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EDITORIAL

Ectopic expression of the osteogenic master gene *RUNX2* in melanoma

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Abstract

The transcription factor *RUNX2* is the osteogenic master gene expressed in mesenchymal stem cells during osteogenic commitment as well as in pre-osteoblasts and early osteoblasts. However, *RUNX2* is also ectopically expressed in melanoma and other cancers. Malignant melanoma (MM) is a highly metastatic skin cancer. The incidence of MM has increased considerably in the past half-century. The expression levels and mutation rates of genes such as *BRAF*, *KIT*, *NRAS*, *PTEN*, *P53*, *TERT* and *MITF* are higher in melanoma than in other solid malignancies. Additionally, transcription factors can affect cellular processes and induce cellular transformation since they control gene expression. Recently, several studies have identified alterations in *RUNX2* expression. In particular, the regulation of *KIT* by *RUNX2* and the increased expression of *RUNX2* in melanoma specimens have been shown. Melanocytes, whose transformation results in melanoma, arise from the neural crest and therefore show "stemness" features. *RUNX2* plays an important role in the re-activation of the MAPK and PI-3K/AKT pathways, thus endowing melanoma cells with a high metastatic potential. In melanoma, the most frequent metastatic sites are the lung, liver, brain and lymph nodes. In addition, bone metastatic melanoma has been described. Notably, studies focusing on *RUNX2* may contribute to the identification of an appropriate oncotarget in melanoma.

Key words: *RUNX2*; Mesenchymal stem cells; Epitelial mesenchymal transition; Bone; Melanoma

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Core tip: In addition to its physiological expression in osteogenic cells, *RUNX2* ectopic expression is also reported in several cancers. Melanoma cells, which arise from the neural crest, are not typical epithelial cells and

exhibit "stemness" features. For this reason the epithelial mesenchymal transition process may be enhanced in melanoma cells. *RUNX2* expression in such context increases the migration and invasiveness of melanoma cells; it can therefore be considered a new oncotarget in melanoma.

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INTRODUCTION

In recent times, the incidence of malignant melanoma (MM) has increased. The increased number of MM patients may be ascribed to lifestyle and environmental changes. Unfortunately, the mortality rate of MM is very high, as MM is highly metastatic and genetically resistant to apoptosis. The surgical treatment of skin lesions before cell spreading is considered a successful therapeutic approach. However, after metastasis and dissemination of cells has occurred, the therapeutic tools are limited. Despite an initial positive response to treatment, melanoma cells become resistant to chemotherapy[1]. Many studies performed in animal models and in cell culture have identified the complex mechanism involved in the metastatic progression of melanoma. Mutations in master transcription regulators such as *BRAF, KIT, NRAS, PTEN, P53, TERT*, and *MITF* have been found frequently[2]. In particular, the microphthalmia-associated transcription factor (*MITF*) is an important oncogene, and it plays a critical role in melanoma transformation due to its role in melanocyte proliferation and differentiation[3]. However, transcription factors in general may affect cellular processes and thereby induce cellular transformation. Among them, *RUNX2*, the master gene of osteogenic differentiation, has been shown to be involved in the transformation and metastatic progression of melanoma[4-6]. *RUNX2*, together with *RUNX1* and *RUNX3*, belongs to the *RUNT*-related gene family[7]. These heterodimeric transcription factors have a DNA-binding "A" subunit and a non-DNA binding "B" subunit. The genomic and cDNA structures of the *RUNT* family members are evolutionarily conserved[8]. *RUNX2* is located on human chromosome 6; its coding sequence is organized in eight exons and controlled by two promoters. Isoforms may originate by the alternative use of promoters or by the alternative splicing of exons; however, the DNA-binding domain remains invariant[7]. *RUNX2* induces the commitment of mesenchymal stem cells to the osteogenic lineage by acting either as a monomer or as a heterodimeric complex, which shows a higher DNA binding activity[7]. Interestingly, differences in *RUNX2* expression are associated with the skeletal differences between modern humans and Neanderthals[9]. Otto *et al*[10] using an in vivo model, showed that the ossification process is lacking in *RUNX2* KO mice. *RUNX2* is expressed not only in mesenchymal stem cells to commit these cells to osteogenic differentiation but also in pre-osteoblasts and early osteoblasts. It is then downregulated, as its continuous expression would prevent osteoblast maturation. This aspect is important for bone remodelling. In acromegaly patients, who are characterized by an excessive production of GH and IGF-1, a high fracture risk occurs; we have demonstrated that RUNX2 gene overexpression induces bone loss and impairs bone remodelling in these patients[11].

RUNX2 OVEREXPRESSION IN CANCER

The *RUNX* family members are frequently deregulated in human cancer. Runx proteins are involved in critical cellular processes such DNA repair, apoptosis, hypoxia, the inflammatory response, EMT, stem cell functions, and oncogene-induced senescence, and they interact with different signalling pathways such as the RAS-ERK, WNT, TGFβ, BMP and Notch pathways[12]. In addition, *RUNX2* is involved in osteolytic diseases, neoangiogenic processes, and invasion and metastasis of solid tumours[4]. *RUNX2* can be associated with HDA6 and can thereby prevent the pro-apoptotic activity of P53 through its deacetylation[13]. *RUNX2* is ectopically expressed in several solid tumours such as breast cancer, pancreatic cancer, prostate cancer, lung cancer, and ovarian epithelial cancer; therefore, we have proposed *RUNX2* as a mesenchymal stem marker for cancer[14,15]. Interestingly, we observed higher levels of *RUNX2* expression in cancer patients with bone metastases compared to patients without bone metastases[15]. In thyroid cancer patients, we observed higher expression of *RUNX2* in cancer tissue as well as in circulating mRNA compared to those levels of *RUNX2* expression in controls[16]. In addition, *RUNX2* expression was higher in cancer patients with microcalcifications compared to patients without microcalcifications[16]. Finally, it has been reported that *RUNX2* induces its target genes SDF-1, CXCR7 and BSP, promoting bone homing[17].

RUNX2 IN MELANOMA

Ectopic expression of *RUNX2* in melanoma tissue and in melanoma cell lines has been shown to be associated with bone sialoprotein (BSP)[18]. BSP has been associated with tumour invasiveness[18]. *RUNX2* also regulates several matrix metalloproteinases that are involved in melanoma progression; therefore, *RUNX2* may be regarded as a common regulator of both BSP and metalloproteinases[19]. This finding could also explain the expression of other bone proteins, such as osteopontin and osteocalcin, in melanoma[20,21]. TGFβ, which induces metastasis formation in advanced melanoma stages[22], can upregulate the expression of *RUNX2* in melanoma

cell lines[23]. In contrast, the tumour suppressor p14ARF reduces *RUNX2* expression[24]. Loss-of-function *p14ARF* mutations lead to increased expression of *RUNX2* and melanoma progression[24]. Ectopic expression of *RUNX2* in melanoma has been associated with invasiveness; it has been demonstrated that *RUNX2* overexpression mediates the migration ability of melanoma cells[6]. Notably *RUNX2* stimulates *VEGF* gene expression[25] and the upregulation of angiogenesis, consequent to *RUNX2* overexpression, may represent a critical step for tumor metastasis.

Furthermore, *RUNX2* overexpression upregulates *SOX9, SNAI2* and *SMAD3*. These transcription factors are involved in EMT as well as in cytoskeletal remodelling and thus enhance the motility and invasive potential of cancer cells[17]. In melanoma, the overexpression of *EGF-R, PDGFRβ, AXL* and *IGF-1R* induces the reactivation of the MAPK and PI3K/AKT pathways, which are involved in migration and invasion processes[26]. The reactivation of these pathways is due to tyrosine kinase receptor-based autocrine loops, in which *RUNX2* plays a pivotal role[5]. The MAPK and AKT pathways regulate most physiological processes, such as proliferation, differentiation and cell survival. It has been suggested that the MAPK and AKT pathways are strongly associated with each other in melanoma; the link between the PI3K/AKT (AKT pathway) and MAPK/ERK1/2 (MAPK pathway) cascades is well known[27]. AKT enhances RUNX2 expression either directly or by inactivating RUNX2 regulators. Similarly, RUNX2 activates the PI3K/AKT pathway by regulating different components[4]. This reciprocal activation induces tumour progression and aggressiveness. In addition, RUNX2 promotes the crosstalk between MAPK and AKT through EGFR, as has been shown in human mammary epithelial cells[28] through the reduction of ERK-mediated inhibition of EGFR and the AKT pathway. The lung, liver, brain and lymph nodes are the most frequent metastatic sites for melanoma cells[29]. Since melanocytes arise from the neural crest, they are not typical epithelial cells; they show "stemness" features. This last finding could partially explain the high metastatic potential of melanoma[29]. Solid malignancies such as breast, prostate and lung cancers form metastases in bone. However, metastatic bone diseases from MM are under-investigated. The data in the literature report an incidence of skeletal metastases in MM patients that ranges from 0.85% to 18.6%[30]. Recently, Zekri *et al*[31], managing MM patients at two referral cancer centres in England, showed that bone metastases occur in 4.1% of patients at all stages of MM. In addition, another study showed that 17.2% of metastatic melanoma patients had metastases in bone tissue.

CONCLUSION

MM accounts for less than 5% of cutaneous malignancies. However, its incidence has been increasing considerably in the past half-century. Therefore, extensive research focusing on the genes involved in melanoma transformation and on the modulation of these genes is needed. Several studies have highlighted the involvement of RUNX2, the master gene of osteogenic differentiation, in melanoma. The invasiveness and metastatic features conferred by RUNX2 seem to be related to its ability to promote EMT. In addition, melanocytes are not typical epithelial cells, and their stemness features could explain melanoma invasiveness. Since metastatic melanoma has a poor prognosis, new and more effective therapeutic tools should be developed in order to implement current therapies. In this context, the exploitation of molecules actually involved in melanoma, such as RUNX2, represents an important frontier for the identification of new oncotargets. Transcription factors have been ignored thus far by the pharmaceutical industry; nonetheless, many studies have identified their central role in cellular transformation. We believe that future efforts directed towards unravelling the complex roles of RUNX2 may contribute to the identification of new therapeutic tools that improve the quality of life of melanoma patients.

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REVIEW

Stem cell therapy for faecal incontinence: Current state and future perspectives

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Abstract

Faecal continence is a complex function involving different organs and systems. Faecal incontinence is a common disorder with different pathogeneses, disabling consequences and high repercussions for quality of life. Current management modalities are not ideal, and the development of new treatments is needed. Since 2008, stem cell therapies have been validated, 36 publications have appeared (29 in preclinical models and seven in clinical settings), and six registered clinical trials are currently ongoing. Some publications have combined stem cells with bioengineering technologies. The aim of this review is to identify and summarise the existing published knowledge of stem cell utilization as a treatment for faecal incontinence. A narrative or descriptive review is presented. Preclinical studies have demonstrated that cellular therapy, mainly in the form of local injections of muscle-derived (muscle derived stem cells or myoblasts derived from them) or mesenchymal (bone-marrow- or adipose-derived) stem cells, is safe. Cellular therapy has also been shown to stimulate

the repair of both acute and subacute anal sphincter injuries, and some encouraging functional results have been obtained. Stem cells combined with normal cells on bioengineered scaffolds have achieved the successful creation and implantation of intrinsically-innervated anal sphincter constructs. The clinical evidence, based on adipose-derived stem cells and myoblasts, is extremely limited yet has yielded some promising results, and appears to be safe. Further investigation in both animal models and clinical settings is necessary to drawing conclusions. Nevertheless, if the preliminary results are confirmed, stem cell therapy for faecal incontinence may well become a clinical reality in the near future.

