



A systematic review of translation and experimental studies on internal anal sphincter for fecal incontinence

Minsung Kim¹, Bo-Young Oh¹, Ji-Seon Lee³, Dogeon Yoon³, Wook Chun^{3,4}, Il Tae Son^{1,2}

¹Department of Surgery, Hallym University Sacred Heart Hospital, Hallym University College of Medicine, Anyang; ²Institute for Regenerative Medicine, Hallym University Sacred Heart Hospital, Hallym University College of Medicine, Anyang; ³Burn Institute, Hangang Sacred Heart Hospital, Hallym University College of Medicine, Seoul; ⁴Department of Surgery, Hangang Sacred Heart Hospital, Hallym University College of Medicine, Seoul, Korea

The complexity in the molecular mechanism of the internal anal sphincter (IAS) limits preclinical or clinical outcomes of fecal incontinence (FI) treatment. So far, there are no systematic reviews of IAS translation and experimental studies that have been reported. This systematic review aims to provide a comprehensive understanding of IAS critical role in FI. Previous studies revealed the key pathway for basal tone and relaxation of IAS in different properties as follows; calcium, Rho-associated, coiled-coil containing serine/threonine kinase, aging-associated IAS dysfunction, oxidative stress, renin-angiotensin-aldosterone, cyclooxygenase, and inhibitory neurotransmitters. Previous studies have reported improved functional outcomes of cellular treatment for regeneration of dysfunctional IAS, using various stem cells, but did not demonstrate the interrelationship between those results and basal tone or relaxation-related molecular pathway of IAS. Furthermore, these results have lower specificity for IAS-incontinence due to the included external anal sphincter or nerve injury regardless of the cell type. An acellular approach using bioengineered IAS showed a physiologic response of basal tone and relaxation response similar to human IAS. However, in both cellular and acellular approaches, the lack of human IAS data still hampers clinical application. Therefore, the IAS regeneration presents more challenges and warrants more advances.

Keywords: *Internal anal sphincter; Fecal incontinence; Cellular treatment; Acellular treatment*

INTRODUCTION

Fecal incontinence (FI) can be a devastating and stigmatizing disease for elderly patients [1-3]. Age is associated with deteriorated anorectal functions presenting with lower anal resting and squeeze pressures, anal sphincter denervation, increased rectal compliance and decreased rectal sensation [4-6]. In addition, dementia, mobility impairment, comorbid chronic diseases, and

bowel disturbances including diarrhea and constipation are the most common independent risk factors for FI [7]. Nontraumatic internal anal sphincter (IAS) weakness observed frequently in elderly FI patients is different in pathophysiology from patients with external anal sphincter (EAS) injuries during delivery [8, 9] or iatrogenic injuries during perianal fistula or anal fissure surgery [10, 11]. Unlike in younger FI patients, in elderly FI, age-related phenotypes are characterized by lower resting pressure, however, 2 have similar squeeze pressure [12].

It is also known that resting pressure maintained by the basal tone of IAS is a main clinical parameter of FI [13, 14]. Compared to the voluntary contractility of skeletal structures of EAS, IAS, conjoined at the end of the gastrointestinal (GI) tract, has autonomic nerve fibers distinct from somatic fibers entering the EAS via the pelvic plexus [15]. Therefore, as an intrinsic property, the smooth muscle (SM) of IAS plays a critical role in maintaining basal tone independent of extrinsic nerve stimulation. Various studies related to restoration of SM cells (SMCs) of IAS as FI treatment have been evolved. *In vitro* and *in vivo* experimental studies

Received: Apr 21, 2022 • Revised: May 9, 2022 • Accepted: May 10, 2022
Correspondence to: Il Tae Son, M.D.

Department of Surgery, Hallym Sacred Heart Hospital, Hallym University College of Medicine, 22 Gwanpyeong-ro 170beon-gil, Dongan-gu, Anyang 14068, Korea

Tel: +82-31-380-1614, Fax: +82-31-380-1619

E-mail: 1tae99@hallym.or.kr

ORCID: <https://orcid.org/0000-0003-1781-9189>

© 2022 The Korean Society of Coloproctology

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<https://creativecommons.org/licenses/by-nc/4.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

using bioengineered human IAS have reported that implantation of intrinsically innervated human IAS constructs generated spontaneous basal tone with myogenic and neuronal components [13, 14]. Furthermore, FI cell treatments [16-19] using bone mesenchymal stem cells (MSCs), adipose stem cells (ASCs), myoblasts, and a combined treatment strategy [20-24] have been reported functional improvement in the injured sphincter. However, these studies for cell therapy showed a bias and lacked data for clinical use as reported by the previous systematic reviews [25-27]. Data on the regeneration of IAS associated with basal tone was also limited, and most cell therapy studies were performed on animal models for the skeletal muscle of EAS or nerve damage [25, 27, 28].

As an intrinsic force of the sphincter SM, the basal tone is generated from the cross-bridge movement of myosin and actin filaments on 20-kDa myosin regulatory light chain phosphorylation (p-MLC₂₀) [29]. Several studies have demonstrated that RhoA and Rho-associated, coiled-coil containing serine/threonine kinase (ROCK) is the major molecular determinant of basal tone in IAS [30, 31]. Recently, it has been covered that multifactorial pathways are involved in the modulation of basal tone including calcium transients or aging-associated IAS dysfunction (AAID) [32, 33]. Some authors have suggested a need for an integrative approach for the uncovered molecular mechanism with a new genesis of spontaneous basal tone in IAS [34]. Furthermore, the IAS relaxation, which is critical for the rectoanal inhibitory reflex (RAIR) in response to defecation, is highly dependent on nerve activity with inhibitory neurotransmitters (NTMs) compared to the ability of IAS to develop spontaneous tone, independently [32, 33].

However, the complexity in the molecular mechanism of myogenic features in IAS might limit preclinical or clinical outcomes of FI cell therapy or regenerative medicine. These treatment modalities should entail the molecular basis of SMCs, which generate basal tone as an intrinsic property and the relaxation in IAS [31]. So far, there are no systematic reviews for translation studies on IAS-based FI treatment covering molecular pathways, regenerative medicine, and cell therapy. This study aims to perform a systematic review of the literature for a comprehensive understanding of the IAS as a critical role in incontinence in FI patients and provide the interrelationship of those studies.

METHODS

Literature search

To identify all the available translation and experimental studies on the IAS in FI published and indexed up to January 31, 2022, a systematic search was conducted in PubMed, Cochrane Library, EMBASE, Medline, and Web of Science. This strategy included *in vitro* and *in vivo* (animal or human) translation studies related to cell therapy, regenerative medicine, tissue engineering, and preclinical study. Medical Subject Headings (MeSH) terms and Emtree terms were used in PubMed and EMBASE, respectively, together with separate words or word combinations in the title or abstract.

Type of article reviewed and data extraction

Automatic deduplication of the retrieved articles was performed twice with EndNote (ver. X9.0.1, Clarivate Analytics, Philadelphia, PA, USA); first per the searched database, and then based on the relevant titles with the assessment of IAS SMCs-related terms including the basal tone, the relaxation, cell therapy, and regeneration medicine. Studies including anatomy and histology were excluded. Two independent reviewers extracted the data from the designed extraction form. Translation studies for SMCs of IAS were classified using keywords as follows: calcium, Rho/ROCK, AAID, oxidative stress, renin-angiotensin-aldosterone, cyclooxygenase (COX), and inhibitory NTM (Table 1). The following data were collected: tissue sample for harvest, mediator, pathway, the action of mechanism in translation studies; cell type, animal model, type of sphincter injury, confirmation of incontinence, implantation, scaffolding, cell tracking, and outcomes measurements in the experimental studies for IAS treatment.

RESULTS

Intrinsic molecular pathway of basal tone in internal anal sphincter

Calcium

Calcium (Ca²⁺) activation in Ca²⁺/calmodulin/myosin light chain kinase (MLCK) pathway plays a critical role in the initial phasic stage of IAS tone development via G protein-coupled receptor (GPCR) activation, and in promoting the p-MLC₂₀ [31]. Ca²⁺-related factors (endothelin, Ca²⁺-free solutions and Ca²⁺-channel blockers, Ca²⁺ oscillations, Ca²⁺ transients [CTs], and Ca²⁺-mediated signaling cascade) can determine the myogenic intrinsic property of the basal tone in IAS directly, rather than extrinsic nerve and hormone stimulation. The intracellular mechanism of endothelin is the biphasic action characterized by an initial brief relaxation followed by a sustained contraction via inhibition of selective protein kinase C (PKC) inhibitor H-7 or calmodulin inhibitor W-13 [35]. Furthermore, a recent study reported an impaired global rise of Ca²⁺ in animal models using mice with knockout SM-specific myosin phosphatase target subunit 1 (MYPT1), which anchors myosin light chain phosphatase (MLCP) to p-MLC. The study demonstrated that maintaining IAS basal tone requires Ca²⁺-mediated MLCK activation [34]. Three channels are involved in generating the Ca²⁺ rise; Ca²⁺-releasing ryanodine receptors/channels (RyRs), transmembrane member 16A (TMEM16A) Ca²⁺-activated Cl (ClCa) channels, and L-type voltage-dependent Ca²⁺ channels (VDCCs). Therefore, altering any one of these channels impaired IAS basal tone similar to the MLCK deletion. This suggests the independence of molecular mechanisms for the initial phase of IAS tone development from the later phase of maintenance of the basal tone. The study also showed the potential of a new treatment targeting a global rise in Ca²⁺-mediated signaling cascade (RyR-TMEM16A ClCa channel-L-type VDCC) [34].

