed genetic zebrafish models of inherited arrhythmia syndromes, with an emphasis on LQTS, SQTS, catecholaminergic polymorphic ventricular tachycardia (CPVT), BrS and arrhythmogenic (right ventricular) cardiomyopathy (ARVC/AC(M)). We discuss the methods used for the generation and phenotyping of these models, as well as the strengths and weaknesses of these approaches.

2 Methods

The PubMed database was searched in February 2021 for articles which contained "zebrafish" in the title or abstract, as well as at least one of the following keywords: "primary electrical disorders", "PED", "cardiac arrhythmia", "channelopathy", "Brugada", "long QT syndrome", "LQTS", "catecholaminergic polymorphic ventricular tachycardia", "CPVT", "short QT syndrome", "SQTS", "arrhythmogenic right ventricular cardiomyopathy", "ARVC", "arrhythmogenic cardiomyopathy" or "ACM". The titles of all identified papers were screened for relevance and all titles which did not concern ventricular electrophysiology or ventricular arrhythmia were excluded. The abstracts and subsequently the full content of the remaining articles were screened for relevance to the included disease phenotypes and/or inherited

cardiac arrhythmia genes. Additional relevant articles were identified from the reference lists of the selected publications. Literature reviews and editorials were screened for relevant references, but only original research papers were included in the final selection.

3 Results

3.1 *The Current Status of Published Reports on Zebrafish Models of Inherited Arrhythmia*

The initial search strategy yielded 253 articles, which were filtered to a total of 32 after screening (Arnaout et al. 2007; Asimaki et al. 2014; Berchtold et al. 2016; Brodehl et al. 2019; Da'as et al. 2019; Giuliodori et al. 2018; Hassel et al. 2008; Heuser et al. 2006; Huttner et al. 2013; Jou et al. 2013; Juang et al. 2020; Kapoor et al. 2014; Koopman et al. 2021; Kopp et al. 2005; Langenbacher et al. 2005; Langenbacher et al. 2020; Langheinrich et al. 2003; Leong et al. 2013; Martin et al. 2009; Meder et al. 2011; Moriarty et al. 2012; Pott et al. 2018; Ramachandran et al. 2013; Rottbauer et al. 2001; Smeland et al. 2019; Sondergaard et al. 2015; Tanaka et al. 2019; Tessadori et al. 2018; Thorsen et al. 2017; van den Boogaard et al. 2012; Warren et al. 2001; Zhou et al. 2016). There was a trend towards an increased publication rate over the last 15 years. Most of these studies used zebrafish embryos whereas 11 studies (Arnaout et al. 2007; Asimaki et al. 2014; Brodehl et al. 2019; Hassel et al. 2008; Huttner et al. 2013; Juang et al. 2020; Koopman et al. 2021; Langenbacher et al. 2020; Meder et al. 2011; Smeland et al. 2019; Warren et al. 2001) included phenotyping of juvenile and/or adult zebrafish, which was performed either by patch-clamp analysis in isolated cardiomyocytes (Asimaki et al. 2014; Brodehl et al. 2019; Koopman et al. 2021; Smeland et al. 2019; Warren et al. 2001) or by an electrocardiogram assessment (ECG) (Arnaout et al. 2007; Hassel et al. 2008; Huttner et al. 2013; Juang et al. 2020; Langenbacher et al. 2020; Meder et al. 2011). LQTS was the most frequently studied condition, accounting for nine out of these 32 articles (28%, Fig. 1).

3.2 *Technical Approaches for the Generation of Genetically Modified Zebrafish Disease Models*

Both forward and reverse genetic approaches have been used for the generation of zebrafish models of inherited arrhythmia. Forward genetics aim at the discovery of the genetic basis for a known phenotype. In reverse genetics, a specific genetic change is generated to assess its phenotypical effects. One of the main hurdles in the application of both techniques in zebrafish is related to an additional duplication of

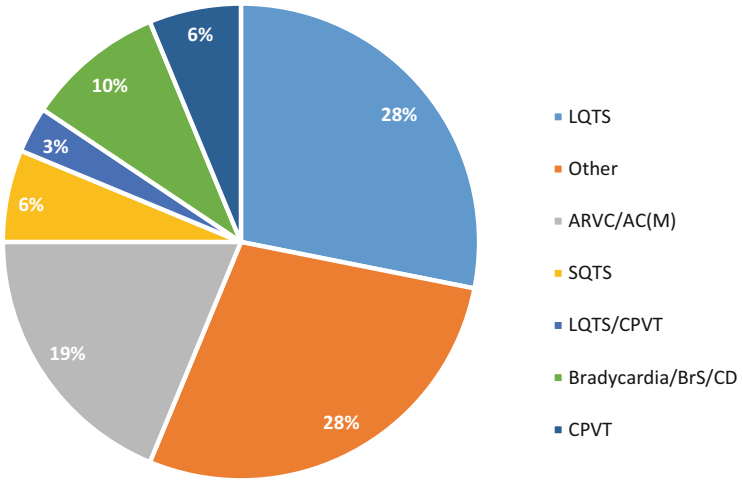

Fig. 1 Percentage distribution of studied disorders (total number of studies: 32). LQTS: long QT syndrome, ARVC/AC(M): arrhythmogenic (right ventricular) cardiomyopathy, SQTS: short QT syndrome, BrS: Brugada syndrome, CD: conduction disease, CPVT: catecholaminergic polymorphic ventricular tachycardia. "Other" conditions included ABCC9-related Intellectual disability Myopathy Syndrome (AIMS) (Smeland et al. 2019), Cantu syndrome (Tessadori et al. 2018), studies on relevant cardiac arrhythmia genes with complex (Huttner et al. 2013; Koopman et al. 2021; Langenbacher et al. 2005, 2020; Rottbauer et al. 2001) or insufficiently characterised (Ramachandran et al. 2013) electrophysiological phenotypes and mutants whose causal mutation has not been fully characterised (Warren et al. 2001)

the zebrafish genome. As a consequence there are many human genes for which there are two orthologues in the zebrafish. If only a single copy of a gene is modified the phenotype can still be rescued due to compensation by its paralogue.

3.2.1 Forward Genetics

The first zebrafish models of inherited arrhythmia have been generated by large-scale forward mutagenesis screens (Table 1), where zebrafish were exposed to mutagens (e.g. ethylnitrosourea) and crossed out. Their offspring was screened for directly observable abnormalities of the heart rhythm (Rafferty and Quinn 2018). The causal genetic variants could subsequently be mapped and cloned (Rafferty and Quinn 2018).

The forward mutagenesis screens have led to the discovery of several mutants with a cardiac arrhythmia phenotype, such as the *island beat* (*isl*) (Rottbauer et al. 2001), *breakdance* (*bre*) (Kopp et al. 2005), *reggae* (*reg*) (Hassel et al. 2008), *hiphop* (*hip*) (Pott et al. 2018) and *grime* (Koopman et al. 2021) mutants (see Table 1 and "Genetic zebrafish models for inherited arrhythmias" for the description of the causal genes and phenotypes related to these mutations). Another mutant

Table 1 Summary of the main techniques used for the generation of genetically modified zebrafish disease models

Method	Advantages	Disadvantages	Cardiac arrhythmia disease models
Forward mutagenic screens	Hypothesis free Possibility to study effect of specific mutations Stable if not embryonically lethal	Not possible to target specific genes Mapping required for identification of causal genes Subtle phenotypes can be missed	*Isl* (*cacna1c*) (Rottbauer et al. 2001), *bre* (*kcnh6a*) (Kopp et al. 2005), *reg* (*kcnh6a*) (Hassel et al. 2008), *hip* (*atp1a1a.1*) (Pott et al. 2018), *slow mo* (unknown gene) (Warren et al. 2001), *grime* (*tmem161b*) (Koopman et al. 2021)
Morpholino knockdown	Phenotype observed in injected embryos No mutagenesis required	Less representative of human mutation Lack of genetic compensation Transient Off-target effects	*Kcnh6a* (Jou et al. 2013; Langheinrich et al. 2003; Tanaka et al. 2019), *slc4a3* (Thorsen et al. 2017), *cacna1c* (Ramachandran et al. 2013), *dsc2* (Heuser et al. 2006), *dspa and dspb* (Giuliodori et al. 2018), *plakoglobin1-a/jupa and plakoglobin-1b/jupb* (Martin et al. 2009), *pkp2* (Moriarty et al. 2012), *mog1* (Zhou et al. 2016), *atp1a1a.1* (Pott et al. 2018)
mRNA injections	Phenotype observed in injected embryos No mutagenesis required Possible to inject mRNA of human genes (e.g. to test specific variants) Can be combined with morpholino to silence endogenous gene expression	Less physiological Transient	*KCNH2/hERG* (Jou et al. 2013; Tanaka et al. 2019), *mog1* (Zhou et al. 2016), *SCL4A3* (Thorsen et al. 2017), *plakoglobin1-a/jupa and plakoglobin-1b/jupb* (Martin et al. 2009), *DSC2* (Heuser et al. 2006), *dspa and dspb* (Giuliodori et al. 2018), *kcnj2-12* (Leong et al. 2013), *CALM1* (Berchtold et al. 2016; Da'as et al. 2019; Sondergaard et al. 2015)
CRISPR/TALE knockout	Stable model More representative of human disease Relatively high mutagenic efficiency	Phenotype usually not visible in first generation Off-target effects Not possible to study effect of individual	CRISPR: a*bcc9* (Smeland et al. 2019), *gstm.3* (Juang et al. 2020), *tmem161b* (Koopman et al. 2021) TALE: *mcu*

(continued)

Table 1 (continued)

Method	Advantages	Disadvantages	Cardiac arrhythmia disease models
		genetic variants (e.g. gain-of-function/ dominant negative)	(Langenbacher et al. 2020)
CRISPR knock-in	Stable model Most representative of human disease Possible to study effect of individual genetic variants (e.g. gain-of-function/dominant negative)	Low mutagenic efficiency Phenotype not visible in first generation Off-target effects	*Abcc9*, *kcnj8 and pln* (Tessadori et al. 2018)
Tol2 transposon	Stable model Possible to introduce human genes/reporter genes High mutagenic efficiency Tissue/organ specific expression Can be used for enhancer assay Possible to study effect of individual genetic variants (e.g. gain-of-function/dominant negative)	Random insertion site Does not alter endogenous genes	*SCN5A* (Huttner et al. 2013), *JUP* (Asimaki et al. 2014), *ILK* (Brodehl et al. 2019), enhancer assays (Kapoor et al. 2014; van den Boogaard et al. 2012)

zebrafish line, called *slow mo* (Baker et al. 1997) was also discovered by this method, but the causal gene has not yet been identified.

Although mutagenesis screens have provided a useful identification method for essential genes involved in normal cardiac electrophysiology in zebrafish, some important genes were likely not identified due to compensation by the unaffected paralogue gene. Subtle phenotypes, which would require more extensive characterisation, could also easily be missed. Additionally, the random mutation process employed for forward genetics is less suitable for targeted translational studies. These disadvantages have paved the way for the application of reverse genetic methods in zebrafish for further modelling of inherited arrhythmia.

3.2.2 Reverse Genetics

Transient Zebrafish Models

In transient models, it is possible to temporarily alter gene products without directly affecting the genomic DNA. This can be achieved by reducing the expression of endogenous genes by morpholino injections and/or inducing the expression of exogenous gene products by mRNA injections (Table 1). Morpholinos are

oligomeric nucleotide analogues, which can reduce gene expression by binding complementary (pre)mRNA sequences leading to a gene knockdown. Morpholinos are able to either block ribosomal translation by binding the start codon or 5' untranslated region of an mRNA molecule or alter splicing by binding a splice-site and inhibiting pre-mRNA processing (Bill et al. 2009). The degree of translational inhibition varies among morpholino constructs (Kamachi et al. 2008) and can be quantified (e.g. by antibodies or RT-qPCR).

mRNA injections can be used to express zebrafish or human genes, which can be either wildtype or contain (potentially) pathogenic variants. This can be combined with morpholinos to simultaneously silence the endogenous zebrafish orthologue and assess the capacity of the injected mRNA to rescue the phenotype. However, this approach can be complicated by an inhibition of the translation of the injected mRNA by the morpholino, leading to an interference with the phenotype rescue (Joris et al. 2017).

Both morpholino knockdown and mRNA injections have the advantage of being easy to implement and providing a fast readout, as the phenotype can be observed already in the injected embryos. However, the knockdown effect is transient and can therefore only be assessed at the larval stage. Moreover, transient models are intrinsically less appropriate for the study of mutation-dependent pathomechanisms, as their phenotype is not induced by genomic alterations. Biased results have been reported, due to more pronounced phenotypes occurring by the use of morpholinos, compared to stable knockout (KO) mutants (Rossi et al. 2015). This effect appears to be related to the lack of engagement of genetic compensation, triggered by nonsense mediated decay, which is not activated by morpholinos (El-Brolosy et al. 2019). Additionally, off-target effects have been observed for morpholinos, which manifest as an unintentional reduction in the expression of non-targeted genes (Eisen and Smith 2008; Joris et al. 2017; Tessadori et al. 2020).

The use of morpholinos thus requires careful experimental design consideration. Additional validation can be performed by comparing the phenotype of morphants generated by morpholinos targeting different loci in the same gene and phenotype rescue experiments (Stainier et al. 2017). Nonetheless, a definite validation of a morpholino phenotype requires a comparison with a stable genetic KO model.

Stable Models

The generation of stable genetic disease models has been greatly facilitated by the discovery of CRISPR-Cas9 (Jinek et al. 2012). This technique, derived from the bacterial defence system against bacteriophagic viruses, is based on the combination of a DNA cleaving enzyme (Cas9) and a customisable RNA sequence (guide RNA or gRNA), which limits the Cas9 activity to a pre-specified genomic locus (Fig. 2). These cleavage events are usually resolved by the error-prone endogenous cellular DNA repair process of non-homologous end joining, often resulting in insertions and deletions (indels) at the cleavage site (Fig. 2) (Adli 2018). The gRNA based genomic localisation mechanism is the main advantage of CRISPR compared to

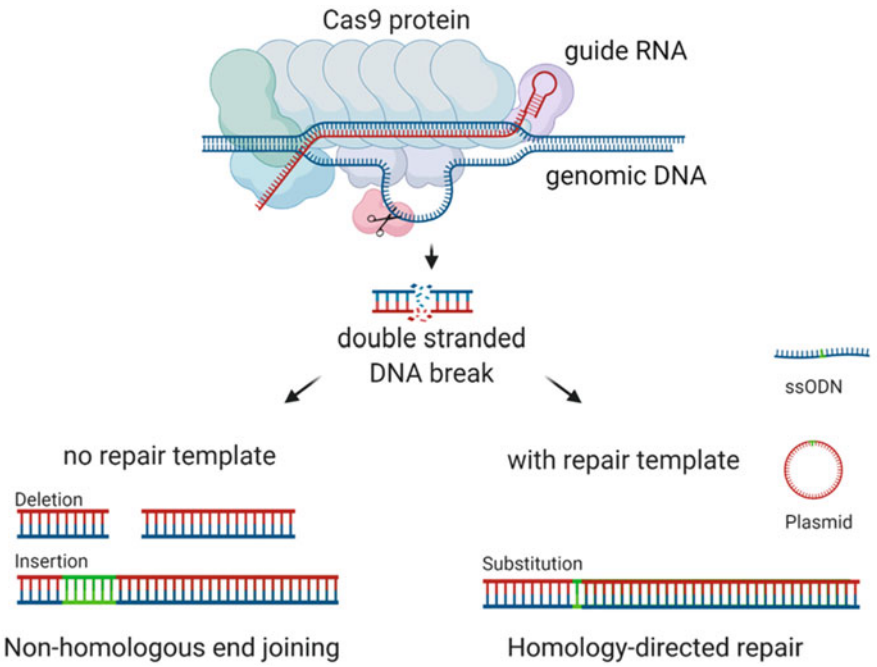

Fig. 2 Principles and components of genomic modification by CRISPR-Cas9. ssODN: single-stranded oligo DNA nucleotides. Created with BioRender.com

previous approaches to genetic editing, such as transcription activator-like effectors (TALEs). These techniques rely on proteins for the direction of their cleavage activity, which renders them more challenging to customise, compared to CRISPR (Adli 2018).

When applied in zebrafish, Cas9 mRNA or protein, together with a gRNA, can be injected into fertilised zebrafish eggs at the one-cell stage (Albadri et al. 2017). The injected "founder" fish develop a variable ("mosaic") genomic sequence at the cleavage site due to different repair outcomes in individual cells during development. Some of the indel events will induce gene KO, either by disrupting the reading frame ("frameshift") in early exons or by altering DNA segments encoding essential protein components. The phenotypic effects of the KO can be difficult to assess in the founder generation due to mosaicism. When founder fish are raised to adulthood, potential germline transmitters can be bred to obtain offspring with a uniform genetic code. These stable mutants provide the opportunity to study the effect of the KO both at the larval and adult stage, as well as over multiple generations.

The generation of zebrafish CRISPR KO mutants is generally quite feasible (Table 1) (Varshney et al. 2015). Nonetheless, it is more time-consuming compared to gene knockdown and mRNA injections, as the phenotype is usually not observed in the injected embryos themselves. Some methods, such as injections with multiple gRNAs targeting different loci in the same gene (Wu et al. 2018) and/or injections

with an alternative form of gRNA (two-RNA component, crRNA:tracrRNA, duplex guide RNA) (Hoshijima et al. 2019), are being developed to compensate for this drawback by increasing the proportion of cells with gene KO in the founder generation.

The CRISPR/Cas9 technique is also capable of generating missense variants (knock-in, Table 1 and Fig. 2) by engaging another type of DNA repair process called homology-directed repair (Fig. 2) (Albadri et al. 2017). By providing an external repair template (usually either a plasmid or a single-stranded deoxynucleotide), it is possible to introduce specific base pair substitutions (Albadri et al. 2017; Boel et al. 2018; Prykhozhij et al. 2018; Tessadori et al. 2018). However, this method still remains challenging as its efficiency is rather low with knock-ins occurring in only up to 1–4% of targeted alleles (Boel et al. 2018).

Similar to morpholinos, off-target effects are also a risk with the use of CRISPR-Cas9. These manifest as DNA cleavage events, which occur at genomic sites other than the targeted region. CRISPR-Cas9 off-target effects can be mitigated by careful in silico design of the gRNA and breeding out the germline transmitters (Rafferty and Quinn 2018). The additional duplication of the zebrafish genome can also be a challenge for the application of CRISPR-Cas9. To study the function of a specific human gene, one often needs to generate mutations in both orthologues in the zebrafish.

3.2.3 Expression of Exogenous Genes

The Tol2 transposon method can stably insert exogenous genes into the zebrafish genome (Table 1) (Kawakami 2007). This method, based on autonomous mobile genetic elements identified in medaka fish, allows for the insertion of up to 11 kilo base pairs of DNA flanked by specific Tol2 sequences into random sites in the genome. Overall, up to 50–70% of the injected fish will produce transgenic offspring (Kawakami 2007). By coupling the transgene to a cell-type specific promoter, its expression can be limited to a certain tissue or organ. The Tol2 transposon method has been used to insert wildtype and mutant human genes (Asimaki et al. 2014; Brodehl et al. 2019; Huttner et al. 2013) and reporter proteins (van Opbergen et al. 2018) into the zebrafish genome (Table 1).

Apart from modelling functional alterations in protein-coding genes, zebrafish have also been used to characterise the function of putative enhancer sequences for cardiac arrhythmia genes (Kapoor et al. 2014; van den Boogaard et al. 2012). Enhancers are non-coding regulatory DNA sequences which interact with the activity of transcription factors to influence the expression of distally located genes (van den Boogaard et al. 2012). Enhancer assays make use of the Tol2 transposon method to insert putative enhancer sequences coupled to a promoter and a gene encoding a fluorescent protein. The regulatory effect of the enhancer sequence can be evaluated by monitoring the tissue expression of the fluorescent protein in the developing embryo. Similarly, zebrafish signalling pathway reporter lines are developed by inserting known pathway specific regulatory elements coupled to a

promoter and a gene encoding a fluorescent protein (Moro et al. 2013). These lines can be used to study factors which are expected to alter the activity of the pathway of interest (e.g. in a genetically altered zebrafish model for ARVC/AC(M) (Giuliodori et al. 2018)).

3.3 Electrophysiological Approaches in Zebrafish Phenotyping

3.3.1 Methods to Assess the Electrophysiological Properties of the Zebrafish Heart

The characterisation of the zebrafish cardiac AP and its underlying currents has been performed by patch-clamp measurements on isolated cardiomyocytes and extracted hearts derived from adult (Abramochkin et al. 2018; Brette et al. 2008; Nemtsas et al. 2010; Zhang et al. 2011) and embryonic (Alday et al. 2014; Jou et al. 2010) zebrafish. Isolated cardiomyocytes can be derived by enzymatic digestion (typically with a mixture of collagenase and trypsin) of dissected adult (Abramochkin et al. 2018; Brette et al. 2008; Nemtsas et al. 2010) or embryonic (Alday et al. 2014) zebrafish hearts.

The electrophysiological characterisation of cardiomyocytes can be done by the patch-clamp method. In the clamp configuration, the recording electrode is brought into a close, high-resistance contact with the cell membrane (a seal), covering a patch of the membrane (Sakmann and Neher 1984). Several recording configurations are possible: a cell-attached, permeabilised and inside-out and outside-out isolated patches (Gurney 2000; Sakmann and Neher 1984). The whole-cell patch-clamp configuration is the most conventional mode, where the recording electrode forms a low resistance contact with the intracellular environment of the cell. Although this configuration disturbs the natural intracellular environment, it also allows to modify it. It has a high signal-to-noise resolution and is useful for characterisation of specific ion conductances.

The whole-cell configuration can be used for the voltage-clamp mode as well as the current-clamp mode (Wickenden 2014). In the current-clamp mode the current passing across the cell membrane is controlled to record the resulting changes in membrane voltage. This method measures the changes in the membrane potential and can be used for the characterisation of the zebrafish cardiac resting and action potentials. This can be assessed in zebrafish on both an isolated heart (Jou et al. 2010; Nemtsas et al. 2010) and single cardiomyocytes (Brette et al. 2008; Nemtsas et al. 2010; Rottbauer et al. 2001).

The voltage-clamp mode, where the voltage across the cell membrane is controlled and the resulting currents are recorded, is the way to assess membrane ion channels and electrogenic ion transporters. Importantly, due to technical limitations, it is not possible to control the voltage over a large membrane surface or over many electrically coupled cells (de Roos et al. 1996). This space phenomenon arises from

the resistance of the cytoplasm and the intercellular contacts, which induces a voltage drop over distance (de Roos et al. 1996). Because of this limitation, membrane voltage will no longer be uniformly distributed, and thus the cell, or several electrically coupled cells, cannot be efficiently voltage-clamped. This is especially important for the heart, where cardiomyocytes are tightly coupled electrically, creating an electrical syncytium. Therefore, voltage-clamp measurements of ionic current can only be performed in isolated cardiomyocytes or membrane patches (Fig. 3a) (Nemtsas et al. 2010; Skarsfeldt et al. 2018). In zebrafish, the voltage-clamp mode has been used to characterise the ionic currents of cardiomyocytes derived from enzymatic digestion of zebrafish hearts (Abramochkin et al. 2018; Alday et al. 2014; Brette et al. 2008; Nemtsas et al. 2010).

The membrane potential can also be assessed by a conventional sharp electrode impaled in the myocardium or isolated cardiomyocytes (Wickenden 2014). Although this impalement can be quite stressful for the cell, the method measures the average membrane potential of several neighbouring cells. Patch-clamp and sharp microelectrodes have been used for the phenotypical assessment of several inherited cardiac arrhythmias in zebrafish (Table 2) (Asimaki et al. 2014; Brodehl et al. 2019; Jou et al. 2013; Koopman et al. 2021; Rottbauer et al. 2001; Smeland et al. 2019; Warren et al. 2001). To date, these remain the most sensitive techniques for the characterisation of the cardiac membrane potential and specific ionic conductance. However, these methods are difficult to apply in vivo.

ECG recording is an important method, which provides information on the electrical properties of the entire heart and can be performed non-invasively in vivo (Milan et al. 2006). Indeed, ECG of adult (Hassel et al. 2008; Huttner et al. 2013; Langenbacher et al. 2020; Meder et al. 2011) and embryonic (Pott et al. 2018; Rottbauer et al. 2001; Tanaka et al. 2019; Thorsen et al. 2017) zebrafish are proven a popular and informative phenotyping tool in models for cardiac arrhythmia (Table 2). Similar to humans, the zebrafish ECG signal displays a P-wave, a QRS-complex and a T-wave (Fig. 3b). The ECG signal can expose arrhythmias as well as other electrophysiological or morphological abnormalities in the heart, e.g. QTc prolongation and hypertrophy. Because of their non-invasive nature, ECG recordings can be performed over a longer time span and are therefore useful for the detection of relatively rare arrhythmic events. ECG recordings in zebrafish require anaesthesia and the signal quality can be further improved by impaling needle electrodes or by opening the dermis and pericardial sac prior to the procedure in adult fish (Liu et al. 2016), although this intervention evidently increases the invasiveness of the procedure.

Important electrophysiological parameters, such as atrio-ventricular conduction delay and depolarisation and repolarisation abnormalities, can be derived from the ECG signal. ECG recordings show significant inter-experimental variability (Liu et al. 2016). For instance, measurements of the QTc interval in adult wildtype zebrafish can range from 250 ms to 600 ms between experiments (Liu et al. 2016). These differences can be attributed to several factors, including electrode placement, experimental temperature, nature or concentrations of anaesthetics and variable calculation methods for the QTc interval (such as the Bazett formula (Chablais

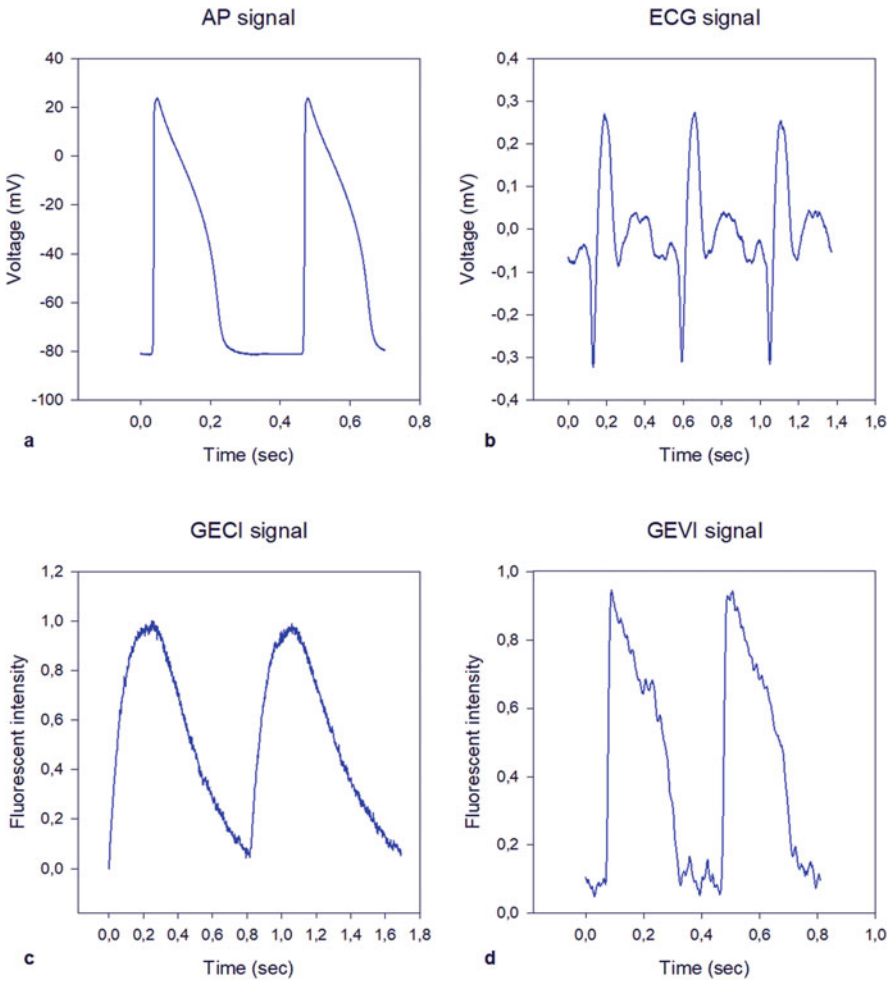

Fig. 3 Overview of signals obtained by different electrophysiological phenotyping tools. (**a**) Patch-clamp recording of a cardiac action potential from a cardiomyocyte derived from the adult zebrafish heart. (**b**) Electrocardiogram (ECG) recorded from a zebrafish embryo at 3 days post fertilisation by surface glass electrode (Thorsen et al. 2017). (**c**) Fluorescent signal from the zebrafish embryo ventricle at 3 days post fertilisation representing a calcium transient, obtained by a genetically encoded calcium indicator (GECI) with a light sheet microscope; (**d**) Fluorescent signal from the ventricle of a zebrafish embryo at 3 days post fertilisation representing the cardiac action potential, obtained by a genetically encoded voltage indicator (GEVI) with a light sheet microscope

et al. 2011; Hassel et al. 2008; Tsai et al. 2011) or custom-made calculations (Arnaout et al. 2007; Milan et al. 2006)).

Table 2 Summary of the main techniques for the electrophysiological characterisation of zebrafish

Method	Advantages	Disadvantages	Examples of arrhythmia disease models
Patch-clamp	Extremely sensitive Ionic current and membrane potential measurements possible Embryo/larvae and adult	Labour intensive Need for specialised equipment and expertise Mostly single cell Difficult to apply in vivo Interference with intracellular environment	*KCNH2/hERG* (Jou et al. 2013), *isl* mutant (Rottbauer et al. 2001), *JUP* (Asimaki et al. 2014), *ILK* (Brodehl et al. 2019)
Electrocardiography (ECG)	In vivo Minimally disruptive Compatible with human phenotype Overview of functioning of entire heart Long recording time possible Embryo/larvae and adult	Require anaesthesia No direct measurement of action potential characteristics Inter-experimental variability	*KCNH2/hERG* (Tanaka et al. 2019), *atp1a1a.1* (Pott et al. 2018), *slc4a3* (Thorsen et al. 2017), *cacna1c* (Rottbauer et al. 2001), *gstm.3* (Juang et al. 2020), *mcu* (Langenbacher et al. 2020)
(High speed) light microscopy video recordings	In vivo Easy to perform Limited technical requirements	Limited information (only atrial and ventricular heart rate and duration of systole/diastole) Only feasible in embryos and optically translucent lines (e.g. Casper strain) Embryo/larvae only	*CALM1* (Da'as et al. 2019; Sondergaard et al. 2015), *mog1* (Zhou et al. 2016), *kcnj2-12* (Leong et al. 2013), *bre* mutant (Kopp et al. 2005), *dsc2* (Heuser et al. 2006), *dspa and dspb* (Giuliodori et al. 2018), *plakoglobin1-a/jupa and plakoglobin-1b/jupb* (Martin et al. 2009), *pkp2* (Moriarty et al. 2012), *slc4a3* (Thorsen et al. 2017)
Calcium and voltage dyes	Some specialised technical requirements Representation of most characteristics of cardiac action potentials (especially for voltage dyes) Detection of localised action potential alterations Possible to map	In vitro (explanted hearts) Need to compensate for motion artefacts Not cell/tissue specific Increased background noise Need for fluorescence microscopy set up No information on	*Atp1a1a.1* (Pott et al. 2018), *bre* mutant (Meder et al. 2011), *reg* mutant (Hassel et al. 2008), *slc4a3* (Thorsen et al. 2017)

(continued)

Table 2 (continued)

Method	Advantages	Disadvantages	Examples of arrhythmia disease models
	conduction pattern and speed Embryo/larvae and adult	absolute membrane voltage or upstroke velocity Less suitable for prolonged measurements (photobleaching and phototoxicity)	
Genetically encoded calcium and voltage indicators	In vivo Cell-type specific Stable transgenic transmission Representation of most characteristics of cardiac action potentials (especially for GEVI) Detection of localised action potential alterations Possible to map conduction pattern and speed	Need to compensate for motion artefacts Need for fluorescence microscopy set-up No information on absolute membrane voltage or upstroke velocity Less suitable for prolonged measurements (photobleaching and phototoxicity) Need to insert indicator into the zebrafish genome Embryo/larvae only	*Kcnh6a* (Arnaout et al. 2007) *tg*(cmlc2:gCaMP)^{s878} line expressing a GECI) *Tmem161b*^{uq4ks}/tg (myl7:chimeric VSFP-butterfly CY) expressing a GEVI and *tmem161b*^{uq4ks}/tg (myl7:Gal4FF;UAS:GCaMP6f) line expressing a GECI (Koopman et al. 2021)

GEVI genetically encoded voltage indicator, *GECI* genetically encoded calcium indicator

3.3.2 Imaging Techniques

The transparency of zebrafish larvae enables the observation of atrial and ventricular contractions under a light microscope, whether or not in combination with (high-speed) video recordings. Although this method only provides information on the heart rate and the atrio-ventricular synchrony, it has been the only method applied in several inherited arrhythmia studies, likely due to its ease of use and lack of technical requirements (Table 2) (Da'as et al. 2019; Kopp et al. 2005; Langheinrich et al. 2003; Leong et al. 2013; Sondergaard et al. 2015; Zhou et al. 2016). The obtained video recordings can be further translated to systole/diastole duration and ventricular morphology.

More options for imaging-based electrophysiological phenotyping are feasible by use of fluorescent microscopy. Calcium and voltage sensitive dyes are able to report changes in free cytosolic calcium concentration or membrane potential, respectively, as a fluorescent signal. The calcium sensitive Calcium Green dextran dye has been used previously to optically map embryonic zebrafish hearts in inherited arrhythmia models ex vivo (Table 2) (Hassel et al. 2008; Meder et al. 2011; Pott et al. 2018).

Other studies have described voltage mapping of ex vivo embryonic (Peal et al. 2011) and adult (Lin et al. 2014; Tessadori et al. 2012) hearts with the RH-237 dye (Lin et al. 2014) as well as the di-4 ANEPPS (aminonaphthylethenylpyridinium) dye (Peal et al. 2011; Tessadori et al. 2012). Importantly, due to different emission spectra, it is also possible to combine calcium and voltage dyes in a single experiment (Lin et al. 2015).

The main disadvantages of calcium and voltage sensitive dyes are the difficulty to use them in vivo and their lack of cell-specificity. The uptake of these dyes is not limited to excitable tissues and other surrounding cell types will also be loaded, thus generating significant background noise. To compensate for these drawbacks and enable cell-type specific in vivo optical mapping, genetically encoded calcium and voltage indicators (GECI and GEVI, respectively; Table 2) have been employed in zebrafish (Arnaout et al. 2007; Chi et al. 2008; Hou et al. 2014; Koopman et al. 2021; Tsutsui et al. 2010; van Opbergen et al. 2018; Weber et al. 2017).