Key words: Faecal incontinence; Anal sphincter; Cell implantation; Tissue engineering; Cell therapy; Stem cells

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Core tip: Faecal incontinence is very frequent and is associated with severe consequences for patients. Available treatment outcomes are not optimal, particularly in the long-term. Stem cells, with or without bioengineering, could improve these results, as demonstrated in other clinical settings. We present a descriptive review of the published literature about faecal incontinence and stem cells, and discuss the existing limitations and concerns. Preclinical studies have confirmed the feasibility and safety of stem cells, and show some interesting results; the limited clinical experience confirms the safety and potential efficacy. However, further studies are needed to obtain clear conclusions.

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INTRODUCTION

Faecal incontinence (FI) is a highly prevalent nonfatal illness associated with considerable embarrassment, anxiety and poor quality of life. In a systematic review by McMillan, it was estimated to occur in 11%-15% of adults[1], and Nelson found it in 2.2% of the general population and 47% of institutionalised individuals[2]. It is difficult to know its real prevalence due to its psychosocial repercussions, as patients tend not to report it to their physicians and physicians rarely ask about it.

The physical repercussions are limited, but psychosocial ones are devastating, which include the loss of self-confidence, disability, body image alteration, social isolation, anxiety, depression, *etc.* A British study observed a four-fold increase in anxiety and five-fold increase in depression, with a significant association of both with faecal incontinence[3]. Furthermore, it could also lead to job loss and is the second highest cause of institutionalisation. Studies focusing on the quality of life reflect significant repercussions in multiple components, such as physical[4,5] and mental[5]; generally, the greater the incontinence, the greater the deterioration it causes ($P < 0.01$)[5]. A Spanish study showed an independent association between quality of life and declining mental health (OR 2.088 and $P = 0.017$)[6].

The economic impact is high and very difficult to estimate, but it consists of direct (diagnostic test, treatments, care, *etc*.) and indirect (job production, secondary treatments, such as for psychological consequences, *etc.*) costs. Indirect costs are harder to calculate and account for more than half[7]. In a Seattle study, annual healthcare costs increased up to $2897 in 2005 according to multivariate analyses (pads, barriers or institutionalisation not included)[4]. In a Dutch study, global costs increased yearly by 2169€ for each patient[7].

The prevalence is higher among women, the elderly, people with poor health or physical limitations, and those residing in nursing homes. Other risk factors include pelvic radiation, pregnancy, pelvic injury associated with vaginal delivery, anorectal surgery, diarrhoea, faecal impaction, some neurological conditions, and diabetes.

Bowel continence is a very developed function that depends on complex sensory and motor interactions between the rectum, anus, external anal sphincter (EAS), internal anal sphincter (IAS), puborectal muscle, and their vascularisation and innervation. When one or more of these structures or interactions are disrupted to such a degree that the others are unable to compensate, incontinence appears. Therefore, FI is a multifactorial disease. The most frequent morphological alteration found in almost 60% of patients is a sphincter lesion, with most of them being obstetric (30%-40%). Sphincter lesions during delivery range from 11%[8] to 26.9%[9], increase with every pregnancy, and cause incontinence in 76.8%-82.8% of patients[9].

Although sacral neuromodulation has been growing exponentially in recent years, surgery remains the treatment of choice for the most severe or refractory cases, mainly when sphincter lesions are present. There are a lot of surgical techniques, but sphincter repair is the most successful for sphincter injuries. Sphincter repair has shown good results in the short-term: Excellent-good in 66%, moderate in 22%, and poor in 12% of patients[10]. However, these outcomes do not persist in the long-term; Halverson and Hull found that 54% were fully incontinent, and only 14% were fully continent 69 mo later[11]. Similarly, the review by Glasgow and Lowry, with 16 publications comprising almost 900 reparations, observed an almost constant decline in initially subjective "good" outcomes in the long-term. Despite these worsening results over time, most patients remained satisfied (also in their quality of life). No failure predictive factors were found[12]. The

reasons for this decay are not well understood.

Stem cell (SC) therapy has been demonstrated to be safe and obtain promising results in a wide variety of clinical and experimental settings: Haematological, cardiovascular, neurological, digestive, traumatic, endocrine, renal and metabolic conditions are some examples. The most commonly used are haematopoietic stem cells (HSCs)[13], mesenchymal stem cells (MSCs)[14-16] or adipose-derived stem cells (ASCs)[17-19]. For example, ASCs have been tried and had favourable outcomes in environments that are particularly unfavourable for wound healing, such as experimental colitis[20], sepsis[21], anal fistula[22-29], Crohn's patients[30], experimental colonic[31,32], and tracheal[33] anastomoses.

Based on the published literature, as well as on our group's experience with FI treatment and using ASCs in experimental and clinical settings (having conducted or participated in more than six clinical trials with autologous or allogeneic ASCs for digestive fistula), our aim was to review published literature related to stem cell therapy for FI, , and currently ongoing clinical trials. To the best of our knowledge, there is only one review on this field from Gräs *et al*[34], which also includes tissue engineering studies published prior to June 2015.

SEARCH

We performed an exhaustive search of the published literature in the United States National Library of Medicine database ("PubMed") using the following terms: "faecal incontinence", "anal incontinence", "stem cells", "progenitor cells", "cellular therapy" and "cell therapy". Only studies published in indexed peer-reviewed journals were selected. "Similar articles" in PubMed and references of the selected studies were also analysed to detect potentially includable articles. Related to bioengineering, only publications combining it with SCs were considered for this review. The United States National Library of Medicine official registry of clinical trials "ClinicalTrials.gov" (http://www.clinicaltrials.gov) and the EU Clinical Trials Register (http://www.clinicaltrialsregister.eu) were searched using the same terms to detect ongoing registered clinical trials. Both searches were performed on April 1st 2018.

The high variability of FI models, the cellular products employed, and the methodology of applying it or evaluating their results, make it impossible to perform a meta-analysis. Therefore, a narrative or descriptive review is presented.

STEM CELLS APPLIED FOR FAECAL INCONTINENCE: A BRIEF OVERVIEW

The pioneering report in this field was in an animal model from Lorenzi *et al*[35] in 2008. From that point onwards, several articles have been published, mostly using animal models. However, only a few experiences with humans have gradually appeared since 2010.

We have identified a total of 36 publications eligible for a deeper analysis. Twenty-nine are preclinical studies on animal models, some combining SCs with bioengineering strategies, that try to create a biocompatible and implantable EAS or IAS construct. Seven publications are on humans. Also, six registered clinical trials were found that are "active" or apparently "ongoing". In the following sections, we are going to analyse and summarise the publications, ordering them using the internet publication date or "Epub" date.

ANIMAL STUDIES PUBLISHED

In the 29 selected preclinical animal studies, high heterogeneity on employed animals, faecal incontinence models, type of repair, kind of SC applied, and response evaluation system were applied. Overviews of the following aspects are presented in Tables 1-3: injury model and repair (Table 1), kind of SC employed (Table 2) and bioengineering strategies combined with SCs (Table 3). The types of animal used and the adjuvants employed to SCs are mentioned later.

The first publication was by Lorenzi *et al*[35] in 2008. They performed a left lateral selective sphincterotomy in male rats without specifying its length. The authors divided the animals into four groups of eight. Two received sphincter-injected BM-MSCs after non-overlapping repair (autologous: group C, or allogeneic associated with immunosuppressive drugs: group D) and were compared with groups of sham injury and saline injection (A), and injury, repair and saline injection (B). There were no relevant complications or *exitus*. After 30 d under histologic examination, a significant decrease in muscle tissue was observed at the site of repair, but morphometric analysis of groups C and D revealed a significantly greater muscle area than in group B ($P < 0.05$), but a significantly lower area than in group A. In functional assays, with *in vitro* contractility, a significantly better response to electrical stimulation and relaxing capability appeared in groups C and D compared with B ($P < 0.05$). No significant differences were found between groups C and D.

In the same year, Kang *et al*[36] published an investigation using cryoinjury in Sprague-Dawley female rats, without specifying the damaged volume (although the probe is applied against the right sphincter hemisphere). The authors studied injection with microscopic guidance of 3×10^6 autologous muscle-derived stem cells (MDSCs) into the sphincter damaged zone. Fifteen rats were divided into three groups: control (A); cryoinjury (B); and cryoinjury and cell therapy (C). Evaluations were performed one week after the injury. In muscle strip *in vitro* contractility assays, cryoinjury significantly decreased contractility and MDSCs increased its amplitude without reaching statistical significance. Upon histological examination, they found labelled cells in all animals at the MDSC

Table 1 Faecal incontinence models employed in published preclinical studies and their types of reparation systems

Surgical injury	Crioinjury	Pudendal nerve crush	No injury
Section	2	1[43]	3
Anterior: 2			
Left lateral: 9[43]			
Posterior subtotal: 3			
Proctoepisiotomy: 1			
25% excision: 4			
50% excision (IAS: 1) (both: 3)			
Total excision EAS: 1			
Type of reparations employed			
Surgical repair	**No repair**	**Substitution**	**Not applicable**
6	Crioinjury: 2	2	3
Randomized 2[39,64]	Section: 9[39,43,64]		
	Excision: 7		

Numbers indicate the number of published studies. EAS: External anal sphincter; IAS: Internal anal sphincter.

Table 2 Origin of stem cells used in published preclinical studies, classified by organ origin and transplant type

Kind of stem cells employed				
Muscle progenitors	**Bone marrow SCs**	**ASCs**	**Enteric neural**	**USCs**
Myoblasts: 6	BM-MSCs: 10[35,38,60]	Aut: 1	Aut: 1	Xenog: 1[38]
Muscle SCs: 9	Mononuclear: 1[60]	Xenog: 1	Xenog: 1	
Autologous/syngeneic	**Allogeneic**		**Xenogeneic**	
11[35]	17[35,38]		3[38]	

Numbers indicate the number of published studies. ASCs: Adipose-derived stem cells; USCs: Umbilical cord stem cells; BM-MSCs: Bone marrow-derived mesenchymal stem cells; Aut: Autologous; Xenog: Xenogeneic.