Table 1. Translation study of molecular pathway for IAS

Study	Year	IAS harvest	Mediator	Pathway	Action of mechanism
Calcium (Ca²⁺)					
Chakder et al. [35]	1999	NA	Endothelins 1 and 2	PKC and the Ca ²⁺ -calmodulin pathways	Endothelin-induced contraction of IAS, via inhibition of selective PKC inhibitor H-7 or calmodulin inhibitor W-13
Zhang et al. [34]	2016	SM-specific MYPT1, TMEM16A, MLCK knockout mice	Global rise in Ca ²⁺	RyR-TMEM16A-VDCC signaling module	MLCK activation by a global rise in Ca ²⁺ via a RyR-TMEM16A-VDCC signaling module sets a basal tone of IAS
Cobine et al. [36]	2020	SM-GCaMP mice	Spatiotemporal properties of Ca ²⁺ transients	L-type VDCCs	Conduction of CTs rising by slow wave from distal to proximal IAS leading to the maintenance of basal IAS
Lu et al. [32]	2021	SMC-specific TMEM16A deletion mouse	SCaO	RyR-TMEM16A-VDCCs pathway	IAS basal tone generated by RyR-TMEM16A-VDCCs signaling module mediated by 2 oscillating Ca ²⁺ signals (SCaOs and ACaOs)
Rho/ROCK					
Rattan et al. [37]	2006	Sprague-Dawley rats	ROCK inhibitor Y-27632	RhoA/ROCK pathways	Selective ROCK inhibitor (lower doses of Y-27632) relax IAS independent of the NOS/cGMP pathway
Patel et al. [38]	2007	Male Sprague-Dawley rats	RhoA-GTP, ROCK II, MLC ₂₀ , phospho-MYPT1, phospho-MLC ₂₀	RhoA/ROCK pathways	Upregulation of RhoA/ROCK maintains spontaneous tone in IAS
de Godoy et al. [40]	2007	H-ras ^{+/+} mice	H-ras	Inhibitory RhoA/Rho kinase machinery	H-ras decreases basal tone in IAS via inhibiting RhoA translocation to the plasma membrane, reducing activation of the Rho kinase isoform ROCK II
Patel et al. [41]	2007	NA	GGTI-297	RhoA prenylation blockade (translocation of RhoA to the SMC membrane)	The inhibitory effect of GGTI-297 maintains a basal tone of IAS via decreasing prenylation of RhoA
Rattan [42]	2010	Sprague-Dawley rats	HMGCR1	RhoA prenylation leading to RhoA/ROCK translocation	Relaxation of IAS by HMGCR1 simvastatin mediated via decreased downstream of RhoA prenylation and ROCK activity
Singh et al. [43]	2011	Human IAS	PDBu	RhoA and ROCK II pathway	PDBu-induced IAS contractility via activation of RhoA/ROCK
Rattan et al. [39]	2012	Human IAS	ROCK- and PKC selective inhibitors Y 27632 and Gö 6850	RhoA/ROCK pathways	Activation of RhoA/ROCK and downstream signaling determines basal tone in IAS via MLCP inhibition
Rattan et al. [44]	2015	Human IAS tissues	Extracellular signal of TXA ₂ , PGF ₂ α	RAS and arachidonic acid pathways	End products (TXA ₂ , and PGF ₂ α) of both RAS and arachidonic acid pathways causes an increase in the IAS tone via triggering of RhoA/ROCK
Rattan et al. [46]	2015	Male Sprague-Dawley rats	SM22	Actin-binding properties of SM22 interfering with actin-myosin interaction	Phosphorylation of SM22 in ROCK inhibits SM22-actin interaction leads to basal tone as in IAS
Singh et al. [45]	2017	Rat	miRNA-139-5p	ROCK2 pathway	Overexpression of miRNA-139-5p causes a decrease in the IAS tone
AAID					
Singh et al. [49]	2016	Fischer rats (F344 of 6-, 18-, and 26-mo-old age)	miRNA133a	RhoA signaling pathway	Aging-associated miRNA133a and its target gene (<i>RhoA</i> , <i>ROCK2</i> , <i>MYOCD</i> , <i>SRF</i> , and <i>SM22</i>) via regulating RhoA signaling pathway express IAS SM phenotype in the aging
Mohanty et al. [48]	2019	Fischer 344 rat	Thromboxane A ₂ /ANG II type	GPCR	Downregulation of GPCR via thromboxane A ₂ and ANG II type 1 receptors desensitization, lysosomal degradation associated with an aging-related decrease in the basal tone of IAS
Singh et al. [47]	2020	Sprague-Dawley rats	BDNF	1. RhoA/ROCK pathway via TrkB/TXA ₂ -R and AT ₁ -R activation 2. NANC relaxation via NO and soluble GC	BDNF-augmented increase in the IAS tone via activation GPCR linked to RhoA/ROCK signaling and NANC Relaxation
Singh et al. [33]	2021	Male Fischer 344 rats (6-mo-old [young group] and 26-mo-old [old group])	TrkB antagonist	GPCR-coupled agonist-stimulation by activation of RhoA/ROCK and NANC stimulation	BDNF rescues AAID via RhoA/ROCK and decreases the nitrenergic NANC inhibitory neurotransmission.

(Continued to the next page)

Table 1. Continued

Study	Year	IAS harvest	Mediator	Pathway	Action of mechanism
Oxidative					
Krishna et al. [51]	2014	Sprague-Dawley rats (20–22-wk-old male)	HO-1	Hemin/HO-1 system	HO (predominantly HO-2 isoform) in neurally mediated relaxation of IAS increases basal tone, and the fibroelastic properties via regulating RhoA/ROCK pathway
Singh et al. [52]	2014	Sprague-Dawley rats; 4–6 mo (adult) and 24–30 mo (aging)	LY83583	LY83583-mediated a decrease in RhoA/ROCK signal transduction	Oxidative stress is associated with aging-associated decrease in IAS tone via disruption of RhoA/ROCK and downstream signaling cascade
Singh et al. [50]	2015	Adult Sprague-Dawley rats	LY-83583	nNOS inhibition and RhoA/ROCK pathway	Bimodal effect of oxidative stress (lower vs. higher concentration = 0.1 nM–10 μM vs. 50–100 μM): lower concentrations leads to an increase in IAS tone via nNOS inhibition and RhoA/ROCK activation by LY-83583
RAS and COX					
De Godoy et al. [53]	2004	Male Sprague-Dawley rats	ANG II	ACE	Biosynthesis of Ang II-related peptides by ACE activity modulates basal IAS tone via AT ₁ -R activation
De Godoy et al. [54]	2005	Male Sprague-Dawley rats	ANG II precursor angiotensinogen	RAS pathway	RAS regulates basal tone in IAS partially via biosynthesis and releases ANG II by activation of AT ₁ -R
De Godoy et al. [55]	2006	Rat	ANG II	Internalization of subtype I receptor(s) (AT ₁ -R) in the plasma membrane and externalization of subtype II receptor(s) (AT ₂ -R) in the cytosol	Translocation of AT ₁ - and AT ₂ -Rs by higher concentrations of ANG II leads to relaxation of the IAS
Inhibitory NTM					
Moumimi et al. [62]	1988	NA	EFS and exogenous VIP	GC and adenylate cyclase	EFS induces relaxation of SM in IAS mediated via guanosine 5'-cyclic monophosphate
Rattan et al. [73]	1992	NA	NO	NANC inhibitory pathway	Inhibitory NANC by NO-mediated IAS relaxation
Rattan et al. [74]	1992	NA	NO	NANC inhibitory pathway	NO or NO-like substance is an important mediator of IAS relaxation in response to NANC nerve stimulation
Chakder et al. [69]	1992	Mice IAS	NO, VIP, superoxide	Superoxide dismutase	IAS relaxation by NO was suppressed by superoxide and reversed by superoxide dismutase
O'Kelly et al. [67]	1993	Human IAS tissue	NO	NANC inhibitory pathway	NO-mediate neurogenic relaxation of the human IAS
Rattan et al. [77]	1995	Mice IAS	Recombinant hemoglobin	NO pathway	Recombinant hemoglobin suppresses IAS relaxation induced by NO
Rattan et al. [63]	1997	NA	PACAP	N-type Ca ⁺⁺ -channel blocker ω-conotoxin	Dual effect: contraction of IAS via the activation of PACAP receptor at P-containing nerve terminals. IAS relaxation by PACAP direct action at nerve terminals of the myenteric inhibitory neurons
Chakder et al. [64]	1998	NA	PACAP	NANC inhibitory pathway	PACAP mediated IAS relaxation via the activation of PACAP1/VIP receptor via presynaptic release of PACAP and VIP by NO
Kubota et al. [58]	1998	Canine	Transmural field stimulation	Membrane hyperpolarization with relaxation	Membrane hyperpolarization relaxes IAS via EFS in the transitional and upper region of IAS
Banwait et al. [72]	2003	Rat	β ₃ -AR	Endothelial NOS	IAS SM relaxation via partly transduced NOS by β ₃ -AR activation
Acheson et al. [66]	2003	Sheep and human IAS	L-arginine, D-arginine	ph and osmolality	L-arginine independent of NO reduce IAS tone
Jones et al. [68]	2003	nNOS knockout mice	NO, antagonists of VIP, ATP, HO	nNOS, nicotinamide adenine dinucleotide phosphate diaphorase HO	NO induces the RAIR primary, and other inhibitory neurotransmitters compensate for the absence of NOS
Rattan et al. [59]	2005	wild-type (WT), HO-2 knockout (HO-2 ^{-/-}) and nNOS knockout (nNOS ^{-/-}) mice	CO, NO, VIP	NANC inhibitory pathway, and nNOS pathway	Inhibitory NANC mediated via activation of nNOS and partly VIP relax IAS. CO is not associated with inhibitory NANC relaxation, which directly relaxes IAS