Similar to dyes, GECI and GEVI are capable of reporting calcium and membrane voltage changes as a fluorescent signal (Fig. 3c–d). In practise, the genetic code for these indicators can be inserted into the genome of zebrafish. The myocardium-specific expression of the indicators is achieved by cardiac-specific promoters, such as the cardiac myosin light chain 2 (cmlc2 or myl7) promoter (Huttner et al. 2013). A recently developed zebrafish line with myocardium-specific expression of a GECI and a GEVI was used for the characterisation of drug-induced changes to the atrial and ventricular heart rate, activation pattern, conduction velocity and action potential duration (van Opbergen et al. 2018). By coupling GECI and GEVI with powerful microscopic techniques, such as light sheet imaging, it also becomes possible to optically map the electrical signals of the entire zebrafish heart (Weber et al. 2017). Although voltage dyes provide overall better brightness and kinetics than GEVI in vitro (Milosevic et al. 2020), due to the difficulty to load dyes intracellularly in vivo, GEVI outperform dyes in vivo (Mutoh et al. 2015).

The measurement of intracellular calcium concentrations by both dyes and genetically encoded indicators is a relatively well-established approach. The full optical characterisation of the cardiac membrane voltage has proven technically challenging. While calcium sensors are localised intracellularly, voltage sensors need to be incorporated in the cell membrane, and therefore tend to be dimmer. Moreover, some components of the membrane voltage signal, such as the AP rise, occur in a very short time span compared to the calcium transient. Voltage imaging thus requires both sensitive and high-speed imaging techniques and sensors with a fast response time. Despite these challenges, voltage imaging remains worthwhile as it provides a detailed characterisation of the cardiac AP. The development of GEVIs is a quickly evolving field and the newest GEVI are very promising due to their vastly improved brightness and kinetics (Shen et al. 2020).

Motion artefacts generated by the beating heart are an important hindrance to the use of both dyes and genetically encoded indicators. Currently, the most commonly used method to compensate for this problem relies on the inhibition of cardiac contraction by induction of electro-mechanical uncoupling. This can be done either pharmacologically, by exposing zebrafish embryos to the myosin ATPase activity

inhibitor blebbistatin (Jou et al. 2010) or the inhibitor of actin polymerisation cytochalasin D (Wang et al. 2013), or by blocking the expression of the *tnnt2* gene with the silent heart morpholino (Weber et al. 2017). Photobleaching is another challenge intrinsic to fluorescent imaging. This degradative process leads to an irreversible loss of the fluorescent intensity of a fluorophore upon each excitation. Fluorescent indicators susceptible to photobleaching are less suitable for prolonged imaging. Lastly, the application of fluorescent imaging can result in damage to cells and tissues (i.e. phototoxicity). The use of fluorescent indicators in vivo needs to be applied carefully with the minimal fluorescent intensity and exposure time required for the experiment.

3.4 Genetic Zebrafish Models for Inherited Arrhythmias

3.4.1 Long QT Syndrome

KCNH2/hERG

Similar to humans, the rapidly activating outward rectifying potassium current (I_{Kr}) functions as the main repolarising current in zebrafish cardiomyocytes, although its kinetics are slightly different (Verkerk and Remme 2012). However, I_{Kr} is not produced by the zebrafish *KCNH2/hERG* orthologue, but by the *kcnh6a* gene, which is primarily expressed in the central nervous system in humans (Vornanen and Hassinen 2016). Nonetheless, the zebrafish Kcnh6a protein shows 60% amino acid identity to the human KCNH2 and several studies have successfully phenotyped I_{Kr}-related LQTS (LQTS type 2) in zebrafish (Table 3) (Jou et al. 2013; Kopp et al. 2005; Langheinrich et al. 2003; Meder et al. 2011; Tanaka et al. 2019).

Forward mutagenesis screens have yielded the *bre* mutant with a p.(Ile59Ser) substitution in the *kcnh6a* gene. This *bre* mutation leads to a loss-of-function through an inhibition of protein trafficking to the cell membrane (Meder et al. 2011). Homozygous *bre* mutant zebrafish embryos have a normal morphology and show an intermittent dysregulation of atrio-ventricular synchrony with a single ventricular heart beat for each two atrial contractions (2:1 heart block) and a frequent embryonic lethality by 7 days post fertilisation (Kopp et al. 2005). The occasional adult survival of homozygous *bre* mutants suggests that both genetic and possibly environmental factors can influence the severity of the zebrafish arrhythmia phenotype. Similar variability in lethality is also observed in families with inherited cardiac arrhythmia (Cerrone et al. 2019).

This cardiac arrhythmia phenotype could be pharmacologically rescued by improving protein trafficking to the cell membrane by administration of cisapride (a prokinetic agent which was found to improve trafficking of mutant *KCNH2* channels) (Ficker et al. 2002) or chemical chaperones (Meder et al. 2011). Two additional pharmacological compounds, which modulate the *bre* phenotype, have been identified in a large chemical screen (2-MMB and the steroid flurandrenolide)

Table 3 Summary of the existing models of inherited arrhythmia,

Disorder	Gene	Genetic modification technique	Electrophysiological phenotyping technique	Electrophysiological phenotype	Reference
LQTS	*KCNH2* = *hERG* (human) *kcnh6* = *zerg* (zebrafish)	Forward mutagenesis screen (*bre* mutant, p.Ile59Ser substitution)	Light microscopy video recordings (embryo)	2:1 heart block Frequent embryonic lethality	(Kopp et al. 2005)
			Calcium dye in explanted hearts (embryo) ECG (adult)	2:1 heart block (embryo) QT prolongation (adult)	(Meder et al. 2011)
		Forward mutagenesis screen (p.Ile462Arg (S213) and p. Met521Lys (S290) substitutions) Morpholino knockdown	Patch-clamp on explanted hearts + GECI (Tg(cmlc2: gCaMP)^{s878}) (embryo) ECG (adult)	Homozygotes: Silent ventricle and embryonic lethality (embryo) Heterozygotes: 2:1 heart block, drug-induced APD prolongation (embryo) QT prolongation (adult)	(Arnaout et al. 2007)
		Morpholino knockdown	Light microscopy video recordings (embryo)	Irregular heart rate 2:1 heart block Silent ventricle	(Langheinrich et al. 2003)
		Morpholino knockdown + *KCNH2* mRNA overexpression	ECG (embryo)	2:1 heart block Ventricular asystole QT-interval prolongation	(Tanaka et al. 2019)
			Light microscopy video recordings + patch-clamp (limited number for validation) (embryo)	2:1 heart block Prolonged APD	(Jou et al. 2013)
	KCNJ2 (human) *kcnj2-12* (zebrafish)	*kcnj2-12* mRNA overexpression	Light microscopy video recordings (embryo)	Increased ratio of dead and malformed embryos	(Leong et al. 2013)
	Na+/K+-ATPase, *atpa1a.1*	Morpholino knockdown + forward mutagenesis screen (*hip* mutant)	Paced ECG + calcium dye (Calcium Green dextran) (embryo)	*Hip* mutants: irregular heart rate, prolonged QTc interval, partial heart block	(Pott et al. 2018)

				Morpholino: morphological abnormalities and decreased systolic function (high dose), reduced and irregular heart rate block (low dose)	
	NOS1AP	Enhancer screen	–	–	(Kapoor et al. 2014)
SQTS	*KCNH2* = *hERG* (human) *kcnh6* = *zerg* (zebrafish)	Forward mutagenesis screen (*reg* mutant)	Calcium dye (Calcium Green dextran) with pacing, sharp microelectrode (embryo) ECG (adult)	Sino-atrial block, atrial fibrillation (embryo) Shortened QTc interval (adult)	(Hassel et al. 2008)
	SLC4A3 (AE3)	Morpholino knockdown	Light microscopy video recordings + ECG (embryo)	Shortened QT-interval	(Thorsen et al. 2017)
CPVT	*CALM1*	mRNA overexpression	Light microscopy video recordings and/or recordings of GFP-labeled Tg(myl7:GFP) hearts (embryo)	No phenotype or increased heart rate compared to controls (either at rest or induced by epinephrine)	(Da'as et al. 2019), (Berchtold et al. 2016), (Sondergaard et al. 2015)
ARVC/AC(M)	*DSC2*, *DSP*, *JUP*, *PKP2*	Morpholino knockdown + mRNA overexpression	Light microscopy video recordings (embryo)	Bradycardia, heart failure, abnormal cell–cell junctions	(Giuliodori et al. 2018), (Martin et al. 2009), (Moriarty et al. 2012), (Heuser et al. 2006)
	JUP	Tol2-mediated insertion of human wildtype and mutant *JUP*	Patch-clamp on isolated cardiomyocytes (adult)	Increased resting membrane potential, decreased maximal depolarisation rate, decreased sodium current density	(Asimaki et al. 2014)
	ILK	Tol2-mediated insertion of human wildtype and variant *ILK*	Light microscopy video recordings (embryo) Patch-clamp on ex-vivo embryonic hearts	Decreased survival, no action potential abnormalities	(Brodehl et al. 2019)

(continued)

Table 3 (continued)

Disorder	Gene	Genetic modification technique	Electrophysiological phenotyping technique	Electrophysiological phenotype	Reference
Other	*CACNA1C*	Forward mutagenesis screen (*isl* mutant)	Patch-clamp on isolated cardiomyocytes, ECG (embryo)	Abnormal ventricular morphology, no ventricular heartbeat, electrically unresponsive ventricles	(Rottbauer et al. 2001)
		Morpholino knockdown	Light microscopy observation (embryo)	No ventricular heartbeat	(Ramachandran et al. 2013)
	SCN5A	Morpholino knockdown	Light microscopy observation (embryo)	Severe morphological cardiac defects	(Chopra et al. 2010)
		Tol2-mediated insertion of human wildtype and mutant *SCN5A* +/− morpholino knockdown	Light microscopy video recordings, ECG (embryo) ECG (adult)	Higher mortality, mild bradycardia, episodes of sinus pause (embryo) Increase in the duration of the PR and QRS intervals (adult)	(Huttner et al. 2013)
	SCN5A + *SCN10A*	Enhancer screen	–	–	(van den Boogaard et al. 2012)
	GSTM3	CRISPR-Cas9 knockout	ECG (adult male)	Prolongation of PR and QRS interval after administration of flecaïnide Inducibility of ventricular arrhythmia by flecaïnide or programmed extra-systolic stimulation	(Juang et al. 2020)
	MOG1	Morpholino knockdown + mRNA overexpression	Light microscopy video recordings (embryo)	Cardiac morphological defects, bradycardia (knockdown) or tachycardia (overexpression)	(Zhou et al. 2016)
	Abcc9	CRISPR-Cas9 knockout of *abcc9* gene	Light microscopy video recordings (embryo) Patch-clamp (adult)	Morphological abnormalities, decreased mobility, heart failure (embryo) Absent K_{ATP} current (adult)	(Smeland et al. 2019)

		CRISPR-Cas9 knock-in of *abcc9* and *kcnj8* genes	Light microscopy video recordings (embryo)	Enlarged hearts and an enhanced cardiac output	(Tessadori et al. 2018)
	Ncx1h	Forward mutagenesis screen (*tre* mutant) Morpholino knock-down + mRNA overexpression	Light microscopy video recordings (embryo)	Atrial and ventricular fibrillation	(Langenbacher et al. 2005)
	Mcu	TALE knockout of *mcu* gene	ECG (adults)	Impaired cardiac function, abnormal QRS morphology, diminished R amplitude, episodes of sinus arrest	(Langenbacher et al. 2020)
	Tmem161b	*Grime* mutant (p.Cys466*), CRISPR knockout of *tmrm161b* gene	GEVI (tg(myl7:chimeric VSFP-butterfly CY)) and GECI (tg(myl7:gal4FF; UAS:GCaMP6f)) (homozygous embryos) Patch-clamp (heterozygous adult)	Sino-atrial and atrio-ventricular abnormalities, faster and increased calcium release (homozygous embryos) Early afterdepolarisations, abnormal AP duration (shorter APD20 but longer APD50 and APD90), increased I_{Ks} and I_{CaL} currents (heterozygous adults)	(Koopman et al. 2021)

(Peal et al. 2011). The pharmacological effects of these drugs appear unrelated to trafficking (Peal et al. 2011). Flurandrenolide acts on the cardiomyocytes through the glucocorticoid receptor, but the exact mechanisms of action are unclear for both 2-MMB and flurandrenolide. *Bre* mutants that survive into adulthood display a prolonged QTc interval (Meder et al. 2011). Experimental pharmacological rescue of this defect in vivo illustrates the potential of zebrafish studies for research into personalised medical treatments.

Two other *kcnh6a* mutants, p.(Ile462Arg) and p.(Met521Lys) missense mutations (called *S213* and *S290*, respectively), were also discovered through forward mutagenesis screens. These mutant embryos displayed a more severe phenotype compared to the *bre* mutants, with silent ventricles and complete embryonic lethality in the homozygous state (Arnaout et al. 2007). Heterologous expression studies in Xenopus oocytes showed a complete lack of function for isolated mutant channels and dominant negative effects when co-expressed with the wildtype allele (Arnaout et al. 2007). Heterozygous mutant embryos showed a 2:1 heart block upon exposure to the I_{Kr} blocking drug terfenadine, as well as a prolonged action potential duration at 90% of repolarisation (APD90), while adult heterozygotes had a prolonged QTc interval (Arnaout et al. 2007).

These findings from forward mutagenesis screens revealed a typical and easily observable feature of the LQTS phenotype in zebrafish, i.e. the 2:1 heart block. It is likely that this phenomenon is caused by increased ventricular refractoriness, rather than increased atrio-ventricular delay (Arnaout et al. 2007). The more severe phenotype observed in the *S213* and *S290* mutants compared to the *bre* mutants is an apt example of how allelic heterogeneity can act as a modifier of disease severity, which highlights the importance of allelic series variant modelling.

Several studies have also looked into the effect of morpholino knockdown of the *kcnh6a* gene, which resulted in a phenotype with an irregular heart rate, an atrio-ventricular block or a silent ventricle (Langheinrich et al. 2003). Co-injection of either the zebrafish *kcnh6a* (Arnaout et al. 2007) or the human *KCNH2* (Jou et al. 2013; Tanaka et al. 2019) mRNA could partially rescue normal cardiac function. This co-injection method was used to develop an in vivo assay to distinguish between benign and disease-causing *KCNH2* variants by examining the ability of *KCNH2* mRNA carrying these variants to rescue the LQTS phenotype (Jou et al. 2013). The zebrafish assay achieved a negative predictive value of 90% and a positive predictive value of 100% compared to heterologous expression (Jou et al. 2013). This assay has the advantage of being in vivo, easy to execute and high throughput, with a phenotype which could be observed directly under a light microscope in the injected embryos.

KCNJ2

Mutations in the *KCNJ2* gene encoding the inward rectifier potassium channel 2 are the cause of LQTS type 7, also called Andersen-Tawil syndrome. A bioinformatical and transcriptional approach has identified the zebrafish *kcnj2-12* gene as the

zebrafish *KCNJ2* orthologue, although with a lower cardiac expression compared to human (Leong et al. 2013). Subsequent overexpression experiments with injections of human wildtype or mutant *KCNJ2* mRNA at identical concentrations showed an increased ratio of dead and malformed embryos in the mutant group. The cardiac phenotype was not thoroughly investigated, as heart rate was the only cardiac variable examined in this study (Table 3) (Leong et al. 2013).

KCNQ1 and KCNE1

The expression of the slowly activating potassium current (I_{Ks}), generated by the protein products of the *KCNQ1* (alpha subunit) and *KCNE1/MINK* (beta subunit) genes, was difficult to confirm in adult zebrafish (Abramochkin et al. 2018) and is yet to be confirmed in zebrafish embryos. In zebrafish, there is pronounced reduction in the expression of the *KCNE1/MINK* beta subunit, which leads to a decreased current amplitude and altered kinetics with a rapid activation and decreased beta-adrenergic responsiveness (Abramochkin et al. 2018). The reduced expression of the beta subunit also alters the protein conformation of the channel complex and thereby its drug sensitivity. These differences make zebrafish a less attractive model for LQTS caused by *KCNQ1* and *KCNE1* mutations (i.e. LQTS type 1 and 5, respectively).

Other LQTS Genes

Apart from the classical potassium channels, other genes that influence the length of the QTc interval have been studied in zebrafish (Kapoor et al. 2014; Pott et al. 2018). Forward mutagenesis screen revealed an LQTS phenotype in zebrafish embryos induced by missense mutations (*hip* mutants) in the *atpa1a.1* gene, encoding Na+/K +-ATPase (Table 3) (Pott et al. 2018). *Hip* mutants displayed a reduced and irregular heart rate, a prolonged QTc interval as well as a partial heart block. In contrast to the previously discovered complete loss-of-function *atpa1a.1* null alleles (*heart and mind* (Shu et al. 2003) and *small heart* (Yuan and Joseph 2004) forward screen mutants), the *hip* mutation showed no impact on cardiac morphology and systolic function, suggesting a partial, rather than complete, loss of function (Pott et al. 2018). Similarly, morpholino experiments have demonstrated an effect on the heart rate only at lower dosages and additional morphological defects with increasing injection dosages (Pott et al. 2018).

Zebrafish were also used as an in vivo screening tool for the enhancer activity of several non-coding variants in the *NOS1AP* gene locus with suspected QT-regulatory functions, which were identified by a genome-wide association (GWAS) study (Table 3) (Kapoor et al. 2014).

3.4.2 Short QT Syndrome

KCNH2/hERG

Similarly to LQTS, the first zebrafish SQTS mutant was discovered by means of a forward mutagenesis screen and concerned a p.(Leu499Pro) missense mutation in the *kcnh6a* gene (*reg* mutant, Table 3) (Hassel et al. 2008). This mutation resulted in a gain of function of the channel protein due to faulty channel inactivation. *Reg* mutant embryos displayed a phenotype with an intermittent loss of the heartbeat, which could be rescued or converted to an LQTS phenotype by injection of a morpholino targeted against *kcnh6a* or an I_{Kr} blocking agent (terfenadine) (Hassel et al. 2008). Both homozygous and heterozygous adult *reg* mutants displayed shortened QTc intervals compared to wildtype controls (Hassel et al. 2008).

SLC4A3

Another zebrafish SQTS model involves the Solute Carrier Family 4 Member 3 (*SLC4A3*) gene, which encodes a Cl^--$HCO3^-$-exchanger (AE3, Table 3) (Thorsen et al. 2017). This is the first anion membrane transporter which was shown to be involved in cardiac arrhythmia. Heterozygous loss-of-function mutations in *SLC4A3* were identified as a cause of SQTS by whole exome sequencing in an affected family and subsequent heterologous expression experiments (Thorsen et al. 2017). This effect was recapitulated by morpholino-induced knockdown in zebrafish embryos, which led to an elevated intracellular pH and a shortened QT-interval (Thorsen et al. 2017).

3.4.3 Catecholaminergic Polymorphic Ventricular Tachycardia

CALM1

Three studies have described the development and characterisation of calmodulin mRNA overexpression models (Table 3) (Berchtold et al. 2016; Da'as et al. 2019; Sondergaard et al. 2015). Human mutations in the *CALM1* gene are involved in both the CPVT phenotype (CPVT type 4) and the LQTS phenotype (LQTS type 14). Two studies found that zebrafish embryos injected with *CALM1* variants previously identified in patients with CPVT had a faster heart rate either at rest (Da'as et al. 2019) or upon exposure to epinephrine (Sondergaard et al. 2015). Additionally, the heart rate in the mutant group was irregular in comparison with controls (Da'as et al. 2019). In the third study, several *CALM1* variants associated with CPVT, LQTS and idiopathic ventricular fibrillation were tested, however only the mutation associated with LQTS gave rise to a phenotype with a reduced resting heart rate (Berchtold et al. 2016). As the electrophysiological phenotyping in all studies was limited to the

observation of the heart rate in transient overexpression models, further exploration is needed to expand on the zebrafish CPVT phenotype.

3.4.4 Arrhythmogenic Right Ventricular Cardiomyopathy

So far, four desmosomal genes implicated in ARVC/AC(M) (*DSC2*, *DSP*, *JUP* and *PKP2*) have been investigated in zebrafish by morpholino knockdown combined with mRNA overexpression (Table 3) (Giuliodori et al. 2018; Heuser et al. 2006; Martin et al. 2009; Moriarty et al. 2012). Knockdown for all studied genes led to a bradycardia phenotype, which was rescued by wildtype mRNA injections of the corresponding gene. When tested, injection of mutant mRNA did not lead to rescue and as such confirmed pathogenicity (Heuser et al. 2006). The morphant embryos showed signs of heart failure with diminished cardiac contractions, pericardial oedema and blood pooling. Electron microscopy confirmed abnormal cell–cell junctions in *dsc2* (Heuser et al. 2006), *dsp* (Giuliodori et al. 2018), *jup* (Martin et al. 2009) and *pkp2* (Moriarty et al. 2012) morphants. The studies on *dsp* and *jup* also examined signalling pathway alterations and uncovered abnormalities in the Wnt/β-catenin pathway (Giuliodori et al. 2018; Martin et al. 2009). These results await confirmation in genetic loss-of-function models.

The c.2057delTG mutation in the *JUP* gene is associated with an autosomal recessive form of ARVC/AC(M), with associated changes in the hair and skin (Naxos syndrome) (Asimaki et al. 2014). The effects of this mutation have been investigated in a zebrafish model with cardiomyocyte-specific overexpression of mutant human *JUP* generated with the Tol2 method (Asimaki et al. 2014). Zebrafish expressing this mutation displayed enlarged hearts with signs of heart failure by 4–6 weeks of age (Asimaki et al. 2014). Electrophysiological abnormalities were also detected with an elevated resting membrane potential, a decreased maximum rate of depolarisation (dV/dT max) and a reduction in sodium current density (Asimaki et al. 2014). This zebrafish model was also used in a chemical screen to identify a small molecule (SB216763 (SB2)), annotated as a GSK3β inhibitor, that rescues the disease phenotype (Asimaki et al. 2014). This pharmacological effect was later confirmed in a mouse model of ARVC/AC(M) (Chelko et al. 2016).

Finally, two variants (p.His33N and p.His77Tyr) in integrinlinked kinase (*ILK*), a non-desmosomal gene involved in cell–matrix interactions, were modelled in zebrafish by cardiomyocyte-specific overexpression of variant human *ILK* via the Tol2 method (Brodehl et al. 2019). Both variants were identified in families with ARVC/AC(M) (Brodehl et al. 2019). The p.His77Tyr embryos showed decreased survival after 5 days post fertilisation, with only 20% reaching adulthood (Brodehl et al. 2019). Although fractional shortening appeared to be mildly decreased in the p. His77Tyr line in the embryonic stage, no morphological abnormalities were detected on histology for both lines (at 3 and 12 days post fertilisation) (Brodehl et al. 2019). Patch-clamp studies on ex-vivo embryonic hearts displayed no abnormalities.

3.4.5 Other Conditions and Relevant Genes

CACNA1C

The *CACNA1C* gene encodes the alpha subunit of the cardiac L-type calcium channel, which is involved in several inherited cardiac arrhythmia conditions (LQTS, BrS and SQTS) (Bezzina et al. 2015). Similar to humans, the zebrafish AP plateau phase is maintained by calcium current. In zebrafish, this current is the product of both the L-type (I_{CaL}) and the T-type (I_{CaT}) calcium channels, while in healthy human adults, the T-type calcium current is only found in conductive tissue and the sino-atrial node (Haverinen et al. 2018; Zhang et al. 2011). The sarcolemmal calcium channels also play a role in the excitation-contraction coupling, as well as the action potential upstroke in zebrafish embryos (Alday et al. 2014; Bovo et al. 2013).

Forward mutagenesis screens have yielded zebrafish embryos with a silent ventricle and abnormal and uncoordinated contractions in the atrium, called the *isl* mutants (Table 3). This phenotype was mapped to homozygous nonsense mutations in the *cacna1c* gene (p.(Gln1077*) and p.(Leu1352*)) (Rottbauer et al. 2001). Similar observations were made in *cacna1c* knockdown morphants (Ramachandran et al. 2013). Voltage-clamp studies in zebrafish cardiomyocytes demonstrated an absence of the L-type calcium current in *isl* mutants (Rottbauer et al. 2001). Ventricles of *isl* mutants showed hypoplasia and were electrically unresponsive to external pacing (Rottbauer et al. 2001). Knockdown of *cacnb2.1*, the zebrafish orthologue of the main beta subunit of the cardiac L-type calcium channel in humans, resulted in cardiac developmental abnormalities (i.e. reduced cell proliferation and abnormal ventricular cell size and shape), bradycardia and heart failure (Chernyavskaya et al. 2012). These experiments clearly demonstrate that the L-type calcium channel is essential for normal cardiac development and AP generation in zebrafish.

SCN5A

The human *SCN5A* gene encodes the main cardiac sodium channel (Nav1.5). Mutations in *SCN5A* are encountered in BrS, cardiac conduction disorders, LQTS type 3 and dilated cardiomyopathy (Bezzina et al. 2015). In zebrafish, the cardiac sodium channel is encoded by two orthologues (*scn5Laa* and *scn5Lab*). The cardiac sodium current density is lower in zebrafish compared to humans, which likely contributes to the lower AP upstroke velocity observed in zebrafish (approximately 90 V/s in the adult zebrafish ventricle compared to 180 V/s in the human ventricle) (Brette et al. 2008; Nemtsas et al. 2010). The zebrafish *scn5Laa* and *scn5Lab* genes appear to play a role in embryonic cardiac differentiation by regulating early cardiac gene expression. Morphological abnormalities with a small heart size due to a reduced number of cardiomyocytes were observed upon morpholino knockdown

of either *scn5Laa* or *scn5Lab*, although these findings need to be confirmed in a stable KO model (Table 3) (Chopra et al. 2010).

The p.(Asp1275Asn) mutation in the *SCN5A* gene, leading to a loss-of-function of the Nav1.5 channel, was reported in multiple families with atrial dysrhythmia, conduction disturbances and dilated cardiomyopathy (Huttner et al. 2013). In order to study the effects of this mutation in zebrafish, stable transgenic lines with expression of either wildtype or mutant human *SCN5A* were generated by the Tol2 method (Table 3) (Huttner et al. 2013). Zebrafish expressing the p.(Asp1275Asn) *SCN5A* construct displayed a higher incidence of mortality, mild bradycardia and episodes of sinus pause, as well as an increased duration of the PR and QRS intervals, compared to non-transgenic controls and transgenic fish injected with the wildtype *SCN5A* construct (Huttner et al. 2013).

Morpholino knockdown of the endogenous *scn5Laa* and *scn5Lab* genes in these transgenic humanised fish led to severe morphological cardiac defects in approximately 79.8% of wildtype *SCN5A* and 86.9% of p.(Asp1275Asn) *SCN5A* embryos (Huttner et al. 2013). This only partial rescue seems to suggest that the endogenous zebrafish genes, rather than human *SCN5A*, remain the main contributors to the sodium current in humanised zebrafish. However, this effect has not yet been replicated in a stable genetic KO model and the lack of rescue could therefore still be attributed to morpholino off-target effects. Moreover, apart from *SCN5A* variant modelling, zebrafish have also been used to identify regulators of the *SCN5A* and *SCN10A* genes in an enhancer assay (Table 3) (van den Boogaard et al. 2012).

GSTM3

A recent study aimed at the discovery of novel disease-causing genes by copy number variant analysis identified deletions of the *Glutathione S-transferase (GSTM3)* gene as a possible mechanism for BrS in a Taiwanese patient cohort (Juang et al. 2020). This gene is involved in cellular defence against oxidative stress. Adult male *gstm.3* KO zebrafish generated by CRISPR-Cas9 showed no ECG abnormalities at rest (Juang et al. 2020). When exposed to a sodium channel blocking agent (flecainide), both the heterozygous and homozygous *gstm.3* KO zebrafish displayed a pronounced prolongation in the PR and QRS intervals compared to wildtype zebrafish, as well as an increased incidence of ventricular arrhythmia.

The ventricular arrhythmia appeared to subside upon administration of the class I antiarrhythmic agent quinidine (Juang et al. 2020). Although quinidine acts on multiple signalling pathways in the heart, its therapeutic effect in BrS is mainly attributed to inhibition of the transient outward potassium current (I_{to}) (Zhou et al. 2010). However, this current is absent in zebrafish cardiomyocytes (Nemtsas et al. 2010). Thus the electrophysiological basis for the beneficial effect observed in *gstm.3* KO zebrafish is not clear. Moreover, both heterozygous and homozygous *gstm.3* KO zebrafish were more likely to develop ventricular arrhythmia when

induced by programmed extra-systolic stimulation compared to wildtype zebrafish (Juang et al. 2020).

Nuclear Import Protein MOG1

The *MOG1* gene functions as a regulator of Ran, a small GTPase with an important role in transport through the nuclear pore complex (Zhou et al. 2016). Apart from its main function, *MOG1* has also been found to influence membrane trafficking of the Nav1.5 channel and at least one dominant negative mutation in *MOG1* has been associated with BrS (Zhou et al. 2016). In zebrafish embryos, *mog1* appears to have a role in the regulation of the heart rate and cardiac morphogenesis, as observed in morpholino-induced knockdown and mRNA overexpression experiments (Table 3) (Zhou et al. 2016). Overexpression experiments with human *MOG1* mRNA containing a variant associated with BrS led to a decreased heart rate. Unfortunately, more detailed reports on cardiac functioning in zebrafish *MOG1* models are not available, as the electrophysiological phenotyping in this study was limited to observations of the heart rate.

K_{ATP}-Associated Genes

In humans, K_{ATP} is composed of the pore-forming Kir6.x protein (encoded by *KCNJ8* and *KCNJ11* genes) and the sulfonylurea subunit (encoded by the *ABCC9* and *ABCC8* genes). Recently, two families with a loss-of-function splice site mutation in the *ABCC9* gene were identified. Homozygous carriers of this mutation displayed a phenotype with a spectrum of morphological, central nervous system and musculoskeletal abnormalities, as well as cardiac systolic dysfunction (*ABCC9*-related Intellectual disability Myopathy Syndrome, AIMS) (Smeland et al. 2019). *Abcc9* zebrafish KO models generated by CRISPR-Cas9 mimicked the human phenotype with morphological abnormalities, decreased mobility and decreased systolic function (Table 3) (Smeland et al. 2019). Gain-of-function mutations in K_{ATP} genes have previously been associated with Cantú syndrome, which is characterised by hypertrichosis, distinctive facial features and cardiovascular abnormalities (Tessadori et al. 2018). Similar to the human phenotype, zebrafish knock-in models of Cantú syndrome mutations showed enlarged hearts and an enhanced cardiac output (Table 3) (Tessadori et al. 2018).

Sodium-Calcium Exchanger (NCX) and Mitochondrial Calcium Uniporter (MCU)

The cardiac sodium-calcium exchanger, responsible for the extrusion of calcium from the cardiomyocyte after contraction, is encoded by several isoforms, of which *NCX1* shows the highest expression in the heart. The *ncx1h* gene has been identified

as the zebrafish *NCX1* orthologue. Both morpholino *ncx1h* knockdown models and forward screen derived loss-of-function *ncx1h* mutants (*tremblor* or *tre*) show ventricular and (even more pronounced) atrial fibrillation (Table 3) (Langenbacher et al. 2005). The phenotype could be rescued by injections with wildtype *ncx1h* mRNA.

In a follow-up study, the synthetic compound efsevin was found to lead to a rescue of the *tre* phenotype through modulation of the mitochondrial calcium uptake (Shimizu et al. 2015). These findings encouraged further research into the zebrafish cardiac calcium homeostasis, which led to the development of a zebrafish line with a TALE-based knockout of the mitochondrial calcium uniporter (*mcu*) (Langenbacher et al. 2020). Adult mutant zebrafish displayed an impaired cardiac function with ECG abnormalities (abnormal QRS morphology, diminished R amplitude and episodes of sinus arrest) (Langenbacher et al. 2020).

Transmembrane Protein 161b (tmem161b)

The *grime* (*uq4ks*) mutant was discovered in a forward mutagenesis experiment designed for the identification of genes implicated in the regulation of cardiac rhythm (Koopman et al. 2021). This mutant line displayed skipped ventricular beats, irregular beats, a slower heart rate and lethality by 15 days post fertilisation (Koopman et al. 2021). This phenotype was mapped to a homozygous missense mutation leading to a premature stopcodon in the *tmem161b* gene (p.Cys466*) (Koopman et al. 2021). The mutation was further confirmed by replicating the phenotype in zebrafish embryos with compound heterozygosity for the p.Cys466* variant and a CRISPR generated knockout allele (Koopman et al. 2021).

Fluorescent imaging and patch-clamp experiments enabled a more thorough examination of the pathophysiology of the cardiac arrhythmia observed in *grime* mutants, as well as the mechanism of cardiac rhythm regulation by *tmem161b*. GEVI (tg(myl7:chimeric VSFP-butterfly CY zebrafish line) and GECI (tg(myl7:gal4FF; UAS:GCaMP6f zebrafish line) experiments in homozygous embryos revealed sino-atrial and atrio-ventricular abnormalities with a faster and increased calcium release (Koopman et al. 2021). Current-clamp experiments on cardiomyocytes derived from adult heterozygotes (homozygotes were not viable up to adulthood) showed an abnormal AP duration (shorter APD20 but longer APD50 and APD90) and early afterdepolarisations (Koopman et al. 2021). This effect was unravelled by voltage-clamp experiments which revealed increased I_{Ks} and I_{CaL} currents (Koopman et al. 2021).

4 Conclusion

Zebrafish cardiac disease modelling presents several challenges, both in the fields of genetic modelling (non-orthology, duplicated genes) and cardiac anatomy and electrophysiology (single atrium and ventricle, altered I_{Ks} kinetics, absence of I_{to}). Nonetheless, zebrafish models have clearly proven successful in reproducing several human inherited cardiac arrhythmia phenotypes. There is a distinct phenotype in zebrafish models for LQTS, SQTS, AIMS and Cantú syndrome. The zebrafish models for CPVT and ARVC/AC(M) are also promising. However, caution is warranted in the interpretation of findings derived from transient morpholino models, as long as these results have not been confirmed with stable genetic mutants.

The lack of a clear phenotype in some inherited arrhythmia models can be attributed to the use of less in-depth phenotyping tools, e.g. light microscopy video recordings as an assessment of electrophysiological characteristics. However, more recent phenotyping tools, such as ECG and optical mapping with genetically encoded indicators, are very promising as they can be applied relatively easily in vivo and provide biologically relevant information on the electrical properties of the entire heart. We anticipate that future models will closely resemble human disease.