Table 3 Bioengineering strategies used with stem cells in published preclinical studies, and scaffolds employed as stem cell carriers to improve their function

Bioengineering models	
[46,55,56]	Polycaprolactone beads
[51]	IAS muscle cells + human ENPC + bilayer collagen and laminin hydrogel
[57]	Polyethylene glycol-based hydrogel matrix scaffold
[58]	Decellularized EAS
[76]	IAS cells + enteric neural progenitor cells (biosphincter)
[65]	Polyacrylamide hydrogel carrier (Bulkamid)
[61,63]	Gelatin scaffold

ENPC: Enteric neural progenitor cells; EAS: External anal sphincter; IAS: Internal anal sphincter.

injection site, confirming survival and tolerability (there were no immune responses in any animal), and also found differentiated muscle masses with variable orientations, suggesting partial myofiber (smooth and skeletal muscle) regeneration.

In 2009, Saihara *et al*[37] isolated allogeneic myoblasts from female F344 rats (at 1-4 wk), implanted them into nude mice, and evaluated myoblast evolution in subcutaneous tissue, damaged thigh muscles and healthy levator ani. Myoblasts were most efficiently obtained from more juvenile rats. SCs were capable of forming myotubes *in vitro* and in subcutaneous fat at 3 wk, and became integrated into damaged muscles with myofiber formation at 4 wk. Nevertheless, in healthy muscle, myoblasts survive in smaller numbers, surround the muscle without integrating into it, and form myotubes but not myofibers. Therefore, injury stimulus may be fundamental to myofiber formation.

Aghaee-Afshar *et al*[38] published the first rabbit model in the same year, applying surgical damage (EAS lateral sectioning) without repair. Two weeks later, seven animals per group received human umbilical cord stem cells (uSCs, 10^4), allogeneic rabbit BM-MSCs (10^4), culture medium or saline solution. These groups were also compared with three non-injured animals, all of which were evaluated before damage, before treatment and two weeks later. Clinical results: complete sphincter competence was found in four out of seven patients with BM-MSCs compared with two out of seven with uSCs, and partial competence in two out of seven with culture medium. On the electromyograph, there was a significant decrease in peaks per second

after the injury, and a significant increase in BM-MSCs compared with pre-treatment values and controls; an insignificant increase appeared in uSCs, and no increase appeared in other groups. Both kinds of SCs were able to survive at the injury site. Histopathologic evaluation showed a normal or muscle-dominant sphincter structure in all animals receiving BM-MSCs, and a fibrous-dominant structure in most animals receiving hUCM as well as in all animals without SCs. Authors do not mention the percentage of implanted cells that survived, and do not confirm their differentiation into myofibers or their "normal" histology.

In 2010, White *et al*[39] published the first randomised study with 120 Sprague-Dawley virginal female rats. The authors performed a transection of EAS with a 7 mm incision (in this species, EAS is about 3-4 mm in longitudinal length). Animals were first randomly allocated to repair or no repair groups, and then each group received injected allogeneic pre-confluence myogenic stem cells (3.2×10^6 on saline) or saline solution. If a repair was performed, a two-layer 5-0 braided polyglactin interrupted suture (1 mm apart) was applied to the rectal mucosa, and EAS were approximated with two single interrupted stitches. Injections were applied under microscopic guidance in EAS ends (before repair, if scheduled). Animals were sacrificed at 1, 3 and 13 wk, and EAS contractility was studied in muscle strips *in vitro*. Seven days after injury, contractile function had severely declined, which was independent of repair. Twitch tension, maximal tetanic contraction, and maximal force in response to electrical stimulation improved significantly with time after sphincter repair. Injected SCs in repaired sphincters resulted in significantly superior ($P < 0.001$) contractile function at both 7 d and 90 d compared with saline. In non-repaired animals, contractile function did not improve with or without SCs. Repair and surgery could cause short-term functional deterioration, and indicators of denervation did not change between groups. The authors propose that SCs need some favourable conditions to work (preserved innervation, muscle apposition), as demonstrated by the minimal effect on non-repaired animals.

In the same year, Craig *et al*[40] analysed the feasibility and safety of allogeneic rat myoblasts injected into the intact EAS of four non-pregnant female Sprague Dawley rodents. Here, 1.5 or 4.5×10^6 labelled cells, divided across three sites, were injected under electromyographic guidance between three and nine o'clock. Ten days later, the authors detected labelled cells within the EAS using immunofluorescence assays. To them, this demonstrated that myoblasts integrate into the host tissue.

Additionally in 2010, Kajbafzadeh *et al*[41] published a paper on rabbits. A surgical subtotal external sphincterotomy (9 mm longitudinal) was performed in the posterior part through an 8 mm longitudinal incision, with only the skin sutured. Three weeks later, autologous MDSCs (7×10^7, nine animals) or saline buffer (12 animals) were injected into section borders. Sphincter electromyography (EMG) and manometry (ARM) were performed immediately before injury, as well as 14, 28, and 60 d after injection in three animals per group. Animals were sacrificed at every interval for histology studies. The three remaining animals from the control group received EMG and ARM after 6 mo. Related to clinical presentation, after the injury, all rabbits demonstrated a flaccid sphincter and occasional loose faecal consistency; this persisted during the 6 mo follow-up in the control group, but recovered after four weeks in the SC group. Upon histological evaluation, circular fibers around lesions in the control group became atrophied, and inflammatory infiltrate, fibrosis and a muscular gap persisted at all of the follow-ups. With SCs, myotubes appeared at 2 wk, and myofibers with variable disposition at 4 wk. At two and 4 wk, labelled cells were detected in all of the grafted sphincters, and there was less CD3+ cell infiltration at 4 wk (null at eight) with very few CD34+ cells appearing. These two last results confirm that findings cannot be explained by bone marrow-derived cell infiltration). A higher proliferative index was also identified with SCs. Upon functional examinations, injury promoted a decrease of approximately 87% in basal pressures. ARM and EMG showed a significant ($P > 0.001$) improvement in the mean anal canal pressure and electrical activity, both at rest and after stimulation, since 4 wk after cell injection (74.8% and 60%-80% of normal values, respectively), which did not appear in the saline group. These values grew in the SC group during the evaluated period. No significant differences were noted in the control group with regard to functional and pathological parameters over time.

The following publication was issued by Pathi *et al*[42] in 2012, and first compared local and systemic SCs. They performed the same injury and repair as White *et al*[39] for 204 nulliparous Lewis rats compared to 20 non-injured animals. Operated rats randomly received one of the following: Local and intravenous phosphate buffered saline (PBS), local allogeneic BM-MSCs (4×10^6 labelled SCs injected on each side of the reparation) with intravenous PBS and intravenous allogeneic BM-MSCs (4×10^6 labelled) with local PBS. Animals were studied at 24 and 48 h and seven and 21 postoperative days, using genetic sphincter expression by quantitative reverse transcription polymerase chain reaction (IL-10, IL-6, TGFβ1, TNFα, CPH-A, COX2, LOX) and with histology and neurophysiology results at 21 d. Upon functional evaluation at 21 d, there was a significant decay in maximal contractile pressure, and an increase in fatigue with PBS; those values were equal to those in non-operated animals in the group receiving local BM-MSCs, and reached intermediate values when systemic BM-MSCs were applied. Upon histological evaluation, when PBS was injected locally (independent from the systemic product), there was a muscular gap replaced by an inflammatory area with fibrosis, and skeletal muscle fibres lost their orientation in the injury borders.

With local MSCs, the correct orientation appeared, and fibers crossed the fibrous area. Labelled cells were detected at 24 and 48 h, but not at seven and 21 d. In wound-healing parameters, pro-inflammatory (COX-2 and IL-6 during 48 h) and anti-inflammatory (IL-10 and TNFα during 21 d) increased transiently after injury in all groups, whereas TFG-β1 (an important mediator of matrix deposition by MSCs) and lysyl oxidase (related with collagen and elastin synthesis) increased significantly at earlier time points with direct MSCs, and in an intermediate manner with systemic MSCs. It was of note that there was a nearly significant (P = 0.057) mortality increase with systemic MSCs related to pulmonary embolisms. The authors concluded that local, but not intravenous, MSCs improved contractility, matrix deposition, and both TGF-β1 and LOX in the acute phase.

In 2013, Salcedo *et al*[43] published the first study that considered pudendal nerve injury using 70 virgin female Sprague-Dawley rats. They applied Zutshi' s surgical injury model, consisting of an incision of EAS and IAS through a precise 3-4 mm incision in the perianal skin[44] and pudendal nerve crush (comprising 30 seconds with a Castroviejo needle holder) as FI models. Animals were randomly assigned to: Surgical sphincterotomy (n = 20), pudendal nerve crush (n = 20), sham sphincterotomy (n = 10, five seconds pressure) and sham pudendal nerve crush (n = 20, dissection only). Then, when they had previously demonstrated significant cytokine level changes (24 h after injury)[45], they applied 2 × 10^6 labelled allogeneic BM-MSCs in PBS that were either injected into each of the four sphincter quadrants or intravenously (five animals for each delivery system per experimental group) and compared them with the same volume of local or intravenous PBS. ARM and EMG were recorded immediately after injury and 10 d after treatment. The authors found that Ⅳ MSCs resulted in a significant increase in resting and peak pressure, as well as EMG amplitude and frequency at 10 d compared to PBS. Local MSCs significantly increased resting pressure and EMG frequency, but not amplitude. There were no improvements with MSCs or PBS after pudendal nerve crush, possibly due to the prompt SC administration prior to denervation changes. With sham surgery, no changes appeared in any group. Labelled cells were not found in MSC-treated animals. The authors concluded that MSCs (local or systemic) could significantly improve ARM and that Ⅳ MSCs significantly improved EM–G after sphincterotomy, but not after pudendal nerve crush.

In 2013, Kang *et al*[46] published the first experiments combining bioengineering and SCs in a dog model of FI. Sphincter injury was induced by the partial extraction of 25% of the posterior IAS/EAS using electrocautery. The dogs were randomly allocated to either the control group, or to the experimental group where they received an injection of porous polycaprolactone beads containing autologous myoblasts into the injury three months later (five dogs per group). The authors evaluated compound muscle action potentials (CMAPs) of the pudendal nerve, ARM, and histopathology three mo after treatment. CMAPs significantly decreased with injury (P = 0.04), but there were no differences between experimental groups (P = 0.49). Resting and squeezing pressures also significantly decreased with injury (P = 0.04) and were higher in the SC group, but without statistical significance. In histological analysis of the control group, there was extensive damage to the muscle fibers with atrophy, cytoplasmic fibrosis and focal interstitial inflammatory cell infiltration. In the therapeutic group, there was a marked foreign body reaction (numerous giant cells and foamy macrophages), with weak staining for α-smooth muscle actin. Therefore, the results did not show firm evidence that injection could improve sphincter function. In the discussion, the authors mentioned that the physical properties of some beads could elicit an adverse immune response or foreign body reaction. These authors also insisted on the advantages of a large animal model to study effects (especially *in vivo*), mentioned the necessity of reinnervation, and emphasised the study's limitation due to the very low number of studied animals.