(Continued to the next page)

Table 1. Continued

Study	Year	IAS harvest	Mediator	Pathway	Action of mechanism
McDonnell et al. [60]	2008	BALB/c mice	P2Y1 receptors and apamin-sensitive K ⁺ channels	Purinergic inhibitory neural pathway	Membrane hyperpolarization via purinergic transmission relaxes IAS
Koyuncu et al. [78]	2008	Rabbit IAS	Isosorbide dinitrate, sodium nitroprusside	NANC inhibitory pathway	NO leads to IAS relaxation via the NANC pathway, but nitrate tolerance was not developed
de Godoy et al. [56]	2009	Knockout mice with selective deletion of COX-1 or COX-2 (COX-1 ^{-/-} and COX-2 ^{-/-} mice)	COX-1	COX-I pathway	Prostanoids produced via COX-1 provide an external trigger for basal tone in IAS
de Godoy et al. [57]	2009	Male Sprague-Dawley rats	Arachidonic acid	COX-I pathway	Arachidonic acid metabolites (PGF2 and thromboxane A2) increases the basal tone of IAS
Acheson et al. [65]	2009	Sheep IAS	NO, noradrenaline	NANC inhibitory pathway	Endogenous noradrenaline acts via postjunctional α ₁ -ARs to antagonize neurogenic relaxations that are largely mediated by NO
Duffy et al. [76]	2012	C57BL/6 and W/W ⁱ mice IAS	ATP	Purinergic inhibitory neural pathway	Purinergic hyperpolarization associated relaxation of IAS independent on intramuscular interstitial cells of Cajal
Keef et al. [75]	2013	VIP ^{-/-} mice	VIP	NANC inhibitory pathway	Ultraslow relaxation and hyperpolarization mediated by VIP leading to prolonged IAS relaxation
Huang [70]	2014	Guinea pig IAS	Thrombin and PAR1 peptide agonists	NO pathway	PAR1 and PAR2 mediate relaxation of IAS
Cobine et al. [71]	2014	Kit ^{cre} opGFP ^{+/+} , C57BL/6 (wild-type), Pdgfra ^{exon1} +/+, smMHC ^{Cre-egfp} , cGKI ^{-/-} mice	GC (GCa, GCβ) and NO	NANC inhibitory pathway (GC-dependent, cGKI independent pathway)	Nitrergic effectors in the PDGFRα-cells induce nitrergic relaxation of IAS mediated by GC within the SIP syncytium
Folasire et al. [61]	2016	Porcine IAS	NO, CO, H ₂ S	NANC inhibitory pathway	Simultaneous release of all 3 gaseous transmitters by EFS induces the relaxations of the IAS

AAID, aging-associated internal anal sphincter dysfunction; ACaO, asynchronized Ca²⁺ oscillation; ACE, angiotensin-converting enzyme; ANG II, angiotensin II; AR, adrenoceptor; AT₁-R, angiotensin II receptor type 1; AT₂-R, angiotensin II receptor type 2; BDNF, brain-derived neurotrophic factor; cGKI, cyclic guanosine monophosphate-dependent protein kinase I; cGMP, cyclic guanosine monophosphate; CO, carbon monoxide; COX, cyclooxygenase; CT, Ca²⁺ transient; EFS, electrical field stimulation; GC, guanylate cyclase; GGTI, geranylgeranyl transferase inhibitor; GPCR, G protein-coupled receptor; GTP, guanosine triphosphate; H₂S, hydrogen sulfide; HMGCR1, HMG-CoA reductase inhibition; MLCP, myosin light chain phosphatase; HO, heme oxygenase; IAS, internal anal sphincter; LY-83583, oxidative stress inducer 6-anilino-5,8-quinoline-dione; miRNA, microRNA; MLC, myosin light chain; MLCK, myosin light chain kinase; MYPT, myosin phosphatase target subunit; NA, not available; NANC, nonadrenergic noncholinergic; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; PACAP, pituitary adenylate cyclase-activating peptide; PAR, proteinase-activated receptor; PDBu, phorbol 12,13-dibutyrate; PDGFR, platelet-derived growth factor receptor; PGF2α, prostaglandin F2α; PKC, protein kinase C; RAIR, rectoanal inhibitory reflex; RAS, renin-angiotensin system; ROCK, Rho-associated protein kinase; RyR, ryanodine receptor; SCaO, synchronized Ca²⁺ oscillation; SM, smooth muscle; SMC, smooth muscle cell; TMEM16A, transmembrane member 16A; TXA2, thromboxane A2; TXA2-R, thromboxane A2-receptor; TrkB, tyrosine kinase receptor B; VDCC, voltage-dependent Ca²⁺ channel; VIP, vasoactive intestinal polypeptide.

Serving as the pacemaker, Ca²⁺-activated Cl⁻ channel TMEM16A also propagates electrical slow waves (SWs) into IAS-SMCs, inducing basal tone via the depolarization and activation of L-type VDCCs and giving rise to CTs in the SMCs. The pharmacological and spatiotemporal properties of CTs in the IAS are very similar to those of SWs with the greatest frequency and amplitude at the distal end of the IAS, suggesting that this is the predominant pacemaker region. Phasic activity by the fastest and highly coordinated pacemaker located at the distal extremity will lead to orally directed contractions that will aid in maintaining continence [36]. The basal tone generated by RyR-TMEM16A Cl⁻ Ca²⁺ channel-L-type VDCC signaling module might also be mediated by oscillating Ca²⁺ signals in SMCs of IAS [32]. The opening of a

small number of RyRs induced Ca²⁺ release with an exhibition of asynchronized Ca²⁺ oscillations (ACaOs) and synchronized Ca²⁺ oscillations (SCaOs) in IAS. In sustained contractions, ACaOs contribute approximately 25%, while SCaOs account for 75%.

Rho/Rho-associated, coiled-coil containing serine/threonine kinase pathway

The basal tone in the IAS is critically dependent on RhoA/ROCK [37]. The balance between the Ca²⁺/calmodulin/MLCK stimulation and MLCP inhibition determined primarily by RhoA/ROCK pathway plays a crucial role in the latter phase of tonic followed by the initial phasic stage of IAS tone development [31, 38]. The latter phase of tonic or sustained contraction in basal tone is de-

pendent on the MLCP inhibition which maintains higher levels of p-MLC₂₀ [39]. RhoA/ROCK which is present throughout the SMCs translocates to the periphery of the SMCs during the sustained contraction phase.

It has been reported that several factors are involved in the regulation of basal tone in IAS via modulating the molecular pathway of RhoA/ROCK in the previous studies [38-45]. The spontaneous tone in the IAS can be induced by upregulation of RhoA/ROCK and PKC in part which mediates its effects via ROCK [38, 39]. Singh et al. [43] reported that phorbol esters, which have been used as PKC activators in SMs, cause contraction of the human IAS SMCs associated with RhoA/ROCK activation rather than the PKC activation. Furthermore, RhoA prenylation is essential for the translocation of RhoA to the cellular membrane and downstream signal transduction of RhoA. Consequently, inhibition of RhoA prenylation by geranylgeranyltransferase I inhibitor, inactivates RhoA/ROCK, and it leads to a decrease in the basal tone in the IAS [41]. Rattan [42] reported that the HMG-CoA reductase inhibitors such as simvastatins relax the IAS SM via decreasing the levels of RhoA prenylation and ROCK activity. de Godoy et al. [40] also reported that H-ras modulates basal tone by inhibiting RhoA translocation to the plasma membrane, reducing activation of the Rho kinase isoform ROCK II. Inhibition of upstream RhoA-Rho kinase signaling produces downstream pre-relaxation events including diminished p-MYPT1 and increased activity of MLCP, resulting in diminished p-MLC₂₀. Thromboxane A₂ (TXA₂), prostaglandin F_{2α} (PGF_{2α}), and angiotensin II (ANG II), which are the end products of arachidonic acid and renin-angiotensin system (RAS) pathway, provide extracellular signals that trigger the activation of RhoA/ROCK for the basal IAS tone in humans [44]. The microRNA (miRNA)-139-5p plays a significant role in the functional phenotypic SMs by repressing RhoA/ROCK pathway in the SMCs, and its low expression in the IAS may increase the basal tone [45]. Phosphorylation of SM22 (transgelin/calponin) protein by ROCK inhibiting SM22-actin interaction leads to the development of basal tone in IAS [46]. The selective ROCK inhibitor which causes relaxation of the IAS might be used for conditions associated with hypertensive IAS [37].