The use of CRISPR-Cas9 to model precise genetic modifications identified in patients will pave the way for research into specific variant based pathological mechanisms and personalised treatments. The use of these techniques will enable the field with detailed characterisation of the electrophysiological alterations in CPVT and ARVC/AC(M) disease models, and most likely demonstrate the zebrafish phenotype of yet unexplored inherited cardiac arrhythmias.

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The Biochemistry and Physiology of A Disintegrin and Metalloproteinases (ADAMs and ADAM-TSs) in Human Pathologies

Deepti Sharma and Nikhlesh K. Singh

Contents

Abstract Metalloproteinases are a group of proteinases that plays a substantial role in extracellular matrix remodeling and its molecular signaling. Among these

D. Sharma and N. K. Singh (✉)
Department of Ophthalmology, Visual and Anatomical Sciences, Integrative Biosciences Center (IBio), Wayne State University School of Medicine, Detroit, MI, USA
e-mail: nsingh2@wayne.edu

metalloproteinases, ADAMs (a disintegrin and metalloproteinases) and ADAM-TSs (ADAMs with thrombospondin domains) have emerged as highly efficient contributors mediating proteolytic processing of various signaling molecules. ADAMs are transmembrane metalloenzymes that facilitate the extracellular domain shedding of membrane-anchored proteins, cytokines, growth factors, ligands, and their receptors and therefore modulate their biological functions. ADAM-TSs are secretory, and soluble extracellular proteinases that mediate the cleavage of non-fibrillar extracellular matrix proteins. ADAMs and ADAM-TSs possess pro-domain, metalloproteinase, disintegrin, and cysteine-rich domains in common, but ADAM-TSs have characteristic thrombospondin motifs instead of the transmembrane domain. Most ADAMs and ADAM-TSs are activated by cleavage of pro-domain via pro-protein convertases at their N-terminus, hence directing them to various signaling pathways. In this article, we are discussing not only the structure and regulation of ADAMs and ADAM-TSs, but also the importance of these metalloproteinases in various human pathophysiological conditions like cardiovascular diseases, colorectal cancer, autoinflammatory diseases (sepsis/rheumatoid arthritis), Alzheimer's disease, proliferative retinopathies, and infectious diseases. Therefore, based on the emerging role of ADAMs and ADAM-TSs in various human pathologies, as summarized in this review, these metalloproteases can be considered as critical therapeutic targets and diagnostic biomarkers.

Keywords A disintegrin and metalloproteinases · ADAM-TSs · Cardiovascular diseases · Colorectal cancer · Proliferative retinopathies

Abbreviations

ACE-2	Angiotensin-converting enzyme 2
AD	Alzheimer's disease
APC	Adenomatous polyposis coli
APP	Amyloid-beta precursor protein
COVID-19	Coronavirus disease 2019
CYS1	Cystin1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor 2
EGR1	Early growth response 1
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
GPCR	G protein-coupled receptor
GRP	Gastrin releasing peptide
HSCs	Hepatic stellate cells
HVR	Hyper variable region
IL-6	Interleukin 6
iRhom	Inactive rhomboid protein

LPS	Lipopolysaccharides
MI	Myocardial ischemia
miR-342	Micro RNA 342
MMPs	Matrix metalloproteinases
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PKC	Protein Kinase C
SARS-CoV2	Severe acute respiratory syndrome coronavirus 2
SNP	Single-nucleotide polymorphism
SVMPs	Snake venom metalloproteases
TGF-α	Transforming growth factor-α
TIMPs	Tissue inhibitors of metalloproteinases
TNFR2	Tumor necrosis factor receptor 2
TNF-α	Tumor necrosis factor α
TrkA	Tropomyosin receptor kinase A
TSP1	Thrombospondin 1
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2

1 Introduction

The extracellular matrix (ECM) is an essential scaffold for the cellular components that initiate biomechanical signals necessary for cellular differentiation, tissue homeostasis, and morphogenesis (Frantz et al. 2010). ECM is important for cell-to-cell and cell-to-matrix interactions for the normal growth and functioning of the organism. Changes in physiological or pathological conditions induce ECM remodeling by enzymes called proteases. ECM remodeling is a sequence of alterations that occur in ECM components which is important for regulation of ECM structure and composition, as well as for expression and secretion of bioactive molecules to affect DNA synthesis, cell differentiation, inflammation, angiogenesis, fertilization, blood coagulation, wound repair, neurogenesis, apoptosis, senescence, and necrosis (Cui et al. 2017). Among various proteases, matrix metalloproteinases (MMPs) or matrixins are the most studied proteases and their proteolytic actions on ECM is important for organogenesis and branching angiogenesis (Cui et al. 2017; Wang and Khalil 2018; Egeblad and Werb 2002). MMPs or matrixins are zinc-dependent metallopeptidases that belong to the metzincin superfamily, other family members include adamalysins, astacins, serralysins, and pappalysins (Djuric and Zivkovic 2017).

A disintegrin and metalloproteinase (ADAMs) and a disintegrin and metalloproteinase with thrombospondin-like motif (ADAM-TSs) belong to the adamalysin family, which are often termed as MDC proteins (metalloproteinase-like, disintegrin-like, cysteine-rich proteins) (Edwards et al. 2008). ADAMs and ADAM-TSs are similar to the MMPs in their metalloprotease domains, except they

have a unique disintegrin domain. The molecular structure of ADAMs and ADAM-TSs is similar, both have a pro-domain, a metalloprotease domain, disintegrin domain, and a cysteine-rich domain. ADAMs have both membrane-associated and secreted forms (due to alternative splicing or cleavage), whereas ADAM-TSs have only secreted forms (as they lack the transmembrane domain and cytoplasmic tail) (Takeda et al. 2012). ADAMs display their proteolytic activity via their metalloproteinase domain that regulates the cleavage of membrane-anchored receptors, growth factors, and cytokines. ADAMs disintegrin domain regulates their adhesive activity, particularly of its binding to integrins (Edwards et al. 2008). The metalloprotease domain of ADAM-TSs processes procollagens I, II, and II and is important for depositing collagen on to the ECM in a tissue-specific manner (Bonnans et al. 2014). ADAM-TSs also cleave various proteoglycans such as aggrecan, versican, brevocan, and neurocan. ADAM-TSs, particularly ADAMTS13 cleaves von Willebrand factor (vWF) and regulates coagulation and thrombotic thrombocytopenic purpura (TPP) (Bonnans et al. 2014).

ADAMs group comprises around 38 members, found in various species (Edwards et al. 2008). The evolution and diversification studies have also found the homologs of ADAMs and ADAM-TSs in other organisms, including fruit fly (*Drosophila melanogaster*), nematodes (*Caenorhabditis elegans*) (Edwards et al. 2008; Souza et al. 2020; Nicholson et al. 2005). Until now 21 ADAMs (13 are proteolytically active) and 19 ADAM-TSs have been classified in the human genome (Zhong and Khalil 2019). In addition to humans, 37 and 34 ADAMs are present in rats and mice, respectively (Takeda et al. 2012; Zhong and Khalil 2019). Most of the ADAMs (1–7, 18, 20, 20–22, 24–30, 32, and 33) express in reproductive tissues, except ADAM-8-12, 15, 17, and 19, which are present in other human tissues. ADAM-TSs 1, 2, 4, 5, 9, and 16 have a wider human tissue distribution and extensive connection with various biological processes. ADAM-TSs 3, 8, 10, and 13 are present in heart, placenta, and brain. ADAM-TSs 7, 10, 13, and 14 are expressed in liver, whereas ADAM-TSs 16, 17, and 20 are mostly expressed in ovary (Porter et al. 2005).

To date, many studies have uncovered the crucial role of ADAMs and ADAM-TSs in tumor formation and embryonic development and pathologies of the eye, lung, heart, liver, kidneys, muscles, and joints. Besides it, ADAMs and ADAM-TSs are considered as potential biomarkers for various pathophysiological conditions such as cancer, inflammation, autoimmune diseases, and cardiovascular diseases (Seals and Courtneidge 2003). Presently, considerable interest is growing to understand the pivotal role of ADAMs and ADAM-TSs family members in human pathology and physiology. In the present manuscript, we are summarizing the biochemistry, regulation, and prospective role of ADAM and ADAM-TSs in various human pathological conditions (Fig. 1), such as Alzheimer's disease, proliferative retinopathies, rheumatoid arthritis, cardiovascular and colon cancer. Despite the implications of many ADAMs and ADAM-TSs across cancers, this review focuses only on the role of ADAMs and ADAM-TSs in colon cancer. Lastly, we will discuss how we can employ ADAMs and ADAM-TSs as potential biomarkers for the detection and management of various human pathologies.

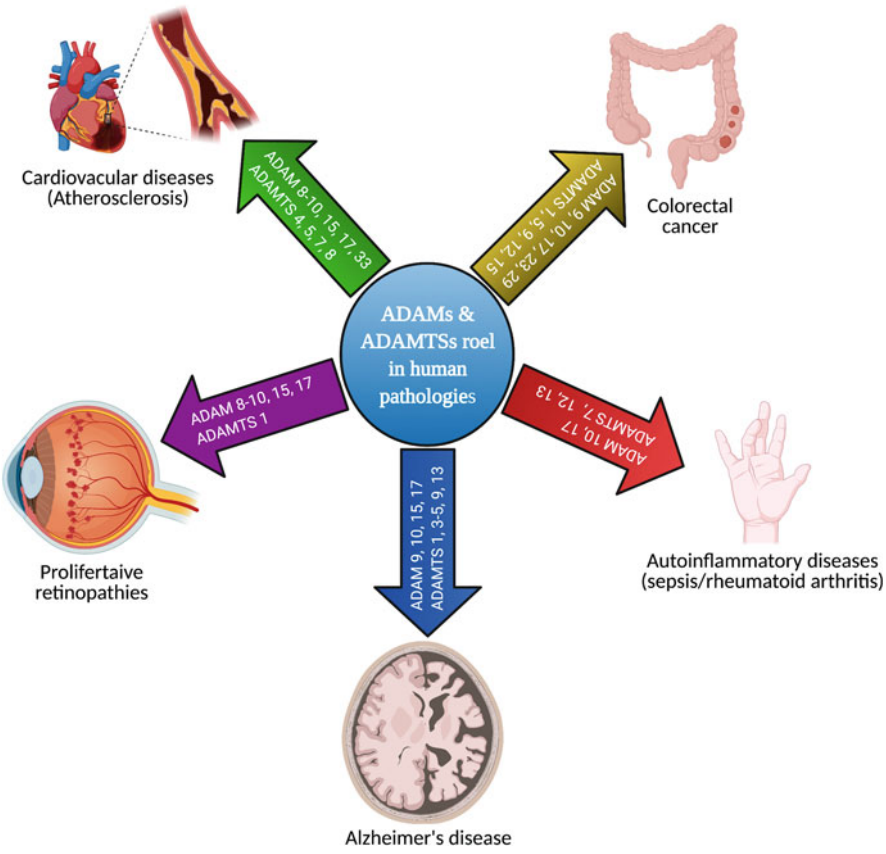

Fig. 1 Role of various ADAMs and ADAM-TSs in various human pathologies

2 Structure of ADAMs and ADAM-TSs Proteins

The adamalysins subfamily members ADAMs and ADAM-TSs have many similarities with snake venom metalloproteases (SVMPs) in protein sequences, spatial structures, and domain organization (Takeda 2016). The basic structure of all adamalysin family members (ADAMs, ADAM-TSs) is similar, as they have pro-domain, metalloproteinase, disintegrin, and cysteine-rich domains (Zhong and Khalil 2019). Despite many structural similarities ADAMs and ADAM-TSs individually possess a lot of structural and domain-specific variability exhibiting their function and tissue-specificity (Fig. 2). Furthermore, the ADAMs and ADAM-TSs are characterized based on their sequence differences within the domains (Table. 1).

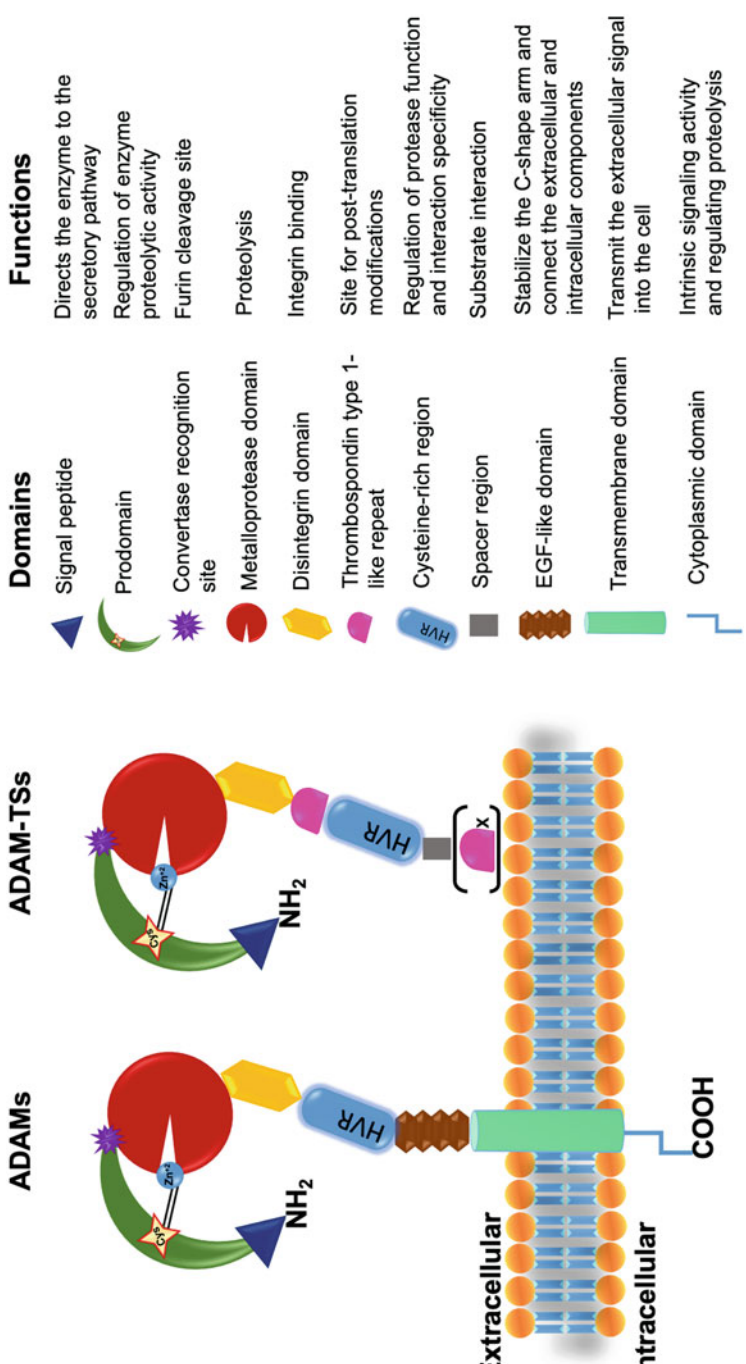

Fig. 2 Basic structure of the prototypical ADAM and ADAM-TS prior to proteolytic removal of the pro-domain at the N-terminus

Table 1 Types of ADAMs and ADAM-TSs based on sequence differences within the domains

Metalloproteases	Members	Type	Structural domains (sequence difference)
ADAMs	1, 8, 9, 12, 13, 16, 19, 20, 21, 24, 26, 28, 30, 33, 40	I	Catalytically active Zn^{2+} binding signature sequence (HExGHxxGxxHD) in M-domain and xCD sequence in D-domain
	2, 7, 11, 18, 22, 23, 27, 29, 32	II	Highly variable catalytical site in M-domain
	15	III	Catalytically active Zn^{2+} binding signature sequence, RGD sequence in D-domain instead of xCD sequence in most ADAMs members
	10, 17	IV	Catalytically active Zn^{2+} binding signature sequence, xCD sequence in D-domain, unlike type I, II, and III EGF-like region is absent
ADAM-TSs	1, 4, 5, 8, 15	I	Comprise of all basic domains
	9, 20	II	TSR modules connected to unique GON-1 domain
	2, 3, 14	III	TSR modules connected to procollagen *N*-Propeptidase
	13	IV	TSR modules connected to complement C1r/C1s, Uegf, Bmp1 (CUB) domain
	7, 12	V	TSR modules connected to mucin/proteoglycan domain and protease and lacunin (PLAC) structure
	6, 10, 16, 17, 18, 19	VI	TSR modules connected to only PLAC domain

2.1 The Pro-Domain

ADAMs and ADAM-TSs metalloproteases consist of an N-terminus peptide sequence which signals the metalloprotease to enter into a secretory pathway to execute its function. The pro-domain follows this signal sequence and is crucial for enzyme maturation. The pro-domain consists of a conserved cysteine residue that interacts with zinc ion (Zn^{2+}) present at the catalytic site, thereby blocking the active site for substrate-binding and cleavage. The pro-domain also remains non-covalently associated with some mature ADAMs and keeps them in an inactive state via the "cysteine-switch" mechanism. In addition to maintaining metalloprotease (ADAMs and ADAM-TSs) enzyme latency, the pro-domain also exhibits multiple subordinate functions such as ensuring stability and proper folding of ADAMs and ADAM-TSs and their entry into the secretory pathway (Edwards et al. 2008; Takeda et al. 2012; Zhang et al. 2016).

2.2 The Metalloprotease Domain

ADAMs and ADAM-TSs enzymes possess their proteolytic activity in the metalloprotease domain adjacent to the pro-domain. The metalloprotease domain retains the highest sequence homology between the metalloproteinases (Djuric and Zivkovic 2017). This domain includes the zinc and water atoms within the catalytic active site required for the hydrolytic processing of protein substrates. This catalytic domain consists of a Zn^{2+} binding motif (HExGHxxGxxHD; where "H" is for histidine, "E" is for glutamic acid, "x" is for variable amino acid, "G" is for glycine, and "D" is for aspartic acid), which comprises three histidine residues and a downstream conserved methionine, which forms a methionine loop or "Met-turn" (Edwards et al. 2008; Blobel 2005). This conservative motif is responsible for the proteolytic cleavage of several receptors, ligands, and ion channels present on the same cell or neighboring cells' membranes. Due to the catalytic activity of the metalloprotease domain, ADAMs and ADAM-TSs can act as sheddases and upstream regulators of various cell signaling pathways. ADAMs and ADAM-TSs not only regulate cell proliferation or apoptosis but also have a role in cell differentiation and tissue remodeling (Arribas et al. 2006).

2.3 The Disintegrin Domain

The disintegrin domain is a ~90 amino-acid long protein fragment of ADAMs and ADAM-TSs. The disintegrin protein is first isolated from snake venom and found to be present in the snake venom metalloproteases (SVMPs). In general, disintegrin protein binds with integrin receptors, thereby preventing its interaction with matrix protein (fibrinogen) and subsequently inhibiting platelet aggregation (Cominetti et al. 2009). The disintegrin domains of SVMPs and ADAMs/ADAM-TSs possess structural similarities (Macêdo et al. 2010). The disintegrin domain not only contributes to the protease activity but also helps in substrate recognition by ADAM and ADAM-TSs. The disintegrin domain in ADAM15 has a characteristic consensus Arg-Gly-Asp (RGD) sequence, which facilitates the binding of ADAMs to integrin receptors (αIIbβ3 and αvβ3) (Lu et al. 2006). Unlike ADAM15, most ADAMs lack the highly conserved RGD recognition sequence and instead have an ECD or xCD sequence (Blobel 1997). Although ADAM-TSs have disintegrin-like domains, there are no reports to show that they interact with integrin receptors (Jones and Riley 2005).

2.4 The Cysteine-Rich and EGF-Like Domain

ADAMs possess the cysteine-rich domain just next to the disintegrin domain, whereas ADAM-TSs comprise a series of thrombospondin type 1 repeat (TSP1) motifs between the disintegrin domain and cysteine-rich domain. In ADAM-TSs, the TSP1 motifs have high sequence homology to that of the region I of thrombospondin 1 and 2 (Lu et al. 2006). This TSP1 motif mediates the binding of ADAM-TSs to the extracellular matrix and contributes to apoptosis and angiogenesis (Kuno and Matsushima 1998; Guo et al. 1997). The function of the cysteine-rich domain present in ADAM and ADAM-TSs structure is not fully known. The ADAM12 cysteine-rich and disintegrin domain promotes the adhesion of myoblasts and fibroblasts (Zolkiewska 1999). Besides it, the cysteine-rich domain of ADAM12 interacts with cell surface syndecan to mediate the cell adhesion process (Iba et al. 2000). Furthermore, the interaction of ADAM13 with extracellular matrix protein fibronectin exhibits the adhesive property of both disintegrin and cysteine-rich domains (Gaultier et al. 2002). The cysteine-rich domain of ADAMs also contains a variable sized loop of 27–55 amino acids with hyper-variable region (HVR). Therefore, the structural information and the involvement of cysteine-rich and disintegrin domains in a cell to matrix or cell to cell interactions suggests them to represent as one functional entity: the "adhesive" domain. In addition, the X-ray crystal structural analysis of ADAM10 has shown that the cysteine-rich domain of ADAM10 blocks the enzyme active site after the pro-domain release, thereby suggesting an additional mode of enzyme regulation (Seegar et al. 2017).

The cysteine-rich domain is succeeded by an epidermal growth factor (EGF)-like region in ADAMs, whereas in ADAM-TSs, it is followed by a spacer region. The EGF-like region connects the extracellular region of ADAMs (that form a characteristic "C-shaped" arm structure) to the transmembrane domain (Takeda et al. 2006). The C-shape arm includes the M (metalloprotease), D (disintegrin), C (cysteine-rich) domain, and a highly variable region of cysteine-rich domain. The spacer region in ADAM-TSs family members is responsible for its interaction with the substrate (de Groot et al. 2009). Therefore, the C-shape architecture of ADAMs and ADAM-TSs structure is essential for their vital functions such as target identification, protein interaction, and proteolytic activity.

2.5 The Transmembrane Domain, Cytoplasmic Tail and Ancillary Domain

Most of the ADAMs are membrane-anchored proteins and thus possess a transmembrane domain located next to the EGF-like region. Due to the absence of the transmembrane domain, ADAM-TSs are mostly secretory proteins. ADAMs C-terminal region consists of an intracellular cytoplasmic tail, whereas ADAM-TSs have an extracellular spacer region followed by 0 to 14 TSP1 motifs. The

cytoplasmic tail is the most variable protein part among the ADAMs family members, both in size and in amino-acid sequence. Proline-rich (PXXP) motifs present within the cytoplasmic tail of ADAMs (ADAMs 7, 8, 9, 10, 12, 15, 17, 19, 22, 29, and 33) facilitate its binding to the proteins containing SH3 (Src homology region-3)-domain (Kang et al. 2000). Besides this, the cytoplasmic tail also contains several (tyrosine, serine, and threonine) residues that are possible sites for phosphorylation (Poghosyan et al. 2002). The transmembrane domain and the cytoplasmic tail of ADAMs interact with proteins that are involved in cell trafficking and intracellular signaling (Stone et al. 1999).

The ADAM-TSs members possess a unique ancillary domain that consists of Thrombospondin type 1 repeats (TSP1) that plays a vital role in enzymes' interaction with ECM components, substrate selection or recognition, and regulation of its enzyme activity. The ADAM-TSs C-terminus ancillary domain is connected to four different domains described (Table 1; Fig. 3) as GON-1 domain, Complement-Uegf-BMP-1 (CUB) domain, protease and lacunin (PLAC) domain, and Mucin-like domain (Porter et al. 2005; Kim and Nishiwaki 2015; Somerville et al. 2003; Bork and Beckmann 1993; Nardi et al. 1999; Somerville et al. 2004).

3 Activation, Inhibition, and Regulation of ADAMs

3.1 Activation

ADAMs family members are generated as inactive pro-enzymes in the endoplasmic reticulum (ER), where the N-terminal pro-domain inhibits the enzymatic cleavage activity via the "cysteine-switch" mechanism (Van Wart and Birkedal-Hansen 1990). Various stimulants such as G-protein coupled receptor (GPCR) ligands, protein kinase C (PKC) activators, Ca^{2+} ionophores, cytokines, and proteinases activate ADAMs. The intracellular enzymatic cleavage of pro-domain via pro-protein convertases (Fig. 4) during their transit through the trans-Golgi network results in the maturation of enzyme (Lum et al. 1998). However, some ADAMs such as ADAM8 and ADAM28 are processed auto-catalytically to their mature form (Schlomann et al. 2002; Howard et al. 2000). The activation process of MMPs is different from ADAMs, as the pro-domain separation and activation occur outside the cell surface (Takawale et al. 2015). The pro-protein convertases, a specialized serine endoproteinase, are primarily responsible for the activation of ADAMs. These pro-protein convertases (furin) predominantly cleave the di-basic consensus RXXR motif at the boundary of the catalytic domain and pro-domain (Wong et al. 2015). Consequently, the Zn^{2+} coordination gets switch toward the metalloprotease domain resulting in the activation of ADAMs which enable it to perform its physiological function and sheddase activity (Anders et al. 2001). Conversely, for some ADAMs like ADAM12, pro-domain remains non-covalently associated with the mature protease following cleavage, thereby influence the functional activity of ADAM12 (Wewer et al. 2006).

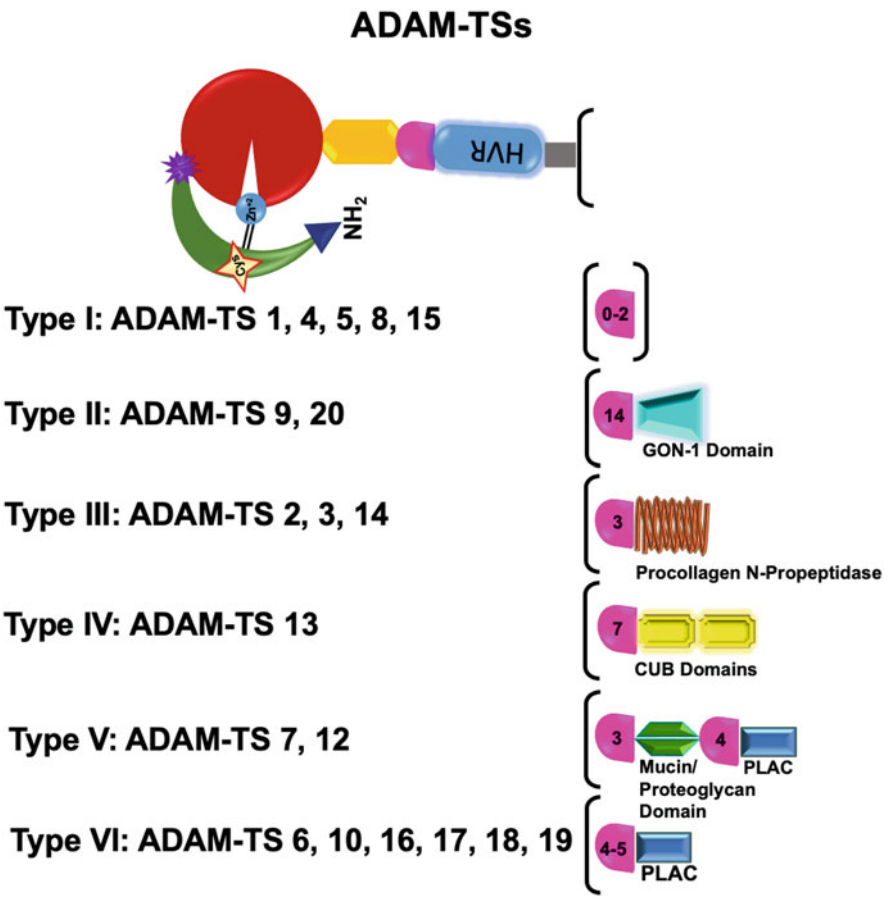

Fig. 3 Basic structure of the prototypical ADAM-TSs prior to proteolytic removal of the pro-domain at the N-terminus

Phosphorylation at the cytoplasmic domain of several membrane-associated proteins is an essential posttranslational modification that is of utmost importance for their activation. Therefore, tyrosine or serine/threonine phosphorylation of various putative residues within the intracellular cytoplasmic tail of ADAMs modulates its activation, adaptor interaction, or trafficking of proteins for substrate identification and interfacing. The cleavage of ADAM12 by furin-peptidase makes it a constitutively active protein that remains intracellular, and the phosphorylation of its cytoplasmic domain helps in its translocation to the cell membrane (Sundberg et al. 2004). Nevertheless, some reports have suggested that ADAM17 and ADAM19 activity depend on their transmembrane domain rather than their cytoplasmic domain (Reddy et al. 2000; Wakatsuki et al. 2004). Some studies have shown that phorbol 12-myristate 13-acetate (PMA) induces ADAM17 phosphorylation at Ser819, but neither its mutation nor deletion of the cytoplasmic domain affected

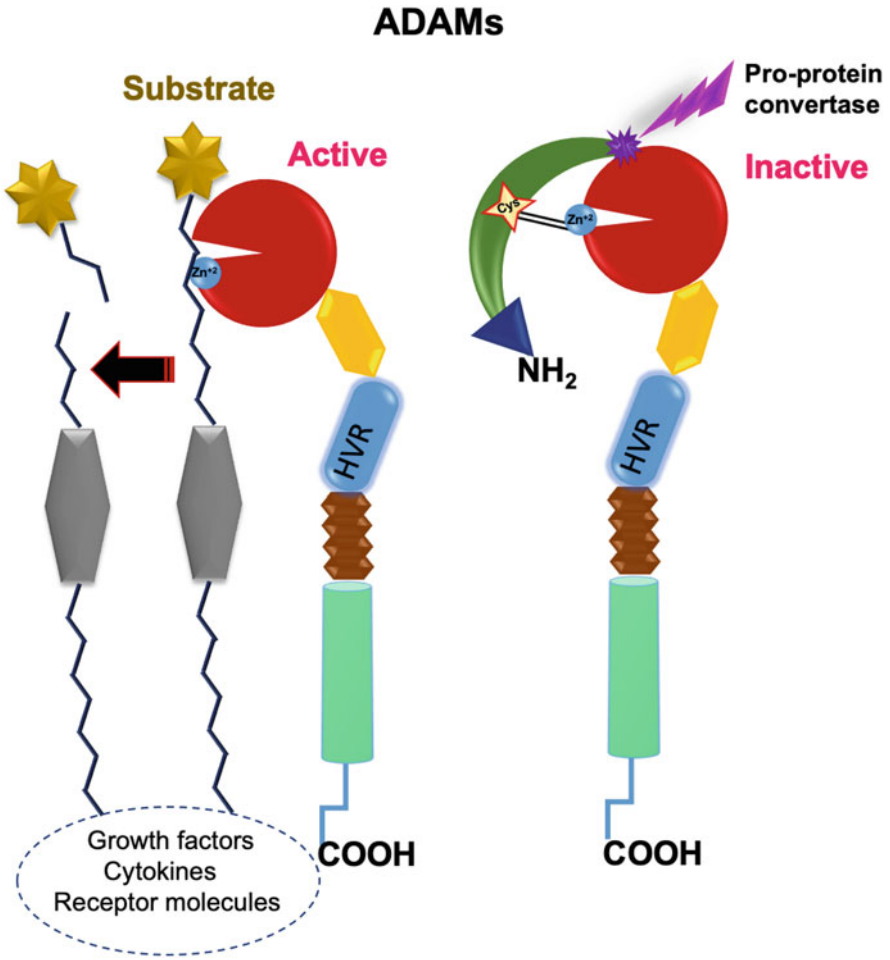

Fig. 4 Activation of ADAMs enzyme through cleavage of the N-terminal pro-domain by pro-protein convertases such as furin. Active ADAMs cleave various membranous substrates

ADAM17 catalytic activity (Fan et al. 2003; Doedens et al. 2003). On the contrary, a critical involvement of the ADAM17 cytoplasmic domain in GPCR-mediated epidermal growth factor receptor activation and signaling is observed in squamous cell carcinoma (Fischer et al. 2003). The gastrin-releasing peptide (GRP)-induced GPCR stimulation results in activation of cSrc-PI3K (phosphatidylinositol 3-kinase)-PDK1 (phosphatidyl inositol-dependent kinase-1) signaling, which leads to phosphorylation and translocation of ADAM17 (Zhang et al. 2006). Furthermore, studies have shown that ADAM17 phosphorylation at threonine 735 (Thr735) residue enhances cleavage of the tropomyosin receptor kinase A (TrkA) neurotrophin receptor in cardiomyocytes and tumor cells (Diaz-Rodriguez et al. 2002; Xu and Derynck 2010; Patel et al. 2014a). Additionally, ADAM17 phosphorylation at Thr735 is

considered necessary for its protein trafficking and maturation (Soond et al. 2005). Recently, a study has reported that src-mediated phosphorylation of ADAM17 at Tyrosine 702 residue is reported as a bona fide phosphorylation site in skeletal myoblasts and mechanically stressed cardiomyocytes, resulting in increased TNF-α-shedding (Niu et al. 2013; Niu et al. 2015).

3.2 Inhibition

ADAMs and ADAM-TSs family members are regulated by physiological and endogenous protein regulators known as TIMPs (tissue inhibitors of metalloproteinases). There are 4 TIMP family members in mammals and one in lower eukaryotes. The inhibitory amino-terminal domain of TIMPs binds non-covalently to the active site of MMPs and forms a tight 1:1 complex (Murphy 2011). TIMPs exhibit a wide range of similarities in their inhibitory function for MMPs, but for ADAMs they are more specific. TIMP1 and TIMP3 inhibit the catalytic activity of ADAM10 in vitro, whereas none of the TIMPs can inhibit ADAM8, 9, and 19 (Amour et al. 2000; Amour et al. 2002; Chesneau et al. 2003). TIMP3 can inhibit the activity of ADAM17, but it requires dimerization of ADAM17 so that it can interact with TIMP3 (Amour et al. 1998). Likewise, TIMP2 and *N*-TIMP3 exhibited potent inhibition for ADAM12-S (secreted splice variant) and transmembrane ADAM12-L (full-length) form (Jacobsen et al. 2008). ADAM33 shows an inhibitory profile distinct from other ADAMs, as its catalytic activity is inhibited weakly by TIMP2 and moderately by TIMP3 and TIMP4 but not by TIMP1 (Zou et al. 2004).