Also in 2013, Jacobs *et al*[47] published the first study with a safety concern. Here, 33 female virgin Sprague Dawley rats received surgical anal sphincter transection and repair, after which 24 underwent the injection of 5.0 × 10^6 allogeneic MDSCs and nine served as the sham control. SC migration to the liver and lung, as well as sphincter histology, were evaluated at 30 d. No evidence of SC migration to the liver or lung was found, but two local growth foci were noted in two animals receiving SCs. Further evaluations of them were consistent with a benign nature; there were no nuclear abnormalities or proliferation. The authors consider that this finding could be explained by the high dose employed, cell trapping, SC overgrowth, and/or paracrine factors. Finally, they concluded that more studies on safety are needed, which could be focused locally since no migration appeared.

Furthermore, Bisson *et al*[48] published in 2013 a cryoinjury study on Fischer rats. The authors verified that the minimal lesion that caused sustainable deficiency was done from 90 degrees, which was repeated after a 24 h interval. Evaluations relied on both an electro-stimulated ARM as well as histology. The experimental groups were: Uninjured controls (n = 11), cryoinjured + PBS (n = 8), and cryoinjured + labelled syngeneic myoblasts injected with microscopic guidance. The novelties included the analysis of different doses and injection sites, and the first long-term follow-up (6 mo); three individual injections of 1 × 10^5 (n = 6), 1 × 10^6 (n = 8), or 1 × 10^7 (n = 6), two at the borders and the last within the lesion; alternatively, a single dose of 1 × 10^6 (n = 6) was injected into a unique site, within or opposite the lesion. Injections were well-tolerated. In the histology, EAS reconstitution

was observed and SCs became integrated and differentiated into mature myofibers. Related to manometry, pressuresincreased over time; after day 30, the SC group had significantly higher pressures compared to PBS controls ($P < 0.001$), and equal pressures to normal rats at day 60. The therapeutic effect persisted over a period of 6 mo. A three-injection system was equally as effective as a single intra-lesion administration at day 60, but an injection opposite the lesion was unable to restore sphincter pressures.

The last publication from 2013 was from Lane *et al*[49], which used Sprague-Dawley rodents. They first established normative EMG EAS parameters. A more radical procedure named proctoepisiotomy, which involved an incision length of 5 mm to include transection of the IAS and EAS, was designed. Then, a layered repair was performed with 6-0 delayed absorbable sutures in a running fashion, followed by an interrupted layer. Animals were randomly assigned to receive myogenic SCs ($n = 24$, 5×10^6 injected under direct visualisation with a dissecting microscope one half to each side of the EAS) or PBS (control group, $n = 9$). The authors evaluated the efficacy by EMG (basal, two and 4 wk) and ARM (basal and 2 wk post-intervention), and measurements of IAS, EAS and total sphincter thickness (millimetres) were also calculated. They found a significant difference between the experimental groups inEMG ($P < 0.01$) and ARM at 2 wk (the SC group recovered basal values), but there were no differences in EMG at 4 wk (both groups returned to baseline). Notably, there were no relevant complications, and measurements of sphincter muscle thickness did not differ between transplant and control rats.

The group of Elmi *et al*[50] published a study in 2014 focusing on SC homing and tracing, employing magnetic resonance imaging (MRI) for the first time in this field. They employed the Kazbafzadeh[41] model of FI in 12 rabbits. Animals were randomly assigned to receive either ultra-small superparamagnetic iron oxide (USPIO)-labelled 9×10^7 autologous MDSCs (experimental group) or saline (control group) at the site of damage 3 wk later. Evaluations were performed with *in vivo* MRI, EMG, and ARM before, 1 h after, and one, two, and 4 wk after the injection. At 4 wk, sphincter sections were obtained for histology; the semi-quantitative analysis of fibrosis, desmin, iron, CD3, and CD68 was performed in two distinct regions according to either the presence (zone Ⅰ) or absence (zone Ⅱ) of signal loss (related to USPIO) on the MRI. Regarding MRI results, signal loss was significant at 1 h, 1 wk, and 2 wk when compared with the pre-injection signal intensity in the SC group, and the maximum signal loss was detected at 1 h followed by a gradual increase during the follow-up (statistical differences at 4 wk appeared compared with those at 1 h). In the control group, there was no statistically significant difference in signal intensity at each time point. In a functional evaluation, a significant improvement in pressure and electrical activity was found in the SC group after 4 wk ($P < 0.001$, 76% of basal values). In the histological studies, atrophic thin circular muscle fibres with fibrosis were seen in the control group, whereas regenerating myofibers staining positively for desmin as well as clusters of iron-positive particles were detectable in the experimental group, mainly in zone Ⅰ areas. A significant decrease in the fibrotic area in zone Ⅰ of the therapeutic group was identified ($P = 0.004$). Minimal infiltration of CD68+ cells and mild CD3+ was reported in both groups. Therefore, iron oxide-enhanced MRI can monitor transplanted SCs.

In the same year, Raghavan *et al*[51] published the development and successful implantation of a bioengineered IAS by employing SCs in rats. Following their studies of bioengineered IAS since 2005[52], the authors created human IAS tissue constructs combining IAS circular smooth muscle cells and human enteric neuronal progenitor cells on a collagen and laminin bilayer hydrogel. Then, constructs were implanted in the perianal region of athymic rats, optimising the implantation with platelet-derived growth factor that was delivered through a microosmotic pump. The implantation was feasible and safe; there were no complications or rejection during the 4 wk follow-up. Implants were viable and had normal morphology, relevant neovascularisation, and normal contractility both *in vitro* and *in vivo*. Treated animals had also normal stooling.

The group of Salcedo and Zutshi from the Cleveland Clinic in Ohio, one of the most important in this field, published a randomized study in 2014[53]. They randomly divided 50 Sprague-Dawley rats into two groups: non-injured ($n = 15$) or injured ($n = 35$). The authors modified their prior injury model to a more aggressive one: An excision of 25% of IAS and EAS through an incision in the ventral aspect, and excision from the ten to two o'clock position under a dissecting microscope. They evaluated the delay to injury administration (24 h or 3 wk) of allogeneic MSCs. Non-injured animals were divided into groups that received either intrasphincteric MSCs ($n = 8$, evaluated at 10 d -5- and 5 wk -3-) or MSCs by serial i.v. infusions ($n = 7$, evaluated at 10 d -5- and 5 wk -2-) 24 h later. Twenty-four hours later, the injury group was divided into groups that received: (1) saline ($n = 10$), either locally ($n = 5$) or by serial i.v. infusions ($n = 5$); (2) MSCs ($n = 10$) into the sphincter ($n = 5$) or by serial i.v. infusions ($n = 5$); or (3) no treatment ($n = 5$). Rats were evaluated with ARM and immunofluorescence 10 d after treatment and at 5 wk. An additional group of ten rats underwent local (five rats) or i.v. (five rats) application of MSCs 3 wk after injury to test the hypothesis that delayed administration will not produce SC homing because of the loss of cytokine signalling. SC administration consisted of the delivery of 5×10^5 labelled allogeneic BM-MSCs in PBS; in i.v. treatments, the same dosage was delivered daily for six consecutive days *via* the tail vein. Related to function, ten days after IM/IV MSC treatment, pressures were significantly increased compared with both the

PBS group and pre-treatment ($P < 0.001$). At 5 wk, there were no significant differences between injury and non-injury, independent of treatment, but pressures were significantly increased after systemic or local MSC administration compared with PBS ($P < 0.001$). Related to histology, when MSCs were supplied, less of a muscular gap, and a marked decrease in fibrosis and scar tissue appeared, with the i.v. infusion showing the least scarring. When MSCs were administered three weeks after injury, there were significant differences only with the pre-treatment values and not with the other experimental groups.

The last publication of 2014 is from Fitzwater *et al*[54], and was a continuation of the White investigation using the same injury and repair procedures[39] in 40 young female Sprague-Dawley rats. Animals were randomised to receive an injection of either PBS or allogeneic MDSCs at the transection site (two injections of 1.6×10^6 at each side) and then euthanised at seven or 90 d (a half each period) for histological evaluation. The authors found sphincter disruption in 100% of the animals in both groups 7 d after injection, but 89% of controls and 78% of SCs had intact sphincters at 90 d. Striated muscle volume increased significantly from 7 to 90 d in both groups, without statistical differences between them at 7 or 90 d. Significant inflammatory infiltrate was seen in both groups at 7 d, and persisted at 90 d, without any differences between groups. However, White *et al*[39] observed a substantial temporal improvement in the contractility of the SC group compared with PBS, so the authors suggest that SCs might improve function without modifying histology.

In 2015, Oh *et al*[55] contributed with two publications about an FI model in mongrel dogs, which consisted of resecting 25% of the posterior part of both sphincters through a perianal incision; no repair was performed and treatments were administered 1 mo after injury. In the first one[55], the authors compared a control group of sham surgery (only skin incision, $n = 5$) with ten injured dogs receiving polycaprolactone beads with PKH-26-labelled autologous myoblasts ($n = 5$) or PBS solution ($n = 5$) injected locally. Three months later, ARM and histopathological studies were performed. Anal pressures were significantly higher in SC-treated dogs than in control dogs, and the PBS group had significantly lower pressures than sham surgery dogs ($P < 0.05$). Contractile pressure in SCs dogs was 49.5% of the average before surgery, whereas it was only 32.8% in the PBS group at the same time. Immunofluorescence confirmed that some myoblasts were differentiated in all animals because labelled cells were detected, as well as some expressed smooth and skeletal muscle markers. In their second publication[56], they randomised ten injured dogs to receive either PKH-26-labeled autologous myoblasts (group A, five dogs) or autologous myoblasts and bFGF-loaded (basic Fibroblast Growth Factor, a muscle differentiation regulator) polycaprolactone beads (group B, five dogs). ARM, pudendal nerve CMAPs and histology were evaluated at 3 mo. They found a significant improvement in ARM and CMAPs in group B compared to A ($P = 0.002$ and 0.001, respectively; in fact, both decreased in group A compared to basal values) and labelled cells were detected in 2/5 (40%) and 5/5 (100%) dogs in the A and B groups, respectively. Therefore, group B treatment improved the recovery, outcomes and SC implantation compared to cell-based therapy alone.