Aging-associated internal anal sphincter dysfunction

Clinically, degenerative transformation of the anal sphincter muscles can be characterized by decreasing IAS tone and increasing response to RAIR and subsequent decreased resting pressure rather than squeezing pressure. Recently, some authors defined AAID with the molecular mechanisms and impeded the development of a specific and safe treatment for AADI-related FI [33, 47]. A previous study reported that aging can induce the downregulation of GPCR via TXA₂ and ANG II type 1 receptors desensitization (TXA₂-R and AT1-R). In their data, the fibroelastic properties (FEP) of the SMs correlated with a decrease in the GPCR expression at gene and protein levels, and an increase in the expres-

sion of GPCR downstream signaling regulatory proteins, RGS2, GRK5, and Arrb2. Therefore, downregulation and compromise in the GPCR recycling lead to a decrease in the IAS tone [48]. An endogenous agent such as a brain-derived neurotrophic factor (BDNF) may also relate to the AAID. As an important member of the neurotrophin family, BDNF which is released primarily in the central nervous system was also released in the peripheral nervous system, especially in the enteric nervous system and in the GI tract SMs. Using different systems, BDNF can rescue AAID via augmentation of GPCR-coupled SM contractility and relaxation. BDNF-mediated increased basal tone can be induced in GPCR-coupled agonist-stimulated IAS by activation of RhoA/ROCK in the SMCs, and via a decrease in the nitrergic nonadrenergic noncholinergic (NANC) inhibitory neurotransmission [33]. BDNF also increased the basal and agonist-stimulated IAS tone via TrkB/TXA₂-R and TrkB/AT1-R signaling, and RAIR-induced IAS relaxation via nitric oxide (NO)/soluble guanylate cyclase (GC) pathway [47].

Furthermore, the miRNA-133a and its gene targets are crucial to the RhoA signaling pathway in relation to contractility and IAS SM phenotype in aging [49]. In their data, it was observed that the basal tone and the FEP of the IAS might be regulated by changes in the transduction molecules, growth and transcription factors, regulators of SMC differentiation, extracellular matrix components, and early markers of SMCs via miRNA-targeting diverse signaling pathways [49]. Most of the AAID studies have been performed on animal models using older rats or compared with younger ones as our review, showing an impossible approach for harvesting sphincter tissue from younger and elderly FI patients.

Oxidative stress

The antioxidant defense mechanisms, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase play a crucial counterregulatory role in ROS levels which are produced during aerobic cellular metabolism. However, with aging, the weakening of the antioxidant defense mechanism can bring about cumulative effects of excessive ROS and results in irreversible cellular damage. As an aging-related risk factor, oxidative stress has been reported to decrease the overall propulsive activity of the gut. A decrease in IAS tone and altered gut motility may be the leading causes of AAID via decreasing phosphorylation of the myosin-binding subunit of MYPT1 and regulatory MLC (MLC₂₀), secondary to RhoA-associated kinase (RhoA/ROCK) inactivation [50-52].

Singh et al. [50, 52] also reported that the effect of extreme oxidative stress decreases IAS tone, via inactivation of RhoA/ROCK II in rats, while a lower level of oxidative stress leads to an increase in IAS tone. Antioxidants such as SOD might play an important role in reversing the aging-associated oxidative damage in IAS. Furthermore, heme oxygenase (HO) degrades heme, producing carbon monoxide, biliverdin, and Fe²⁺ [43]. Krishna et al. [51]

identified the role of HO in neurally mediated relaxation of IAS. It was shown that carbon monoxide (CO), one of the HO products, causes SM relaxation along with hyperpolarization of the SMCs via upregulation of RhoA/ROCKII and downregulation of MYPT1 levels.

Molecular pathway of relaxation in internal anal sphincter *Renin-angiotensin system and cyclooxygenase*

In the pathway of the RAS, renin converts angiotensinogen into angiotensin I, and angiotensin I is converted into ANG II by the angiotensin-converting enzyme (ACE). ANG II activates specific membrane receptors in the target cells, and binds to 2 different subtypes of GPCRs; AT1-R and AT2-R. Previous studies demonstrated that RAS via ANG II biosynthesis inhibitor (ACE inhibitor) and release of ANG II followed by activation of AT1-R and AT2-R, provide a partial regulation of the basal tone in the IAS [53, 54]. ANG II can also produce a bimodal effect, a contraction (at lower concentrations) followed by relaxation (at higher concentrations) [55]. A contraction of IAS is mediated by AT1-R, and relaxation of IAS is mediated by AT2-R. Higher concentrations of ANG II result in the internalization of the AT1-R to the cytosol and the externalization of AT2-R to the plasm membrane. The translocated AT2-Rs for activation exert their inhibitory effect on the SMCs of IAS leading to relaxation. In addition, among the 2 primary isoforms of the COX pathway, COX-1 might play a role in maintaining basal tone by producing PGF 2α , thromboxanes, and ANG II which provides the signal at SMCs of IAS [56, 57]. However, the molecular mechanism between RhoA/ROCK and PKC in the COX-1-sustained tone in the IAS remains unclear.

Inhibitory neurotransmitter

IAS relaxation presented as RAIR in response to defecation is dependent on nerve activity with NTMs [58]. In functional experiments, noradrenaline is the only NTM that has been identified to elicit an excitatory response in the sphincter, while inhibitory roles have been suggested for NO, CO, vasoactive intestinal polypeptide (VIP), acetylcholine and adenosine 5'-triphosphate (ATP), and pituitary adenylyl cyclase-activating peptide (PACAP) [59-64]. These transmitters appear to interact for SM relaxations, especially NO. NO which is synthesized from L-arginine in a reaction catalyzed by NO synthase (NOS), is freely soluble, diffuses rapidly, has a short half-life (3 seconds) [65, 66], and mediates neurogenic relaxation by binding to cytosolic GC and stimulating the production of cyclic guanosine monophosphate (cGMP) [67]. In absence of NOS, other inhibitory NTM including VIP or ATP, heme compensates to maintain an approximately normal response [68]. NO also caused the IAS relaxation after activation by the formation of NO $_3^-$ after contact with the superoxide [69] or via proteinase-activated receptor-1 (PAR1) and PAR2 [70].

The interstitial cells of Cajal (ICC), which are located in close proximity to the nerves throughout most of the GI tract including IAS, participate in nitrergic and cholinergic NTM in the IAS. In

other interstitial cells, platelet-derived growth factor α (PDGFR α) is thought to be responsible for the purinergic component of inhibitory NTM. ICC and PDGFR α cells are both closely associated with neural NOS neurons in the IAS. SMCs from gap junctions with adjacent ICC and PDGFR α cells, give rise to an electrical syncytium referred to as the "SIP syncytium." The nitrergic relaxation in the IAS results from the signals integration by GC within the SIP syncytium, while cGMP-dependent protein kinase I and its downstream targets are responsible for less than half of the relaxation [71]. In addition, endothelial NOS may partly modulate the vascular SMs relaxation in response to activation of the β_3 -adrenoceptor (AR). The stimulation of β -AR causes a release of Gs α , which activates adenylyl cyclase to produce cAMP. Elevated levels of cAMP produce SM relaxation in IAS via activation of cAMP-dependent protein kinase A [72]. A NO-like substance (CO, and VIP) as an inhibitory NTM of NANC nerves, is responsible for the neutrally mediated IAS SM relaxation [67, 73, 74]. Rattan et al. [59] compared the ability of NO, CO, and VIP to relax mouse IAS. VIP, which mediated ultraslow relaxation and hyperpolarization by purinergic and nitrergic NTM, was the most potent, followed by NO. While, longer stimulus trains of electrical field stimulation, a non-purinergic, non-nitrergic relaxation and hyperpolarization, which is mediated by VIP, lead to prolonged IAS relaxation [75], and very high concentrations of CO may not be a relevant relaxation agonist in mouse IAS [59]. The purines such as ATP also contributed to inhibitory NTM in the IAS. Duffy et al. [76] reported that purinergic hyperpolarization associated with IAS relaxation is independent of intramuscular ICC. McDonnell et al. [60] reported that a purinergic pathway significantly contributes to inhibitory motor innervation in the IAS by hyperpolarization of the postjunctional membrane to produce inhibitory junction potentials. Folasire et al. [61] also reported that hydrogen sulfide possibly contributes relaxation of the porcine IAS, and its potency is lower than that of CO. In addition, recombinant hemoglobin 1.1 (rHb1.1) could be caused specific obliteration of NO-induced relaxation of the IAS [77]. However, nitrate tolerance did not develop in IAS SM in the previous study [78].