The mechanism of inhibition by TIMPs involves the (1) chelation of Zn^{2+} ion at the enzyme (ADAMs) active site by the α-amino and carbonyl groups present on the amino-terminal Cys1 of the TIMPs; (2) interaction of Ser/Thr (OH group) with the nucleophilic Glu of the metalloproteinase catalytic cleft, causing displacement of a water molecule, necessary for peptide hydrolysis (Murphy 2011). The mode of inhibition of TIMPs described for MMPs was found to vary among ADAMs family of metalloproteases. In contrast to MMPs, where interaction with the N-terminal domain of TIMPs is required for MMPs inhibition, the c-terminal domains of TIMP1 and TIMP3 interact and inhibit ADAM10 (Rapti et al. 2008; Schlondorff and Blobel 1999). The full-length TIMP3 and its N-terminal domain inhibit the isolated catalytic domain of ADAM17 (Lee et al. 2002, 2003; Wei et al. 2005). However, the addition of C-terminal domains and subsequently only cysteine-rich domains of ADAM17 significantly attenuated the inhibitory potency of the TIMP3 (Moss et al. 2007; Gonzales et al. 2004; Muraguchi et al. 2007). The phosphorylation of ADAM17 cytoplasmic domain by p38MAPK or ERK resulted in the reduction of its dimers, and thus inhibition by TIMP3 (Xu et al. 2012). Other than the TIMPs, some ADAMs (ADAM10 and ADAM17) demonstrate specific and selective inhibition via their isolated pro-domain, which is independent of the "cysteine-switch" mechanism (Moss et al. 2007; Gonzales et al. 2004). RECK (a reversion-inducing cysteine-

rich protein with Kazal motifs) is also an inhibitor of the ADAM10 activity during embryonic brain development (Muraguchi et al. 2007). Also, several pharmacological inhibitors have been developed to target the catalytic Zn^{2+} ion, but these are highly non-specific and unselective for both ADAMs and MMPs inhibition. Among them, some Zn^{2+} chelators such as hydroxamate and 1,10-phenanthroline are considered potent inhibitors of ADAMs proteolytic activity (Seals and Courtneidge 2003).

In addition, some small synthetic compounds have been reported that show specific inhibition to ADAMs activity. The hydroxamate-based inhibitors such as INCB3619 and INCB7839 inhibit ADAM10 and ADAM17 activity in cell-based experiments with better selectivity and bioavailability (Duffy et al. 2011; Fridman et al. 2007; Zhou et al. 2006). The knockout cell studies have found that INCB4298, a selective inhibitor of ADAM-17 blocks the shedding of heregulin, transforming growth factor-alpha (TGFα), heparin-binding epidermal growth factor (HB-EGF), and androgen receptor (AR). However, the ADAM10-selective inhibitor INCB8765 blocks EGF ligand processing (Hundhausen et al. 2003; Zocchi et al. 2016). TAPI-1, TAPI-2, and Batimastat (BB-94) and GW280264X have been reported as potential inhibitors of ADAM-17 (TACE) and mediate their inhibitory effect by blocking the shedding of cytokine receptors, but also affect the activity of MMPs and ADAM10 (Wetzel et al. 2017). Another small molecule inhibitor GI254023X is considered a selective inhibitor of ADAM10, but it additionally targets ADAM17, MMP2, and MMP9 (Zhou et al. 2006; Mathews et al. 2011). Zocchi et al. have recently reported that newly synthesized hydroxamate inhibitors, LT4 and MN8, have higher specificity for ADAM10 over ADAM17 and MMPs in a Hodgkin lymphoma (HL) cell line. The in vitro and cell-based assays used to study the inhibitory profile of ADAM8 demonstrated that BB-94, GW280264, FC387, and FC143 exhibit an inhibitory effect on ADAM8 activity, whereas GM6001, TAPI2 and BB2516 (Marimastat) and GI254023 showed significantly low and negligible inhibition (Schlomann et al. 2019). Furthermore, monoclonal antibodies (mAb 8C7) raised against the substrate-binding pocket within the ADAM10 C-domain demonstrated the specific blocking of Ephrin uptake and cleavage in a cell-based model (Atapattu et al. 2012). The monoclonal antibodies (mAb 8C7) based blocking was more efficient than the GM6001 (Atapattu et al. 2012).

In addition to synthetic compounds, some natural compounds such as Rapamycin and Triptolide have also been shown to inhibit ADAM10. Zhang et al. demonstrated that rapamycin treatment significantly decreases the activation of ADAM10, thereby increases the levels of the β-carboxyl-terminal fragment of β-amyloid precursor protein in vitro and in vivo (Zhang et al. 2010a). Furthermore, natural compound Triptolide (diterpenoid epoxide) obtained from Tripterygium wilfordii also significantly decreases the ADAM10 expression upon treatment in U937 and MCF-7 cells (Soundararajan et al. 2009).

3.3 Regulation

The posttranslational modifications such as phosphorylation and glycosylation regulate the function and structure of ADAMs family members. ADAM8 contains three *N*-glycosylation sites, which are necessary for its processing, cell surface localization, stability, and activity (Srinivasan et al. 2014). In breast cancer cells, the splice variants of ADAM9, such as transmembrane (ADAM9-L), and the secreted variant (ADAM9-S) were found to possess glycosylation sites (Fry and Toker 2010). Although one *N*-glycosylation sites in ADAM12 and 5*N*-glycosylation sites in ADAM15 have been reported, neither of the glycosylation sites in ADAM12 and ADAM15 exhibits any functional significance (Kodama et al. 2004; Krätzschmar et al. 1996). Conversely, substrate and inhibitor binding along with the catalytic activity of ADAM17 have been extremely influenced by glycosylation. Human ADAM17 exhibits a high level of glycosylation in the mammalian cell which potentially reduces its sheddase activity towards various TNFα-based substrates along with its significant inhibition by non-zinc binding inhibitor (Chavaroche et al. 2014). In addition, some ADAMs (ADAM8, 9, 10, 12, and 17) substrates also exhibit various degrees of glycosylation which influence their physiological functions (Minond et al. 2012).

Furthermore, the gene expression of ADAMs is regulated epigenetically, primarily in cancer cells. In general, the known developmental effects of epigenetic regulation (DNA methylation) involve long-term silencing of gene expression. Likewise, Z-DNA-mediated epigenetic silencing of ADAM12 has been observed in breast cancer cells (Ray et al. 2013; Nakao et al. 2014). Histone deacetylation has been found essential for the TGFβ1-induced expression of ADAM19 in ovarian cancer (Chan et al. 2008), whereas inhibition of histone deacetylation induces expression of ADAM19 in monocytic THP-1 cells (Ehrnsperger et al. 2005). Also, the methylation status of the promoter region of the ADAM33 gene has been shown to tightly regulate its expression in a cell type-specific manner (Yang et al. 2008).

The interaction of ADAMs with MMPs also regulates their function. Therefore, ADAMs act as substrates for MMPs or sheddases. For instance, it has been reported that ADAM12 influences the redistribution and activity of MT1-MMP (membrane-tethered MMP), thus forming a ternary protein complex with integrin αvβ3 at the cell surface (Albrechtsen et al. 2013). Furthermore, MT1-MMP has been stated as a critical negative modulator of ADAM9 proteolytic activity as it forms a complex with ADAM9 and FGFR2, thus protecting ADAM9-mediated FGFR2 ectodomain shedding. Also, loss of ADAM9 completely restores the defective FGFR2 signaling and largely rescued the impaired calvarial osteogenesis in MT1-MMP-deficient mice embryos (Chan et al. 2012). Likewise, MMP-7 processes proADAM28s (65 kDa) into active ADAM28s of 42- and 40-kDa forms, which selectively digest insulin-like growth factor binding protein-3 (Mochizuki et al. 2004).

It has been revealed that MMP7 controls the transcription of ADAM12 downstream of the angiotensin II-induced signaling pathway, resulting in the development of hypertension and cardiovascular hypertrophy (Wang et al. 2009a). Also,

ADAM17 has a role in cancer (prostate) cell invasion by shedding of TGF-α, which consequently results in activation of the EGFR-MEK-ERK pathway and increased levels of MMP-2 and MMP-9 (Xiao et al. 2012). In addition, the lentiviral RNAi-mediated silencing of the ADAM17 gene inhibits the TNF-α/NF-κB signaling along with the MMP9 expression in LPS treated A549 lung epithelial cells (Li et al. 2013a). During cardiovascular disease development, ADAM17 regulates MMP2, a mediator of angiotensin II-induced hypertension (Odenbach et al. 2011).

The tetraspanins and rhomboids, a superfamily of multi-transmembrane proteins, are associated with ADAM10 and ADAM17, respectively (Matthews et al. 2017). These proteins regulate their maturation, trafficking to the cell membrane, and activity (Matthews et al. 2017). Among all, only six tetraspanins (Tspan5, 10, 14, 15, 17, and 33) have been co-immunoprecipitated with ADAM10 in stringent lysis buffers (Dornier et al. 2012; Haining et al. 2012). The catalytic activity of ADAM10 is regulated by anti-tetraspanin mAbs that further stimulate the shedding of epidermal growth factor (EGF) and tumor necrosis factor-α (TNF-α) (Arduise et al. 2008). In vitro studies involving the knockdown of highly expressed tetraspanins in various primary cells and cell lines showed a reduction in ADAM10 enzyme activity and its trafficking to the plasma membrane (Dornier et al. 2012; Haining et al. 2012; Prox et al. 2012). Furthermore, six different tetraspanins reported till now exhibit different subcellular localizations. In addition, each tetraspanin regulates ADAM10 differently, such as some are involved in stimulation or suppression of ADAM10 sheddase activity and some in trafficking to intracellular compartments, and others to the plasma membrane (Dornier et al. 2012). iRhom1 and iRhom2, two non-protease rhomboids, have been reported as the regulators of ADAM17. iRhom2 is expressed majorly in hematopoietic cells, however, iRhom1 is more widely expressed except hematopoietic cells (Issuree et al. 2013; Christova et al. 2013). Studies using iRhom2 knockout mice have reported a reduced release of the proinflammatory cytokine, TNF-α upon LPS stimulation (Adrain et al. 2012; McIlwain et al. 2012). In addition, these mice failed to control Listeria monocytogenes infection (Adrain et al. 2012; McIlwain et al. 2012). These results indicate that iRhom2 directly regulates the catalytic activity of ADAM17, a major sheddase of TNF-α. Furthermore, iRhom1/2 double knockout mice have shown a considerable reduction in the mature ADAM17 levels and EGFR phosphorylation (Li et al. 2015). Lastly, the regulatory mechanisms of ADAMs, as briefly illustrated in Fig. 5, enhance our understanding of their role and mode of action in health and disease conditions.

4 ADAM-TSs Regulation

The ADAM-TSs are secreted as extracellular metalloproteases that do not contain EGF-like, cytoplasmic, and transmembrane domains. ADAM-TSs has a multi-domain structure that includes N-terminal signal peptide, a pro-domain, metalloprotease domain, disintegrin domain, a thrombospondin type 1 repeat, spacer region, and cysteine-rich domain. Their C-terminal region is composed of ancillary

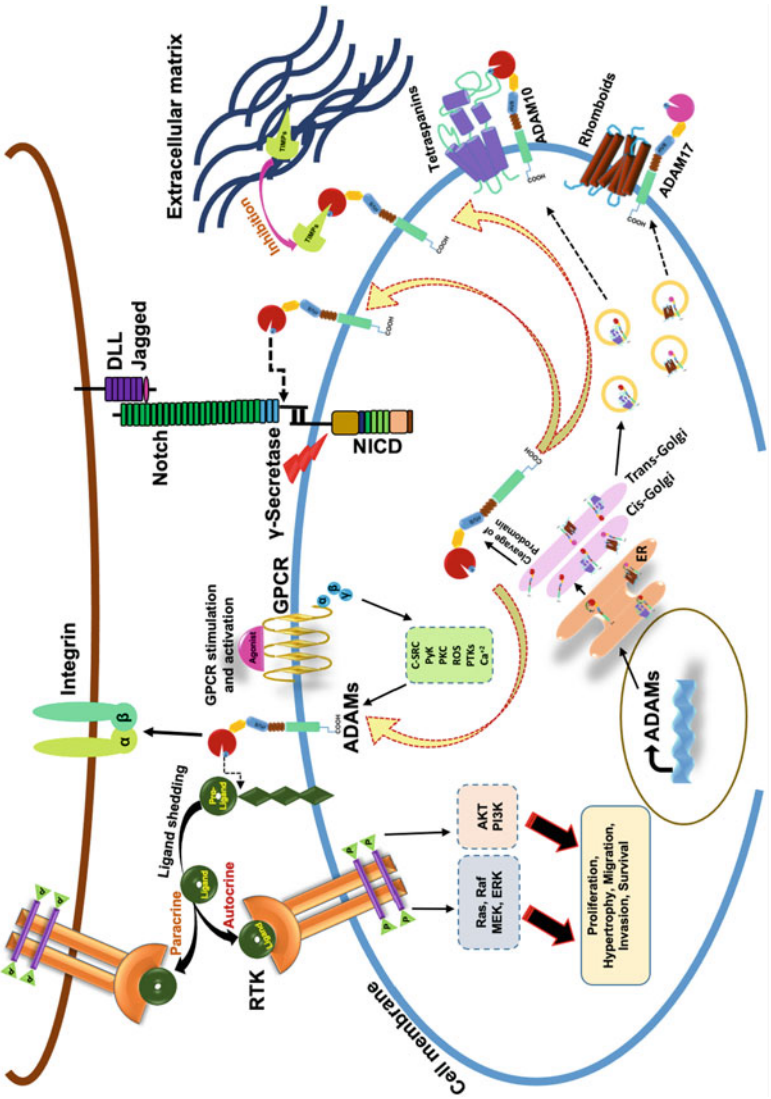

Fig. 5 Basic synthesis, regulation, and function of ADAMs. The pro-domain of ADAMs is cleaved by pro-protein convertase in the Golgi resulting in translocation of active ADAMs to the plasma membrane. The cytoplasmic domain of ADAMs is phosphorylated by different intracellular signaling mediators via stimulation of GPCRs with an agonist. Ligand generated by ADAMs proteolytic cleavage mediates autocrine and paracrine activation of RTK. ADAMs also shows non-proteolytic function via their interfacing with integrin dimers. ADAM10 and ADAM17 have been shown to be regulated by tetraspanins and rhomboids, respectively. ADAMs (e.g., ADAM10) are well-known for their involvement in the canonical Notch signaling via ectodomain shedding of Notch

Fig. 5 (continued) receptor after binding to one of its ligands located on a neighboring cell. The resulting membrane-anchored stub further undergoes cleavage by the γ-secretase complex. ADAMs also get inhibited by endogenous inhibitors (TIMPs)

domain following thrombospondin type 1 repeat domains that determine its substrate specificity, localization, and interaction of the protease. Unlike ADAMs, ADAM-TSs do not demonstrate any binding with integrins, despite having a disintegrin-like domain.

4.1 Activation

Like ADAMs, the ADAM-TSs metalloprotease zymogens get activated after cleavage of N-terminal pro-domain by pro-protein convertase (Fig. 6) at consensus sequence RX(K/R)R in trans-Golgi or at the cell surface (Colige et al. 2005; Somerville et al. 2004; Koo and Apte 2010). Unlike other ADAM-TSs, the ADAM-TS13 pro-domain is unusually short, poorly conserved, and lacks cysteine-switch motif, indicating that the pro-domain is not required for folding or secretion and enzyme latency (Majerus et al. 2003). Furthermore, the processing of proADAM-TS1 and proADAM-TS4 to their mature form occurs intracellularly in the trans-Golgi network, involving two separate proteolytic actions that affect their localization, ECM binding, and activity (Rodriguez-Manzaneque et al. 2000; Wang et al. 2004). The proADAM-TS5 is processed extracellularly, whereas proADAM-TS9 is processed at the cell surface by pro-protein convertase, thus distinct from those of other ADAM-TSs proteases (Longpre et al. 2009; Koo et al. 2007). Studies have shown that pro ADAM-TS2 not only gets activated via conventional N-terminal cleavage by pro-protein convertase but also through the autocatalytic

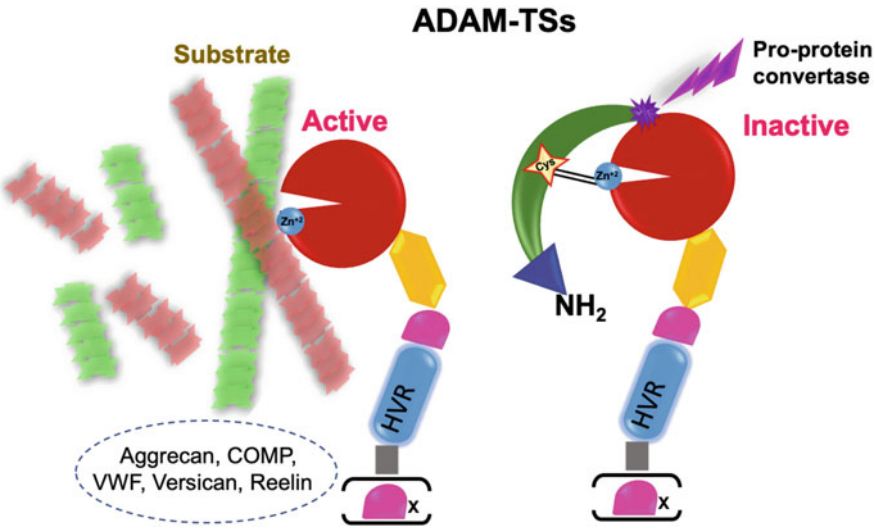

Fig. 6 Activation process ADAM-TSs enzyme through cleavage of the N-terminal pro-domain by pro-protein convertases such as furin. Active ADAM-TSs cleave various extracellular proteins as substrates

cleavage of its C-terminal end (Colige et al. 2005; Bekhouche and Colige 2015). The autocatalytic activation by C-terminal cleavage has also been reported for ADAM-TS1, 4, 8, 9, and 12, however, best characterized in ADAM-TS1 and -4 (Porter et al. 2005).

4.2 Inhibition

ADAM-TSs are inhibited by TIMPs, where ADAM-TS4 and ADAM-TS5 are potently inhibited by TIMP3, similar to ADAM17 (Lim et al. 2010). TIMP3 has also been shown to be an effective inhibitor of ADAM-TS2 and ADAM-TS4 (Wang et al. 2006; Hashimoto et al. 2001). TIMP2 and TIMP3 catalytically inhibit ADAM-TS1, but not TIMP1 or TIMP4 (Rodriguez-Manzaneque et al. 2002). In addition to TIMP3, α2-macroglobulin is also shown to inhibit ADAM-TS4 and ADAM-TS5 (Tortorella et al. 2004).

It was also reported that the use of chondroitin sulfate E and heparan sulfate increases the inhibitory affinity of TIMP-3 towards ADAM-TS5 (Troeberg et al. 2014). Paplilin, an extracellular matrix glycoprotein has also been shown to inhibit ADAM-TS proteinase (Kramerova et al. 2000). Furthermore, the anti-ADAM-TS5 monoclonal antibody, GSK2394002, was used as a therapeutic agent for osteoarthritis (OA) (Larkin et al. 2015; Apte 2016).

In addition, a series of cis-1(S)2(R)-amino-2-indanol-based compounds have been reported as selective inhibitors for the catalytic domain of aggrecanases, ADAM-TS4 and -5 over other metalloproteases (Tortorella et al. 2009). Calcium pentosan polysulfate (CaPPS), from beechwood, reported as a multifaceted exosite inhibitor of aggrecanases, interacts with the noncatalytic spacer domain of ADAM-TS4 and the cysteine-rich domain of ADAM-TS5. CaPPS also increased the cartilage level and affinity of TIMP-3 for ADAM-TS4 and -5 (Troeberg et al. 2008; Takizawa et al. 2008). In addition, a series of cis-1(S)2(R)-amino-2-indanol-based compounds have been reported to selectively bind to the catalytic domain and inhibit aggrecanases, ADAM-TS4 and ADAM-TS5 (Tortorella et al. 2009). Calcium pentosan polysulfate (CaPPS), from beechwood, is a multifaceted exosite inhibitor of aggrecanases that interacts with the noncatalytic spacer domain of ADAM-TS4 and the cysteine-rich domain of ADAM-TS5. CaPPS also increased the cartilage level and affinity of TIMP-3 for ADAM-TS4 and -5 (Troeberg et al. 2008; Takizawa et al. 2008). Also, granulin-epithelin precursor (GEP), a secreted growth factor, binds to ADAM-TS7 and ADAM-TS12 and inhibits the cleavage of cartilage oligomeric matrix protein. In addition, GEP inhibits the expression of these aggrecanases (Guo et al. 2010). Furthermore, hypermethylation of ADAM-TS1, 8, 9, 12, 18, and 19 leads to silencing of its activity in tumor cells (Lind et al. 2006; Moncada-Pazos et al. 2009; Lung et al. 2008; Jin et al. 2007).

4.3 Regulation

The posttranslational modifications of ADAM-TSs, such as glycosylation and proteolytic cleavage of ancillary domains, regulate their localization, secretion, activation, and catalytic functions (Kelwick et al. 2015). All ADAM-TSs except ADAM-TS4 exhibit N-terminal glycosylation, with *N*-glycosylation of ADAM-TS9 pro-domain is essential for its secretion (Apte 2009). Unlike other ADAM-TSs, ADAM-TS13 has thrombospondin type 1 repeats which contain a consensus sequence for O-fucosylation, which is functionally significant for ADAM-TS13 secretion and ensures proper protein folding (Ricketts et al. 2007). Also, proteolytic processing by membrane-type 4-matrix metalloproteinase within the C-terminal domains of ADAM-TS4 is essential for its activation (Gao et al. 2004).

5 ADAMs and ADAM-TSs Role in Cardiovascular Diseases

The ADAMs and ADAM-TSs play a crucial role in cardiovascular diseases (CVD) such as atherosclerosis, hypertension, coronary artery disease, myocardial infarction, and heart failure.

The ADAMs and ADAM-TSs play a crucial role in cardiovascular diseases (CVD) such as atherosclerosis (Table 2). Atherosclerosis is a chronic inflammatory disease in which narrowing of arteries occurs due to the abnormal deposition of lipids and inflammatory cytokines in blood vessels. During atherogenesis, ADAMs not only promote the recruitment and differentiation of inflammatory cells, but also act as sheddases for various crucial mediators such as growth factors, cytokines, chemokines, and adhesion molecules. For instance, junctional adhesion molecule-A (JAM-A) and vascular endothelial (VE)-cadherin, which regulates leukocyte transendothelial migration and vascular permeability, are a substrate for ADAM10 or ADAM17 (Ponnuchamy and Khalil 2008; Koenen et al. 2009; Schulz et al. 2008). Furthermore, CX3CL1 (fractalkine) and CXCL16, described as substrates for ADAM10 and ADAM17, mediate adhesion and migration of leukocytes through the vascular wall (Hundhausen et al. 2007; Ludwig and Weber 2007). ADAM17 is responsible for ectodomain shedding of adhesion molecules like vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 (Tsakadze et al. 2006; Garton et al. 2003). These adhesion molecules play critical roles during different stages of atherosclerosis development. Recently, a distinct association between ADAM17 substrates and recurring atherosclerosis in human subjects has been reported, emphasizing the positive role of ADAM17 activity in predicting cardiovascular events (Rizza et al. 2015). Also, in atherosclerotic cerebral infarction (ACI) patients, the rs653765 polymorphism is positively correlated with ADAM10 promoter activity and expression (Li et al. 2013b). Furthermore, ADAM10 role in vascular diseases has been elucidated, where a significant increase in ADAM10 expression was observed during plaque development from early to advanced, and to

Table 2 Functions of ADAMs and ADAM-TSs involved in cardiovascular disease (CVD)

ADAMs/ ADAM-TSs	Substrates	Pathological effect in cells/tissues	Reference
ADAM8	L-selectin, PSGL-1, TNF-α, TNFR-1, VCAM-1	↑ Expression in atherosclerotic development and myocardial infarction (MI), neutrophils, and macrophages Diagnostic/prognostic biomarker	(Sun et al. 2012; Holloway et al. 2010; Levula et al. 2009; Raitoharju et al. 2011; Kessler et al. 2015)
ADAM9	TNF-α, EGF, HB-EGF	Interaction with integrin Monocyte homing, migration, or proliferation in aorta, carotid, and femoral arteries	(van der Vorst et al. 2017)
ADAM10	VE-cadherin, JAM-A, Fractalkine, CXCL16	↑ Vascular permeability ↑ Leukocyte adhesion and trans-endothelial migration, plaque fibrosis, contribute to atrial dilation	(Gao et al. 2004; Ponnuchamy and Khalil 2008; Koenen et al. 2009; Schulz et al. 2008; Rizza et al. 2015; Li et al. 2013b; Donners et al. 2010; Eerenberg et al. 2016)
ADAM12	HB-EGF	↑ Levels in arteriovenous fistula (AVF) patients	(Arndt et al. 2002)
ADAM15	EGF, TGF-α, HB-EGF, BTC and EPR	Interaction with integrin Monocyte homing, migration, or proliferation in aorta, carotid, and femoral arteries contribute to atrial dilation	(van der Vorst et al. 2017; Oksala et al. 2009; Bültmann et al. 2011; Sun et al. 2010; Eerenberg et al. 2016)
ADAM17	ICAM-1, VCAM-1, TNF-α, TNF receptors I and II, TGF-α, L-selectin, IL-6 receptor, M-CSF receptor 1,	↑ Inflammation, leukocyte recruitment Monocyte homing, migration, or proliferation in aorta, carotid, and femoral arteries Atheroprotective in myeloid and Atheroprogressive in epithelial cells ↑ Hypertension, role in post-MI repair	(Gao et al. 2004; Schulz et al. 2008; Hundhausen et al. 2007; Ludwig and Weber 2007; Tsakadze et al. 2006; Garton et al. 2003; van der Vorst et al. 2015; Holdt et al. 2008; Nicolaou et al. 2017; Zheng et al. 2016; Fan et al. 2015; Canault et al. 2006; Zhao et al. 2015; Wang et al. 2009b; Zhu et al. 2018; Vuohelainen et al. 2011)
ADAM33	KL-1, tumor necrosis factor-related activation-induced cytokine	↑ Expression by VSMC in the arterial wall (including the intima, media, and adventitia), inflammatory	(Sun et al. 2012; Holloway et al. 2010)

(continued)

Table 2 (continued)

ADAMs/ ADAM-TSs	Substrates	Pathological effect in cells/tissues	Reference
		cells and in atherosclerotic lesions	
ADAM-TS1	Versican	Promotes aortic VSMC migration, ↑ serum levels in acute myocardial infarction (AMI)	(Theodorou et al. 2017; Jönsson-Rylander et al. 2005; Wågsäter et al. 2008; Norata et al. 2004; Fan et al. 2015)
ADAM-TS2	Fibrillar procollagens	↑ Levels in AMI and failing human hearts and hypertrophic murine hearts	(Hirohata et al. 2017; Mishra et al. 2010)
ADAM-TS4	Versican, aggrecan, α2-macroglobulin	↑ Expression in macrophages Expression upregulated during the development of atherosclerosis in $LDLR^{-/-}ApoB^{100/100}$ mice ↑ Plasma levels in coronary artery disease (CAD).	(Theodorou et al. 2017; Jönsson-Rylander et al. 2005; Rizza et al. 2015)
ADAM-TS5	Biglycan, versican	Proteoglycan turnover and lipoprotein retention	(Didangelos et al. 2012)
ADAM-TS7	COMP	VSMC migration and intimal thickening after vascular injury ↑ Plasma levels in CAD and AMI. Inhibits re-endothelialization	(Bongrazio et al. 2000; Chen et al. 2011; Yu et al. 2016; Mead and Apte 2018; Reilly et al. 2011; Lee et al. 2012)
ADAM-TS8	Aggrecan	Expressed in macrophage enrich areas of human atherosclerotic carotid plaques and coronary unstable plaques	(Theodorou et al. 2017; Jönsson-Rylander et al. 2005)
ADAM-TS13	Von Willebrand factor (VWF)	Low activity in intramyocardial hemorrhage	(Wu et al. 2015)
ADAM-TS18	Aggrecan	ADAM-TS18 deficiency causes increase	(Shen et al. 2017)

ruptured atherosclerotic plaques (Donners et al. 2010). Due to the embryonic lethality of ADAM10 full knockout mice, the effect of ADAM10 deletion on atherosclerosis has been evaluated using myeloid-specific ADAM10-knockout mice. The myeloid-specific deletion of the Adam10 gene increases plaque fibrosis and reduces macrophage accumulation but has a negligible effect on plaque size (van der Vorst et al. 2015). The metalloprotease ADAM17 has been attributed as a unique genetic factor of atherosclerosis vulnerability. Quantitative trait locus mapping in

mice demonstrated that elevated levels of ADAM17 are associated with decreased lesion formation (Holdt et al. 2008). Additionally, an atheroprotective role of ADAM17 has been observed in ADAM17-deficient mice, in which the endogenous TNFR2 signaling in vascular cells has been overactivated due to reduced shedding of membrane-anchored TNFα and TNFR2 (Nicolaou et al. 2017). However, in rats elevated level of ADAM17 along with increased expression of TNF-α has been found associated with cardiac remodeling after acute myocardial infarction (Zheng et al. 2016). Cardiomyocyte-specific ADAM17 knockdown in mice subjects demonstrated that ADAM17 plays an important role in post-myocardial infarction (MI) repair by suppressing activation of VEGFR2 and impairing angiogenesis, thus limiting left ventricular dilation and dysfunction (Fan et al. 2015). In mice, ADAM17 expression is found to be linked with lesions in atherosclerosis-prone sites (aortic sinus and arch), thereby contributing to the increased levels of soluble TNF receptors (TNFR1 and TNFR2) in the plasma, parallel to atherosclerosis progression (Canault et al. 2006). Also, lentiviral-mediated ADAM17 gene silencing in abdominal aortic plaques of rabbits enhances plaque stability via down-regulating ERK-NF-κB signaling and upregulating TGF-β1 signaling (Zhao et al. 2015). Recently, cell-type-specific and curative effects of ADAM17 deficiency on atherosclerosis have been demonstrated in myeloid and epithelial cells. The authors used $ApoE^{-/-}LysMCreADAM17^{fl/fl}$, and $ApoE^{-/-}BmxCreADAM17^{fl/fl}$ mice to demonstrate that ADAM17 expression in myeloid cells is atheroprotective and atheroprogressive in endothelial cells (van der Vorst et al. 2017). The above results suggest the cell-specific role of ADAM17 in cardiovascular diseases.

In addition to ADAM17, high levels of ADAM9 and ADAM15 have been reported in macrophages present in advanced atherosclerotic plaques (Oksala et al. 2009). In addition, overexpression of ADAM15 in western diet-fed rabbits attenuates the progression of atherosclerosis (Bültmann et al. 2011). In contrast, some reports have shown that enhanced expression of ADAM15 in endothelial cells induces endothelial permeability, promoting monocyte and neutrophil transmigration (Sun et al. 2010, 2012). These observations indicate the physiological significance of ADAM15 in atherosclerosis progression, even though the underlying mechanisms are yet unclear. Moreover, ADAM33 expression has been found in the inflammatory cells of human atherosclerotic lesions, and single nucleotide polymorphisms (SNP) of both ADAM8 and ADAM33 genes are shown to be associated with pathogenesis and development of atherosclerosis (Holloway et al. 2010; Levula et al. 2009). Likewise, ADAM8 polymorphism (rs2275725) is associated with elevated ADAM8 serum levels and myocardial infarctions (MI) risk (Raitoharju et al. 2011). Recently, a study reported that although a significant increase in ADAM8 expression was observed in the active human plaques lesion area, no significant change in atherosclerotic lesion area was observed in hematopoietic or whole-body ADAM8 deficient mice (Theodorou et al. 2017).

The presence of ADAM-TSs is reported in intimal thickenings and advanced atherosclerotic lesions. ADAM-TSs contribute to lipid retention and affect the adhesion and recruitment of macrophages. ADAM-TS1 exhibits its potential role in atherogenesis by cleaving extracellular matrix (ECM) proteins and inducing

vascular smooth muscle cells (VSMC) migration and neointima formation (Jönsson-Rylander et al. 2005). The expression of the ADAM-TS proteases, particularly ADAM-TS1 and ADAM-TS4 have shown their importance in atherosclerosis by inducing the cleavage of versican, a vital constituent of the vascular ECM. Furthermore, ADAM-TS4, 5, and 8 expressions were also observed in human carotid artery lesions and advanced coronary plaques. The ADAM-TS4, 5, and 8 are highly expressed in macrophage populated areas of atherosclerotic plaques, while ADAM-TS1 expresses predominantly in endothelial and smooth muscle cells (Wågsäter et al. 2008; Norata et al. 2004). ADAM-TS1 mediates tumor necrosis factor-α (TNF-α) and lipopolysaccharide (LPS) induced inflammation in endothelial cells (Bongrazio et al. 2000). Also, shear stress-dependent induction of ADAM-TS1 expression suggests a potential role for ADAM-TS1 vessel structure maintenance under normal flow conditions (Bongrazio et al. 2000). An atheroprotective role of ADAM-TS5 has been observed in ADAM-TS5-deficient mice, where its deficiency leads to the accumulation of proteoglycans (biglycan and versican) and its binding to lipoproteins in atherosclerosis (Didangelos et al. 2012). ADAM-TS7 is involved in VSMC migration and neointima development in response to vascular injury during atherogenesis (Wang et al. 2009b).

Hypertension is considered a primary risk factor for CVD. ADAMs and ADAM-TSs have shown their association with hypertension. In vitro and in vivo studies showed that ADAM17-knockdown suppressed the angiotensin II-induced hypertension and end-organ damage (Shen et al. 2017). On the contrary, ADAM-TS18 deficient mice showed augmented metabolic disorders and a higher risk of cardiovascular disease and hypertension (Zhu et al. 2018).

ADAMs and ADAM-TSs also play a significant role in coronary artery disease, myocardial infarction, and heart failure. Increased circulating levels of ADAM17 substrates have been detected in patients with established atherosclerosis (Rizza et al. 2015). High plasma levels of ADAM-TS4 were observed in patients with coronary artery disease (Zha et al. 2010), and statin therapy reduces it (Zha et al. 2010; Chen et al. 2011). Elevated plasma ADAM-TS7 levels were observed in patients with coronary artery disease, which subsequently promotes atherosclerosis (Yu et al. 2016; Mead and Apte 2018). Furthermore, $Ser^{214}Pro$ substitution in the ADAMTS-7 pro-peptide reduced ADAM-TS7 proteolytic activity and was associated with coronary artery disease (Reilly et al. 2011). Studies using ADAM-TS7 knockout mice showed the potential role of ADAM-TS7 in post-injury vascular intimal hyperplasia (Kessler et al. 2015).

Rat cardiac transplantation model showed an elevated expression of ADAM8 in myocardial infarction (MI) over controls (Vuohelainen et al. 2011). In cardiomyocyte-specific ADAM17 deficient mice, lower survival, higher cardiac rupture rates, increased left ventricular dilation, and decreased ejection fraction have been observed over control mice, demonstrating the protective role of ADAM17 in post-MI repair (Fan et al. 2015). ADAM-TS1 is expressed optimally in normal tissues, but its serum levels were found elevated in patients with acute myocardial infarction (Hirohata et al. 2017). Tissue samples from patients with acute myocardial infarction (AMI) showed augmented expression of ADAM-TS2, −3,

and -13 in culprit plaques and most likely in endothelial cells and macrophages (Lee et al. 2012). Furthermore, elevated plasma levels of ADAM-TS7 show an association of ADAM-TS7 with ventricular remodeling after AMI (Wu et al. 2015). Studies performed using patients with intramyocardial hemorrhage (IMH) showed significantly lower activity of ADAM-TS13. However, no significant change in infarct size or IMH has been observed after the intracoronary administration of recombinant ADAM-TS13, indicating a negative correlation between ADAM-TS13 and infarct size (Eerenberg et al. 2016).