In the same year, Montoya *et al*[57] published a bioengineering investigation with Sprague-Dawley female rats. Eighty rats underwent midline transection of both AS by a 7 mm full-thickness incision without repair. After 2 wk, the edges were re-exposed and animals were randomly assigned to receive the following treatment by injection (20 animals per treatment): (1) nothing (non-repaired control, NRC); (2) a polyethylene glycol-based hydrogel matrix scaffold combined with PBS (PBS/hydrogel); (3) a hydrogel matrix scaffold combined with allogeneic pre-confluence MDSCs (3.2×10^6 cells, a half in each edge, SC/hydrogel); and (4) type I collagen. Then, animals were sacrificed 4 or 12 wk later (ten and ten animals from each group, respectively), and their sphincters were analysed for contractile function, disruption, and striated muscle volume. Time-matched unoperated controls were utilised for each of the two time points ($n = 10$ each period). In functional analysis, after 4 wk, maximal electrical field-stimulated contractions were significantly decreased in all four non-repaired groups compared with non-injured; however, contractions were improved in SC/hydrogel group relative to NRC (significant), PBS/hydrogel, or collagen groups. NRC and PBS/hydrogel deteriorated at 12 wk, while SC/hydrogel maintained improvement. Related to morphology, striated muscle volume increased significantly *vs* NRC from 4 to 12 wk for PBS/hydrogel (65%) and SC/hydrogel animals (63%). At 12 wk, SC/hydrogel animals had greater striated muscle volumes than all other treatment groups ($P = 0.001$); no differences appeared at 4 wk. At 12 wk, all NRC showed disruption, while only 20% of SC/hydrogel ($P = 0.048$) and 0% of collagen-treated ($P = 0.008$) were disrupted. There was also remarkably little inflammation at 4 and 12 wk with SC/hydrogel or collagen, with occasional giant multinucleated cells and small vascular channels on intervening fibrosis between muscular endings. Therefore, a compatible matrix may facilitate SC survival, differentiation, or function, leading to functional recovery despite morphological disruption.

In 2015, Kajbafzadeh *et al*[58] published another bioengineering model with rabbits. The EAS of 16 rabbits were resected, decellularised and transplanted into the terminal rectum of the incontinent rabbits 6 mo later. Animals were divided into two groups: 1 ($n = 8$) receiving injected 7×10^7 autologous myogenic satellite cells into the implant; and 2 ($n = 8$) without injection. Histological evaluation at 3-mo intervals and EMG with electrical stimulation after two years (the longest follow-up published) were performed. In the

histological evaluation, no evidence of inflammation or rejection was observed and the transplanted EAS appeared normal; there were no morphological differences, but all immunohistochemical markers in the SC group revealed significant enhancement three and 6 mo after surgery ($P < 0.001$) without significant differences between 12 and 24 mo. In the functional evaluation of both groups, grafted EAS contracted in response to needle and electrical signals to both the muscle and pudendal nerve; more signals were always detected in group 1, but no statistical study about this issue was provided.

In 2016, Sun *et al*[59], also from Zutshi's team, further expanded the concepts of delayed repair and SC homing. First, the authors investigated the best electrical stimulation parameters in an SD rat model; secondly, they evaluated the most efficient delivery route for allogeneic BM-MSCs, randomly allocating SD rats into three groups: Intravascular ($n = 20$), intraperitoneal ($n = 8$), or direct (intramuscular) injection ($n = 14$). In both experiments, *in vivo* cytokine expression and luciferase-labelled sphincter cell imaging were employed. A significant ($P = 0.03$) increase was found in MSC retention at the site of electrical stimulation with direct intramuscular injection (not in the other groups) compared to sham-stimulated animals. Finally, 16 SD rats underwent a ventral excision of 50% circumference of AS and then randomly received (four animals each group): (1) no treatment; (2) daily electrical stimulation for 3 d; (3) 3 d stimulation followed by 10^6 MSCs at the injury site the third day; and (4) 3 d stimulation with two injections of 10^6 MSCs the first and third days three weeks later. Function was assessed before and 4 wk after intervention when histologic assessment was also done. In the results, there was significantly more new muscle in the injured area four weeks after intervention, and there was also a significantly improved anal resting pressure in group 3 compared with all other groups.

Also in 2016, Mazzanti *et al*[60] (from Lorenzi' s group) published a study with 32 Lewis rats using Lorenzi's injury and primary repair models[35]. There were four experimental groups: Sphincterotomy and repair plus intrasphincteric injection of saline (A), *in vitro*-expanded allogeneic BM-MSCs (B), minimally-manipulated allogeneic BM mononuclear cells (MNCs, C) and the fourth underwent sham operation (D). At day 30, histologic, morphometric, *in vitro* contractility, and functional analyses were performed. Both SCs improved muscle regeneration: A large gap in the muscular layer filled with dense connective tissue and mast cells appeared in group A, which was almost completely repaired in the SC groups that contained numerous small clusters of smooth muscle cells irregularly interspersed in the fibrosis. Moreover, SC groups showed increased contractile function compared to saline ($P < 0.05$). No significant difference was observed between the two SCs used. GFP+ (Green Fluorescent Protein) cells remained in the injury proximity for up to 30 d post-injection. The authors concluded that both kinds of SCs are similar in terms of efficacy.

In 2017, Sun *et al*[61] published an interesting paper combining cytokines, bioengineering and SCs in an attempt to mimic acute injury conditions by homing SCs with cytokines, since healing at a time distant to injury, as in clinical situations, is a huge challenge. Thirty-two female Sprague Dawley rats underwent 50% excision of the AS complex; three weeks later, four interventions were randomly allocated ($n = 8$): (1) no intervention; (2) 100 μg plasmid -expressing stromal derived factor 1 (SDF-1); (3) plasmid and 800000 allogeneic BM-MSCs (injected at injury area); and (4) plasmid with a gelatine scaffold mixed with cells (same dose) injected 3 d later. The authors analysed ARM before and 4 wk after intervention, when histology was also studied. Related to function, the three intervention groups had a significantly greater change in resting pressure compared with the control group. In histology, plasmid and plasmid with cells groups showed increased muscle mass and architectural organisation, whereas controls showed disorganised architecture and less muscle. There was also significantly less fibrosis at the injury sites in the plasmid and plasmid plus cells groups compared with the control group. Therefore, the local delivery of the SDF-1 plasmid with or without local MSCs enhanced sphincter muscle regeneration long after injury, thereby improving functional outcome.

Also in 2017, Bohl *et al*[76] developed a passive FI model in rabbits and studied bioengineered IAS. The injury consisted of an IAS hemicircumferencial sphincterectomy through a ventral curvilinear incision. Autologous biosphincter innervated constructs were produced using IAS biopsy and small bowel biopsy to obtain enteric neural SCs, employing the methodology of Gilmont *et al*[62]. Six constructs were obtained from each animal and were supplemented with neural differentiation medium (Neurobasal-A). Each rabbit received four biosphincters (with two million smooth muscle cells and 800000 neural progenitors). Twenty female rabbits divided into three groups were used: Non-treated (6): Injury without treatment; Treated (10): Injury followed by the implantation of biosphincters conforming a ring in the intersphincteric space 6-8 wk later (only eight were finally evaluated); and sham group (4): Injury followed by re-accessing the surgical site without more manoeuvres. ARM was used before and after injury and one and 3 mo after treatment; histology was also analysed. After the injury, all rabbits had significantly decreased basal tone and loss of both Recto-Anal Inhibitory Reflex (RAIR) and anal hygiene; these findings were sustained at 3 mo in groups A and C. In group B, both parameters were restored and significantly higher at one and 3 mo. In histological evaluation, smooth muscle reconstruction and continuity were observed in group B compared with the others; innervation and vascularisation of implants were also observed.

The same year, Sun *et al*[63] hypothesised that

regenerating at a time remote from injury requires the re-expression of cytokines to attract SCs. Here, 56 female Sprague-Dawley animals underwent the same procedure as in their previous paper (50% ventral excision)[59,61] and three weeks later were randomly allocated to four groups (14 animals per group): (1) no treatment; (2) 100 μg of SDF-1 plasmid injected locally; (3) local injection of plasmid and 8×10^5 BM-MSCs 3 d later; and (4) plasmid and a gelatine scaffold mixed with BM-MSCs 3 d later. The protein expression of cytokines CXCR4 and Myf5 was investigated 1 wk after treatment (n = 6 per group) and the resting animals received ARM, histology, immunohistochemistry and morphometry 8 wk after treatment. Related to functional results, all of the groups receiving the plasmid had significantly higher anal pressures than controls, with no differences between groups receiving the plasmid. In morphology, all of the groups receiving the plasmid had significantly more organised muscle architecture than controls, with no differences between therapeutic groups. Also, animals receiving plasmid alone had significantly greater muscle (smooth and skeletal) in the defect (P = 0.03) than either animals with injury alone (P = 0.02) or those receiving the plasmid, cells, and scaffold (P = 0.03). Significantly less fibrosis appeared with plasmid alone. There were no differences in CXCR4 or Myf5 levels at 1 wk. The authors concluded that an SDF-1 plasmid may be sufficient to repair an injured anal sphincter, even long after the injury and without either MSCs or scaffold treatments.

In the first 3 mo of 2018, three publications have appeared. The first is from our research team, and is the pioneer study employing both autologous (syngenic) ASCs and biosutures for FI[64]. First, anorectal normal anatomy was studied on Wistar and BDIX female rats. Then, an injury model consisting of a 1 cm extra-mucosal myotomy beginning at the anal verge in the anterior middle line was defined and characterised histologically and functionally (ARM). After injury, 36 BDIX rats were randomised to three groups for: (1) cell injection (10^6 labelled ASCs) without repair; (2) biosuture repair (two sutures with 1.5×10^6 GFP-ASCs); and (3) conventional suture repair and cell injection. Functional, safety and morphological studies were conducted during 1 wk. Biosutures became covered with 820000-860000 ASCs, with 100% viability, but some ASCs remained adhered after suture use. ARM showed spontaneous, consistent, rhythmic contractions, taking the form of "plateaus" with multiple twitches that were very heterogeneous in their frequency, mean duration and mean number of peaks. With the injury, both sphincters were completely sectioned, and in ARM, the described activity was replaced by a gentle oscillation of basal line without a pattern. Surprisingly, these findings appeared irrespective of repair or treatment received. ASCs survived in this potentially septic area for at least 7 d: 84% of animals had GFP+ cells, mainly in the muscular section area or in the interposed tissue, forming "conglomerates" with the injections (groups 1 and 3) or wrapping the biosutures. ASCs were also able to migrate to the damaged zone. No relevant adverse events, mortality or unexpected tissue growths were found.

The following publication was from Kuismanen *et al*[65] with Sprague-Dawley rats and with the novelty of employing xenogeneic human ASCs supplemented with human platelet lysate. For injury, the authors mimicked an acute fourth grade sphincter tear by sectioning both AS and anal mucosa, and then repaired them plane by plane with 6-0 poliglecaprone running sutures using magnifying loupes. Injections (at 30° and 330° on a superimposed clock face) were administered prior to perianal skin closure. They also tested whether ASC efficacy could be improved by adding a polyacrylamide hydrogel carrier called Bulkamid. Female virgin rats were randomised into four groups (n = 14-15/group): hASCs (3×10^5) in saline, or Bulkamid and saline, or Bulkamid alone. Evaluation methods: ARM before and two (n = 58) and four weeks after injury (n = 33), micro-computed tomography, and histology. In functional evaluation, both the median resting and peak pressure were significantly higher at 2 and 4 wk in the ASC groups compared with the other groups, and both grew more during the evaluation period; there was no difference between the ASC-carriers (saline *vs* Bulkamid). In the morphological evaluation, no ASCs were recognised at either 2 or 4 wk, and there was no difference in muscle continuity, fibrosis, or collagen formation between the four groups. Bulkamid-hydrogel was well integrated with minor foreign body reaction. The inflammation was scored considering cell infiltration, oedema, haemorrhage and necrosis, as described by Nolte *et al*[66], and there was significantly more inflammation in the hASC-groups, especially in the saline-ASCs. The authors also found a good correlation between histology and micro-CT, so they suggested this for non-destructive morphometric analysis on the whole injured area.