Internal anal sphincter treatment in fecal incontinence: cellular vs. acellular

Inoue et al. [16] reported that cell treatment using ASC sheets in an animal model showed a significant improvement of the anal pressure in the ASC sheet transplantation group compared with the control group after 8 weeks of cell implantation. Histologically, in the vicinity of the remaining SM, reproduction of SM was detected with an increased level of SMC-specific markers. Cell treatment using human ASCs (hASCs) injections combining them with polyacrylamide hydrogel carrier, bulkamid showed the functional improvement in anorectal manometry, low inflammatory response, and tissue integration on micro-computed tomography [19]. Salcedo et al. [17, 18] also reported a significant increase in resting anal sphincter pressure and peak pressure, anal

sphincter electromyography amplitude and frequency, and a marked decrease in fibrosis and scar tissue in an animal model using an intramuscular or intravascular injection of MSCs. Oh et al. [20] showed the functional differentiation of polycaprolactone beads containing autologous myoblasts labeled with PKH-26 fluorescent dye in animal models using posterior resection of IAS and EAS. But it remained weak and unclear for an association between functional improvement of resting pressure and the differentiation of myoblasts into SMCs of IAS. Sarveazad et al. [79] reported the efficacy of co-application of laser and hASC therapy showing improved resting anal pressure and enhanced myogene-

sis and angiogenesis with increased expression level. All the previous incontinence models included incision of EAS or adding pudendal nerve crush [16-20, 79]. In the observed histologic augmentation of smooth muscular layers in IAS, but in cell tracking, no newly differentiated SMCs of implanted stem cells were found at the injury site under fluorescence *in situ* [16], micro-computed tomography [19], confocal microscopy, or immunofluorescence assay using green fluorescent protein labeling [17, 18] (Table 2).

In contrast to cellular treatment, scaffolding or gut engineering using natural biomaterial has been employed for IAS malfunctions [13, 14, 80-83]. Some authors developed a 3-dimensional

Table 2. Treatment of IAS for fecal incontinence; cellular and acellular approaches

Study	Year	Cell type	Animal (No. of animal)	Type of sphincter injury/confirmation of incontinence	Implantation/factors/scaffolding	Cell tracking	Outcome
Inoue et al. [16]	2018	ASC sheets	Female Sprague-Dawley rats (n = 18)	Sphincterotomy by the removal of the left semicircle in both the IAS and EAS via a posterior incision/ Not confirmed	None	Fluorescence <i>in situ</i> hybridization	Anal manometry, histology
Salcedo et al. [17]	2013	Mesenchymal stem cell	Age-matched female Sprague-Dawley rats (n = 70)	Incising the IAS and EAS 2–3 mm deep+pudendal nerve crush/Confirmed via microscopy	IM or IV injection	Green fluorescent protein	Anal manometry, electromyography, immunofluorescence analysis
Salcedo et al. [18]	2014	Mesenchymal stem cells	Age-matched female Sprague-Dawley rats (n = 50)	Excision of 25% of the IAS and EAS muscle/Confirmed via microscopy	IM or serial IV injections	Green fluorescent protein	Anal manometry, immunofluorescence, histology
Kuismanen et al. [19]	2018	hASC	Sprague-Dawley female virgin rats (n = 60)	Acute fourth grade EAS and IAS muscle and mucosa) and sewed back with 6-0 poliglecaprone/Not confirmed	Polyacrylamide hydrogel carrier, Bulkamid	Micro-computed tomography	Anal manometry, micro-computed tomography imaging, 3D imaging, histology
Oh et al. [20]	2015	Autologous myoblasts	Male mongrel dogs (19–22 kg; 10 wk old) (n = 15)	Resecting 25% of the posterior IAS and EAS by electrocautery/Anal manometry and CMAP confirmed	Polycaprolactone beads	Fluorescent dye PKH-26	<i>In vitro</i> contractility, CMAP, histology
Sarveazad et al. [79]	2019	hASC	Male rabbits (n = 7)	Grade 4 tear at EAS and IAS/ Confirmed via histology, percentage of collagen, muscle	Laser (660 nm, 90 sec, Dil solution immediately after sphincterotomy, daily, 14 days)		Anal manometry, immunofluorescence, histology, collagen analysis, VEGFA, Ki67 mRNA, vimentin mRNA gene expression profiling
Hecker et al. [80]	2005	SMCs from rabbits IAS	<i>In vitro</i>	None	3D cylindrical IAS ring/fibrin gel and 5-mm diameter SYLGARD mold	None	<i>In vitro</i> physiologic functionality (generating spontaneous basal tone, kinetics, and dose-response curve of force generated by IAS ring), histology
Somara et al. [81]	2009	SMCs from human IAS	<i>In vitro</i>	None	3D bioengineered ring model	None	<i>In vitro</i> physiologic functionality (contractile properties and force generation in response to acetylcholine, PKC inhibitor calphostin-C, Rho/ROCK inhibitor Y-27632, permeable Rho/ROCK inhibitor c3-exoenzyme, and PKC activator PDBu)

(Continued to the next page)

Table 2. Continued

Study	Year	Cell type	Animal (No. of animal)	Type of sphincter injury/confirmation of incontinence	Implantation/factors/scaffolding	Cell tracking	Outcome
Raghavan et al. [13]	2011	SMCs from mouse IAS	RAG1 ^{-/-} mice (NA)	None	Implantation of bioengineered IAS construct in back of mice/IM-FEN cells	None	<i>In vivo</i> myogenic and neuronal components (basal tone; spontaneous generation, relaxation, and recovery by VIP-ergic, EFS, cholinergic, KCl-induced), histology
Raghavan et al. [83]	2010	SMCs from Human and rabbits IAS	C57BL/6J mice	None	Implant subcutaneously on the dorsum of mice/microosmotic pump+fibroblast growth factor-2	None	<i>In vivo</i> physiologic functionality (generation of spontaneous basal tone; contraction and relaxation by responding to cholinergic, nitrenergic, and VIP-ergic stimulation and potassium chloride), histology
Raghavan et al. [14]	2014	SMCs from human IAS	Rat	None	Implant surgically into the perianal region/Innervation of enteric neuronal progenitor cells from the human colorectum	None	<i>In vivo</i> physiologic functionality (relaxation of IAS ring in response to EFS, VIP), immunohistochemical analysis

ASC, adipose stem cell; CMAP, compound muscle action potential; EAS, external anal sphincter; EFS, electrical field stimulation; hASC, human adipose stem cell; IAS, internal anal sphincter; IM, intramuscular; IM-FEN, immortomouse fetal enteric neuronal; IV, intravenous; mRNA, messenger RNA; NA, not available; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; RAG1, recombination activating gene 1; ROCK, Rho-associated protein kinase; SMC, smooth muscle cell; VEGFA, vascular endothelial growth factor A; VIP, vasoactive intestinal polypeptide; 3D, 3-dimensional.

(3D) bioengineered ring (BR) model constructed from isolated human IAS SMC [80-82] (Table 2). Bioengineering an *in vitro* 3D fibrin-based model consisting of a homogenous population isolated from human sphincteric SMC on a fibrin gel might provide a favorable cellular environment to cell survive tissue expansion compared to the previous cell treatment [16, 18-20, 22, 69]. The 3DBR showed an increase in contractile protein expression profiles of PKC, RhoA, CPI-17, and HSP27 which have the specificity of basal tone generated by IAS circular SM at rest [81]. In the IAS reconstructs, the basal tone was maintained with developing spontaneous tone (0.68 ± 0.26 mN), and concentration-dependent tone response to bethanechol (a muscarinic agonist), K⁺ depolarization, and isoproterenol (β -adrenoceptor agonist), Y-27632. It also had higher levels of RhoA/ROCK, PKC-potentiated inhibitor, or inhibitory phosphoprotein for myosin phosphatase (CPI-17), MYPT1, and MLC₂₀ compared to rectal SM [84]. In intrinsically innervated human IAS models using enteric neuronal progenitor cells isolated from the human colorectum, also demonstrated that myogenic and neuronal components of the bioengineered IAS response physiologically to VIP-ergic, electrical stimulation, cholinergic, KCl-induced neurotransmission with relaxation, and contraction of basal tone [13]. However, it is limited due to complications such as infection and erosion related to foreign body reaction at perineal implantation [14], and no a clinical trial on bioengineered IAS reconstructs has been reported.

The interrelationship between the molecular mechanism and regenerative treatment for internal anal sphincter

Several translation studies have revealed the molecular mechanism of SMC in IAS as intrinsic properties, which can be characterized by Ca²⁺, RhoA/ROCK, AAID, oxidative stress, RAS, COX, and inhibitory NTM. However, we observed that previous cellular treatments [16-20, 79] did not show a relationship between SMCs-related histologic or functional outcomes and basal tone or relaxation-related molecular pathways in injured IAS regardless of the cell type or confirmation of IAS-incontinence. We considered that it might be due to the complexity of the molecular mechanisms for basal tone and relaxation of IAS as intrinsic properties (Fig. 1). While the bioengineered IAS reconstructs as acellular approach models have a potential for *in vivo* response and functional properties of 3DBR similar to human IAS dependence on RhoA/ROCK [84].