An increased expression of ADAM10 and ADAM15 at both the transcript and protein levels has been observed in patients with atrial fibrillation (AF), demonstrating their role in structural remodeling of the fibrillating atria (Arndt et al. 2002). ADAM12 levels increased significantly in arteriovenous fistula (AVF) mice model under oxidative stress conditions. Furthermore, ADAM12 levels were significantly decreased after treatment of AVF mice with hydrogen sulfide donor molecule (NaHS) (Mishra et al. 2010). In addition, an increased expression of ADAM-TS2 was observed in failing human hearts and hypertrophic murine hearts. The deletion of ADAMTS2 in mice significantly enhanced pressure overload-induced cardiac hypertrophy, and ADAMTS2 overexpression in cardiac tissues attenuated this phenotype, suggesting a protective function of ADAM-TS2 in the development of cardiac hypertrophy under pathological conditions (Wang et al. 2017).

6 ADAMs and ADAM-TSs Role in Colorectal Cancer

Several reports have suggested a role for ADAMs and ADAM-TS families in the etiopathogenesis of colorectal cancer (Table 3). The hepatic stellate cells (HSCs) secrete a spliced variant of ADAM9 (ADAM9-S), which promotes colon carcinoma cell invasion. Furthermore, the colon carcinoma cell invasion depends on both the protease activity of ADAM9 and its binding to the α6β4 and α2β1 integrins on the plasma membrane of colon carcinoma cells (Mazzocca et al. 2005). The ADAM9 overexpression enhances growth factor-mediated VE-cadherin internalization and cell–cell contact disruption in HT29 human colon cancer cells (Hirao et al. 2006). The tissue microarray analysis of colorectal cancer (CRC) showed a significant correlation of Adam10 gene expression with late stage of cancer (Knosel et al. 2005). Also, proteomic characterization of the tetraspanin web identified ADAM10 as one of the components of the tetraspanin network (Le Naour et al. 2006). Furthermore, expression of ADAM10 along with L1-CAM (neuronal cell adhesion receptor) in human CRC cells confers to the metastatic capacity in CRC cells to the liver, thus significantly contributing to the development of the invasive stage of colon cancer (Gavert et al. 2007). The overexpression of ADAM 10 and C-erbB-2 in gastric cancer lesions plays a key role in gastric cancer invasion and metastasis (Wang et al. 2011a). The increased expression of ADAM17 in liver metastases than primary colorectal tumor cells has revealed the potential role of ADAM17 in the metastatic process (Lin et al. 2007). Also, ADAM17 has shown a

Table 3 Functions of ADAMs and ADAM-TSs involved in colorectal cancer

ADAMs/ ADAM-TSs	Substrates	Pathological effect in cells/tissues	Reference
ADAM9	Laminin, gelatin, E-cadherin	Promotes carcinoma invasion and colonizes the liver	(Wang et al. 2017; Mazzocca et al. 2005)
ADAM10	L1-CAM	*Adam10* gene correlates with a late stage of cancer Component of tetraspanin web Enhanced metastasis in colon cancer cells Tumor progression and prognostic biomarker	(Hirao et al. 2006; Knosel et al. 2005; Le Naour et al. 2006; Gavert et al. 2007)
ADAM17	TGF-α, amphiregulin, heregulin	Promotes metastatic invasion, colonization, tumor progression and drug resistance in CRC tumors	(Wang et al. 2011a; Lin et al. 2007; Kyula et al. 2010)
ADAM23	No protease activity available	Gene down-regulated in CRC cells	(Schmidt et al. 2018; Choi et al. 2009)
ADAM29	No protease activity available	Gene undergoes mutation	(Wang et al. 2011b)
ADAM-TS1	No protease activity available	Gene epigenetically deregulated, early tumorigenesis biomarker	(Sjoblom et al. 2006; Lind et al. 2006)
ADAM-TS5	No protease activity available	Gene epigenetically deregulated, mRNA expression down-regulated	(Ahlquist et al. 2008)
ADAM-TS9	No protease activity available	Tumor-suppressor protease	(Kim et al. 2011)
ADAM-TS12	No protease activity available	Tumor-suppressor protease, prognostic biomarker	(Zhang et al. 2010b; Moncada-Pazos et al. 2009)
ADAM-TS15	No protease activity available	Tumor-suppressor protease	(Wang et al. 2011c)

significant role in drug resistance mechanisms during colorectal cancer chemotherapy (5-fluorouracil) treatment. The in vitro and in vivo studies have shown an increase in ADAM17 activity after chemotherapy treatments, therefore, blocking ADAM17 activity using siRNA in conjunction with chemotherapy may have therapeutic potential for the treatment of CRC (Kyula et al. 2010). Furthermore, the study using $APC^{Min/+}$ and $ADAM17^{ex/ex}$ mouse models demonstrated that shedding of IL-6 via ADAM17 is a prerequisite for IL-6 trans-signaling that induces β-catenin–dependent tumorigenesis in CRC. Therefore, knockdown of ADAM17 activity resulted in abrogation of tumor formation (Schmidt et al. 2018). In colorectal

cancer cell lines, an aberrant silencing of Adam23 gene has been reported due to epigenetic modification, resulting in abnormal cell–cell interactions, and increased cell migration and metastasis (Choi et al. 2009). However, in another study downregulation of DNA-methyltransferase-1 (DNMT1) by restoring the expression of miR-342 resulted in ADAM23 reactivation (Wang et al. 2011b). The elucidation of the genome sequence of human CRC tissues revealed that Adam29 gene undergoes mutations that probably affect the cellular functions, including transcription, adhesion, and invasion (Sjoblom et al. 2006).

The microarray screening has identified ADAM-TS1 as one of the genes deregulated epigenetically in colorectal tumorigenesis (Lind et al. 2006). Therefore, methylated ADAM-TS1 is considered a suitable marker for the early detection of colorectal cancer (Ahlquist et al. 2008). The methylation profiling-based studies on bead-chip arrays have exhibited hypermethylation of ADAM-TS5 gene promoter in CRC (Kim et al. 2011). A large group study of CRCs conducted using a high-resolution melting method showed a significant correlation between ADAM-TS9 promoter methylation and its decreased expression (Zhang et al. 2010b). ADAM-TS12, a novel anti-tumor protease with an anti-proliferative effect on tumor cells, is epigenetically silenced in colon cancer cell lines and tumor tissues (Moncada-Pazos et al. 2009). Furthermore, ADAM-TS12 plays a vital role in inhibiting tumor progression and has been considered a potential prognostic biomarker for colorectal cancer (Wang et al. 2011c). The presence of genetically inactive ADAM-TS15 in various in vitro and in vivo colon cancer studies revealed that ADAM-TS15 markedly promotes tumor growth and invasion. Further, microarrays analysis showed a negative correlation between ADAM-TS15 expression and histopathologic differentiation grade of colon carcinomas (Viloria et al. 2009).

7 ADAMs and ADAM-TSs Role in Autoinflammatory Diseases (Sepsis/Rheumatoid Arthritis)

The ADAMs and ADAM-TSs also influence various autoinflammatory diseases like sepsis and rheumatoid arthritis (Table 4), caused by an abnormal innate immune system. The role of ADAM17 in sepsis is shown in mice, where conditional deletion of ADAM17 in myeloid cells protects the mice from endotoxin shock and has considerably low serum TNF levels compared to control animals (Horiuchi et al. 2007). However, in the acute pulmonary inflammation model of mice, ADAM10 has been reported essential for chemokine-induced migration of monocytic cells and neutrophils, thereby stimulating accumulation of leukocytes in alveoli and the development of pulmonary edema (Pruessmeyer et al. 2014). The ADAM10 variant with rs653765 polymorphism in the promoter region is associated with the progression of severe sepsis in humans (Cui et al. 2015). This polymorphism functionally activates ADAM10 gene expression and concomitantly elevates relevant substrates (Cui et al. 2015). Also, the above ADAM10 functional variant confers the

Table 4 Functions of ADAMs and ADAM-TSs involved in autoinflammatory diseases (sepsis/rheumatoid arthritis)

ADAMs/ADAM-TSs	Substrates	Pathological effect in cells/tissues	Reference
ADAM10	TNF-α, Fractalkine (CCL2, CXCL1, CX3CL1 and CXCL16), sIL6R, JAM-A, JAM-C	Promotes leukocyte recruitment and edema formation in a mice model of acute pulmonary inflammation ADAM10 genetic polymorphism associated with progression of sepsis ↑ Expression in RA	(Horiuchi et al. 2007; Pruessmeyer et al. 2014; Cui et al. 2015; Moss et al. 2008)
ADAM17	TNF-α, TGF-α	Sheddase activity promotes rheumatoid arthritis and endotoxin shock ↑ Expression in RA	(Viloria et al. 2009; Chen et al. 2019; Dreymueller et al. 2012; Lin et al. 2016; Patel et al. 1998; Charbonneau et al. 2007)
ADAM-TS7	COMP	↑ Expression in cartilage and synovium of patients with RA	(Isozaki et al. 2013)
ADAM-TS12	COMP	↑ Expression in cartilage and synovium of patients with RA Genetic polymorphisms are associated with RA	(Isozaki et al. 2013; Liu 2009)
ADAM-TS13	vWF	Significantly lower levels in patients with sepsis and pediatric sepsis syndrome Prognostic biomarker	(Liu et al. 2020; Levi et al. 2018)

progression of sepsis in a large group of patients, modulates the EGR1/ADAM10 pathway, and influences the severity of sepsis (Chen et al. 2019). These results illustrate the clinical significance of ADAM10 in the pathogenesis and development of sepsis. ADAM17 also plays a critical role in endotoxin-mediated acute pulmonary inflammation. An increased ADAM17 expression in endotoxin-treated lung microvascular endothelial cells enhances the ectodomain shedding of chemokines and the junctional adhesion molecules. Furthermore, endotoxin-triggered vascular permeability, edema formation, pulmonary leukocyte recruitment, and the release of TNF-a & IL-6 have also been abrogated in endothelial-specific adam17-knockout mice (Dreymueller et al. 2012). ADAM12 is expressed in naïve T-cells and acts as a costimulatory molecule to activate and induce the proliferation of T-helper 1 (Th1) cells (Liu et al. 2020).

The decreased level of ADAM-TS13 in sepsis patients leads to thrombotic microangiopathy, clinically manifesting as a syndrome with multiple organ dysfunction, most importantly brain and kidneys, and potentially influencing almost all organs (Levi et al. 2018). Furthermore, a decrease in ADAM-TS13 activity has

been reported in different pediatric sepsis syndromes, including sepsis, severe sepsis, and septic shock. The ADAM-TS13 activity is negatively correlated with the severity of pediatric sepsis, whereas decreased ADAM-TS13 activity on day 1 has been found related to increased risk of mortality (Lin et al. 2016).

Rheumatoid arthritis (RA) is an autoimmune and chronic inflammatory disease that causes joint inflammation and tissue destruction that results in functional damage. The first evidence associating ADAMs in RA has demonstrated the upregulation of ADAM17 mRNA in arthritis-affected cartilage compared to normal cartilage (Patel et al. 1998). Furthermore, hypoxia-inducible factor-1 α (HIF-1 α) and TNF-α mediated enhancement of ADAM17 mRNA levels have been reported in RA-affected joints (Charbonneau et al. 2007). The high efficacy in the treatment of preclinical mouse models of RA with specific ADAM17 inhibitors, such as TMI-2 (Wyeth) and BMS-561392 (Bristol Myers Squibb), further supports the role of ADAM17 in arthritis (Moss et al. 2008). Significantly elevated levels of ADAM10 mRNA and protein in human rheumatoid arthritis synovial tissue suggest the involvement of ADAM10 in the pathogenesis of rheumatoid arthritis (Isozaki et al. 2013). ADAM-TS7 and ADAM-TS12 are found associated with cartilage oligomeric matrix protein (COMP) degradation in vitro and are also overexpressed in the synovium and cartilage of rheumatoid arthritis patients. The α2-macroglobulin and granulin-epithelin precursors (GEP) were reported as endogenous inhibitors of ADAM-TS7 and ADAM-TS12 (Liu 2009). Furthermore, rs10461703 genetic polymorphisms of ADAM-TS12 are associated with the development of RA (Nah et al. 2012).

8 ADAMs and ADAM-TSs Role in Alzheimer's Disease

Alzheimer's disease (AD) is a progressive brain disorder in which toxic amyloid-β (Aβ) peptides get accumulated in the brain. Amyloid-β (Aβ) peptide is produced when β- and γ-secretase cleave amyloid precursor protein (APP). Alternative cleavage of the APP by the α-secretases (i.e., ADAMs) is neuroprotective and hence prevents the development of AD (Table 5). Several ADAMs, including ADAM9, ADAM10, and ADAM17 are suggested to possess the α-secretase activity and may consequently contribute to neuroprotection (Asai et al. 2003; Kuhn et al. 2010). Furthermore, while ADAM10 has both constitutive and regulated α-secretase activity, ADAM9 and ADAM17 have only regulated secretase activity (Lammich et al. 1999; Postina et al. 2004). The in vivo studies have shown that even a moderate neuronal overexpression of ADAM10 strongly enhanced α-secretase cleavage of APP, delays formation of plaque, and reduces cognitive defects observed in a transgenic AD mouse model (Endres et al. 2014). A small clinical study reported an enhanced ADAM10 α-secretase activity and a significant increase in APPs-α levels in patients receiving oral administration of synthetic retinoid acitretin (Endres et al. 2014). A declined ADAM10 levels in patients with AD indicates the importance of ADAM10 in the molecular pathogenesis of AD (Colciaghi et al. 2002).

Table 5 Functions of ADAMs and ADAM-TSs involved in Alzheimer's disease

ADAMs/ ADAM-TSs	Substrates	Pathological effect in cells/tissues	Reference
ADAM9	Amyloid precursor protein (APP)	Regulated α-secretase activity, neuroprotective	(Nah et al. 2012; Kuhn et al. 2010; Bernstein et al. 2003)
ADAM10	APP, notch	Constitutive α-secretase activity, neuroprotective Delays plaque formation and alleviates cognitive defects	(Asai et al. 2003; Kuhn et al. 2010; Lammich et al. 1999; Postina et al. 2004; Endres et al. 2014; Colciaghi et al., 2002)
ADAM12	APP	Interacts with FISH protein, influenced susceptibility to late-onset AD	(Slack et al. 2001; Malinin et al. 2005)
ADAM15		Expresses in few diffuse plaques Linked via interaction with integrin and/or Src protein tyrosine kinases	(Colciaghi et al. 2002)
ADAM17	APP	Role in regulated and constitutive α-secretase activity in cultured cells	(Asai et al. 2003; Kuhn et al. 2010; Lammich et al. 1999; Hotoda et al. 2002; Buxbaum et al. 1998)
ADAM-TS1	Aggrecan, Versican	Overexpression of ADAM-TS1 as marker protein for neurodegeneration	(Satoh et al. 2000)
ADAM-TS3	Reelin	Protease cleaves and inactivates Reelin in the cerebral cortex and hippocampus	(Krstic et al. 2012)
ADAM-TS4	Reelin, APP	Facilitates large fraction of insoluble Aβ peptides generation Protease cleaves Reelin	(Clark et al. 2001; Miguel et al. 2005; Ogino et al. 2017)
ADAM-TS5	Reelin	Protease cleaves Reelin	(Miguel et al. 2005)
ADAM-TS9		Remodeling the basement membrane and ECM	(Harold et al. 2007)
ADAM-TS13	vWF	Overexpression of ADAM-TS13 attenuates BBB disruption, increased micro-vessels, capillary perfusion, and cerebral blood flow	(Walter et al. 2019)

Immunoreactive staining showed that ADAM10 is associated with diffuse and neuritic plaques, whereas ADAM15 is observed in diffuse plaques. These findings suggest a direct involvement of ADAM10 in the pathology of AD, whereas ADAM 15 might be influencing the disease through its interaction with integrins and/or tyrosine kinases, particularly src tyrosine kinases (Bernstein et al. 2003). The overexpression of soluble and an alternatively spliced form of ADAM9 in COS cells results in enhanced phorbol ester-mediated digestion of APPs-α, suggesting α-secretase-like activity of ADAM9 (Hotoda et al. 2002). Additionally, in vitro

studies reported ADAM17 as an α-secretase, where disruption of the Adam17 gene and inhibition of ADAM17 enzyme activity eliminates regulated and constitutive α-cleavage of APP, respectively, in cultured cells (Buxbaum et al. 1998; Slack et al. 2001). Amyloid-β peptide (Aβ) is a causative agent for Alzheimer's disease (AD), and various in vitro studies have shown that ADAM12 mediate the neurotoxic effect of Aβ and influenced susceptibility to late-onset of AD (Malinin et al. 2005; Harold et al. 2007).

Unlike ADAMs, little is known regarding the role of the ADAM-TS in neurodegenerative disorders such as AD. Cloning and characterization of rat ADAM-TS9 fragment from a beta amyloid-treated astrocyte cDNA library indicate the possible role of ADAM-TS9 in the events leading to Alzheimer's disease (Clark et al. 2001). The Aβ induces the expression of ADAM-TS4 in cultured rat astrocytes indicating induced ECM degradation in the AD brain (Satoh et al. 2000). The frontal cortex of adult brains from AD patients showed more than five-fold overexpression of ADAM-TS1, but ADAM-TS5 levels were comparable to controls (Miguel et al. 2005). ADAM-TS4 and ADAM-TS5 have been recognized as Reelin (extracellular signaling protein) cleaving enzymes in a mouse model of AD (Krstic et al. 2012). Recently, it has been observed that ADAM-TS3 cleaves the N-terminal site of Reelin in the cerebral cortex and hippocampus, thereby negatively regulating Reelin. Therefore, for the prevention or treatment of AD, inhibition of ADAM-TS3 could be considered as a potential therapeutic strategy (Ogino et al. 2017). In autopsy brain samples from AD patients, metalloprotease ADAM-TS4 has been reported for the generation of a large fraction of insoluble Aβ peptides truncated at the N-terminus with Aβ4–x peptides (Walter et al. 2019). Furthermore, virus-mediated expression of ADAM-TS13 in the brain of APPPS1 mice is beneficial, as it reverses the vascular phenotype, AD-type pathologies, and behavioral deficits (Cao et al. 2019). These effects may be due to increased Aβ clearance from the brain to plasma, which may be due to improved blood-brain barrier (BBB) function (Bradley et al. 2007).

9 ADAMs and ADAM-TSs Role in Proliferative Retinopathies

The development of new blood vessels from existing vasculature is a critical process involved during tissue repair and any abnormality in the above process leads to pathological conditions such as retinal neovascularization. Retinal neovascularization is a leading cause of blindness in humans and is a clinical manifestation of several eye diseases including hypoxia-induced neovascularization, proliferative diabetic retinopathy, retinopathy of prematurity, and macular degeneration (Bradley et al. 2007; Chen and Smith 2007).

Several studies have shown the role of ADAMs and ADAM-TSs (Table 6) in pathological retinal neovascularization. The ADAM9 contribution to pathological neovascularization has been evaluated using a murine model of retinopathy of

Table 6 Functions of ADAMs and ADAM-TSs involved in proliferative retinopathies

ADAMs/ ADAM-TSs	Substrates	Pathological effect in cells/ tissues	Reference
ADAM8	CD31, Tie-2, Flk-1, Flt-1, EphrinB2, EphB4, VE-cadherin, KL-1, E-selectin, neuregulin-1β2	ADAM8 negatively regulates retinal neovascularization	(Horiuchi et al. 2003)
ADAM9	EphB4, Tie-2, Flk-1, CD40, VCAM, VE-cadherin	↑Expression in endothelial cells in pathological vascular tufts in the OIR model Important role in ischemia-induced retinal neovascularization	(Chen and Smith 2007)
ADAM10	Notch	ADAM10-deficient mice died at 9.5 days of embryogenesis Adam10 gene deletion in endothelial cells affects vascular structures in developing and adult mice	(Edwards et al. 2008; Guaiquil et al. 2010; Hartmann et al. 2002)
ADAM15	Notch1 and -4, PECAM-1, VE-cadherin, TIE-2, membrane-type 1 MMP, Kit-ligand	*adam15*$^{-/-}$ mice show a strongly reduced angiogenic response in a model of hypoxia-induced proliferative retinopathy	(Guaiquil et al. 2009)
ADAM17	VE-cadherin, V-CAM, EphB4, EMMPRIN, IGFR1 or PECAM, HB-EGF	ADAM17 inactivation in endothelial cells significantly reduced pathological neovascularization in a mouse model for retinopathy of prematurity	(Glomski et al. 2011)
ADAM-TS1		ADAM-TS1, an endogenous regulator of endothelial cell proliferation Inhibits angiogenesis in vivo and suppresses endothelial cell proliferation in vitro	(Weskamp et al. 2010)

prematurity where the level of ADAM9 expression predominantly increased in endothelial cells of the pathological vascular tufts. Also, the upregulated ADAM9 sheddase activity was largely dependent on reactive oxygen species production (Guaiquil et al. 2009). Likewise, in the oxygen-induced retinopathy (OIR) model, elevated levels of ADAM15 were observed in endothelial cells compared to the wild-type controls, and mice deficient in ADAM15 showed a significant reduction in neovascularization (Horiuchi et al. 2003). On the contrary, ADAM8-deficient mice showed enhanced neovascularization in retinas following the OIR model, which suggests that ADAM8 inhibits neovascularization (Guaiquil et al. 2010). The ADAM10-deficient mice died at 9.5 days of embryogenesis with numerous defects in the cardiovascular system and vasculogenesis, indicating the importance of

ADAM10 on Notch signaling and (neo)vessel formation (Edwards et al. 2008; Hartmann et al. 2002). Furthermore, mice (ADAM10-Tie2-Cre) with endothelial cell-specific inactivation of ADAM10 showed severe vascular abnormalities in the retina and various specialized vascular compartments during development (Glomski et al. 2011). Similarly, endothelial cell-specific inactivation of ADAM17 resulted in a significant reduction in tube formation and retinal neovascularization in a mouse model of retinopathy of prematurity (Weskamp et al. 2010).

In the OIR mice model, VEGF rapidly and strongly stimulates the expression of ADAM-TS1 in an endothelial cell in a PKC-dependent manner, thereby inhibiting endothelial cell proliferation and angiogenesis. Also, the deletion of endogenous ADAM-TS1 in endothelial cells results in increased endothelial cell proliferation indicating its role as a negative regulator of retinal neovascularization (Zhenhua et al. 2006).

10 ADAMs Role in Infectious Diseases

ADAMs have a prominent role in infectious diseases via pathogen/viral recognition and clearance, along with cytokine release and leukocyte recruitment (Table 7). ADAMs are mainly involved in infectious diseases through catalytic ectodomain

Table 7 Functions of ADAMs involved in infectious diseases

ADAMs/ ADAM-TSs	Substrates	Pathological effect in cells/ tissues	Reference
ADAM8		ADAM8 promotes leukocytes recruitment	(Kononchik et al. 2018; Dreymueller et al. 2017)
ADAM9		Upregulated during hepatitis B virus–related hepatocellular carcinoma metastases	(Olvera-Garcia et al. 2016)
ADAM10	Notch1, pattern-recognition receptors (PRRs), viral receptors	Uptake and clearance of pathogens, promotes viral recognition and entry	(Aljohmani and Yildiz 2020; Kneidl et al. 2012; Etzerodt et al. 2010; Gopal et al. 2015; Cappenberg et al. 2019; Kondratowicz et al. 2011; Mikulicic et al. 2019; Oliviero et al. 2017)
ADAM17	Notch1, viral receptors, ACE2, TNF-α, IL-6R	Uptake and clearance of pathogens, promotes viral recognition and entry	(Aljohmani and Yildiz 2020; Kneidl et al. 2012; Etzerodt et al. 2010; Gopal et al. 2015; Cappenberg et al. 2019; Kondratowicz et al. 2011; Mikulicic et al. 2019; Oliviero et al. 2017; Yan et al. 2020; Lambert et al. 2005)

shedding of their numerous substrates such as adhesion molecules, junction molecules, chemokines, and cytokines (Aljohmani and Yildiz 2020). ADAM10 and ADAM17 influence the uptake and clearance of pathogens by shedding of pattern-recognition receptors (PRRs), particularly receptor for advanced glycation end products (RAGE), CD163, and L-selectin (Kneidl et al. 2012; Etzerodt et al. 2010; Gopal et al. 2015; Cappenberg et al. 2019). In addition to bacterial entry, ADAM10 and ADAM17 are involved in viral recognition and entry via shedding of various viral receptors facilitating their cell/nuclear entry and replication (Kondratowicz et al. 2011; Mikulicic et al. 2019; Oliviero et al. 2017; Kononchik et al. 2018). Furthermore, ADAM8 and ADAM9 protein expression was found to be upregulated during viral infection (Dreymueller et al. 2017; Ma et al. 2009; Olvera-Garcia et al. 2016; Xiang et al. 2017). Besides this, ADAMs are shown to protective against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) infection. During SARS-CoV2 infection, angiotensin-converting enzyme II (ACE2) is a cellular receptor for the virus' spike (S) protein (Yan et al. 2020). ACE2 is a critical shedding substrate for ADAM17 and is highly expressed in the lung and myocardium (Lambert et al. 2005). Therefore, overexpression of ADAM17 might protect us against SARS-CoV2 infection via shedding of ACE2. ACE2 inhibition prevents SARS-CoV2 entry and blocks the circulation of virus particles (Patel et al. 2014b).

There are clear shreds of evidence available that SARS-CoV2 virus entry is facilitated by proteolytic cleavage of the S glycoprotein via furin, an endoprotease, just after the binding of virus spike proteins with ACE2 (Walls et al. 2020). Notch1, a key regulator of furin expression at the transcriptional level and is a direct substrate of both ADAM10 and ADAM17 (Weskamp et al. 2010). Therefore, activation of Notch 1 via ADAM10/ADAM17 shedding results in upregulation of Notch targeting genes such as furin (Qiu et al. 2015). Consequently, blocking of Notch1 signaling via inhibition of ADAM10/ADAM17 sheddase activity may downregulate the furin expression and might provide a potential approach to prevent SARS-CoV2 entry and infection (Rizzo et al. 2020). Furthermore, high serum levels of TNF-α and IL-6 are reported as predictive biomarkers for COVID-19 patients (Del Valle et al. 2020). Both TNF-α and IL-6R are the potent substrates of ADAM17. Hence inhibition of ADAM17 sheddase activity might serve as a critical preventive measure against SARS-CoV2 infection. In vitro and in vivo studies have shown that inhibition of ADAM17 markedly decreases the SARS-CoV2 infection and also attenuates its severe clinical outcome (Haga et al. 2010). The role of ADAM proteases during SARS-CoV2 infection is still in its infancy, therefore careful evaluation is required for its potential therapeutics.

11 Conclusion and Future Perspectives

ADAMs and ADAM-TSs are the metalloproteases involved in the extracellular matrix remodeling and degradation, but in recent years they are also shown to regulate the development and pathology of various diseases. They are structurally

related to MMPs in terms of their domains except for ADAM-TSs, which have thrombospondin motifs instead of a transmembrane domain. The ADAMs regulate cell phenotype and behavior by ectodomain shedding and influence cell–cell communication via adhesive interactions. The ADAMs are widely related to various human pathologies, as they regulate multiple cellular functions and physiological conditions. Unlike ADAMs, our fundamental understanding of ADAM-TS proteins' functions in human development and pathologies is still in its infancy. The association of ADAM-TS proteases with various diseases will continue to emerge, and we might understand their significance in days to come.

Although the in vitro regulation and functions of the ADAMs are known, further research is needed to confirm the physiological relevance of ADAMs and ADAM-TSs in vivo. Despite satisfactory evidence presented on the role of ADAMs and ADAM-TSs enzymes, further studies are greatly required to determine their complete mechanism of action, their activators, and inhibitors. Studies are needed to understand the significance of their downstream signaling molecules in disease development and progression. Although a reduction in ADAMs or ADAM-TSs levels causes adverse outcomes, abnormal upregulation or overexpression of these enzymes can lead to detrimental effects. Therefore, enormously competent and promising approaches are required to design novel therapeutics targeting ADAMs and ADAM-TSs. There are redundancy and overlap in the function of ADAMs and ADAM-TSs due to their crosstalk with various other proteins. Therefore, targeting multiple members of these families may exhibit sufficient effects. Consequently, cell- and tissue-specific functions and their physiological levels along with the activation process of these enzymes should also be considered in designing therapies.

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A Review: Uses of Chitosan in Pharmaceutical Forms

Olimpia Daniela Frenț, Laura Vicaș, Tunde Jurca, Stefania Ciocan, Narcis Duteanu, Annamaria Pallag, Mariana Muresan, Eleonora Marian, Adina Negrea, and Otilia Micle

Contents

Vicaș Laura, Jurca Tunde, Ciocan Stefania, Pallag Annamaria, Muresan Mariana, Marian Eleonora, Negrea Adina and Micle Otilia contributed equally with all other contributors.

O. D. Frenț, L. Vicaș, T. Jurca, A. Pallag, and E. Marian
Department of Pharmacy, Faculty of Medicine and Pharmacy, University of Oradea, Oradea, Romania
e-mail: emarian@uoradea.ro

S. Ciocan, N. Duteanu (✉), and A. Negrea
Politehnica University of Timisoara, Timisoara, Romania
e-mail: roxana.birjovanu@student.upt.ro; narcis.duteanu@upt.ro; adina.negrea@upt.ro

M. Muresan and O. Micle
Department of Preclinical Discipline, Faculty of Medicine and Pharmacy, University of Oradea, Oradea, Romania

Abstract Chitosan is a natural polysaccharide widespread in nature. It has many unique and attractive properties for the pharmaceutical field: it is biodegradable, safe, hypoallergenic, biocompatible with the body, free of toxicity, with proven anticholesterolemic, antibacterial, and antimycotic action. In this review we highlighted the physical, chemical, mechanical, mucoadhesive, etc. properties of chitosan to be taken into account when obtaining various pharmaceutical forms. The methods by which the pharmaceutical forms based on chitosan are obtained are very extensive, and in this study only the most common ones were presented.

Keywords Biocompatible · Biodegradable · Chitosan · Pharmaceutical forms · Polysaccharide

Abbreviations

DNA	Deoxyribonucleic acid
HMWC	High molecular weight chitosan
HPLC	High-performance liquid chromatography
IR	InfraRed
LD	Lethal dose
LMWC	Low molecular weight chitosan
MMWC	Medium molecular weight chitosan
MRI	Magnetic resonance imaging
O/W	Oil/water
PVA	Polyvinyl alcohol
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
UV	Ultraviolet
W/O	Water/oil
W/O/W	Water/oil/water

1 Introduction

Chitosan is a biopolymer discovered in 1859 by Rouget, in 1894, Hoppe Seyler gave it the name *chitosan,* and in 1950 its chemical structure was elucidated (Baldrick 2010; Khor 2001). Chitosan can be used in pharmaceutical field because it is a

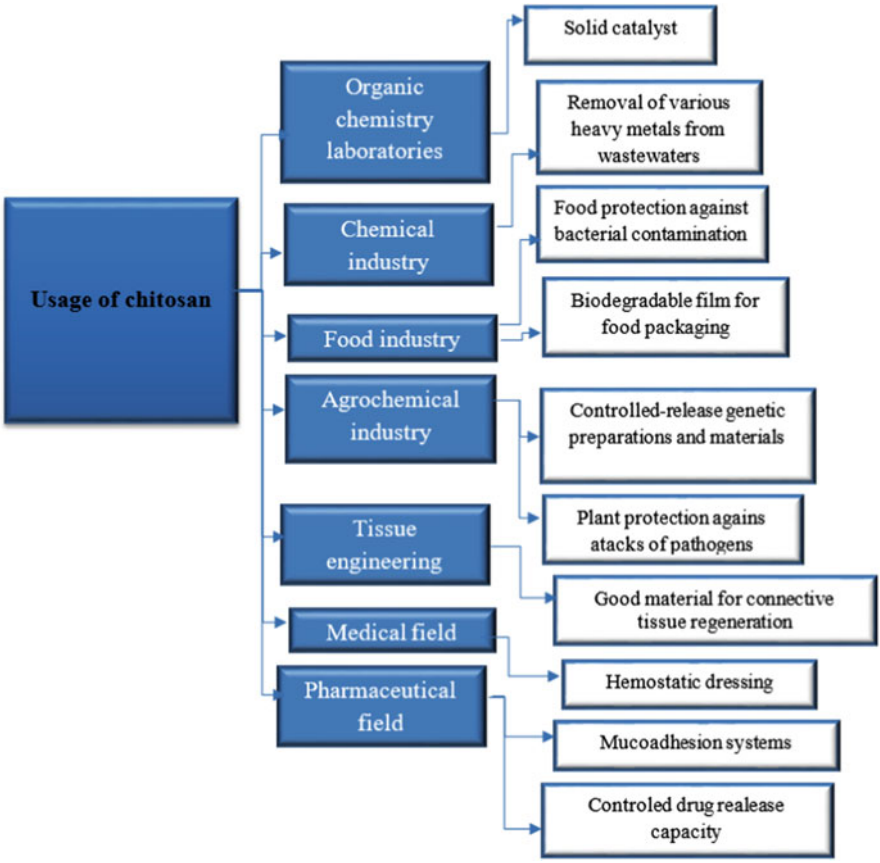

Fig. 1 Chitosan usage (Buşilă et al. 2015; Crini 2005; Jayakumar et al. 2010b; Kashyap et al. 2015; Kim et al. 2005; Malerba and Cerana 2018; Mendes et al. 2016; Quiñones et al. 2010; Yağız et al. 2016)

nonallergenic, biocompatible, nontoxic, biodegradable biopolysaccharide with antimicrobial, antitumor, anticholesterolemic action (Dong et al. 2015; Hamed et al. 2016b; Ozcelik et al. 2014; Schneiderman and Hillmyer 2017). Chitosan has a high capacity to release the drug due to the cationic character and primary amino groups (Salomon et al. 2017). Amino groups are also responsible for other properties: mucoadesion and gelling "in situ" (Andersen et al. 2015; Chen et al. 2004; Elgadir et al. 2015).

The properties of chitosan make it useful in different fields as it is shown in Fig. 1.

2 Synthesis of Chitosan

The source of chitosan is chitin, a mucopolysaccharide, widespread in nature (Khor 2001; Ko et al. 2002; Muzzarelli et al. 2012; Periayah et al. 2016). Chitin has an orderly, fibrillated structure, a high degree of crystallinity and polymorphism due to intra- and intermolecular hydrogen bonds (Xu et al. 2015).