The most recent publication is from Li *et al*[67], the pioneer evaluating electroacupuncture (with a galvanic stimulation) combined with SC therapy. The authors employed Zutshi's surgical injury[44] without repair. Sixty Sprague-Dawley rats were randomly divided into five groups of 12: (1) sham-operated control; (2) injured; (3) injury plus electroacupuncture (EA); (4) injury plus allogeneic BM-MSCs; and (5) injury plus BM-MSCs and EA. EA was performed once a day for six consecutive days by inserting an acupuncture needle bilaterally 5 mm at the ST36 point and connecting them to a low-frequency electronic pulse instrument. BM-MSCs were administered with a single injection of 9.6×10^6 SCs in the caudal vein. Animals not receiving EA underwent needling at ST36 connected to an acupuncture apparatus and animals not receiving BM-MSCs were given a normal saline injection. Only morphological analyses were performed on days 1, 3, 7 and 14. In histology, BM-MSCs and EA associated with

neovascularisation, fibroplasia and less inflammation, and both combined obtained the strongest effects; also BM-MSCs and EA significantly increased capillary density, with the BM-MSC + EA group having the highest values. Sarcomeric α-actinin expression was significantly higher at day 14 in groups 3–5 compared to 2 (injury only), and in group 5 compared with 3 and 4 (P = 0.009 and P = 0.005, respectively), suggesting that tissue repair was higher in the BM-MSC+EA group. Similar results were observed for SDF-1 and MCP-3 expression, suggesting the promotive effects of EA on the homing of BM-MSCs. The authors concluded that the combination of EA and BMSC is more effective.

In a brief analysis, there is high heterogeneity in faecal incontinence models (different surgical sections, variable partial excisions, total excision, cryoinjuries and pudendal nerve crush) and in injury managements (repair or not, substitution). The two most employed SCs include: muscle progenitors (including MDSCs and myoblasts, more committed and derived from the previous, 15 studies) and bone marrow cells (10); allogeneic or autologous use is similar (17 and 11 studies, respectively, one uses both types). Muscle progenitors are less well-defined in the literature compared with MSCs; there is no consensus defining MDSCs and myoblasts as opposed to MSCs and ASCs, so the cellular products employed in publications could be more heterogeneous and could combine different cell lines. Thirteen studies randomly assigned treatments. Murine models are primarily employed (mainly for accessibility and lower cost: 21 studies), however bigger animal models have grown in the last years (looking for greater human similarity: five studies with rabbits and three with dogs have been published). More than one third of published studies have combined SCs and bioengineering with favourable results, and eight have employed different adjuvants to enhance SC function, implantation or survival (2 SDF-1 and one study for each one of the following: human platelet lysate, PDGF, bFGF, anal electrical stimulation, electroacupuncture, and neural differentiation medium). The publications are summarised in Table 4.

All investigations, except two, confirm the safety and absence of relevant adverse events. There is one alert with local injection (two local benign foci of growth in nearly 400 published injected animals)[47] and another with systemic (mortality increment associated with pulmonary embolisms)[42], possibly due to the high doses employed.

In general, good and encouraging morphological and functional results have been observed, as well as data suggesting regeneration aspects. There are only three studies[54,63,64] that find no differences using SCs or control products (placebo[54,64] or active[63]), and another one putting it in doubt[61]. The majority have confirmed SC survival in this potentially septic area, but some have not been able to find cells that retain SC labelling[42,43]. Most publications only perform short or at least medium-term follow-up (three-6 mo), with only one long-term follow-up (2 yr) published[58]. There are also many doubts concerning the mechanisms of action of SCs in this field.

We think that many more studies are needed to draw concrete conclusions. To date, publications indicate safety and suggest a very interesting potential efficacy, but more are required to confirm these promising results.

HUMAN STUDIES PUBLISHED

There are seven publications regarding SC administration in humans for FI, including 89 patients (55 receiving SCs). There was one study not focused on FI, one case report, three observational studies (two with the same patient cohort) and two randomised controlled trials. Employed SCs have been myoblasts (five studies, all autologous) and ASCs (one autologous and one allogeneic). An overview of these published investigations is presented in Table 5.

A Phase Ⅱ study for complex perianal fistula by García-Olmo *et al*[24] analysed FI in patients operated upon at their centre. Five out of 13 (38.46%) from the experimental group (fistulae treated with ASCs plus fibrin glue) had FI and three improved (60%), compared to three out of 13 (23.08%) in the control group (fibrin glue) who did not improve[24]. The evaluation was purely subjective, and the study was not designed to accomplish this objective. These results should therefore be evaluated with caution.

The first specific publication was the observational study from Frudinger *et al*[68]. The authors injected autologous myoblasts into the EAS from ten female patients with non-operated anterior lesions that were refractory to conservative treatment. Attempting to optimise SC integration, patients received anal electrical stimulation 15 min per day for 10 wk prior to implantation and 28 d after it. Cell dosage is not perfectly described; the authors performed 12-14 0.5 mL injections of a solution containing 20.16 × 10^6 SC/mL under ultrasonic guidance in a semi-circular array, including EAS divided ends and the intervening scar. No adverse events appeared. There were significant decreases in the Wexner scale (13.7 unities), daily defecations (0.4), and incontinence episodes per week (8) at the one year follow-up. Related to function, voluntary pressure grew significantly at one and 6 mo, but later decayed to basal values at 12 mo; maximal and median resting pressure also significantly decreased (7 and 6mmHg respectively) between six and 12 mo. Morphologically, there were no important changes in ultrasonography during the follow-up. Quality of life improved significantly during all the studies. The authors concluded that the treatment is feasible, safe, well-tolerated, and improved symptomatology

Table 4 Overview and concise review of different published studies related to faecal incontinence and stem cell therapy in animal models

Ref.	Animal	*N*	Randomized	Type of SC	Compared to	FI model	Repair?	Treatment	Effect measure	Follow up	Principal Results	Security concerns
[35]	Rats	32	No	AUT/ALLOG BM-MSCs	Sham injury Injury + SSF	Surg section	Surg	Inj IE	Histology *In vitro* contractility	30 d	↑ muscular area ↑ Electric response and relaxing	No
[36]	Rats	15	No	MDSC AUT	No injury Crioinj/crioinj + SCs	Crioinjury	No	Inj IE	Histology *In vitro* contractility	7 d	SC survive + myofibre differentiation ↑ contractility (NSS)	No
[37]	Rats	??	No	Myoblast ALLOG	Subcutaneous levator ani thig muscle	No	No	Inj levator ani	Histology	??	SC survivor injury necessary for myofibre formation	No
[38]	Rab-bits	31	No	hUSCs SYNG BM-MSCs ALLOG	Culture medium Saline	Section	No	Inj IE 2 wk later	Clinic EMG Histology	2 wk	BM-MSC: better continence ↑ act SS ↑ muscle	No
[39]	Rats	120	Yes	MDSC ALLOG	Saline	Surg section EAS	Surg	Inj IE	Contractility	13 wk	↑ SS contractility 7/90 d only repaired	No
[40]	Rats	4	No	Myoblasts ALLOG	None	No	No	Inj IE	Histology	10 d	SC survival and integration in sane host tissue	No
[41]	Rabbits	21	No	MDSC AUT	Saline	Surg section EAS	No	Inj IE 3 wk later	Clinic Histology EMG + MAR	2 mo 6 mo (control)	↑ continence since 4w Miotube + myofibre (4wk), SC Survival, ↓ Cd3 and cd34 cells, ↑ proliferate ↑ SS MAR and EMG since 4wk and grew	No
[42]	Rats	224	No	BM-MSCs ALLOG local/systemic	PBS local/Syst	Surg section EAS	Surg	Inj IE/systemic	Mollecular Histology Neurophisiology	21 d	Local: ↑ECM acute phase ↑ fibers SS detected 24-48 h (no later) ↑ activity	↑ mortality nearly SS systemic
[43]	Rats	70	Yes	BM-MSCs ALLOG local/systemic	PBS local/Syst/ Sham injuries	Surg section PNC	No	Inj IE/systemic	MAR + EMG	10 d	IM/IV improve MAR, IV MAR non after PNC No SC survivor	No
[46]	Dogs	10	No	Myoblast AUT + bioengineering	SC/nothing	Excision 25% AS	No	Inj IE 3 mo later	CMAP/MAR Histology	3 mo	↑ MAR (non SS) Foreign body reaction	No
[47]	Rats	33	No	MDSCs ALLOG	Sham control (9 *vs* 24 rats)	Surg section	Surg	Inj IE	Migration lung-liver AS histology	30 d	No migration	2 benign local foci

[48]	Rats	45	No	Myoblast SYNG	Uninjured crioinj + PBS	Crioinjury	No	Inj IE	Histology/MAR	2 mo (histo) 6 mo (function)	Restitutio (60 d), SC integrated ↑ MAR 30 d, SS from 60 d	No
[49]	Rats	33	Yes	MDSC ALLOG	PBS	Surg section (Proctoepisio)	Surg	Inj IE	MAR + EMG Histology	4 wk	Improve SS EMG + MAR 2wk not 4wk No differences in sphincter thickness	No
[50]	Rabbits	12	Yes	MDSC AUT	Saline	Surg section EAS	No	Inj IE 3wk later	MRI/MAR + EMG Histology	4 wk	Labelled cells in MRI + areas, iron + myofibre ↑ ES MAR y EMG	No
[51]	Rats	??	No	Neural enteric progenitors XENOG	No injury/Crio/ Crio + SCs	NO	No	BE: NPC + IAS cells + bilayer	Histology/EMG	4 wk	↑ neovascularization normal functioning	No
[53]	Rats	50	Yes	BM-MSCs ALLOG local/systemic	Saline Uninjured	Excision 25% AS	No	Inj IE/serial Ⅳ 24 h/3 wk later	MAR Histology (immunofluoresc)	5 wk	-↑ P 10d MSCs, 5wk MSC > Saline but no differences with uninjured Histology: ↓gap, fibrosis, scar/ Delayed 3wk no efficacy	No
[54]	Rats	40	Yes	MDSC ALLOG	PBS	Surg section	Surg	Inj IE	Histology	3 mo	No differences between groups	No
[55]	Dogs	15	No	Myobl AUT + PCL beads	PBS Uninjured	Excision 25% AS	No	Inj IE 1mo later	MAR Histology	3 mo	↑ SS MAR (50% basal) SC survival + differentiation	No
[56]	Dogs	10	Yes	Myoblast AUT (A)	(B) Myob aut + PCL beads with bFGF	Excision 25% AS	No	Inj IE 1 mo later	MAR/CMAP Histology	3 mo	↑ SS MAR + CMAP B > A SC en 40% (A) *vs* 100% (B)	No
[57]	Rats	80+ 20	Yes	MDSC ALLOG + hidrogel	Nothing PBS-hydrogel Collagen/No injury	Surg Section	No	Inj IE	Contractility Histology	3 mo	↑Contract and ↑ all F-U in SC-Hydrogel ↑ SS Muscle SC-Hydrogel; ↓ inflammation SC-Hydrogel and collagen	No