The best option of cell source and phenotype is still a questionable issue in the field of GI regeneration [85]. The myogenic features SMCs isolated from IAS are different from those of skeletal muscle cells from EAS in the human anal sphincter [86]. Therefore, cell treatments using cell types originated from skeletal muscle might be unsuitable for restoration of dysfunctional IAS clinically, which is composed of SMCs characterized by intrinsic force contracted at rest, with small-amplitude slow contractions and slow relaxation. These implied that only one study using autologous skeletal-muscle-derived cells showed a significant improvement in resting pressure after cell implantation, but it did not

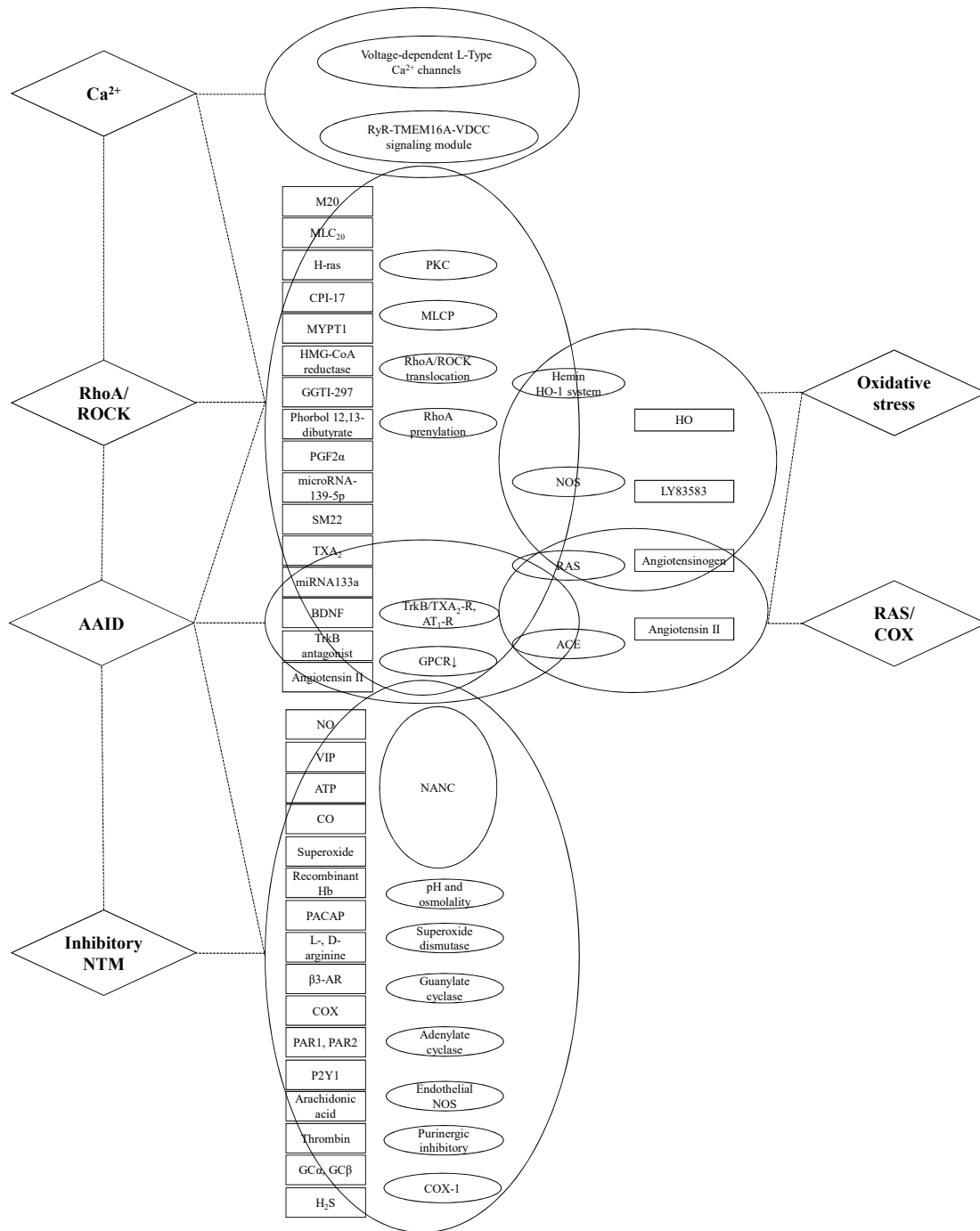


Fig. 1. The interrelationship diagram of molecular mechanisms for basal tone and relaxation of internal anal sphincter for fecal incontinence. AAID, aging-associated internal anal sphincter dysfunction; ACE, angiotensin-converting enzyme; AR, adrenoceptor; ATP, adenosine 5'-triphosphate; BDNF, brain-derived neurotrophic factor; CO, carbon monoxide; COX, cyclooxygenase; GC, guanylate cyclase; GGTI, geranylgeranyl transferase inhibitor; GPCR, G protein-coupled receptor; HO, heme oxygenase; MLC₂₀, 20-kDa myosin regulatory light-chain; MLCP, myosin light chain phosphatase; MYPT1, myosin phosphatase target subunit 1; NO, nitric oxide; NOS, nitric oxide synthase; NTM, neurotransmitter; PACAP, pituitary adenylate cyclase-activating peptide; PAR1, proteinase-activated receptor-1; PGF2α, prostaglandin F2α; PKC, protein kinase C; RAS, renin-angiotensin system; ROCK, Rho-associated, coiled-coil containing serine/threonine kinase; RyR, ryanodine receptor/channel; TMEM16A, transmembrane member 16A; TXA₂, thromboxane A₂; VDCC, voltage-dependent Ca²⁺ channel; VIP, vasoactive intestinal polypeptide.

demonstrate the physiologic relationship between those outcomes and basal tone of IAS as a role to maintain resting pressure [87]. However, according to a recent study, myogenic progenitor cells derived from the skeletal muscle can be differentiated into SMCs *in vitro* culture of transforming growth factor β 1 and heparin-containing medium, with showing contractile proteins expression profiles and functional voltage-gated ion channels associated with SM lineage commitment [88].

Moreover, in the included studies, experimental evidences performed on real human IAS tissue were only 5, and most animal models used rats or mice with contractile protein-specific gene deletion or inhibitory drug. Harvest of viable SMCs of IAS isolated human anal sphincter might be quite difficult because only patients who underwent abdominal perineal resection operation can provide intact anal sphincter. A larger-scale human anal sphincter also needs a new approach for expansion capacity, for example, 3D scaffolding or spheroid culture, and organoids, reflecting that the previous studies have been performed on small size animals like rats or mice. In limitation, we could not perform an interrelationship analysis based on experimental methods for between included molecular mechanisms and regenerative treatment for IAS. Further study needs with an interrelationship analysis for those.

CONCLUSIONS

This study implicates the complexity of the molecular mechanisms for basal tone and relaxation of IAS as intrinsic properties, which change from normal continence into incontinence in FI patients influenced by multifactorial etiology. Many translation studies have revealed the key pathway of IAS for the functional improvement of resting pressure and RAIR in FI. However, experimental evidence of FI cellular treatment has lower specificity for IAS, and a lower interrelationship between the molecular mechanisms of IAS and functional outcomes of cell treatments. Furthermore, there are no reports on lab-grown engineered gut tissues in patients with FI. The field of IAS regeneration presents more challenges and warrants more advances.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

FUNDING

None.

REFERENCES

1. Read NW, Abouzekry L, Read MG, Howell P, Ottewell D, Donnelly TC. Anorectal function in elderly patients with fecal impac-

tion. *Gastroenterology* 1985;89:959-66.

- Kim CN. Usefulness of patient-reported outcome measures and anorectal physiologic tests in predicting clinical outcome for fecal incontinence. *Ann Coloproctol* 2019;35:289-90.
- Irei Y, Takano S, Yamada K. Propiverine hydrochloride as a treatment for fecal incontinence. *Ann Coloproctol* 2020;36:88-93.
- Fox JC, Fletcher JG, Zinsmeister AR, Seide B, Riederer SJ, Bharucha AE. Effect of aging on anorectal and pelvic floor functions in females. *Dis Colon Rectum* 2006;49:1726-35.
- Bharucha AE, Daube J, Litchy W, Traue J, Edge J, Enck P, et al. Anal sphincteric neurogenic injury in asymptomatic nulliparous women and fecal incontinence. *Am J Physiol Gastrointest Liver Physiol* 2012;303:G256-62.
- Bannister JJ, Abouzekry L, Read NW. Effect of aging on anorectal function. *Gut* 1987;28:353-7.
- Bharucha AE, Dunivan G, Goode PS, Lukacz ES, Markland AD, Matthews CA, et al. Epidemiology, pathophysiology, and classification of fecal incontinence: state of the science summary for the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) workshop. *Am J Gastroenterol* 2015;110:127-36.
- Bharucha AE. Fecal incontinence. *Gastroenterology* 2003;124:1672-85.
- Pla-Mart V, Martín-Arévalo J, Martí-Fernandez R, Moro-Valdezate D, Garcia-Botello S, Espi-Macias A, et al. Long-term evolution of continence and quality of life after sphincteroplasty for obstetric fecal incontinence. *Ann Coloproctol* 2020;38:13-9.
- Pinsk I, Czeiger D, Lichtman D, Reshef A. The long-term effect of standardized anal dilatation for chronic anal fissure on anal continence. *Ann Coloproctol* 2021;37:115-9.
- Usui A, Ishiyama G, Nishio A, Kawamura M, Kono Y, Ishiyama Y. Two-stage complete deroofting fistulotomy approach for horse-shoe fistula: successful surgery leaving continence intact. *Ann Coloproctol* 2021;37:153-8.
- Meyer I, Blanchard CT, Markland AD, Gibson EG, Richter HE. Fecal incontinence symptoms and impact in older versus younger women seeking care. *Dis Colon Rectum* 2019;62:733-8.
- Raghavan S, Gilmont RR, Miyasaka EA, Somara S, Srinivasan S, Teitelbaum DH, et al. Successful implantation of bioengineered, intrinsically innervated, human internal anal sphincter. *Gastroenterology* 2011;141:310-9.
- Raghavan S, Miyasaka EA, Gilmont RR, Somara S, Teitelbaum DH, Bitar KN. Perianal implantation of bioengineered human internal anal sphincter constructs intrinsically innervated with human neural progenitor cells. *Surgery* 2014;155:668-74.
- Gagnard C, Godlewski G, Prat D, Lan O, Cousineau J, Maklout Y. The nerve branches to the external anal sphincter: the macroscopic supply and microscopic structure. *Surg Radiol Anat* 1986;8:115-9.
- Inoue Y, Fujita F, Yamaguchi I, Kinoue H, Kawahara D, Sakai Y, et al. Improvement of anal function by adipose-derived stem cell sheets. *Dig Surg* 2018;35:64-9.
- Salcedo L, Mayorga M, Damaser M, Balog B, Butler R, Penn M,