Chitosan is the major component of tendons, arthropods, of the outer shell of insects, echinoids, annelids, molluscs, non-mathelmints lining their digestive, excretory, and respiratory systems and the cell wall of brown algae, *Chlorella* green algae, filamentous fungi and *Mucor rouxii* yeasts (Ahmed and Ikram 2015; Dev et al. 2010; Muzzarelli et al. 2012; Peniche et al. 2008; Raafat and Sahl 2009).

The greatest amount of chitosan is obtained from chitin, isolated from the shells of marine crustaceans, resulting from the processing of seafood (Heidari et al. 2018; Safari et al. 2016). After obtaining chitosan, it is purified in three stages using chemical (deproteinization, demineralization, and depigmentation/depigmentation) or biological (enzymatic, fermentation) treatments (Ahmed and Ikram 2015; Heidari et al. 2018; Rasti et al. 2016).

Several steps are taken in the process of obtaining chitosan to achieve a high degree of deacetylation and due to the harsh reaction conditions, severe depolymerizations of chitosan can also occur (Fiamingo et al. 2016).

Jorge A by M. Delezuk et al. obtained chitosan with superior mechanical properties, from β-chitin by ultrasonic irradiation. Processing was carried out in a single stage lasting 30 min at a temperature of 50–80°C (Delezuk et al. 2011).

Y. S. Puvvada et al. synthesized low molecular weight chitosan suitable for pharmaceutical use in shrimp exoskeleton, as is depicted in Fig. 2 (Puvvada et al. 2012; Weska et al. 2007).

In order to obtain high-quality chitosan Pranee Lertsutthiwong et al. found that it is necessary to take into account during the process of its extraction from chitin: concentration of chemicals used, soaking time, demineralization time (deproteinization), and temperature. A higher concentration of NaOH 5% or a longer treatment with 4% HCl leads to stronger hydrolysis and lower viscosity. The solubility of chitosan after 1 day of deacetylation with NaOH 5% was greater than 90%. After 3 days of deacetylation at 40°C, a chitosan with 70% degree of deacetylation was obtained. After 8 days of deacetylation, the degree of deacetylation of chitosan was 80% (Rojsitthisak et al. 2002). Therefore, if the period of deacetylation is increased and the temperature is constant and the concentration of NaOH is taken into account, then the degree of deacetylation of the chitosan increases (Viarsagh et al. 2010). A degree of deacetylation greater than 80% at low temperature (40°C) cannot be achieved in a single stage (Rojsitthisak et al. 2002). If deacetylation is carried out twice, then a deacetylation degree of 88% at 40°C could be achieved. Deacetylation of chitin with NaOH 5% at 90°C at a stage for 5 h resulted in an 88% degree of deacetylation (Rojsitthisak et al. 2002).

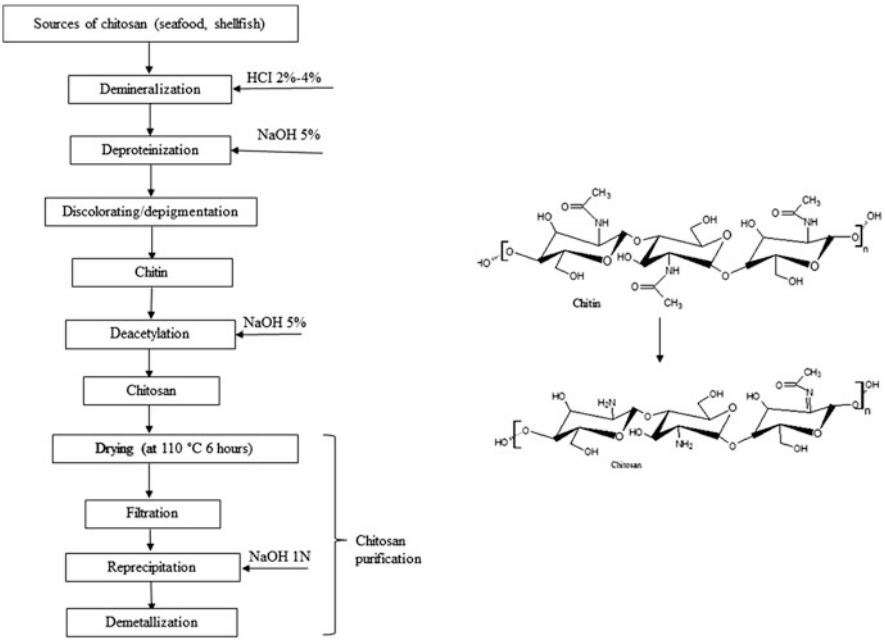

Fig. 2 Chitosan preparation starting from chitin (Puvvada et al. 2012; Weska et al. 2007)

3 Chemical Structure

Chitosan with the chemical name poly-β-(1,4)-2-amino-2-deoxy-D-glucose is a linear chain copolymer, consisting of glucosamine and N-acetylglucosamine groups, available in varying degrees of deacetylation (Dash et al. 2011; Ko et al. 2002; Ojagh et al. 2010; Sheskey et al. 2017). Chitosan has a crystalline and rigid structure, consisting of two repetitive units, joined by glycosamidic β (1 → 4) bonds, N-acethyl-2-amino-2-D-glucopiranosis, and 2-amino-2-deoxy-D-glucopiranosis (Fig. 3) (Dash et al. 2011; Shukla et al. 2013).

The crystallinity of chitosan is inversely proportional to the kinetics of biodegradation. The distribution of acetyl groups along the chain affects its crystallinity and

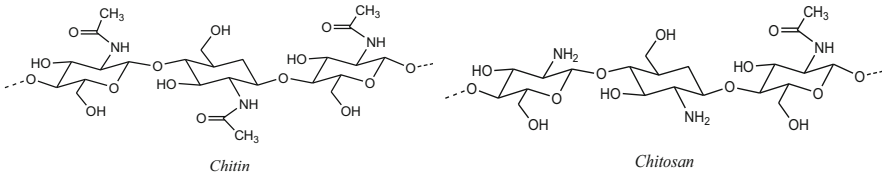

Fig. 3 Chemical structure of chitin and chitosan

Table 1 Physical modification of chitosan

Physical modification			
Method used	Polymer used	Newly obtained compound	Reference
Mixing	Polyvinyl alcohol (PVA)	Nanoparticles of Ag wrapped in chitosan mixed with PVA (antimicrobial effect)	(Abdelgawad et al. 2014)
Mixing	Polyvinylpyrrolidone (PVP)	Chitosan, ZnO, and PVP nanocomposite	(Karpuraranjith and Thambidurai 2017)
Mixing	Polyethylene oxide	Chitosan films	(Alexeev et al. 2000)

biodegradation rate, so that smaller chains will be degraded more rapidly than chains with higher molecular mass (Croisier and Jérôme 2013). Studies on chitosan have highlighted its crystalline, orthorhombic structure which depends on the different preparation of chitosan films (Naito et al. 2016). E. L. Mogilevskaya et al. have highlighted by X-ray diffraction that chitosan has a very high degree of deacetylation and lower crystalline regions, if the chitin from which it is obtained is subjected to the process of plastic deformation (grinding) (Mogilevskaya et al. 2006).

Due to the presence of the primary amino group, from position C-2 of each deacetylated unit, chitosan has a helical, hydrophile structure that helps to achieve inter- and intramolecular interactions (Lee et al. 2013). In positions C-6 and C-3, there are two primary and secondary, functional, reactive and repetitive hydroxyl groups that allow the physical (e.g. Table 1) or chemically chitosan to be modified, under mild conditions to improve its physical, chemical, mechanical, or biological properties (Periayah et al. 2016; Raafat and Sahl 2009; Safari et al. 2016; Shukla et al. 2013).

Chitosan may undergo several chemical modification in amino grouping such as: acetylation, alkylation, grafting, metal chelation, condensation with aldehydes and ketones, etc. and in hydroxyl grouping: O-acetylation, etherification, esterification, etc. with formation of chitosan derivatives (Cheung et al. 2015; Xu et al. 2015). By chemical modification of the structure of chitosan in the primary amino grouping, chitosan derivatives have been obtained so far: phosphorylated chitosan, hydroxyalchil chitosan, chitosan thiolate, chitosan sulfate, trimethylated chitosan, carboxyalchil chitosan, chitosan compounds with sulfonamide structure (with antioxidant action superior to chitosan) etc.

The major component of chitosan is glucosamine which is synthesized in the body from glucose, and then is taken over by cells using glucose transporters (Baldrick 2010; Reine et al. 2016). Glucosamine is essential for the cartilage synthesis of glycoproteins and glycosaminoglycans (chondroitin sulfate, hyaluronic acid, and keratin sulfate) that help maintain the health of cartilage and synovial fluid (Dahmer and Schiller 2008; Huskisson 2008; Jerosch 2011; Kirkham and Samarasinghe 2009). Glucosamine should be supplemented in the body at the time of installation of osteoporosis (Martel-Pelletier et al. 2008).

4 Physicochemical Properties of Chitosan

4.1 Degree of Deacetylation

The degree of deacetylation of chitosan refers to the ratio (number) of groups D-glucosamine and N-acetyl-D-glucosamine (Gámiz-González et al. 2017). The degree of deacetylation of chitosan must be greater than 50% if it is intended to be used in the medical or pharmaceutical field (Cho et al. 2005). This degree of deacetylation depends on the other physicochemical and mechanical properties: solubility, molecular weight, viscosity, etc. (Hafdani and Sadeghinia 2011; Heidari et al. 2018; Ravi Kumar 2000).

There are many analytical methods used to determine the degree of deacetylation: spectroscopic (IR, MRI, UV), conventional (titrations, conductometry, potentiometry, ninhydrin analysis, absorption of free amino grouping using picric acid), destructive (elemental analysis, acid or enzyme hydrolysis followed by colorimetry or HPLC chromatography and thermal analysis) (Heidari et al. 2018).

Infrared technique (IR) is the most widely used method of qualitative evaluation and comparison because the results of other methods can be influenced by certain factors. An inconvenience to destructive methods is that it requires long periods of time for measurements (Kasaai 2009). Determination of the degree of deacetylation for several chitosan samples by derived ultraviolet spectrometry using several solvents confirmed good method accuracy, with the variation of typical coefficients being around 1% (da Silva et al. 2008). The deacetylation of chitosan determined by the thermal analysis method was 76.2 ± 0.36 for the temperature of 35°C (Martínez-Camacho et al. 2010). The molecular mass of chitosan is between 10 and 1,000 kDa (Malafaya et al. 2007).

4.2 Molecular Mass

Chitosan by molecular mass can be divided into three categories: low molecular weight chitosan (LMWC) $<$ 100 kDa, chitosan with medium molecular weight (MMWC) 100–1,000 kDa, and chitosan with high molecular weight (HMWC) $>$ 1,000 kDa. Molecular mass may affect its antimicrobial properties (Hafdani and Sadeghinia 2011) and physicochemical properties (Raafat and Sahl 2009).

Chitosan, which has a high molecular mass, cannot cross the biological membrane of bacterial cell but is adsorbed to surface of the cell by blocking the transport of nutrients through the membrane, so producing cellular lysis. Chitosan with low molecular mass ($<$5,000 kDa) penetrates the bacterial cell, inhibits the messenger RNA synthesis, arrives with the bacterial nutrients where it bounded to bacterial DNA, disrupts the bacterial membrane by facilitating the flow of DNA from cells and so causes cell death (Divya et al. 2017; Huang et al. 2004). Chitosan with a low

molecular weight of between 3.5 and 15.8 kDa can be used to obtain water-soluble chitosan nanoparticles loaded with protein-based active substances. Medium molecular mass chitosans are suitable for the administration of medicinal products as they have increased mucoadesion and ensure controlled release of active substances from the pharmaceutical form. High molecular weight chitosan has poor mucosal adhesion and controlled release of the drug (Kumirska et al. 2011). Many studies show that the high molecular weight of chitosan affects its solubility in water. The higher the amount of organic or inorganic acid used to hydrolyze chitosan, the lower molecular weight of the hydrolyzed chitosan and the higher water solubility. J Santoso et al. obtained chitosan with an average molecular weight of 166.34 kDa with a dissolution capacity of 53.66% and a degree of 92.92% deacetylation by chemical hydrolysis at 60°C for 90 min using 5% acetic acid (Santoso et al. 2020).

Nidal A. Qinna, et al. have shown that the molecular mass of chitosan and the degree of deacetylation play an important role in choosing the way of administration of the pharmaceutical form, in the absorption of a drug passing through the intestinal epithelium, without causing adverse reactions on the stomach (Qinna et al. 2015). So, lower the degree of deacetylation and molecular mass, lower the toxicity, faster the action is installed and the absorption is higher (Abdul Khalil et al. 2016; Qian et al. 2006; Schipper et al. 1996).

4.3 Solubility

Pure chitosan has p*K*a = 6.3, is positively charged and insoluble in water, alkaline medium or organic solvents, but is soluble in acidic medium (Ahmed and Ikram 2015; Hong-liang et al. 2010; Raafat and Sahl 2009; Yadav and Chauhan 2017). Low solubility in water can be improved by: modification of the chemical structure, amino and hydroxyl groups from chitosan structure are sites capable of forming many bonds (amide, ester, and Schiff bases with other compounds) or by shaking with various organic acids (acetic acid, pyruvic acid, citric acid, malic acid, glyoxal acid, glycolic acid, ascorbic acid, formic acid) or inorganic (nitric acid, hydrochloric acid, perchloric acid, phosphoric acid) for the formation of soluble salts with pseudoplastic and viscoelastic properties (Ahmed and Ikram 2015; Anitha et al. 2009; Benhabiles et al. 2012; Dev et al. 2010; Fan et al. 2009; Raafat and Sahl 2009; Yadav and Chauhan 2017).

Viscosity of chitosan salts increases with increased concentration, decreases with temperature, and increases with its deacetylation (Sheskey et al. 2017). Chitosan sulfate is a water-soluble salt that exhibits anticoagulant and antioxidant activity (due to the chelating capacity of free radicals) (Abraham et al. 2018). The solubility of chitosan in water depends on: the degree of deacetylation, the distribution of the acetyl groups, and the degree of polymerization. By properly controlling these parameters solubility can be improved. Min Fan et al. improved the solubility of chitosan compared to that of chitin and commercial chitosan by using a process of

ice-thawing and deacetylation in an aqueous solution of lithium hydroxide and urea (Fan et al. 2009).

Another way to increase the solubility of chitosan in water is to change its molecular weight. Chito-oligosaccharides obtained by depolymerization have increased solubility in water and antimicrobial, anti-inflammatory, neuroprotective, antioxidant, etc. (Ojagh et al. 2010).

There are studies showing that the derivative of chitosan with polyethylene glycol increases its solubility in water, but polyethylene glycol can prevent the active groups of chitosan from being prevented, thus blocking its intrinsic properties (Anitha et al. 2011). Meenakshi Malhotraşi et al. modified the method of preparation of chitosan nanoparticles grafted with polyethylene glycol, protected the amino groups in position C2 of chitosan, by the use of phthalic anhydride, and for the etherification reaction they used sodium hydride. Tests on neural cells have suggested that nanoparticles are nontoxic and have the ability to carry genes (Kim et al. 2013).

Solubility may also be increased by chemical reticulation with glutaraldehyde, formal aldehyde, sodium tripolyphosphate, vanillin, or other reticulation chemical agents. The use of vanillin as a reticulation agent is beneficial because, unlike other chemical agents, it is not toxic to organism (Benhabiles et al. 2012). Reticulation of chitosan with glutaraldehyde leads to the production of toxic chitosan hydrogels that limit their medical use. By replacing glutaraldehyde with monoaldehyde, e.g. cinamil-imina, biocompatible hydrogels of cinamil-imino-chitosan are obtained, which absorb large amounts of water and encapsulate large amounts of the medicinal substance (Craciun 2018).

Another advantageous method of increasing water solubility is by introducing the carboxymethyl grouping into the structure of chitosan and increasing pH to neutral or alkaline, without influencing its other characteristics. So O-carboxymethyl chitosan obtained is an amphiprotic, nontoxic, biodegradable, biocompatible compound with antimicrobial and antifungal properties (Ahmed and Ikram 2016).

4.4 Mucoadhesion

Chitosan is bioadhesive and easily binds to negative loaded surfaces such as mucous membranes (Puvvada et al. 2012). Chitosan mucoadhesion is the property that is taken into account in oral forms of administration to solve bioavailability problems. The stay of the drug in the body can be prolonged if the drug has adhesion to the membranes (Hanif et al. 2019). Increasing the stay time of chitosan-based medicinal products systems at places of application or absorption is advantageous, as it prolongs the action of the active substance and ensures the sustained release of the drug substance (Ways et al. 2018).

Mucoadhesion is carried out in two stages, by binding to mucin glycoproteins through weak ion bonds (Hanif et al. 2019; Roy et al. 2009):

– wetting of the polymer and the mucosa membrane when contact with mucin is initiated and
– activation and plasticization of the mucoadhesive system under the influence of moisture, allowing mucoadhesive molecules to release.

Chitosan intensifies penetration by opening junctions of the intestinal epithelium (Wu et al. 2005), making possible both paracellular and transcellular transport (Mohammed et al. 2017). The mucoadhesion of chitosan (Pusateri et al. 2003) is due to the presence of protonable amino groups in its structure, which in acidic medium are positively charged and interact with negatively charged sialic acid (Croisier and Jérôme 2013).

Claus-Michael Lehrsi et al. tested mucoadhesive properties in various polymers and observed that chitosan has superior mucoadhesion to hydroxypropyl cellulose and carboxymethyl cellulose (Lehr et al. 1992). For a polymer to have mucoadhesive properties it must have large cohesive properties. In chitosan, cohesion is quite poor, but can be improved by forming complexes with anionic compounds (Elgadir et al. 2015) or by obtaining hydrophilic thiomers, with free thiol groups, capable of forming intra- and intermolecular disulfuric bonds within the polymeric network. This greatly increases the cohesion and stability of drug distribution systems (tablets, micro and nanoparticles, hydrogels, etc.). Thiomers make covalent, strong bonds with mucus glycoproteins. Into the thiomers class are included: iminotiolan-chitosan, chitosan-thioglycolic acid, chitosan-tioethylamine, etc. (Shah et al. 2017).

4.5 Pseudoplastic Properties

Due to its structure, chitosan together with his derivatives can be used in the preparation of hydrogels (Ahmed 2015; Badwan et al. 2015). Hydrogels are prepared from macromolecular substances, forming three-dimensional reticulated networks, being, soluble or not in water, hydrophiles, but which swell in water, due to the ability to absorb water (Ahmadi et al. 2015; Huang et al. 2017; Muț et al. 2018).

Hydrogels intended for topical application have many advantages: the effect is located at the site of application, allows the use of drug substances with short half-life or which if administrated orally have poor absorption and low bioavailability. The main advantage of topical administration of drugs that have increased oral toxicity is the concentration of the drug substance in the affected area without producing adverse effects on other organs. The benefits of topical formulations can also be given by the properties of the excipients used: biocompatibility, biodegradability, non-irritability, innocuity, and low cost (Anitha et al. 2009; Iurciuc-Tincu et al. 2020; Muț et al. 2018; Popovici and Lupuleasa 2017). The therapeutic efficacy of a topical formulation depends on both the nature of the vehicle and the physicochemical properties of the active substance (Bharat et al. 2003).

Chitosan has a high molecular weight, has pseudoplastic properties being a good viscosity-raising agent in the acidic environment. Chitosan hydrogels have the

ability to absorb water, to present themselves in the form of three-dimensional, hydrophilic reticulated networks (Anitha et al. 2009; El-Leithy et al. 2010). The release of the medicinal substance from the hydrogel is carried out by means of diffusion and swelling control processes (Agnihotri et al. 2004). The release of the active substance from the gel matrix is rapid, especially for hydrophilic medicines, due to the high water content of the hydrogel (Aiedeh et al. 1997).

Hydrogels made up of natural polymers and hydrophiles such as chitosan can be frequently used as ointment bases, in the formulation of dermatological and cosmetic preparations. Chitosan-based hydrogels have been extensively studied over the past decade and have attracted attention due to the benefits of their specific characteristics: biocompatibility, low toxicity, biodegradation, hydrophilic character, the presence of amino groups and cationic character, these characteristics make chitosan a perfect polymer for successful production of hydrogels or other pharmaceutical forms (Huang et al. 2017; Muț et al. 2018). Hydrogels generally have a higher degree of permeability than creams and ointments. The vast majority of drugs incorporated in hydrogels do not pass through the skin, but act locally, on the surface of the skin or throughout the epidermis. Hydrogels have good viscosity, satisfactory bioadhesion and do not irritate or sensitize the skin (Bharat et al. 2003). Chitosan hydrogel can be used for the preparation of controlled-release gels, on the skin of drug substances or for the preparation of gels intended for application to the oral or rectal mucosa (Popovici and Lupuleasa 2017).

Chitosan can be successfully used together with gelatin and glycerol phosphate when obtaining thermosensitive hydrogels with controlled release of latanoprost to treat glaucoma in rabbits. Intraocular pressure decreased significantly over 8 days and then remained within normal limits (Cheng et al. 2014).

By modifying the structure of the chitosan with the catechol Xu J. et al. obtained a derivative of catechol-chitosan which they reticulated with genipine (genipine is a nontoxic reticulation agent). As a model drug substance they used lidocaine. In vivo studies have shown that this hydrogel adheres to the oral mucosa of the rabbit, and lidocaine was detected in the rabbit serum at a concentration of 1 ng/ml. It has also been shown that hydrogel did not cause inflammation of the rabbit's oral mucosa. So, this study demonstrated that chitosan can be used for the production of mucoadhesive and biocompatible hydrogels intended for the administration of drug substances on the oral mucosa (Fonseca-Santos and Chorilli 2018; Xu et al. 2015).

Diclofenac/chitosan hydrogel has been shown to be used successfully on the rectal mucosa without producing irritation. Encapsulation of diclofenac in chitosan microspheres before its incorporation into the hydrogel administration system is effective because it reduces irritation in the rectal mucosa (El-Leithy et al. 2010).

The mechanism of hydrogel formation is carried out by physical processes involving reversible interactions between polymers or chemicals where polymers are formed established by irreversible interactions (Ahmadi et al. 2015; Croisier and Jérôme 2013).

The ability to form gels may or may not be influenced by pH and temperature. The inflating properties of chitosan are dependent on pH so the rate of its swelling

decreases sharply with the increase in pH (Popovici and Lupuleasa 2017). Temperature can also influence the physicochemical and rheological properties of the gel, so Jaepyoung Cho et al. studied the structure of chitosan gel formed at high temperature. Obtained structure was partially thermoreversible at low temperature. The increase in temperature did not influence the pH values of the system, but increased conductivity. Increased ion strength, in the presence of glycerophosphate, increased the hydrophobic effect, creating favorable conditions for the formation of gel (Cho et al. 2005). Salicylaldehyde is a nontoxic, temperature-sensitive reticulation agent that reticulates with chitosan forms salicylaldehyde/chitosan hydrogels with antimicrobial and antifungal properties. This hydrogel has good mechanical and thixotropic properties and rapid swelling capacity (Iftime et al. 2017).

The ability to form the gel can be influenced by other polymeric macromolecules which by interpenetration with chitosan form the gel. New studies have shown that chitosan can be used successfully to obtain semi-permanent networks of chitosan and polyethylene oxide. The hydrogel obtained has an increased swelling capability, but it can be modified according to the mass fraction and molecular mass of polyethylene oxide (Popovici and Lupuleasa 2017).

5 Safety of the Chitosan Usage in Pharmaceutical Practice

Chitosan has biological properties, for example it is biodegradable, biocompatible, and safe for human body. By chemically modifying the fundamental skeleton of chitosan, it is derived from biodegradable, biocompatible, and safe derivatives that can be used in the pharmaceutical and biomedical industries (Ahmed and Ikram 2015; Raafat and Sahl 2009). Chitosan can be easily degraded by a large number of enzymes and degradation products are nontoxic. Chitosan is biocompatible with living tissues and does not cause allergic reactions. Under action of ferments chitosan degraded, forming different oligosacgharides, which are completely absorbed by human body (Agnihotri et al. 2004). Degradation of chitosan to oligosaccharides can be achieved by: ionizing radiation, ultrasound, acid hydrolase, oxidoreductive and enzymatic degradation (Aranaz et al. 2009).

Chitosan is well tolerated by the human body, does not cause local side effects (skin, on the eye membrane, and nasal) or unwanted systemic effects. This property is very important when it is intended to be used as an implantable biomaterial (Raafat and Sahl 2009) or in various applications of the medical field (Raafat and Sahl 2009). Both biodegradation and biocompatibility depend on the degree of deacetylation of chitosan, when the degree of deacetylation varies between 50 and 60% its biodegradation is disrupted and when the degree of deacetylation is more than 90% it can be used for anticancer applications (Ramasamy et al. 2017).

Compared to other natural polysaccharides, chitosan has lower toxicity (Agnihotri et al. 2004). In vivo toxicity studies have shown that ORAL LD = 50 (lethal dose) in mice is 16 g/kg body/day, higher than sodium chloride (Fang and Bhandari 2010) or sucrose and is contraindicated for people allergic to shellfish

(Raafat and Sahl 2009). Kirk H. Waibesi et al. have proved its possible local administration of hemostatic bandages based on chitosan to people allergic to shellfish. All patients who had a history of shrimp or clam allergy did not show positive results when testing skin diseases, chitosan powder, nor did they experience any adverse reactions while wearing the bandage (Waibel et al. 2011).

6 Antimicrobial Activity

Studies show that even if it has low oral toxicity, special attention should be paid when used for long periods of time in order not to cause disturbance of the intestinal microflora (Raafat and Sahl 2009). Sarbani Dey Ray pointed out in his study that systemic exposure to chitosan should be limited, requiring thorough studies of its safety as an excipient in parenteral preparations (Ray 2011).

In culture media, chitosan has antimicrobial activity on bacteria: *Staphylococcus aureus, Listeria monocytogenes, Pseudomonas aeruginosa, Escherichia coli, Shigella dysenteriae,* and *Vibrio cholerae* (Martínez-Camacho et al. 2010). The action may be influenced by the type, molecular mass, degree of polymerization of chitosan, microbial organism, and environmental and pH conditions (Hafdani and Sadeghinia 2011).

Chitosan in free polymer form exhibits antifungal activity that can be influenced by molecular mass, degree of substitution, concentration, mushroom types, and types of functional groupings of chitosan derivatives chains. Therefore, chitosan exhibits natural antimycotic activity without the need for chemical changes (Ing et al. 2012).

Antimicrobial action can be increased by associating chitosan with essential oils, which have phenolic groups in their structure and which, in turn, exhibit antibacterial action (essential oils of cloves, thyme, rosemary, sage, cinnamon) (Szymańska and Winnicka 2015).

7 Uses of Chitosan in the Pharmaceutical Field

Chitosan is used in various dietary supplements for weight loss, because many studies made on animals and humans have reported that it reduces both body weight and cholesterol, it has the ability to bind and to absorb fat in the digestive tract and it has characteristics similar to dietary fiber because digestive enzymes are not able to degrade it (Jin et al. 2017; Neyrinck et al. 2009). It is currently only marketed for weight and cholesterol reduction (Batista et al. 2018).

Chitosan belongs to the category of biodegradable polymers with beneficial and safe properties for the body (Goycoolea et al. 2016; Kashyap et al. 2015; Wang et al. 2018; Yuan et al. 2015). The most important characteristics of a biopolymer when it is intended for use in the pharmaceutical or medical field are: biocompatibility and biodegradability, antimicrobial activity, nontoxic and non-carcinogenic, ensuring

the delivery of medicinal products to the target, being economical and harmless (Gopi and Amalraj 2016; Abdul Khalil et al. 2016; Pérez-Recalde et al. 2018; Periayah et al. 2016; Yadav et al. 2015; Yuan et al. 2015).

Chitosan unlike many synthetic polymers is not immunogenic, does not interact with the active substance and can be used in various oral preparations as an excipient (George and Abraham 2006). It can be used for the administration of colon-acting medicines in combination with other polymers, when obtaining chitosan microspheres that have a lower density than gastric fluids and which float in the stomach without affecting the rate of emptying of the stomach for a long period of time, when obtaining tablets, granules, gels, emulsions, microspheres, microcapsules, nanoparticles (Jain and Tiwari 2011; Jayakumar et al. 2010a; Kaushik et al. 2015; Sheskey et al. 2017). Zeeshan Ahmed et al. were able to design chitosan nanoplates on which they inserted various fatty acids (oleic acid, palmitic acid, stearic) and α-cyclodextrin (Ahmed et al. 2018). The antifungal effect of nanoplatelet (oleoyl-chitosan and α-cyclodextrin) with amphotericin B deoxycholate was also highlighted. Studies in vivo and in vitro demonstrated complete healing of the vaginal mucosa of mice infected with *Candida albicans* and *Candida glabrata* (Grisin et al. 2017a, b).

Chitosan can be used as a pharmaceutical excipient because it "has technological-pharmaceutical and biopharmaceutical qualities superior to microencapsulation materials" (Popovici and Lupuleasa 2017).

In the pharmaceutical field, chitosan use of excipient for the production of vector systems of generation I and II medicinal products (transport and target disposal drugs) is studied. In these vector systems the active substance is dispersed into the biodegradable auxiliary substance which is intended to protect the drug substance from degradation and ensures its controlled release from the system (Hamed et al. 2016a; Ines et al. 2008; Popovici and Lupuleasa 2017; Puvvada et al. 2012).

Microparticles and nanoparticles belong to the category of vector sites of drugs. Microparticles are spherical polymer particles, in which one or more drug substances can be dispersed and have a diameter of between 1 and 1,000 μm. Nanoparticles are small colloidal particles, second generation vector therapeutic systems, with a diameter of 10–10,000 nm (Popovici and Lupuleasa 2017; Tiyaboonchai 2003; Zimmer and Kreuter 1995).

Microparticle systems are graded by structural characteristics in microspheres and microcapsules, and nanoparticle systems in: nanospheres or nanopellets and nanocapsules (Popovici and Lupuleasa 2017).

Structure of micro- and nanocapsules is of the tank type, in which active substance particles may be present in liquid or solid state (in microcapsules), dissolved or suspended in a watery or oily environment (at nanocapsules), covered with a polymer membrane (Popovici and Lupuleasa 2017; Tiyaboonchai 2003; Zimmer and Kreuter 1995).

Structure of micro- and nanospheres is of a monolith type (made up of heterogeneous particles), in which the active substance is dissolved or dispersed in the natural/synthetic polymeric matrix, matrix type, without cavities and without distinct walls in which the medicinal substance is included in the polymeric matrix (at nanospheres) (Popovici and Lupuleasa 2017; Tiyaboonchai 2003; Zimmer and Kreuter 1995). Advantages of microspheres usage are presented in Fig. 4.

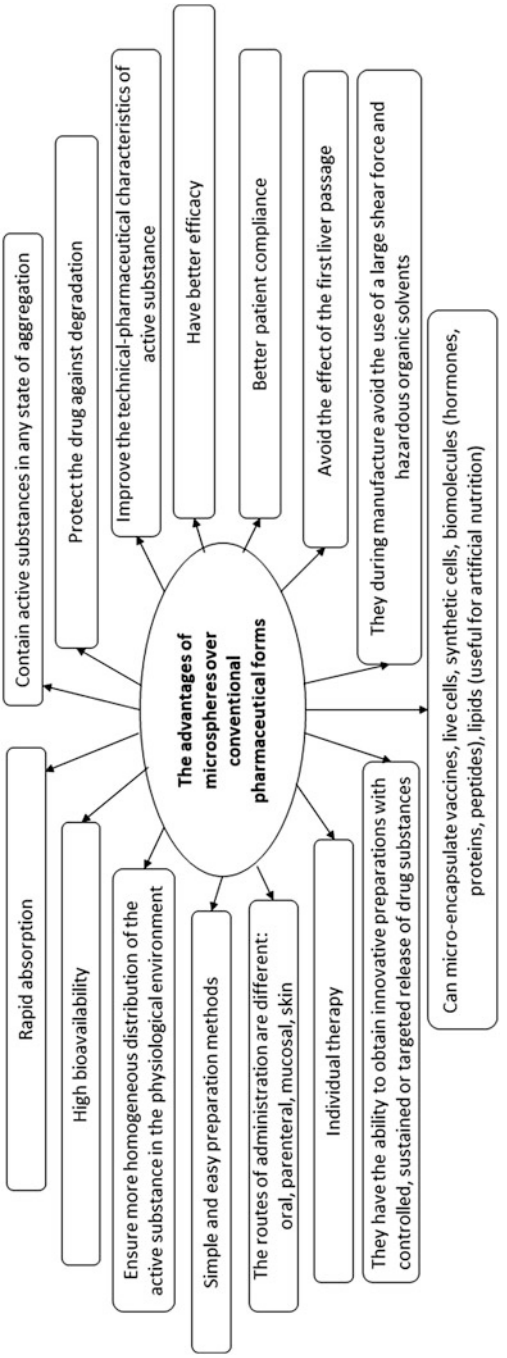

Fig. 4 The advantages of microspheres over conventional pharmaceutical forms (Dev et al. 2010; Lengyel et al. 2019; Popovici and Lupuleasa 2017)

Chitosan can be used as a microencapsulation material in the development of microparticles because in comparison with synthetic polymers it has several properties (Popovici and Lupuleasa 2017).

Microencapsulation method has many advantages and is one of the most important forms of controlled release of drug substances, so by microencapsulation the unpleasant taste and smell of some active substances (furantoin) can be masked and the adverse effects and toxicity of drug can be minimized by obtaining a form of oral dosing (microcapsules) with sustained (Hari et al. 1996), slow and controlled (Bakry et al. 2016) release.

The nanoparticles are intended to ensure the delivery of the drug substance to the target and exhibit almost all the properties of liposomes, including particle size (Wu et al. 2005). Advantages of usage of nanoparticles over liposomes, as drug carriers are presented in Fig. 5.

Factors affecting the formation of nanoparticles, their size and surface load are: molecular mass and degree of deacetylation of chitosan. Effectiveness of the capture of the drug is dependent on p*K*a and the solubility of the drugs caught (Agnihotri et al. 2004; Ines et al. 2008; Popovici and Lupuleasa 2017; Tiyaboonchai 2003). Advantages of usage of nanoparticles over microspheres, as drug carriers are presented in Fig. 6.