[58]	Rab-bits	16	No	MDSC AUT	Only EAS scafold	Total EAS excision	No	EAS sustitution	Histol (every 3 mo) EMG 2 yr	2 yr	No inflammation-reject, improve SS 3-6mo Improve EMG (no statistics provided)	No
[59]	Rats	58	Yes	BM-MSC ALLOG + electrostim	No treatment Elecrostimulation	Excision 50%	No	Inj IE + electrostim	Histology/MAR	4 wk	4wk, electrostimulation + 1 dose MSCs: ↑ muscle in injury area ↑ resting P compared with other groups	No
[60]	Rats	32	No	BM-MSCs ALLOG BM mononuclear	Sham surgery SSF	Surg section	Surg	Inj IE	Histol/ morphometry/ MAR *In vitro* contractility	30 d	SC ↑ regen and SS contractility No differences between SC SC survive 30 d	No
[61]	Rats	32	Yes	BM-MSCs ALLO + SDF-1 (simult/ deferred)	No treatment SDF-1	Excision 50%	No	Inj IE + SDF-1 ± gelatin scaffold	Histology/MAR	4 wk	SDF-1 +/- SCs: ↑ resting P and %muscle and muscle organization and ↓ fibrosis (SS)	No
[76]	Rabbits	20	No	Neural enteric Progenitors AUT	No treatment Sham injury	Excision 50% IAS	No	Sustitution (biosphincter) 6-8 wk later	Histology/MAR	3 mo	Functional improvement since 1mo, SS with others Regeneration, neovascularization and innervation	No
[63]	Rats	56	Yes	BM-MSCs ALLOG + SDF-1 (deferred)	No treatment SDF-1	Excision 50%	No	Inj IE + SDF-1 ± gelatin scaffold	Histology Morphometry MAR Cytoquines	8 wk	Plasmid +/- SCS: ↑ MAR, muscle organization Plasmid: ↑ muscle mass SDF-1 sufficient for repairing without SC+ / -scaffold	No
[64]	Rats	36	Yes	ASCs SYNG	Conventional suture	Surg section	Yes/No	Inj IE biosuture	Histology/MAR	7 d	No functional differences SC survivor and migration to injury	No

[65]	Rats	58	Yes	Human ASCs	SSF Bulkamid (hydrogel)	Surg section	Surg	Inj IE	MAR micro-CT Histology	4 wk	Functional: ↑ SS ASCs and grew: no differences between carriers Morphology: no differences in muscle, > inflammation if ASCs, micro-CT correlation	No
[67]	Rats	60	No	BM-MSCs ALLOG ± electroacupunct	Sham injury Elecroacupunture SSF acupuncture	Surg section	No	Inj IV	Morphology	14 d	SC+EA ↑ vessels, fibroplasia and ↓ inflammation ↑ muscle SS and homing growth factors (SS). Electroacupunct promotes homing	No

AUT: Autologous; ALLOG: Allogeneic; SYNG: Syngeneic; XENOG: Xenogeneic; SSF: Saline solution; Surg: Surgical; Inj: Injection; IE: Intrasphincteric; Crioinj: Cryoinjury; NSS: Non-statistically significant; SS: Statistically significant; ??: Unknown; ECM: Extracellular matrix; AS: Anal sphincter; Proctoepisio: Proctoepisiotomy; NPC: Neural progenitor cells; Immunofluoresc: Immunofluorescence; P: Pressure; PCL: Polycaprolactone; F-U: Followup; Histol: Histology; Simult: Simultaneous; Electrostim: Electrostimulation; Electroacupunt: Electroacupuncture.

with a functional correlation. Five years later, a long-term evaluation was published[69] that analyzed defecatory diaries, blood analyses, quality of life and function annually. No adverse events or changes in blood analyses appeared. Wexner, resting and voluntary contraction pressures, as well as the overall and sub-measures of quality of life, improved significantly ($P < 0.001$) for the entire evaluated period. Reduced defecation frequency and the number of FI episodes also persisted for five years.

Romaniszyn *et al*[70] initially published, as a proof-of concept, a case of autologous myoblast implantation in EAS. Cells were obtained from the quadriceps, and the patient had a traumatic AS rupture refractory to both sphincyteroplasty and biofeedback; an 8-10 mm scar on both AS persisted, and the Faecal Incontinence Severity Index (FISI) score was 20 points. Here, 6×10^8 myoblasts were transplanted under ultrasonographic guidance and distributed on both sides of the muscle scar, on the remaining EAS, and directly into the scar. The procedure was uneventful. Controls took place every 6 wk for three visits, and then after one year. FI improved from the 6th week: it disappeared to solids and soiling but persisted to flatus. Squeezing pressure also improved, and activity in the EMG started to register on the scar area, where there was no activity before implantation. These results motivated them to perform a prospective study on ten patients that was published in 2015[71]. They included patients with FI of different origins with a Wexner (CCI) > ten, as well as low pressures with preserved reflex and innervation. In addition, they excluded patients with Wexner = 20, EAS defects > 90° and denervation. They implanted 3×10^8 myoblasts distributed into three injections: if a defect existed, 0.5 mL for each EAS border, 1 mL in the scar and the remaining volume in normal EAS, and if there was no defect, 3 mL was distributed around the EAS ring. The follow-up was the same as in the pilot study and was completed by nine patients. No muscle biopsies or implantation procedures generated complications. Regarding ARM, no changes appeared at 6 wk, but values gradually increased at 12 and 18 wk (significantly at 18). After 18 wk, significant subjective improvement was obtained in six patients (66.7%), and all patients improved in ARM, five very significantly (55.6%). Upon EMG evaluation, improvement was found in all visits, with the highest values at 12 and 18 wk. Twelve months later, a deterioration of continence was reported by two of the six patients, with good results at 18 wk (also present in ARM and EMG); nevertheless, mean values were

Table 5 Overview of published clinical experience in stem cell therapy for faecal incontinence

Ref.	Study type	*N*	Stem cell	Treat-ment	Compared	Other treatments	Effect measure	F-U	Principal results
[102]	Phase Ⅱ RCT	26	AUT ASCs	Injection fistula	Fibrin glue	No	Subjective	1 yr	Improvement 60% *vs* 23%
[68]	Obser-vational	10	AUT MB	Injection EAS	No	Anal electrical estimulation 10 + 4 wk	Clinical MAR Morphology	1 yr	↓ Wexner and episodes 1 yr, ↑QoL ↑ Voluntary P 1, 6 mo no at 12 Morphology no changes
[69]	Obser-vational	=						5 yr	↓ Wexner and episodes, ↑QoL ↑ P
[70]	Case report	1	AUT MB	Injection EAS	No	No	Clinical MAR + EMG	1 yr	Improved since 6 wk ↑ P and EMG on scar area
[71]	Obser-vational	10	AUT MB	Injection EAS	No	No	Clinical MAR	1 yr	MAR SS 18 wk Clinical: 66% 18 wk and 44.4% 1 yr EMG improvement all F-U
[72]	RCT double-blind	18	ALLO ASCs	Injection EAS	PBS	Surgery	Wexner US EMG	2 mo	No differences on Wexner ↑ SS muscle area and EMG
[73]	Phase II RCT	24	AUT MB	Injection EAS	Placebo	Biofeedback 15 d	Wexner, FIQL MAR, NPS US, MRI	1 yr	SS improve wexner 1 yr, response 60% Partial improvement FIQL 6-12 mo No morphologic differences at 12 mo Transient placebo effect

AUT: Autologous; ALLO: Allogeneic; RCT: Randomized controlled trial; MB: Myoblast; NPS: Neurophysiology; SS: Statistically significant; QoL: Quality of life; P: Pressure; F-U: Follow-up.

still significantly better than before implantation. The remaining four (44.4%) continued with satisfactory results. The authors concluded that more studies are needed to obtain a longer response.

In 2017, a double-blind randomised clinical trial with allogeneic ASCs for sphincter defects was published by Sarveazad *et al*[72]. Twenty patients were randomised, but 18 were analysed (one exclusion by cancer diagnosis before treatment, and one lost in follow-up) in two groups: Both received a non-overlapping sphincteroplasty with 3-0 PDS and then received either 6×10^6 ASCs (nine patients, one-half injected into each end of the muscle) or PBS (nine patients). Two months later, the CCI score, endorectal sonography, and EMG were recorded. No adverse events related to SC were detected. Both groups improved their Wexner scores without differences. In echography and EMG, the ratio of the area occupied by the muscle to the total lesion area showed a significant (P = 0.002) 7.91% increase in the SC group. EMG activity was significantly higher in the therapeutic group (P = 0.002). The authors conclude that ASCs may act as an adjuvant for surgeries that replace fibrous tissue with muscle. The trial was registered at the Iranian Registry of Clinical Trials with the code IRCT2016022826316N2.

Finally This year, Boyer *et al*[73] published a phase Ⅱ randomised placebo-controlled study using autologous myoblasts. They included patients with severe FI (CCI ⩾ 10) due to sphincter deficiency (single defect, multiple disruption or degeneration of EAS; lesions > 30% circumference are excluded) and refractory to medical treatment and biofeedback. In total, 24 patients were included, 12 receiving intrasphincteric injections of SCs and 12 receiving placebo. Eight injections of 100 ± 20 $\times 10^6$ SCs or placebo were made into both the remnant EAS and circumferentially as an outpatient procedure under echography guidance. A seven-day course of antibiotics and a biofeedback re-education program of 15 d were employed, and patients in the placebo group were eligible to receive frozen SCs after one year. The follow-up consisted of visits at six and 12 mo, as well as the completion of CCI (primary endpoint), FIQL scores, ARM, perineal electrophysiological tests, anal sonography, and MRI. Related to the primary endpoint, the median CCI at 6 mo significantly decreased from baseline in both the therapeutic (9 *vs* 15, P = 0.02) and placebo (10 *vs* 15, P = 0.01) groups without differences between them. However, at 12 mo, the median CCI continued to ameliorate in the SC group (6.5 *vs* 15, P = 0.006), while the effect was lost in the placebo group (14 *vs* 15, P = 0.35), with a higher response rate observed in the SC arm (58% *vs* 8%, P = 0.03). After delayed rescue SC injection in the placebo group, the response rate was 60% (6/10) at 12 mo. In secondary endpoints, FIQL did not improve in the placebo arm at both six- and 12-mo, and one and two of its components significantly ameliorated in the therapeutic arm at six and 12 mo, respectively.