- et al. Mesenchymal stem cells can improve anal pressures after anal sphincter injury. *Stem Cell Res* 2013;10:95-102.
18. Salcedo L, Penn M, Damaser M, Balog B, Zutshi M. Functional outcome after anal sphincter injury and treatment with mesenchymal stem cells. *Stem Cells Transl Med* 2014;3:760-7.
 19. Kuismanen K, Juntunen M, Narra Girish N, Tuominen H, Huhtala H, Nieminen K, et al. Functional outcome of human adipose stem cell injections in rat anal sphincter acute injury model. *Stem Cells Transl Med* 2018;7:295-304.
 20. Oh HK, Lee HS, Lee JH, Oh SH, Lim JY, Ahn S, et al. Functional and histological evidence for the targeted therapy using biocompatible polycaprolactone beads and autologous myoblasts in a dog model of fecal incontinence. *Dis Colon Rectum* 2015;58:517-25.
 21. Oh HK, Lee HS, Lee JH, Oh SH, Lim JY, Ahn S, et al. Co-administration of basic fibroblast growth factor-loaded polycaprolactone beads and autologous myoblasts in a dog model of fecal incontinence. *Int J Colorectal Dis* 2015;30:549-57.
 22. Salcedo L, Lian L, Jiang HH, Sopko N, Penn M, Damaser M, et al. Low current electrical stimulation upregulates cytokine expression in the anal sphincter. *Int J Colorectal Dis* 2012;27:221-5.
 23. Sun L, Yeh J, Xie Z, Kuang M, Damaser MS, Zutshi M. Electrical stimulation followed by mesenchymal stem cells improves anal sphincter anatomy and function in a rat model at a time remote from injury. *Dis Colon Rectum* 2016;59:434-42.
 24. Trébol J, Georgiev-Hristov T, Vega-Clemente L, García-Gómez I, Carabias-Orgaz A, García-Arranz M, et al. Rat model of anal sphincter injury and two approaches for stem cell administration. *World J Stem Cells* 2018;10:1-14.
 25. Evers J, Jones JF, O'Connell PR. Systematic review of animal models used in research of origins and treatments of fecal incontinence. *Dis Colon Rectum* 2017;60:614-26.
 26. Trébol J, Carabias-Orgaz A, García-Arranz M, García-Olmo D. Stem cell therapy for faecal incontinence: current state and future perspectives. *World J Stem Cells* 2018;10:82-105.
 27. De Ligny WR, Kerkhof MH, Ruiz-Zapata AM. Regenerative medicine as a therapeutic option for fecal incontinence: a systematic review of preclinical and clinical studies. *Am J Obstet Gynecol* 2019;220:142-54.
 28. Weledji EP. Electrophysiological basis of fecal incontinence and its implications for treatment. *Ann Coloproctol* 2017;33:161-8.
 29. Murthy KS. Signaling for contraction and relaxation in smooth muscle of the gut. *Annu Rev Physiol* 2006;68:345-74.
 30. Somlyo AP, Somlyo AV. Ca²⁺ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* 2003;83:1325-58.
 31. Rattan S. Ca²⁺/calmodulin/MLCK pathway initiates, and RhoA/ROCK maintains, the internal anal sphincter smooth muscle tone. *Am J Physiol Gastrointest Liver Physiol* 2017;312:G63-6.
 32. Lu P, Chen J, Zhang C, Saur D, Baer CE, Lifshitz LM, et al. Oscillating calcium signals in smooth muscle cells underlie the persistent basal tone of internal anal sphincter. *J Cell Physiol* 2021;236:5937-52.
 33. Singh A, Rattan S. BDNF rescues aging-associated internal anal sphincter dysfunction. *Am J Physiol Gastrointest Liver Physiol* 2021;321:G87-97.
 34. Zhang CH, Wang P, Liu DH, Chen CP, Zhao W, Chen X, et al. The molecular basis of the genesis of basal tone in internal anal sphincter. *Nat Commun* 2016;7:11358.
 35. Chakder S, Rattan S. Mechanisms and sites of action of endothelins 1 and 2 on the opossum internal anal sphincter smooth muscle tone in vitro. *J Pharmacol Exp Ther* 1999;288:239-46.
 36. Cobine CA, Hannigan KI, McMahan M, Ni Bhraonain EP, Baker SA, Keef KD. Rhythmic calcium transients in smooth muscle cells of the mouse internal anal sphincter. *Neurogastroenterol Motil* 2020;32:e13746.
 37. Rattan S, De Godoy MA, Patel CA. Rho kinase as a novel molecular therapeutic target for hypertensive internal anal sphincter. *Gastroenterology* 2006;131:108-16.
 38. Patel CA, Rattan S. Cellular regulation of basal tone in internal anal sphincter smooth muscle by RhoA/ROCK. *Am J Physiol Gastrointest Liver Physiol* 2007;292:G1747-56.
 39. Rattan S, Singh J. RhoA/ROCK pathway is the major molecular determinant of basal tone in intact human internal anal sphincter. *Am J Physiol Gastrointest Liver Physiol* 2012;302:G664-75.
 40. de Godoy MA, Patel CA, Waldman SA, Katsuki M, Regan RE, Rattan S. H-ras inhibits RhoA/ROCK leading to a decrease in the basal tone in the internal anal sphincter. *Gastroenterology* 2007;132:1401-9.
 41. Patel CA, Rattan S. RhoA prenylation inhibitor produces relaxation of tonic smooth muscle of internal anal sphincter. *J Pharmacol Exp Ther* 2007;321:501-8.
 42. Rattan S. 3-Hydroxymethyl coenzyme A reductase inhibition attenuates spontaneous smooth muscle tone via RhoA/ROCK pathway regulated by RhoA prenylation. *Am J Physiol Gastrointest Liver Physiol* 2010;298:G962-9.
 43. Singh J, Maxwell PJ 4th, Rattan S. Immunocytochemical evidence for PDBu-induced activation of RhoA/ROCK in human internal anal sphincter smooth muscle cells. *Am J Physiol Gastrointest Liver Physiol* 2011;301:G317-25.
 44. Rattan S, Singh J, Kumar S, Phillips B. Nature of extracellular signal that triggers RhoA/ROCK activation for the basal internal anal sphincter tone in humans. *Am J Physiol Gastrointest Liver Physiol* 2015;308:G924-33.
 45. Singh J, Mohanty I, Addya S, Phillips B, Yong HM, An SS, et al. Role of differentially expressed microRNA-139-5p in the regulation of phenotypic internal anal sphincter smooth muscle tone. *Sci Rep* 2017;7:1477.
 46. Rattan S, Ali M. Role of SM22 in the differential regulation of phasic vs. tonic smooth muscle. *Am J Physiol Gastrointest Liver Physiol* 2015;308:G605-12.
 47. Singh A, Mohanty I, Singh J, Rattan S. BDNF augments rat internal anal sphincter smooth muscle tone via RhoA/ROCK signaling and nonadrenergic noncholinergic relaxation via increased