In microparticles and nanoparticles chitosan can be used as drug delivery substance for: antibiotics, sulfamides, antiparasitic substances, chemotherapy, cytostatics, antivirals, nonsteroidal and steroidal anti-inflammatory agents, antiasthmatics, peptides

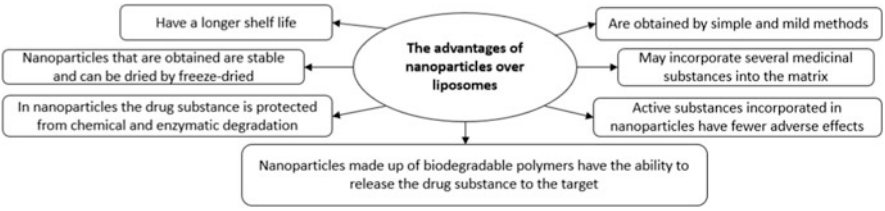

Fig. 5 The advantages of nanoparticles over liposomes (Popovici and Lupuleasa 2017; Wu et al. 2005)

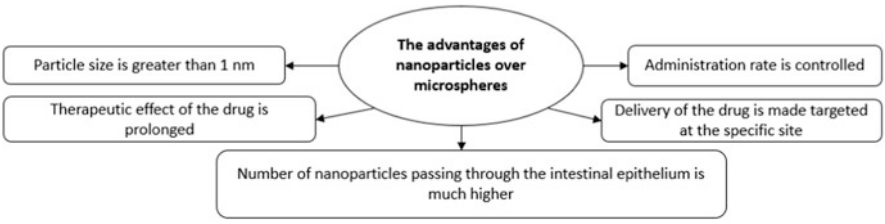

Fig. 6 The advantages of nanoparticles over microspheres (Wu et al. 2005)

and proteins, nucleic acids, calcium channel blockers, beta blockers, fat-soluble vitamins, etc.

Chitosan is a natural, hydrophilous, and biodegradable auxiliary substance, a constituent of the wall of microcapsules and nanocapsules or the mold of microspheres and nanospheres (Popovici and Lupuleasa 2017).

It is very important to know the physicochemical properties of chitosan when obtaining micro or nanoparticles in order to avoid interactions between the active substance and chitosan, to obtain pharmaceutical forms with the desired characteristics and to choose the correct method of obtaining. Chitosan-based microparticles and nanoparticles can be administered orally in various pharmaceutical forms (tablets, capsules, suspensions) with modified, parenteral, mucous, and dermal release (Popovici and Lupuleasa 2017).

Methods by which micro and nanoparticles are obtained are vast and have developed a lot in recent years and this paper has focused on the following: chemical or thermal crosslinking method, ionotropic gelation method, solvent-based methods (solvent injection and solvent evaporation method), methods based on interfacial polycondensation reactions, coacervation (simple and complex), spray method (aerosols) with drying and spraying with solidification, extrusion and spheronization (Ines et al. 2008; Popovici and Lupuleasa 2017; Tiyaboonchai 2003).

8 Methods Used to Obtain Particulate Systems Based on Chitosan

8.1 Chemical or Thermal (Crosslinking) Denaturing Method

The process used for preparation of nano/microparticles is presented schematically in Fig. 7.

Mechanism of the method of chemical crosslinking of chitosan with glutaraldehyde is based on the conjugation of the free amino group of chitosan with glutaraldehyde (Agnihotri et al. 2004). Crosslinking with glutaraldehyde leads to the formation of a stable H/L emulsion. Removal of the organic solvent is done by evaporation at low temperature, the surfactant excess by precipitation with $CaCl_2$, and precipitate by centrifugation. The final nanoparticles freeze (Ines et al. 2008; Tiyaboonchai 2003). During emulsion formation, the size of the final particles, the degree of crosslinking of the agent used, and the mixing speed must be checked (Agnihotri et al. 2004).

In Table 2 are presented some examples of different products obtained from chitosan using different crosslinking methods. At the same time are depicted the practical advantages presented by produced compounds.

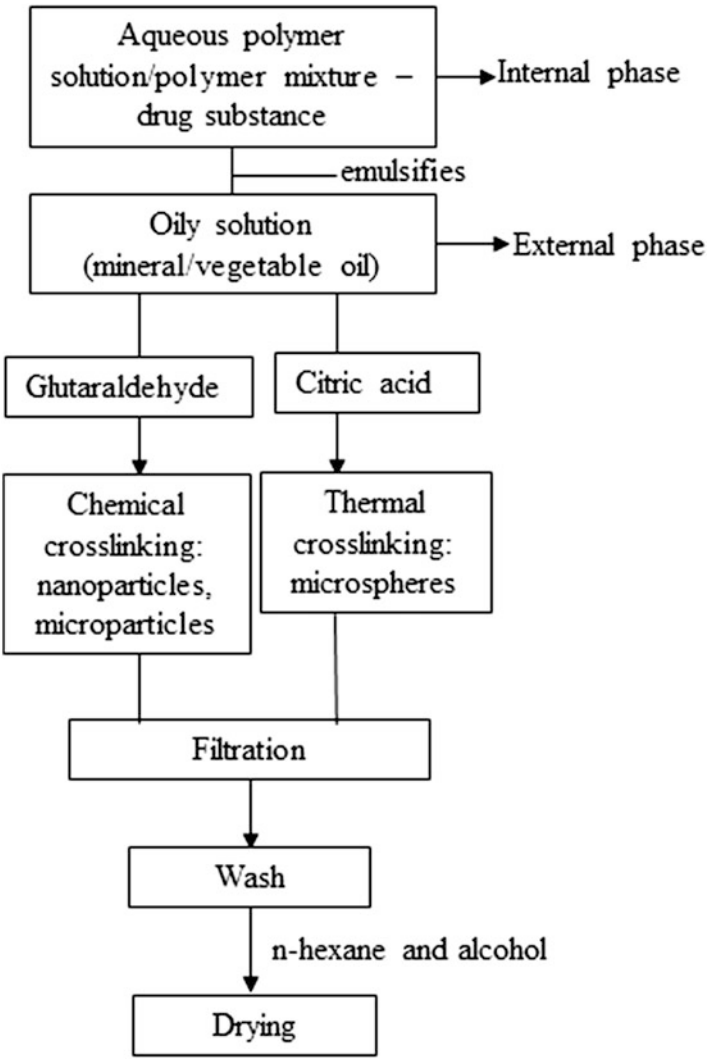

Fig. 7 Preparation of micro and nanoparticles by chemical or thermal denaturation (Agnihotri et al. 2004; Popovici and Lupuleasa 2017)

8.2 Ionotropic Gelation Method

It is a simple, reversible, easy to achieve method of physical reticulation,that avoids side effects, the use of toxic reagents, and is carried out by electrostatic interactions compared to the chemical crosslinking method with glutaraldehyde. The complexing reaction occurs between the positive amino groups of chitosan and the negative

Table 2 Some examples of products obtained by chemical or thermal (crosslinking) denaturing method

Pharmaceutical forms	Incorporated substance with chitosan	Advantages
Nanoparticles	– Propolis nanoparticles by gelation chitosan with Arabic gum and crosslinking with glutaraldehyde (Cavalu et al. 2019)	– Have improved bioavailability, stability at encapsulation and a controlled release of propolis from the polymer matrix into gastric juice and simulated intestinal fluids
Microspheres	– Theophylline, griseofulvin, and acetylsalicylic acid, by reticulation with glutaraldehyde. An aqueous dispersion of chitosan acetate was prepared in paraffin oil, using dioctyl sulfosuccinate as a surfactant (Thanoo et al. 1992)	– Crosslinking density, particles size, active substance may influence the release rates of active substance from the microspheres – Studies in vitro on release rates of microencapsulated active substances have shown that it occurs after a zero-order kinetics – They may be useful for intravenous administration of chemotherapy agents as well as in chemoembolization.
Microspheres	– Crosslinking of chitosan with glutaraldehyde (Shah et al. 2009)	– Increase resistance to chemical degradation of chitosan, but may confer toxicity – This can be reduced by using another, less toxic crosslinking agent, e.g. tripolyphosphate
Microspheres	– Citric acid is used as crosslinking agent in preparation of microspheres by thermal crosslinking. Citric acid is added to an aqueous solution of chitosan that is solubilized into acetic acid. The crosslinking solution of chitosan is cooled and then added to corn oil, maintained at 120°C, under vigorous shaking for 40 min and then filter. Obtained microspheres shall be washed with diethyl ether and dried (Ines et al. 2008)	– To obtain the microparticles with specific properties and performances

groups of tripolyphosphate anion, according to Fig. 8 (Dev et al. 2010; Hashad et al. 2016; Koukaras et al. 2012; Nagpal et al. 2010).

In Table 3 are presented some examples of different gelation methods used for different particle preparation.

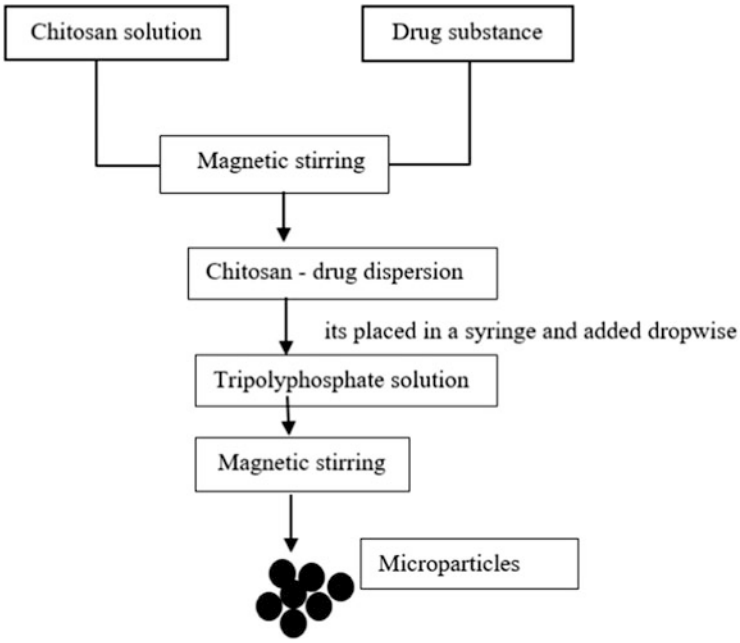

Fig. 8 Preparation of chitosan–tripolyphosphate microparticles by ionotropic gelation (Ines et al. 2008; Mitra and Dey 2011; Tiyaboonchai 2003; Tiyaboonchai and Ritthidej 2003)

8.3 Methods Based on the Use of Solvents

The methods are used to obtain pharmaceutical forms from drug substances with unstable molecules or with bioavailability problems, but due to the toxicity of some solvents used, the methods have limited use.

An advantage of this method is the low temperature at which work is carried out, beneficial for the processing of thermosensitive substances. These methods are divided into: solvent injection and solvent evaporation (Nair et al. 2012).

8.3.1 Solvent Injection Method

This method uses water-miscible (ethanol) or water non-miscible (ether) solvents that are injected into the hydrophile phase (Popovici and Lupuleasa 2017).

The method of injection with miscible solvents with water consists of dissolving/dispersing the drug substance and the lipid substance into an organic solvent miscible with water (ethanol, acetone, isopropanol, etc.) then this solution/dispersion is injected by a needle of a syringe into the water, under shaking. When it comes into contact with water, lipid substances precipitate in the form of nanoparticles by capturing the drug substance. The size of the nanoparticles can be controlled by taking into account: type of lipid, surfactant, solvent and viscosity of the external

Table 3 Some example of ionotropic gelation method

Pharmaceutical forms	Incorporated substance with chitosan	Advantages
Microparticles	– When preparing chitosan microparticles tripolyphosphate is used as an ion crosslinking agent. The dimensions of microparticles (500–710 mm), lead to a 90% increase in the efficiency of felodipine encapsulate (Ko et al. 2002)	– The low pH and high concentration of the tripolyphosphate solution cause the slow release of felodipine from microparticles
Nanoparticles	– The bovine serum albumin can be incorporated into controlled-release nanoparticles if they are between 200 and 400 nm in size (Hong-liang et al. 2010)	– Various drug substances, even proteins and peptides, can be incorporated into nanoparticles – The spherical shape with a smooth surface was demonstrated by in vitro tests
Nanoparticles	– It was incorporated venlafaxine hydrochloride into a suspension of chitosan nanoparticles by crosslinking with tripolyphosphate and coated with polyethylene glycol (Shah et al. 2009)	– Polyethylene glycol is used as a coating material because it is a safe and non-toxic compound that helps the drug substance to be transported to the target in the body – Polyethylene glycol allows the medicinal substance to be put into circulation for longer in physiological fluids and makes the nanoparticle suspension stable for more than 6 months at room temperature
Hydrogel pearls	– Chitosan with polyethylene glycol by crosslinking with tripolyphosphate, using diclofenac sodium as a drug substance (Buranachai et al. 2010)	– The ionotropic crosslinking process with sodium tripolyphosphate leads to encapsulation levels – An efficiency of more than 90% while maintaining a prolonged release of the drug substance (8 h)
Nanoparticles	– Chitosan particles with metal ions, in particular Cu^{2+}, Zn^{2+} using tripolyphosphate (Du et al. 2009)	– In vitro studies have demonstrated that they increase the antimicrobial properties of chitosan

phase (Schubert and Müller-Goymann 2003). This technique mainly uses physiological lipids, which are in solid condition at room temperature and surfactants for emulsification (Popovici and Lupuleasa 2017).

Rahul Nair et al. made an aqueous suspension of solid lipid nanoparticles (SLN) of chitosan and carbamazepine, using the technique of injection with organic solvent (ethanol) using tristearin and phospholipon R as lipid substances and tween 80 as surfactant. The obtained solid lipid nanoparticles of carbamazepine showed high encapsulation efficiency, high physical stability and ensured a controlled release of carbamazepine for a long period of time. The advantage of this method is that physiological lipids are used so we can avoid the use of toxic organic solvents in the preparation process and the scope is vast (cutaneous, oral, intravenous) (Nair et al. 2012).

This method can also be prepared by lecithin nanoparticles using insulin as an active substance. Insulin is a hydrophile substance with a high molecular weight, which must first undergo a physical complexation with phospholipids (e.g., lipoid S75) in order to increase its permeability through the mucous membranes, stability to the enzymatic degradation and to increase its solubility in ethanol so that it can be successfully encapsulated in lecithin chitosan nanoparticles. Insulin encapsulation in chitosan multilayer nanoparticles improved insulin release after oral administration to diabetic rats by 5%, lowering blood glucose levels (Liu et al. 2016).

8.3.2 Solvent Evaporation Method

Micro/nanoencapsulation by solvent escape technique is rapid, helps to obtain small and porous microspheres. This method also has some disadvantages: the organic solvent is responsible for the occurrence of the phenomenon of agglomeration or aggregation of microparticles, large amounts of active substance may be lost in the aqueous phase, and crystals of the drug substance can form on the surface of the microparticles (Popovici and Lupuleasa 2017).

- Microparticles are obtained by dispersing the polymer and the drug substance into a volatile or partially miscible organic solvent with water. The emulsions to be prepared are type O/W for lipophilic drug substance or type W/O/W for hydrophilic drug substances (Trotta et al. 2003).
- If the active substance is hydrophobic, e.g. partially soluble/insoluble in the organic solvent used: the active substance and chitosan is dissolved in a volatile organic solvent and then the solution obtained is added to an aqueous phase in which the polymer is not soluble and contains the emulsifier, under shaking to obtain an O/W emulsion. Continue shaking at a lower speed than at the previous stage to allow evaporation of the solvent from the drops of the internal phase of the emulsion and to allow the formation of microparticles which are suspended in the aqueous phase. Then the microparticles are separated by filtration.
- If the active substance is hydrophile, e.g. soluble in the organic solvent used: the aqueous solution of the active substance is emulsified in the first stage in the volatile organic solvent solution in which chitosan is dissolved. In the next stage the previously obtained W/O emulsion shall be emulsified with a large quantity of aqueous phase which may or may not contain a surfactant resulting in a W/O/W emulsion which is subjected to agitation for evaporation of the volatile solvent, separation by centrifugation several times and then freeze-off (Mohammed et al. 2017; Popovici and Lupuleasa 2017).

Microspheres containing metformin have been prepared by the solvent evaporation method, using a non-aqueous solvent (acetone). The active substance and polymer were mixed with acetone resulting in a suspension. The suspension was then introduced into paraffin oil, under shaking at room temperature, stirring for 4 h to allow the solvent to evaporate. The formed microspheres are repeatedly washed with ether until all the oil is removed and then dried at room temperature (Garud 2012).

8.4 Microencapsulation by Interfacial Polycondensation Reactions

This method involves the condensation of two complementary polymers, each soluble in one of the phases of the biphasic system which consists of two non-miscible liquids. The polycondensation reaction occurs at the interface of the two phases. The formation of dispersion is carried out in the presence of an appropriate stabilization agent.

Steps in the process of obtaining microparticles:

- initial polycondensation
- formation of the primary membrane
- deposition of oligomeric molecules on primary membranes and strengthening the final wall of microcapsules (Popovici and Lupuleasa 2017).

K. Aiedeh et al. incorporated insulin into chitosan microcapsules by interfacial reticulation with ascorbyl palmitate. Chitosan is a hydrophilic polymer and ascorbyl palmitate is the fat-soluble form of vitamin C, which has amphiphilic properties that allow it to be disposed at the H/L emulsion interface. The aqueous solution of chitosan containing insulin is added to 50 ml of paraffin oil. The obtained A/U emulsion is shaken at room temperature 15 min after which 25 ml butanol and 1 g ascorbyl palmitate are added. Continue stirring for another 24 h at room temperature, after which the microcapsules are separated by centrifugation, washed with diethyl ether, and desiccated at constant weight. This method is suitable to produce biodegradable controlled-release systems, as it avoids alteration of the physicochemical characteristics of drug products (Aiedeh et al. 1997).

8.5 Coacervation

Coacervation is a physicochemical process (Agnihotri et al. 2004) "which consists of separating the dispersions of macromolecular colloids into two liquid phases, one of which contains most of the colloidal dispersed phase, and the other is devoid or very poor in colloidal particles" (Popovici and Lupuleasa 2017).

Coacervation is a physicochemical process (Agnihotri et al. 2004) that consists in separating the initial dispersion of one or more macromolecular hydrocolloids into two liquid phases, where a phase containing the largest amount of colloidal particles will surround the active substance and will form the coacervate, and the other phase is missing or very poor in colloidal particles (Kailasapathy 2009). Liquid substances (in the form of emulsion), solids (in suspension form) or live cells may be included in microparticles, provided that the active substances are insoluble or very little soluble in the coacervation medium and are compatible with the polymer used in microencapsulation.

Coacervation according to the factor that determines desolvation is of two kinds: simple or complex coacervation (Agnihotri et al. 2004).

In simple coacervation, the desolvation can take place by

- addition of electrolytes/salting
- addition of non-solvent in the colloidal solution of the polymer
- changing osmotic reports
- change pH
- temperature change (Popovici and Lupuleasa 2017).

Simple coacervation (salting) can obtain microspheres as follows: the particles of drug substances are dispersed in a low pH chitosan solution. A solution with a high pH of 8.5–9.0 ammonium hydroxide is added to the colloidal system in order to form the coacervation drops which are then adsorbed to the surface of the particles of the drug substance. The process of formation of microspheres is carried out under shaking, with high mixing speed, to avoid sticking and forming agglomerates and filtration. This method can produce microparticles up to 10 nm in size (Mohammed et al. 2017; Sahil et al. 2011).

In complex coacervation, desolvation is carried out by the interaction between two polymers incompatible with sodium alginate as it is shown in Fig. 9, carboxymethyl cellulose, gelatin or polyethylene glycol, etc., with different electrical loads (Popovici and Lupuleasa 2017). By this method it is possible to prepare microcapsules (Tiyaboonchai 2003; Tiyaboonchai and Ritthidej 2003), microspheres (Basu et al. 2011), hydrogels (El-Leithy et al. 2010), microparticles (Aelenei et al. 2009).

Some examples of pharmaceutical forms obtained by coacervation are given in Table 4.

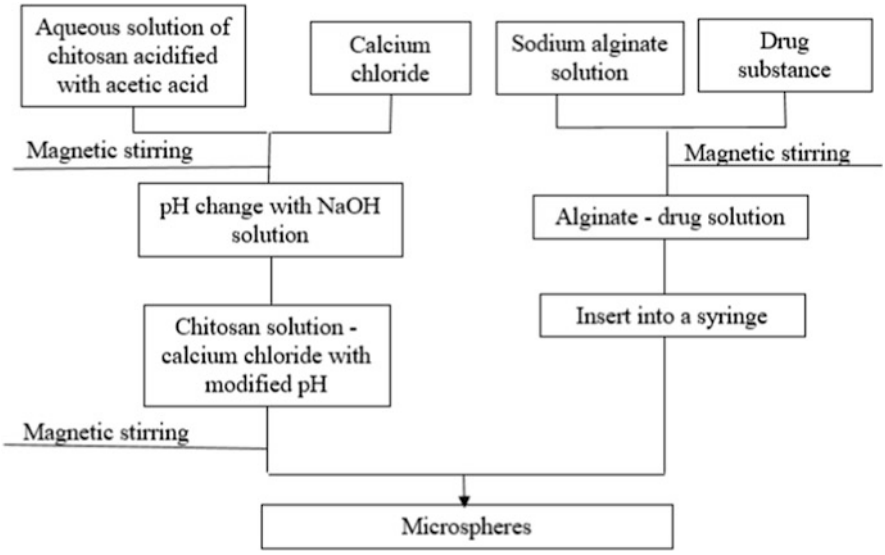

Fig. 9 Procedure for preparing chitosan microspheres by complex coacervation with sodium alginate (Li et al. 2008)

Table 4 Some examples of products obtained by coacervation

Pharmaceutical forms	Incorporated substance	Advantages
Microparticles	– Curcumin and polysaccharides of chitosan with gellan and carrageenan (Fang and Bhandari 2010; Iurciuc-Tincu et al. 2020)	– A controlled release of curcumin from the polymeric matrix – Could be administered orally without gastric juice degrading or influencing the activity of curcumin – Increase the plasma half-life – Bioavailability of curcumin in vivo and in vitro
Microcapsules	– Indomethacin in two colloids chitosan and carboxymethyl cellulose and glutaraldehyde to facilitate the strengthening of microcapsules of chitosan–carboxymethyl cellulose (Tiyaboonchai 2003)	– They increase the patient's acceptance – Reduce adverse effects, plasma concentration fluctuations and frequency of dosages
Complex microspheres	– Tramadol hydrochloride using chitosan and gelatin B (Basu et al. 2011)	– It increases the patient's compliance and helps to overcome the shortcomings of tramadol
Microparticles	– Tannic acid incorporated in chitosan (Aelenei et al. 2009)	– The particle size obtained is much smaller, on the order of nanomers, the incorporated tannic acid is much larger than in the other two samples and has a much better thermal stability
Nanoparticles	– Chitosan by complexation with alginates, cellulose, pectin, carrageenan, acrylic acid, etc. (Buranachai et al. 2010; Ines et al. 2008; Tiyaboonchai 2003)	– The size of the complexes formed can vary between 50 and 700 nm
Microcapsules	– Amoxicillin powder was suspended in an aqueous solution of sodium alginate with the aid of sodium diethyl-sulfosuccinate used as surfactant (Arora and Budhiraja 2012)	–
Nanoparticles	– Insulin with chitosan complexing with alginate (Sarmento et al. 2007)	– A size adequate for absorption in the gastrointestinal tract to diabetic rats – Oral administration of concentrations of 50 and 100 I.U./kg decreased the level of basal glucose by more than 40%, maintaining the state of hypoglycemia over 18 h
Hydrophilic nanoparticles	– Nifedipine with chitosan and alginate (Li et al. 2008)	– The diameter of the nanoparticles obtained was suitable for absorption in the gastrointestinal tract – The release of nifedipine from nanoparticles is thus pH sensitive in the simulated intestinal fluid (pH 6.8) and in phosphate buffer (pH 7.4) the release was rapid and in the simulated gastric fluid (pH 1.5) it was slow

(continued)

Table 4 (continued)

Pharmaceutical forms	Incorporated substance	Advantages
Microspheres	– Chitosan-controlled alginate-based nano-selenium microspheres (Cavalu et al. 2019; Cavalu et al. 2017)	– The degree of crystallinity was low, which is an advantage for controlled release – The matrix of alginate and chitosan prolongs the release time of selenium being a convenient matrix for the delivery of selenium in the duodenum, cecum, and colon – Following the FRAP analysis, it was shown that alginate potentiated the antioxidant effect of nanoparticles

8.6 Spray Method (Aerosolization) with Drying and Spray with Solidification

The difference between these two techniques is that "in the case of spray drying, the rapid hardening of the microparticles is obtained by the rapid evaporation of the solvent from a stream of hot air," and in the case of the solidification spray process "a thermal solidification is achieved, at low temperature values, of a pulverized molten mass" (Popovici and Lupuleasa 2017).

The principle of this method is to convert a liquid (solution, emulsion, or suspension) into a solid (Popovici and Lupuleasa 2017).

The technological process of spray drying takes place in a continuous flow, in a closed circuit, in a single stage, it is economical, flexible, and adapted to prepare particles with various sizes and shapes: powders, granules, suspensions, or agglomerates (Bhise et al. 2008). Microparticles can be produced, prior to dispersion preparation, to retain their special properties, increased solubility, and ability to target the final product (Vehring 2008).

The preparation technique consists first of dissolving chitosan in an aqueous solution of acetic acid. The solid drug substance is dissolved or dispersed in the polymer solution under rapid stirring and then added to a suitable crosslinking agent. This solution or dispersion is atomized in a stream of hot air. Atomization leads to the formation of small free-flowing particles, from which the solvent evaporates instantly. The quality characteristics of the microparticles obtained depend on "nozzle size, spray rate, active substance/coating ratio, degree of dispersion of the active substance, viscosity of the liquid introduced into the spray system, temperature and size of the drying chamber and collecting channel." The separation of the microparticles from the hot air is done with the help of the cyclone separator, and the removal of the solvent is done by vacuum drying (Table 5) (Agnihotri et al. 2004; Popovici and Lupuleasa 2017; Sahil et al. 2011).

Table 5 Some examples of products obtained by spray method with drying and spray with solidification

Pharmaceutical forms	Incorporated substance	Advantages pharmaceutical forms
Nanoparticles	Calcitonin-loaded chitosan (Sinsuebpol et al. 2013)	– Following intratracheal administration in rats with a nanoparticles, quantitative analyses of plasma and pharmacokinetic parameters obtained demonstrated that the system is able to release calcitonin-loaded chitosan nanoparticles through the deep lung region into the systemic circulation
Microparticles	Ionic gelated chitosan can be used to obtain scopolamine microparticles by spray drying technique (Lee et al. 2013)	– Orodispersible tablets have the advantage of complete dissolution in 45 s – Scopolamine is released more slowly by microencapsulation with chitosan than if it's not encapsulated – Microencapsulation can control the rate of release of scopolamine from orodispersible tablets, the drug is protected from premature destruction and avoids the unpleasant taste given by it
Microparticles	Chitosan microparticles with aloe-vera and vitamin E (Pereira et al. 2014)	– The microparticles obtained have mucoadhesive properties, good adhesion to the burned surface due to the large contact surface (as demonstrated by the in vivo scarring test) and ensure the efficient release of components in the injured area
Microcapsules	Chitosan with aqueous extract of Eugenia dysenterica (Mazutti da Silva et al. 2020)	– The microparticles showed good physical and chemical stability for 60 days in the refrigerator – Increased the penetration of catechol (active ingredient of the plant) through the skin facilitating wound healing

Organic chitosan salts (acetate, lactate, and citrate) are amorphous solids that show better sphericity when subjected to high temperatures of the spray drying process (Cervera et al. 2011). This technique has some advantages when it is desired to mask the unpleasant taste of a drug substance (Bora et al. 2008). Chitosan increases the dispersal and pulmonary deposition capacity (demonstrated in vitro) of micron-sized powders that are spray dried (Li and Birchall 2006).

8.7 Extrusion and Spheronization

It is a physical process used mainly for obtaining pellets (small spherical particles with a diameter of 0.2–2 mm, whether or not covered with films, for internal use, obtained from fine powders or granules) and granules (small agglomerated solid particles with irregular, vermicular, cylindrical, spherical shape, with a diameter of 0.2–4 mm, intended for oral administration) (Popovici and Lupuleasa 2017).

The phases of the technological process for pre-release of controlled-release pellets consist in: mixing active and auxiliary substances to obtain a homogeneous powder mixture, wetting, kneading, extrusion, spheronization, drying, coating, conditioning (Chukwumezie et al. 2002; Popovici and Lupuleasa 2017). The obtaining process is simple and fast (Bhaskaran and Lakshmi 2010).

In the extrusion process to obtain pellets the most used excipient is microcrystalline carboxymethyl cellulose. Despite its advantages: the structure of "fringed crystal aggregates," internal porosity, large surface, its ability to prolong the release of drug substances that are poorly soluble, it has also some disadvantages that limit its usage: does not disintegrate, can be chemically incompatible with the drug substance. In recent years, many polymers have been studied to be used instead of carboxymethyl cellulose for the formulation of pellets: chitosan, starch, pectin, dextran, carrageenan, tragacanth, etc. (Chukwumezie et al. 2002; Popovici and Lupuleasa 2017).

Chitosan has some advantages if used as a coating material in the formulation of microcapsules by extrusion: it increases the microencapsulation of alginate, improves the survival of encapsulated probiotic bacteria and keeps the prebiotic intact when it comes into contact with the contents of the gastrointestinal tract (gastric and intestinal juice). Maria Chavarri et al. incorporated the probiotic medium and quercetin (prebiotic) into a sodium alginate solution, which was previously extruded into the chitosan solution. The beads obtained were then subjected to sieving, washing with sterile distilled water and lyophilization (Chávarri et al. 2010).

9 Conclusions

Currently, more and more attempts are being made to use chitosan as a polymer in the pharmaceutical field to obtain microcapsules, microparticles, microspheres, nanocapsules, nanoparticles, films, hydrogels, etc. The methods by which these pharmaceutical forms are obtained are very complex. In order to obtain the safest, long-term, stable, and effective pharmaceutical forms of chitosan, the stability problems of chitosan must also be taken into account. The chitosan molecule can be included in pharmaceutical formulations because it corresponds to the characteristics of biopolymers used in the pharmaceutical field and the chemical structure of chitosan can be physically or chemically modified under mild conditions, improving it is physical, chemical, mechanical, and biological properties.

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Cell-to-Cell Crosstalk: A New Insight into Pulmonary Hypertension

Yan Zhang and Yun Wang

Contents

Abstract Pulmonary hypertension (PH) is a disease with high pulmonary arterial pressure, pulmonary vasoconstriction, pulmonary vascular remodeling, and microthrombosis in complex plexiform lesions, but it has been unclear of the exact mechanism of PH. A new understanding of the pathogenesis of PH is occurred and focused on the role of crosstalk between the cells on pulmonary vessels and pulmonary alveoli. It was found that the crosstalks among the endothelial cells, smooth muscle cells, fibroblasts, pericytes, alveolar epithelial cells, and macrophages play important roles in cell proliferation, migration, inflammation, and so

Y. Zhang and Y. Wang (✉)
Department of Clinical Pharmacology, School of Pharmacy, China Medical University, Shenyang, Liaoning, People's Republic of China
e-mail: ywang28@cmu.edu.cn

on. Therefore, the heterogeneity of multiple pulmonary blood vessels and alveolar cells and tracking the transmitters of cell communication could be conducive to the further insights into the pathogenesis of PH to discover the potential therapeutic targets for PH.

Keywords Alveolar epithelial cell · Crosstalk · Endothelial cells · Fibroblasts · Inflammation · Macrophages · Pericytes · Pulmonary hypertension · Remodeling · Smooth muscle cells

Abbreviations

15-HETE	15-Hydroxyeicosatetraenoic acid
5-HTT	Serotonin transporter
5-LO	5-Lipoxygenase
AASMCs	Aortic artery smooth cells
ADAM17	A disintegrin and metalloproteinase 17
AECs	Alveolar epithelial cells
AKT	Protein kinase B
AT1	Alveolar type I
AT2	Alveolar type II
BMPR2	Bone morphogenic protein receptor 2
C/EBPβ	CCAAT/enhancer-binding protein beta
CCR2	C-C chemokine receptor types 2
CCR5	C-C chemokine receptor types 5
CTGF	Connective tissue growth factor
CXCL1	Murine chemokine 1
CXCL12	Chemokine 12
CXCL8	Chemokine 8
Ecs	Endothelial cells
EGFR	Epidermal growth factor receptor
EndoMT	Endothelial-to mesenchymal transition
ERK	Extracellular signal-regulated kinase
ERK1/2	Signal-regulated kinase 1/2
ET-1	Endothelin-1
FoxM1	Forkhead box M1
FOXO3a	Forkhead box protein O3a
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSK-3β	Glycogen synthase kinase-3β
H2O2	Hydrogen peroxide
HIF1α	Hypoxia-inducible factor-1alpha
HIMF	Hypoxia-induced mitogenic factor
HIV	Human immunodeficiency virus
HMGB1	High mobility group box 1

hPAH	Hereditary pulmonary arterial hypertension
HPH	Hypoxic pulmonary hypertension
IL-6	Interleukin 6
IL-8	Interleukin 8
ILK	Integrin-linked kinase
IPF	Idiopathic pulmonary fibrosis
JAK2	Janus kinase 2
LTB4	Leukotriene B4
LVEF	Left ventricular ejection fraction
MIF	Macrophage migration inhibitory factor
MiR-143	microRNA-143
MiR-92a-3p	microRNA-92a-3p
MMP-2	Matrix metalloproteinase-2
MMP-9	Matrix metalloproteinase-9
mPAP	Mean pulmonary artery pressure
NF-κB	Nuclear factor-Kb
Notch1	Notch receptor 1
Nox4	NADPH oxidase 4
NRGs	Neuregulins
OPN	Osteopontin
p38MAPK	p38 mitogen-activated protein kinases
PAECs	Pulmonary artery endothelial cells
PAH	Pulmonary arterial hypertension
PASMCs	Pulmonary artery smooth muscle cells
PCH	Pulmonary capillary hemangiomatosis
PDGF-B	Platelet-derived growth factor BB
PDK4	Pyruvate dehydrogenase kinase 4
PFKFB3	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PI3K	Phosphatidylinositol-3-kinase
PMVECs	Pulmonary microvascular endothelial cells
PS1	Presenilin 1
PTEN	Phosphatase and tensin homolog
PVOD	Pulmonary veno-occlusive disease
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
Shh	Sonic hedgehog
SMCs	Smooth muscle cells
SOD2	Superoxide dismutase 2
Stamp2	Six-transmembrane protein of prostate 2
STAT3	Transcription 3
TGFα	Transforming growth factor-alpha
TNF-α	Tumor necrosis factor-alpha
TSP1	The secreted protein thrombospondin 1
Wnt5a	Wnt family member 5A
WSPH	World symposium on pulmonary hypertension

1 Introduction

PH is defined as the mean pulmonary artery pressure (mPAP) is 20 mmHg or higher in the resting state. PH is an unwanted disease with the rising incidence rate and death rate, so the researchers pay more and more attention to explore the mechanism and effective treatment of PH (Hansmann 2017). The World Symposium on Pulmonary Hypertension (WSPH) divides PH into five groups according to its symptoms, pathological mechanisms, hemodynamic features, and treatment methods: Group 1: pulmonary arterial hypertension (PAH); Group 2: PH due to left heart disease; Group 3: PH due to lung diseases and/or hypoxia; Group 4: PH due to pulmonary artery obstructions and Group 5: PH with unclear and/or multifactorial mechanisms. The latest PH clinical classification and its causes are presented in Table 1 (Simonneau et al. 2019). The main pathological characteristics of PH include pulmonary vascular contraction and remodeling, inflammation, and microthrombosis. Specially, pulmonary vascular remodeling is becoming more and more important in PH, but there is still a lack of the effective therapy for pulmonary vascular remodeling (Zhao et al. 2021). Endothelin-1, prostacyclin, and nitric oxide are three pathological factors involved in PAH based on the dysfunction of endothelial cells and have been taken as the directions to PAH treatment (Pulido et al. 2016). However, these immediate treatments can't markedly improve the morbidity and the mortality of PH, except lung transplantation (Evans et al. 2021). With the progression of cellular and molecular biology, it is believed that the therapeutic targets for alleviating vascular remodeling, pulmonary contraction, and pulmonary inflammation would be constantly discovered.

In addition, it is gradually recognized of the impacts of the changes on the pulmonary vascular microenvironment of PH, especially the role of the interactions between the cells of the pulmonary vascular wall in the development of PH (Pasha 2014). PH includes abnormalities of vascular cells (endothelial cells, smooth muscle cells, and fibroblasts) and inflammatory cells (Southgate et al. 2020). More and more reports are proved of the roles of endothelial cells (ECs), smooth muscle cells (SMCs), and fibroblasts, as well as platelets and inflammatory cells in PH (Stenmark et al. 2018). However, the role of the interaction between alveolar epithelial cells and other cells in PH is unclear. It should be furtherly explored whether alveolar epithelial cells are involved in pulmonary vascular remodeling, pulmonary vascular contraction, and pulmonary inflammation. The purpose of this review is focused on the roles of crosstalk between cells in the pathogenesis of PH and to explore the potential therapeutic targets to improve the survival rates.

Table 1 Updated clinical classification and causes of PH

Group	Clinical classification	Causes
1	PAH	1.1 Idiopathic PAH 1.2 Heritable PAH 1.3 Drug- and toxin-induced PAH 1.4 PAH associated with: 1.4.1 Connective tissue disease 1.4.2 HIV infection 1.4.3 Portal hypertension 1.4.4 Congenital heart disease 1.4.5 Schistosomiasis 1.5 PAH long-term responders to calcium channel blockers 1.6 PAH with overt features of venous/capillaries (PVOD/PCH) involvement 1.7 Persistent PH of the newborn syndrome
2	PH due to left heart disease	2.1 PH due to heart failure with preserved LVEF 2.2 PH due to heart failure with reduced LVEF 2.3 Valvular heart disease 2.4 Congenital/acquired cardiovascular conditions leading to post-capillary PH
3	PH due to lung diseases and/or hypoxia	3.1 Obstructive lung disease 3.2 Restrictive lung disease 3.3 Other lung disease with mixed restrictive/obstructive pattern 3.4 Hypoxia without lung disease 3.5 Developmental lung disorders
4	PH due to pulmonary artery obstructions	4.1 Chronic thromboembolic PH 4.2 Other pulmonary artery obstructions
5	PH with unclear and/or multifactorial mechanisms	5.1 Hematological disorders 5.2 Systemic and metabolic disorders 5.3 Others 5.4 Complex congenital heart disease

PAH pulmonary arterial hypertension, *PH* pulmonary hypertension, *HIV* human immunodeficiency virus, *PVOD* pulmonary veno-occlusive disease, *PCH* pulmonary capillary hemangiomatosis, *LVEF* left ventricular ejection fraction

2 Fundamental Pathogenesis in the Progression of PH

PH is a cardiopulmonary illness that can influence the pulmonary arterial and venous circulation and induce right ventricle hypertrophy (Hoeper et al. 2017; Kim and George 2019). The pathogenic factors of PH include pulmonary endothelial cell disorders, abnormal vascular wall cell proliferation, inflammation, and multiple gene mutations, which finally lead to right ventricular hypertrophy, cardio myocytes damage, and death (Makino et al. 2011; Tuder 2017; Montani et al. 2016) (Fig. 1). Despite different forms of PH exhibit diverse pathological mechanisms, the previous research results indicated that the crosstalks between the blood vessel wall cells (i.e., SMCs, fibroblasts, and Ecs) are the corporative features of the molecules and the

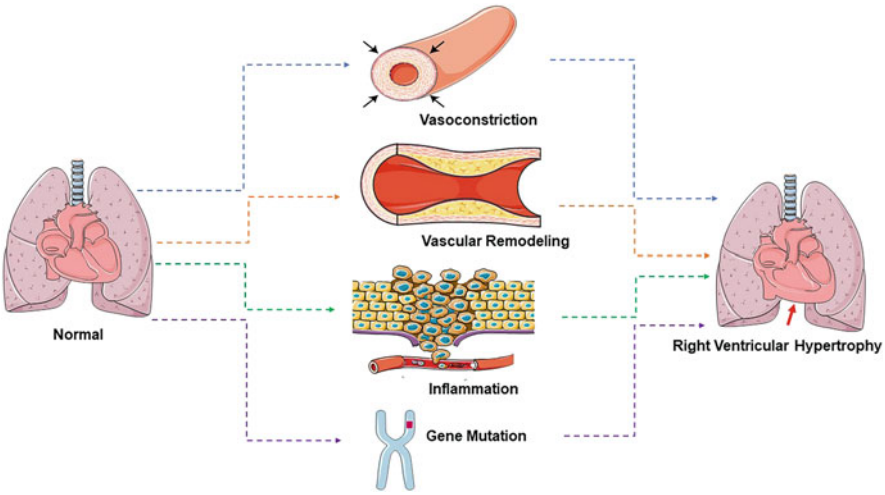

Fig. 1 Basic pathogenesis in the progression of PH. The basic pathogenesis of PH includes vasoconstriction, vascular remodeling, inflammation and gene mutation, which results in pulmonary arterial hypertension and right ventricular hypertrophy

cells involved in pulmonary vascular remodeling, pulmonary vascular contraction, and pulmonary inflammation (Pasha 2014). Therefore, exploring these molecular and cellular pathogenesis of PH will help to find more effective therapeutic targets to control the progression of PH.

3 Pulmonary ECs Crosstalk with SMCs

3.1 EC Regulation of SMC Proliferation

ECs can adjust and control the vascular function. EC dysfunction means the imbalance between vasoconstrictors and vasodilators produced by EC, between activators and inhibitors of SMC growth and migration, and between proinflammatory and anti-inflammatory signals in the PAH (Perros et al. 2015). Specifically, when EC dysfunction, PAH has the following characteristics: pulmonary inflammation, accumulation of inflammatory cells, oxidative/nitrifying stress, changes in vascular cell viability, and proliferation (Evans et al. 2021; Huertas et al. 2014). These characteristics of PAH support the key role of EC dysfunction in the pathogenesis of PAH. It is worth exploring whether the proliferation of SMC is an inherent sign or is caused by the dysfunction of ECs. In the homeostasis of the pulmonary circulation, the interaction between EC and SMC plays an important role (Gao et al. 2016). Under a pathological status, the interaction of the two cells leads to an increase in pulmonary blood vessel tension, which in turn results in a series of pathological manifestations such as increased pulmonary artery pressure, vascular remodeling,

and right ventricular hypertrophy (Guignabert et al. 2015). Hypoxia-induced mitogenic factor (HIMF) plays an important role in EC-SMC crosstalk. HIMF stimulated pulmonary artery ECs (PAECs) to generate and release high mobility group box-1 (HMGB1) by the regulation of autophagy and bone morphogenic protein receptor 2 (BMPR2), and finally made pulmonary artery smooth muscle cells (PASMCs) proliferation (Gao et al. 2016). Dysfunctional PAECs deliver a variety of mediators, for instance, platelet-derived growth factor-BB (PDGF-B), endothelin-1 (ET-1), chemokine 12 (CXCL12), and macrophage migration inhibitory factor (MIF), which can induce forkhead box M1 (FoxM1) expression in PASMCs and activate FoxM1-related to PASMCs proliferation, resulting in pulmonary vascular remodeling and PH (Dai et al. 2018). There is an evidence that ECs can communicate with SMCs through micro-RNA195 to regulate serotonin transporter (5-HTT) to induce the proliferation of SMCs (Gu et al. 2017). Previous studies have shown that the proliferative endothelial cell phenotype is the prominent feature of PAH. The studies suggested that changes in EC phenotype contribute to the occurrence of PAH (Awad et al. 2016). For example, long-term smoking can change the phenotype of human lung microvascular EC. Thereby, inducing EC apoptosis or inherited epigenetic EC dysfunction is related to pulmonary artery remodeling and PH (Petrusca et al. 2014). This crosstalk between ECs and SMCs leads to SMC proliferation and an increase in the thickness of the inner wall of blood vessels.

3.2 SMC Regulation of EC Proliferation and Migration

Recent studies provide a few fascinating findings of SMC regulation of EC proliferation. In SMC-EC co-culture, it was found that BMPR2 activated notch receptor 1 (Notch1) to induce EC proliferation (Miyagawa et al. 2019). Both cells required BMPR2 to produce type IV collagen to activate integrin-linked kinase and result in stabilization of presenilin 1 (PS1) and activation of Notch1. And Notch1 kept the EC proliferative capacity by fortifying mitochondrial mass and tempting 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3 (PFKFB3). Loss of Notch1 aggravated hypoxia-induced PH, which was related to damaged EC proliferation and regeneration, leading to loss of anterior capillary arteries (Miyagawa et al. 2019). This research offered the direct evidence that SMC promoted EC proliferation and rebirth to keep monolayer integrity and vascular homeostasis. Some factors released by SMC entice EC migration. The important factors mediating cell–cell communication are cytokines, chemokines, and cell surface receptors (Hwang 2013). New research reports indicated that exosomes were also significant mediators of cell–cell interactions. A lot of diverse molecules existed in exosomes can be absorbed by recipient cells (Jiao et al. 2018). There is an evidence that microRNA-143 (miR-143) plays an important role in PAH patients and PAH animal models. The exosome miR-143 secreted from PASMCs facilitated the migration of PAECs (Deng et al. 2015). This shows that the exosome-mediated intercellular communication between PASMC and PAEC is of significance in PH.

4 Pulmonary ECs Crosstalk with Non-SMCs

4.1 Fibroblasts

The EC-fibroblast crosstalk acts a specific role in the pathogenesis of PAH as well. Endothelial to mesenchymal transition (EndoMT) is involved in the pathogenesis of many human illnesses such as PAH (Jimenez and Piera-Velazquez 2016). ECs can turn into fibroblast-like cells through EndoMT and have an effect on PAH. ECs polarized fibroblasts into myofibroblasts by releasing ET-1and interleukin 6 (IL-6) and then obtained collagen and extracellular matrix proteins to accelerate pulmonary vascular remodeling (Thenappan et al. 2018; Evans et al. 2021). Adventitia fibroblasts take a part in pulmonary vascular remodeling by means of multiple mechanisms. For instance, the augment of myofibroblasts increased the rigidity of the extracellular matrix, which directly caused the activation of PAEC proliferation (Thenappan et al. 2018). Fibroblast-originated matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), and 15-hydroxyeicosatetraenoic acid (15-HETE) can regulate PAEC proliferation (Liu et al. 2018). The secreted protein thrombospondin-1 (TSP1) in lung fibroblasts disrupted EC–fibroblasts interactions to promote vascular remodeling in PAH (Labrousse-Arias et al. 2016).

4.2 Pericytes

In PAH, the number of pericytes is reduced and pericyte-EC crosstalk is also attributed to pulmonary vascular remodeling. Abnormal pericyte recruitment contributes to the pathogenesis of PAH (Ricard et al. 2014). Studies have shown that Wnt family member 5A (Wnt5a) plays a key role in the crosstalk between pulmonary microvascular endothelial cells (PMVECs) and pericytes, and also contributes to pulmonary vascular remodeling in PAH. The recruitment of pericytes in small blood vessels needed Wnt5a produced by healthy PMVECs. But defects of Wnt5a in PAH PMVECs can cause persistent pulmonary hypertension and right heart failure after hypoxia recovery (Yuan et al. 2019). As a result, PMVECs lack of Wnt5a had a decreasing capacity to recruit pericytes and resulted in a loss of small blood vessels in PAH (Yuan et al. 2019). In addition, Pyruvate Dehydrogenase Kinase 4 (PDK4), which is a gene encoding a mitochondrial enzyme responsible for inhibiting glucose oxidation, is related to the excessive proliferation of pericytes in PAH and improved EC-pericyte crosstalk. The decrease of PDK4 contributes to promote EC-pericyte crosstalk (Yuan et al. 2016). Therefore, genes that regulate and control pericyte-EC crosstalk may become a new therapeutic target to cure the loss of small blood vessels in PAH.

4.3 Inflammatory Cells

The main structural change of PAH is pulmonary vascular remodeling (Liang et al. 2020). And there have been definite reports showing that inflammation plays a major pathogenic factor in pulmonary vascular remodeling (Oliveira et al. 2017). At the same time, the recruitment of inflammatory cells and EC proliferation play an equally important effect in the pathogenesis of PAH (Mumby et al. 2017). It can be seen that inflammatory cells and ECs are inseparable from the development of PAH (Kuebler et al. 2018). Hence, the role of inflammation in PAH is getting more and more attention. Cytokines and chemokines are certain to be the driving factors and contributing factors of the perivascular inflammation in PAH (Le Hiress et al. 2015). Macrophages and lymphocytes may be referred to the course of pulmonary blood vessel remodeling. The recruitment and accumulation of leukocytes were facilitated by granulocyte macrophage colony-stimulating factor (GM-CSF) (Sawada et al. 2014), CXCL12 (Dai et al. 2016), connective tissue growth factor (CTGF) (Pi et al. 2018), IL-6 (Van Hung et al. 2014), and leptin (Xue et al. 2017) released by activated ECs. Other factors released from accumulated leukocytes such as leukotriene B4 (LTB4) derived from macrophages can cause EC apoptosis (Tian et al. 2013), and MIF derived from T cell lymphocytes can cause inflammation of the EC and recruit inflammatory cells (Le Hiress et al. 2015).

5 Alveolar Epithelial Cells Crosstalk with SMCs

5.1 Alveolar Epithelial Cell Regulation of SMC Proliferation

Apart from damage and dysfunction of EC in the pathogenesis of PH, more and more people realize that the alveolar epithelial cells (AECs) play an equally significant role in the development of PH. Some studies found that the activation of endoplasmic reticulum stress after long-term high-altitude exposure accelerates the apoptosis of AECs, which may induce the occurrence and development of high-altitude pulmonary hypertension (Pu et al. 2020). CTGF over-expressed in epithelial cells activates the integrin-linked kinase (ILK)/glucose synthesis kinase-3β (GSK-3β)/β-catenin pathway, resulting in dysfunction of epithelial cell and PASMC remodeling, which finally leads to the occurrence of PH (Chen et al. 2011). Recently, it has also been found that AECs take part in the pulmonary vascular contraction and remodeling of hypoxic pulmonary hypertension (Wang et al. 2021). The function of alveolar epithelial cells is not only a component of gas exchange but also a vital barrier to protect the human body from harm. AECs can quickly repair and regenerate cells to restore a complete alveolar epithelial barrier and then respond to acute lung injury (Zhang et al. 2019). Alveolar type I (AT1) and alveolar type II (AT2) cells are two types of AECs. The main function of AT2 cells is to synthesize and secrete pulmonary surfactants. Furthermore, AT2 cells can be

differentiated into AT1 cells in the homeostasis of the alveoli and the repair process after injury. The AT1 cells constitute the thin air-blood barrier, which is the epithelial component, and the coverage rate reaches 95% of the alveolar surface area (Chen and Liu 2020). According to previous reports, in hypoxic pulmonary hypertension (HPH), the response of pulmonary blood vessels to hypoxia was not like the expansion of blood vessels in the systemic circulation but vasoconstriction (Böger and Hannemann 2020). The alveoli will not make a rise in systemic pressure after being hypoxic, but only made an increase in pulmonary artery pressure. After the alveoli sense hypoxia, it was primarily felt on the alveolar capillary membrane. The alveolar capillary membrane is composed of epithelial and endothelial membranes on the alveolar wall. The hypoxia signal propagated from the alveolar capillaries to the small arteries and then caused the contraction of PASMCs (Hough et al. 2018). Both AECs and ECs show tolerance to hypoxia, but AECs were more sensitive (Wang et al. 2021). It was reported that hypoxia increased the concentration of reactive oxygen species (ROS) in AECs (Grimmer and Kuebler 2017). Hydrogen peroxide (H_2O_2) from AECs dependent on the ROS/superoxide dismutase 2 (SOD2) pathway regulated pulmonary vascular remodeling and contraction. In vitro, it was manifested that AECs not only facilitated the proliferation of PASMCs but also facilitated the proliferation of aortic artery smooth cells (AASMCs) in hypoxia (Wang et al. 2021). Therefore, the pulmonary vascular microenvironment formed by AECs and SMCs was involved in the remodeling and contraction of pulmonary vessels.

5.2 SMC Regulation of AEC Proliferation

In acute lung inflammation, the spread of this inflammation relied on the immunologic function of lung interstitial cells, for example, SMCs (Udjus et al. 2019). SMC released a mass of proinflammatory factors, for instance, cytokines (Tumor Necrosis Factor-alpha [TNF-α], IL-6), chemokines (Chemokine 8 [CXCL8]/Interleukin 8 [IL-8]/murine Chemokine 1 [CXCL1]), and growth factors (neuregulins [NRGs] and transforming growth factor alpha [TGFα]) (Tliba and Panettieri 2009). These inflammatory responses need a disintegrin and metalloproteinase 17 (ADAM 17)-mediated transactivation of ErbB receptors after the application of exogenous TGFα or NRG1. Transactivation of cells mediated by ErbB via binding to epidermal growth factor receptor (EGFR)/ErbB1 or ErbB3 and ErbB4 receptors and shed TGFα or NRGs. Furthermore, ErbB4 has developmental functions and is involved in the synthesis and proliferation of surface active substances in AECs. Therefore, in the pulmonary inflammation, decreased ADAM 17-mediated growth factor in SMC accelerated the transactivation of ErbB4 by means of NRGs, for example, NRG1 activation facilitated the proliferation of AECs and keeps the homeostasis of the cellular environment (Dreymueller et al. 2014). It is worth attention of the crosstalk between AECs and SMCs in pulmonary inflammation of PH.

6 Alveolar Epithelial Cells Crosstalk with Non-SMCs

6.1 Fibroblast

Idiopathic pulmonary fibrosis (IPF), pathologically common interstitial pneumonia, is a long-term disease with unclear etiology (Larson-Casey et al. 2020). The pathogenesis of the disease includes alveolar epithelial injury, abnormal vascular repair, and pulmonary vascular remodeling. The main complications of IPF patient survival include lung cancer and pulmonary hypertension. There were a lot of evidence that one of the important factors leading to IPF is the dysfunction and continuous damage of AECs (Lee et al. 2018; Wang et al. 2020). The migration, proliferation, and activation of mesenchymal cells are caused by active AECs and are accompanied by the formation of fibroblasts/myofibroblasts foci and excessive accumulation of extracellular matrix. Just like that DNA harm in AEC II resulted in osteopontin (OPN) expression via activating extracellular signal-regulated kinase (ERK)-dependent signaling pathways. The induced OPN boosts the proliferation of AEC II and the migration of fibroblasts (Kato et al. 2014). Therefore, the integrity of the alveolar epithelial barrier was ultimately maintained due to the interaction between AECs and fibroblasts, which indicated that crosstalk between AECs and fibroblasts will be closely associated with the development of PH.

6.2 Macrophages

Similarly, the interactions between AECs and macrophages play an equally important regulatory role in the development of pulmonary diseases (Young et al. 2016; Byrne et al. 2016). Macrophages are one of the important regulators of pulmonary inflammatory disease. Macrophages plasticity, specificity, and ability to interact with other cells make them a pivotal factor in the pathogenesis of pulmonary inflammatory disease (Wang et al. 2019). In addition, the crosstalk between macrophages and AECs is a key factor in the pathological process of pulmonary inflammation and fibrosis, which is directly attributed to the contact between macrophages and AECs. AT2 cells can secrete sonic hedgehog (Shh), and its signal transduction facilitated the secretion of OPN in macrophages. OPN acted on macrophages through autocrine or paracrine mode. And OPN fortified the expression of arginase-1 by means of activating the Janus kinase-2/transcription 3 (JAK2/STAT3) signaling pathway, and caused pulmonary fibrosis to occur (Hou et al. 2021). Similarly, the interaction between AECs and macrophages also plays an important role in regulating the progression of lung injury, which is becoming a new research hotspot. Exosomes from AECs can cause pulmonary inflammation and enable the migration and activation of macrophages. MicroRNA-92a-3p (miR-92a-3p) in AECs-derived exosomes mediated cellular communication between AECs and macrophages and facilitated the activation of macrophages by means of controlling Phosphatase and

tensin homolog (PTEN) expression and adjusting the activation of nuclear factor-κB (NF-κB) signaling pathways (Liu et al. 2021). Therefore, the crosstalks between AECs and macrophages probably take part in PH by regulating pulmonary inflammation.

7 Macrophages Crosstalk with SMCs

The accumulation of inflammatory cells is an important feature in vascular injury during PAH. Inflammatory cells (T, B lymphocytes, mast cells, monocytes, and macrophages) take a key part in the PAH process. Macrophages are innate immune cells, composed of recruited/infiltrated monocytes, which are essential for keeping homeostasis and repairing ability. Polarized macrophages may be generally classified into three genres: classically activated macrophages (M1), alternating activated macrophages (M2), and regulatory macrophages (M2b) (Huang et al. 2020). So it is worth noting that macrophages are involved in the pathogenesis of PAH (Bordenave et al. 2020). Early recruitment and replacement activation of macrophages are of great significance in the pathogenesis of PAH (Bai et al. 2019). Macrophage-mediated inflammation is intricate, and the interaction between macrophages and PASMCs can regulate the internal microenvironment of the lung. In the co-culture of m2-macrophages and PASMC in direct contact, we understand that it facilitates the proliferation and migration of PASMC by relying on C-C chemokine receptor types 2/C-C chemokine receptor types 5 (CCR2/CCR5) (Abid et al. 2019). Furthermore, we found that M2b macrophages can also adjust the proliferation, migration, and apoptosis of PASMC by adjusting and controlling the phosphatidylinositol-3-kinase/protein kinase B/forkhead box protein O3a (PI3K/Akt/FoxO3a) pathway (Huang et al. 2020). Six-transmembrane protein of prostate 2 (Stamp2) has been reported to be an important anti-inflammatory protein in macrophages. The lack of Stamp2 can directly affect the interaction between macrophages and SMCs, resulting in aggravation of pulmonary hypertension caused by hypoxia (Batool et al. 2020). And a recent study showed that LTB4 (5-lipoxygenase [5-LO] metabolite) derived from macrophages plays an important effect in causing pulmonary vascular remodeling in PAH (Tian et al. 2013; Peters-Golden and Henderson 2007). 5-LO and LTB4 are related to the evolution of PAH (Peters-Golden and Henderson 2007). In the lung lesions of hPAH patients with Bmpr2 mutations, 5-LO-mediated inflammation similarly converts PAEC into a diseased neointimal phenotype. In rats and patients with Bmpr2 mutations, 5-lo-expressing neointimal lesions might facilitate vascular inflammation and vascular remodeling (Tian et al. 2019). Pulmonary vascular remodeling is featured by the progress of distinct neointimal lesions, covering concentric laminar intima fibrosis and plexiform lesions (Dickinson et al. 2011). These neointimal lesions give rise to intraluminal obstruction due to EC and SMC proliferation, fibrosis, and inflammation, which ultimately leads to irreversible PAH (Steffes et al. 2020; Chan and Loscalzo 2008). Studies showed that activation of PI3K/Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) pathways mediates

LTB 4 through regulating the GSK-3β/β-catenin/cyclin D1 axis to induce PASMCs proliferation, and indicated that this pathway was involved in alleviating vascular remodeling and was beneficial to PAH treatment (Li et al. 2020). The above conclusions proved that the interactions between macrophages and SMCs played an equally significant effect in the pathogenesis and development of PAH.

8 Fibroblasts Crosstalk with Macrophages

Communication mediated by adventitia cells acts a potential role in vascular inflammation and pathogenesis. The adventitia contains many innate immune cells, especially macrophages, fibroblasts, and DCs. The number of fibroblasts accounts for the majority of adventitia cells. The interaction of fibroblasts and macrophages in the adventitia of blood vessels facilitates the transmission of inflammatory signals and the progression of PH (Li et al. 2021). Studies have reported that fibroblasts have the ability to recruit and activate macrophages, leading to vascular inflammation and vascular remodeling, in which lactate and IL-6 play a significant role. Fibroblasts in a large number of animal models and humans with PH regulate macrophage activation (El Kasmi et al. 2014). For example, previous studies have shown that fibroblast paracrine IL-6 and macrophage STAT3 united with hypoxia-inducible factor 1alpha (HIF1α) and CCAAT/enhancer-binding protein beta (C/EBPβ) caused the pathogenesis of PH, which directly led to the occurrence of vascular inflammation and remodeling (El Kasmi et al. 2014). At the same time, studies have found that macrophages can receive and combine the signals sent by fibroblasts and then carry out disparate transcriptomics and metabolomics programming to keep a more stable lung microenvironment during the pathogenesis of PH (Li et al. 2021). LTB4 derived from macrophages aggravated the proliferation, migration, and differentiation of human pulmonary artery adventitia fibroblasts in a dose-dependent manner via its homologous G protein-coupled receptor, BLT1. LTB4 stimulated human pulmonary artery adventitia fibroblasts by up-regulating p38 mitogen-activated protein kinase (MAPK) and NADPH oxidase 4 (Nox4) signaling pathway to promote PH development (Qian et al. 2015). The crosstalks between fibroblasts and macrophages in the microenvironment of the adventitia of blood vessels are expected to play a great therapeutic significance in improving the process of pulmonary vascular remodeling.

9 Concluding Remarks

PH is considered to be a cardiopulmonary disease with pulmonary vascular inflammation, vascular remodeling, and cardiac cell damage (Yeo et al. 2020; Hsu et al. 2020). Cell-to-Cell Crosstalk has been taken as a new and further understanding of the physiology and pathology of PH (Rafikova et al. 2019). Phenotypic changes

resulted in pulmonary vascular remodeling, pulmonary vasoconstriction, and pulmonary inflammation contain: (a) the crosstalk between pulmonary ECs and SMCs that generate a lung microenvironment of proliferation, anti-apoptosis, and vasoconstriction (b) the crosstalk between pulmonary ECs and non-SMCs that generate a lung microenvironment of proliferation, anti-apoptosis, and vasoconstriction (c) the crosstalk between AECs and SMCs that cause a lung microenvironment of proliferation, anti-apoptosis, and vasoconstriction (d) the crosstalk between AECs and non-SMCs that create a lung microenvironment of proliferation, anti-apoptosis, and vasoconstriction (e) the crosstalk between macrophages and SMCs to promote a lung microenvironment of proliferation, anti-apoptosis, and vasoconstriction (f) the crosstalk between macrophages and fibroblasts to promote a lung microenvironment of proliferation, anti-apoptosis, and vasoconstriction. As a result, the imbalance between proliferation and apoptosis, between vasoconstriction and vasodilation, as well as proinflammatory and anti-inflammatory resulted in pathological changes in the inner, middle, and adventitia of the pulmonary blood vessels, narrowing of the vascular lumen, vascular remodeling, pulmonary inflammation, and finally increased pulmonary artery pressure.

The morbidity rate of PH is still very high. Some patients are asymptomatic during the onset of the disease, which is the disadvantage of the early diagnosis and treatment (Strauss et al. 2019). The damage and repair of cells and the complex crosstalk between multiple cells have been added to the pathogenesis of PH (Voelkel et al. 2012). If the intricate interactions between vascular cells and their regulatory signaling pathways are enough realized and are applied in clinic, the pathological state of PH can be changed to the greatest extent and the mortality can be reduced. Therefore, the signaling pathways emphasized in the crosstalk between multiple cells play an important role in many diseases, especially PH. It is summarized of the crosstalk between cells in the pathogenesis of PH (Fig. 2).

In human and rodents PH, there exist the crosstalks between ECs and SMCs (Lin et al. 2019), ECs and pericytes (Yuan et al. 2019), ECs and Macrophages (Sawada et al. 2014), AECs and fibroblasts (Kato et al. 2014), AECs and macrophages (Hou et al. 2021), macrophages and SMCs (Abid et al. 2019), fibroblasts and macrophages (El Kasmi et al. 2014), ECs and fibroblasts (Labrousse-Arias et al. 2016). However, the crosstalk between AECs and SMCs has been studied only in rodent models until now (Wang et al. 2021). In comparison, the researches in rodent models of PH are more involved in cell-to-cell crosstalk than Human PH disease. And other studies found that in HIMF-induced PH, HMGB1-receptor for advanced glycation end products (RAGE) signal transduction is essential for mediating EC and SMC crosstalk, which was confirmed from idiopathic PH patients and rodent PH. However, data from humanized mice were found to further confirm the clinical significance of the HIMF/HMGB1 signal axis (Lin et al. 2019). Therefore, in future research, humanized mice should be more used in the crosstalk between cells for the development of biomedical research and clinical treatment of PH.

Nowadays, the research focused on network medicine can expand the opportunities for personalized treatment of PH (Wang and Loscalzo 2021). Network medicine is the use of effective genomic instruments as well as biostatistics,

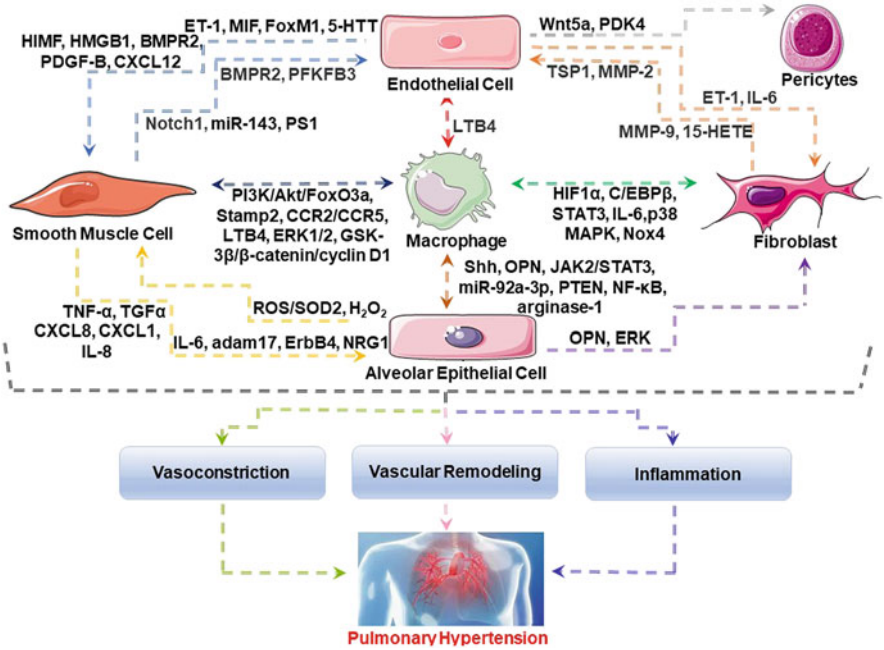

Fig. 2 Cell-to-cell crosstalk in the pathogenesis of PH. The crosstalk between cells (Endothelial cell, Smooth muscle cell, Alveolar Epithelial cell, Fibroblast, Pericyte, and Macrophage) and the regulated signal pathways are involved in the pathogenesis of PH. *NF-κB* nuclear factor-κB, *ET-1* Endothelin-1, *BMPR* bone morphogenic protein receptor, *FoxM1* forkhead box M1, *MIF* macrophage migration inhibitory factor, *BMPR2* bone morphogenic protein receptor 2, *HIMF* hypoxia-induced mitogenic factor, *PDGF-B* platelet-derived growth factor-BB, *CXCL12* Chemokine 12, *HMGB1* high mobility group box-1, *5-HTT* Serotonin transporter, *MMP-9* matrix metalloproteinase-9, *TSP1* The secreted protein thrombospondin-1, *IL-6* Interleukin 6, *15-HETE* 15-hydroxyeicosatetraenoic acid, *PDK4* Pyruvate Dehydrogenase Kinase 4, *GM-CSF* granulocyte macrophage colony-stimulating factor, *CTGF* Connective Tissue Growth Factor, *LTB4* leukotriene B4, *MMP-2* matrix metalloproteinase-2, *PFKFB3* 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase3, H_2O_2 hydrogen peroxide, *SOD2* superoxide dismutase 2, *TNF-α* Tumor Necrosis Factor alpha, *CXCL8* Chemokine 8, *IL-8* Interleukin 8, *CXCL1* murine Chemokine 1, *NRG1* neuregulin1, *TGFα* Transforming growth factor alpha, *adam17* a disintegrin and metalloproteinase 17, *Notch1* notch receptor 1, *ERK* extracellular signal-regulated kinase, *OPN* osteopontin, *PTEN* Phosphatase and tensin homolog, *CCR2* C-C chemokine receptor types 2, *CCR5* C-C chemokine receptor types 5, *PI3K* phosphatidylinositol-3-kinase, *AKT* protein kinase B, *FoxO3a* Forkhead box protein O3a, *Stamp2* Six-transmembrane protein of prostate 2, *HIF1α* Hypoxia-inducible factor-1 alpha, *C/EBPβ* CCAAT/enhancer-binding protein beta, *JAK2* Janus kinase-2, *STAT3* transcription 3, *Wnt5a* Wnt family member 5A, *PS1* presenilin 1, *Shh* Sonic hedgehog, *MiR-143* microRNA-143, *ROS* reactive oxygen species, *miR-92a-3p* microRNA-92a-3p, *ERK1/2* signal-regulated kinase 1/2, *GSK-3β* glycogen synthase kinase-3β, *p38 MAPK* p38 mitogen-activated protein kinases, *Nox4* NADPH oxidase 4

bioinformatics, and dynamic system analysis to find the way of the prevention and treatment of PH (Napoli et al. 2019; Fiscon et al. 2018). For instance, in future research, computational modeling techniques can be used to enhance the interpretation of the relationship between the cells on the pulmonary vessels and the

pulmonary alveoli, and their interaction with neighboring cells. In conclusion, a full understanding of cell-to-cell crosstalk can bring more therapeutic options to the pathogenesis of PH. The important signals in cell-to-cell crosstalk will become the new targets for the prevention and treatment of PH.

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