- NO release. *Am J Physiol Gastrointest Liver Physiol* 2020;318:G23-33.
48. Mohanty I, Singh J, Rattan S. Downregulation of thromboxane A2 and angiotensin II type 1 receptors associated with aging-related decrease in internal anal sphincter tone. *Sci Rep* 2019;9:6759.
49. Singh J, Boopathi E, Addya S, Phillips B, Rigoutsos I, Penn RB, et al. Aging-associated changes in microRNA expression profile of internal anal sphincter smooth muscle: role of microRNA-133a. *Am J Physiol Gastrointest Liver Physiol* 2016;311:G964-73.
50. Singh J, Kumar S, Rattan S. Bimodal effect of oxidative stress in internal anal sphincter smooth muscle. *Am J Physiol Gastrointest Liver Physiol* 2015;309:G292-300.
51. Krishna CV, Singh J, Kumar S, Rattan S. Heme oxygenase-1 up-regulation modulates tone and fibroelastic properties of internal anal sphincter. *Am J Physiol Gastrointest Liver Physiol* 2014;307:G595-601.
52. Singh J, Kumar S, Krishna CV, Rattan S. Aging-associated oxidative stress leads to decrease in IAS tone via RhoA/ROCK down-regulation. *Am J Physiol Gastrointest Liver Physiol* 2014;306:G983-91.
53. De Godoy MA, Dunn S, Rattan S. Evidence for the role of angiotensin II biosynthesis in the rat internal anal sphincter tone. *Gastroenterology* 2004;127:127-38.
54. De Godoy MA, Rattan S. Autocrine regulation of internal anal sphincter tone by renin-angiotensin system: comparison with phasic smooth muscle. *Am J Physiol Gastrointest Liver Physiol* 2005;289:G1164-75.
55. de Godoy MA, Rattan S. Translocation of AT1- and AT2-receptors by higher concentrations of angiotensin II in the smooth muscle cells of rat internal anal sphincter. *J Pharmacol Exp Ther* 2006;319:1088-95.
56. de Godoy MA, Rattan N, Rattan S. COX-1 vs. COX-2 as a determinant of basal tone in the internal anal sphincter. *Am J Physiol Gastrointest Liver Physiol* 2009;296:G219-25.
57. de Godoy MA, Rattan N, Rattan S. Arachidonic acid metabolites follow the preferential course of cyclooxygenase pathway for the basal tone in the internal anal sphincter. *Am J Physiol Gastrointest Liver Physiol* 2009;296:G727-34.
58. Kubota M, Saita S, Szurszewski JH. Membrane properties and the neuro-effector transmission of smooth muscle cells in the canine internal anal sphincter. *J Smooth Muscle Res* 1998;34:173-84.
59. Rattan S, Regan RF, Patel CA, De Godoy MA. Nitric oxide not carbon monoxide mediates nonadrenergic noncholinergic relaxation in the murine internal anal sphincter. *Gastroenterology* 2005;129:1954-66.
60. McDonnell B, Hamilton R, Fong M, Ward SM, Keef KD. Functional evidence for purinergic inhibitory neuromuscular transmission in the mouse internal anal sphincter. *Am J Physiol Gastrointest Liver Physiol* 2008;294:G1041-51.
61. Folasire O, Mills KA, Sellers DJ, Chess-Williams R. Three gaseous neurotransmitters, nitric oxide, carbon monoxide, and hydrogen sulfide, are involved in the neurogenic relaxation responses of the porcine internal anal sphincter. *J Neurogastroenterol Motil* 2016;22:141-8.
62. Moumami C, Rattan S. Effect of methylene blue and N-ethylmaleimide on internal anal sphincter relaxation. *Am J Physiol* 1988;255(5 Pt 1):G571-8.
63. Rattan S, Chakder S. Excitatory and inhibitory actions of pituitary adenylate cyclase-activating peptide (PACAP) in the internal anal sphincter smooth muscle: sites of actions. *J Pharmacol Exp Ther* 1997;283:722-8.
64. Chakder S, Rattan S. Involvement of pituitary adenylate cyclase-activating peptide in opossum internal anal sphincter relaxation. *Am J Physiol* 1998;275:G769-77.
65. Acheson A, Rayment S, Eames T, Munday M, Nisar P, Scholefield J, et al. Investigation of the role of adrenergic and non-nitroergic, non-adrenergic neurotransmission in the sheep isolated internal anal sphincter. *Neurogastroenterol Motil* 2009;21:335-45.
66. Acheson AG, Griffin N, Scholefield JH, Wilson VG. L-arginine-induced relaxation of the internal anal sphincter is not mediated by nitric oxide. *Br J Surg* 2003;90:1155-62.
67. O'Kelly T, Brading A, Mortensen N. Nerve mediated relaxation of the human internal anal sphincter: the role of nitric oxide. *Gut* 1993;34:689-93.
68. Jones OM, Brading AF, Mortensen NJ. Role of nitric oxide in anorectal function of normal and neuronal nitric oxide synthase knockout mice: a novel approach to anorectal disease. *Dis Colon Rectum* 2003;46:963-70.
69. Chakder S, Rattan S. Neurally mediated relaxation of opossum internal anal sphincter: influence of superoxide anion generator and the scavenger. *J Pharmacol Exp Ther* 1992;260:1113-8.
70. Huang SC. Proteinase-activated receptor-1 (PAR1) and PAR2 mediate relaxation of guinea pig internal anal sphincter. *Regul Pept* 2014;189:46-50.
71. Cobine CA, Sotherton AG, Peri LE, Sanders KM, Ward SM, Keef KD. Nitroergic neuromuscular transmission in the mouse internal anal sphincter is accomplished by multiple pathways and postjunctional effector cells. *Am J Physiol Gastrointest Liver Physiol* 2014;307:G1057-72.
72. Banwait KS, Rattan S. Role of nitric oxide in beta3-adrenoceptor activation on basal tone of internal anal sphincter. *Am J Physiol Gastrointest Liver Physiol* 2003;285:G547-55.
73. Rattan S, Chakder S. Role of nitric oxide as a mediator of internal anal sphincter relaxation. *Am J Physiol* 1992;262(1 Pt 1):G107-12.
74. Rattan S, Sarkar A, Chakder S. Nitric oxide pathway in rectoanal inhibitory reflex of opossum internal anal sphincter. *Gastroenterology* 1992;103:43-50.
75. Keef KD, Saxton SN, McDowall RA, Kaminski RE, Duffy AM, Cobine CA. Functional role of vasoactive intestinal polypeptide in inhibitory motor innervation in the mouse internal anal sphincter. *J Physiol* 2013;591:1489-506.
76. Duffy AM, Cobine CA, Keef KD. Changes in neuromuscular transmission in the W/W(v) mouse internal anal sphincter. *Neurogastroenterol Motil* 2012;24:e41-55.

77. Rattan S, Rosenthal GJ, Chakder S. Human recombinant hemoglobin (rHb1.1) inhibits nonadrenergic noncholinergic (NANC) nerve-mediated relaxation of internal anal sphincter. *J Pharmacol Exp Ther* 1995;272:1211-6.
78. Koyuncu A, Bagcivan I, Sarac B, Aydin C, Yildirim S, Sarioglu Y. Lack of nitrate tolerance in isosorbide dinitrate- and sodium nitroprusside-induced relaxation of rabbit internal anal sphincter. *World J Gastroenterol* 2008;14:4667-71.
79. Sarveazad A, Babahajian A, Yari A, Rayner CK, Mokhtare M, Babaei-Ghazani A, et al. Combination of laser and human adipose-derived stem cells in repair of rabbit anal sphincter injury: a new therapeutic approach. *Stem Cell Res Ther* 2019;10:367.
80. Hecker L, Baar K, Dennis RG, Bitar KN. Development of a three-dimensional physiological model of the internal anal sphincter bioengineered in vitro from isolated smooth muscle cells. *Am J Physiol Gastrointest Liver Physiol* 2005;289:G188-96.
81. Somara S, Gilmont RR, Dennis RG, Bitar KN. Bioengineered internal anal sphincter derived from isolated human internal anal sphincter smooth muscle cells. *Gastroenterology* 2009;137:53-61.
82. Hashish M, Raghavan S, Somara S, Gilmont RR, Miyasaka E, Bitar KN, Teitelbaum DH. Surgical implantation of a bioengineered internal anal sphincter. *J Pediatr Surg* 2010;45:52-8.
83. Raghavan S, Miyasaka EA, Hashish M, Somara S, Gilmont RR, Teitelbaum DH, et al. Successful implantation of physiologically functional bioengineered mouse internal anal sphincter. *Am J Physiol Gastrointest Liver Physiol* 2010;299:G430-9.
84. Singh J, Rattan S. Bioengineered human IAS reconstructs with functional and molecular properties similar to intact IAS. *Am J Physiol Gastrointest Liver Physiol* 2012;303:G713-22.
85. Zakhem E, Raghavan S, Suhar RA, Bitar KN. Bioengineering and regeneration of gastrointestinal tissue: where are we now and what comes next? *Expert Opin Biol Ther* 2019;19:527-37.
86. Son IT, Lee HS, Ihn MH, Lee KH, Kim DW, Lee KW, et al. Isolation of internal and external sphincter progenitor cells from the human anal sphincter with or without radiotherapy. *Colorectal Dis* 2019;21:38-47.
87. Frudinger A, Pfeifer J, Paede J, Kolovetsiou-Kreiner V, Marksteiner R, Halligan S. Autologous skeletal-muscle-derived cell injection for anal incontinence due to obstetric trauma: a 5-year follow-up of an initial study of 10 patients. *Colorectal Dis* 2015;17:794-801.
88. Thurner M, Deutsch M, Janke K, Messner F, Kreutzer C, Beyl S, et al. Generation of myogenic progenitor cell-derived smooth muscle cells for sphincter regeneration. *Stem Cell Res Ther* 2020;11:233.