No change was evident for either arm on sonography, MRI or electrophysiological tests at 12 mo. No relevant adverse events were identified relatable to treatment. Therefore, SCs have shown tolerance, safety, and clinical benefits at 12 mo, despite a transient placebo effect at 6 mo.

In a brief analysis of these few publications, all of them confirm the implant safety, the absence of relevant adverse events, and the feasibility of employing SCs; of the 89 patients, 55 received SC-based therapies. Regarding results, encouraging clinical, morphological and functional results have been observed, and data suggesting muscle increase have appeared. Only ten patients from one study have surpassed a long-term evaluation[69]; the habitual follow-up is one year. More randomised and comparative studies, as well as long-term evaluations, are needed to draw conclusions about efficacy.

ONGOING CLINICAL TRIALS

According to both the United States National Institutes of Health worldwide clinical trials registry (accessible from www.clinicaltrials.gov) and the EU Clinical Trials Register (www.clinicaltrialsregister.eu) on 1st April 2018, there were six registered clinical trials about stem cell therapy for FI. Surprisingly, there are no new records since previously performed search one year earlier, which is unusual for SC therapies since they are so extensively studied. We will describe them briefly here:

NCT02292628

A Spanish phase Ⅰ/Ⅱ triple-blinded randomised trial comparing autologous injected ASCs (40×10^6) with placebo in 16 patients.

Inclusion: A unique IAS defect and/or EAS ($\leqslant 100°$). CCI $\geqslant$ 12 and/or at least six episodes per month. FI for at least two years.

Outcome measures: The primary is serious adverse events during 12 mo, and the secondary are changes in FI diary, ARM, CCI or FI quality of life during 12 mo.

Actual situation: Active but not recruiting.

NCT02161003

An Egyptian phase Ⅰ/Ⅱ non-masked single group trial for children with FI after surgery for high imperforate anus.

Treatment: Unspecified dose of autologous MSCs injected all around the sphincter (12 points). Estimated enrolment: 50 patients.

Outcome measures: the primary is FI Score at 24 wk, and the secondary is clinical assessment at 12 wk; maximum daily dry intervals (days 1, 30, 90); pelvic MRI and EMG at 90 and 180 d.

Actual situation: Unknown recruitment status, estimated completion date surpassed and not actualised since June 2014.

NCT01011686

Phase Ⅰ trial in South Korea focused on the security of local autologous ASCs (the registry does not specify the dose or implantation method).

Eligible patients: CCI $\geqslant$ 5, FI for more than 6 mo, AS continuity (ultrasound) and abnormal ARM.

Outcome measures: in primary, there is one about efficacy (CCI) and another about safety (abnormality of laboratory and adverse events), and the secondary consists of ARM and ultrasound. All these measures are evaluated at 4 wk.

Actual situation: Appears as "terminated" without obtaining the desired recruitment for unknown reasons (last data update in 2011). No related results have been published.

NCT02384499

Phase Ⅰ randomised placebo-controlled, unicentric and single-blinded trial with allogeneic ASCs from South Korea and with two phases. Safety study: a dose escalation study: three groups of three patients receive 3×10^7, 6×10^7 or 9×10^7 cells/mL, respectively. Follow-up: physical examination, serologic and immunologic response test, CCI, satisfaction survey, WHO toxicity scale, adverse events, ARM and ultrasound at 1, 4, 8 wk, 4, 6, 9, and 12 mo. Response is assessed at 8 wk to select the best dosage. Efficacy test: It compares the efficacy of ASCs *vs* placebo (0.9% normal saline plus fibrin glue) with six patients in each group. Employs randomised, open-label and single-blind design. Clinical assessment and follow-up are identical to the safety study.

Eligible patients: Failed medical therapy or biofeedback for more than 2 mo with CCI $\geqslant$ 8, continuous sphincter on sonography with decreased pressures on ARM. Cell therapy procedure: 6 mL of fibrin glue plus ASCs solution are prepared; 4 mL are injected at four points in IAS (3, 6, 9 and 12 h), and the other third in the EAS intermediate four positions.

Actual situation: The authors published the study protocol in 2017[74], but the recruitment status is "unknown". The estimated study completion date has been surpassed by more than one year, and the last update of the registry was on March 2015. No related

results have been published.

NCT01949922

A non-masked Danish pilot study in 15 patients. It is not a pure SC trial because it analyzes the injection of autologous muscle fibres and not SCs. However, a small part of the fibres is used for analysing MDSCs number and, therein, the regenerative potential of the sample.

Eligible patients: Patients with CCI ⩾ 9 or affected quality of life after 3 mo with pelvic floor muscle training.

Outcome measures: The primary is efficacy (CCI), and the secondary is safety both at one year. Other: efficacy of pelvic floor muscle training (3 mo); improvements in quality of life, anal reflectometry, 3D ultrasound (1 yr), and correlation between the regenerative potential and effects of the tissue samples (1 yr).

Actual situation: The recruitment status is "unknown", the completion date has passed, and the data has not been actualised within the past two years.

NCT02687672

A phase Ⅰ/Ⅱ trial in Jordan that is unrelated to FI. Designed to treat chronic complete spinal cord injuries by autologous, purified CD34+ and CD133+ HSCs using bone marrow or leukapheresis as sources. The study focuses on safety and efficacy over five years, and includes FI evaluation with a questionnaire as a secondary outcome. Currently active but non-recruiting, and the estimated completion is in December 2021.

In a critical analysis of these "ongoing" trials, it draws attention that some of them are in a non-updated state, have been closed or cancelled without completing recruitment, or for no well-defined causes. This generates some doubt about the methodology, or even worse, the efficacy and safety. No alerts have been publicised about safety, so it therefore cannot be a real concern, however it is better to wait for new trials as well as the completion and publication of the ongoing trials' results.

DISCUSSION

FI is a frequent, chronic and highly limiting condition that mainly affects quality of life and has very important economic implications. Its current treatment is multimodal and progressive. If conservative and pharmacological management fails, a variety of invasive procedures are available: sacral or tibial nerve stimulation, the injection of bulking agents, sphincter repairs, sphincter substitution using the gracilis muscle or an artificial device, and finally, in totally refractory patients, a proximal stoma may be useful. To summarise, these procedures have moderate short-term efficacy and decreasing or unknown long-term efficacy, and many have high morbidity rates and compromised cost-effectiveness. In this context, a cellular therapy based in SCs is an attractive potential alternative.

One of the first problems to be solved in this field is how to obtain an FI model, and its correlation with the clinical problem. Published literature shows a high variability of models, with the most frequently applied being surgery (23 publications), including 15 sections and eight excisions (from 25% to 100% of the sphincter complex).

In section models, Lorenzi *et al*[35] described a left lateral selective sphincterotomy without specifying the length. Zutshi's model[44] consisted of "a precise 3-4 mm incision", which might not cause a total sphincter section. White *et al*[39] performed a selective EAS lesion by a total section of 7 mm followed by rectal mucosa repair. We have described an anterior section of both sphincters of 1 cm in length[64], one of the most extensive sections. Going further, Lane *et al*[49] performed a more aggressive injury defined as a "proctoepisiotomy", but did not describe the technical details or extension. Similar or minor modified procedures have been employed by Mazzanti[60], Salcedo[75], Elmi[50], Pathi[42], Fitzwater[54], Montoya[57], Kuismanen[65] and Li[67].

A Salcedo publication in 2010[75] found that rats receiving Zutshi's injury without repair or treatment presented anal pressure recovery 14 d later, comparable registries to controls after 28 d and bridging fibrosis in histology; these findings were not observed with pudendal nerve transection. This publication made Zutshi's team from Cleveland Clinic Ohio turn to more aggressive procedures. Salcedo *et al*[53] described an excision of 25% of both sphincters (through an incision in the ventral aspect, from the ten to two o' clock position) and later, Sun extended it to an excision of 50%[59]. A similar model of partial excision was employed in dogs by Kang[46] and Oh[55,56] and in rabbits by Bohl[76], and a total excision was employed by Kajbafzadeh[58]. Aiming to minimise the effects of the poorly developed sphincters in rats, some authors have employed bigger animals such as rabbits[38,41,50,58,76] and dogs[46,55,56], but there are only eight published papers compared to twenty-one in rats. Other FI models include cryoinjuries employed by Kang *et al*[36] (they did not specify the muscular volume damaged) and by Bisson *et al*[48], who published that the minimal significant cryoinjury must be of at least 90°.

We can discuss the clinical relevance of these models. Human obstetric trauma is more complex than a simple section during episiotomy or a perineum tear. Sphincter injury may be related to muscle injury, prolonged regional hypoxia, denervation, faulty repair, or a combination of them. Other factors could be added later in life: aging, hormonal changes, surgeries, *etc.* In an effort to reproduce these complex effects, simulated childbirth injury models have been designed. This was first described for urinary incontinence by Resplande *et al*[77] and by Sievert *et al*[78] where an inflated 10F Foley catheter is inserted inside the vagina for 3-4 h; to

simulate labour, an episiotomy and balloon extraction was performed. Later, Healy *et al*[79] published a model for FI using two intrapelvic, retrouterine balloons (six Fr urinary catheters) placed through a 3 cm laparotomy for one hour. More studies on simulated childbirth models are possibly needed.

On the other hand, it is known in clinical settings that immediate repair offers better results, but the most frequent scenario is a repair indicated years later and with chronic local conditions (fibrosis, denervation, atrophies, no inflammation). Thus, in preclinical papers, there are nine delayed treatments (repairing or injections delayed 2[38], 3[41,46,50,53] or 4 wk[55,56] and substitutions delayed 6-8 wk[76] or 6 mo[58]). We may discuss whether the considered periods are sufficient to mimic the chronic setting, as it is likely that only acute and subacute conditions have been tested. In the acute setting, some potential confounding factors have been observed[64] (mucosal tears, faecal contamination, *etc.*) that could compromise SCs survivorship or effects. However, there are also cytokines that are fundamental for SC homing and activation, as has been demonstrated for acute myocardial infarction with the SDF-1 factor[80]. In this field, Salcedo *et al*[45] made some relevant contributions; they studied Stromal derived factor-1 (SDF-1 or CXCL12) and monocyte chemotactic protein-3 (MCP-3), known signals that force homing of BM-MSCs to ischaemic tissue, and their expression following direct injury to the AS and pudendal nerve. They found that direct injury resulted in higher levels soon after injury and for 3 wk, whereas denervation resulted in an overexpression for only 10 d, which may lead to more fibrosis[45]. Therefore, in chronic conditions, these molecules will have normal values and thus could make it difficult for SCs to act. To increase these factors, SCs could be transfected with plasmids or the local production could be stimulated using surgical injury or electrical stimulation, such as in the paper by Sun[59]. The previously mentioned publications[61,63] open up an interesting new research field; the combination of SCs or their vehicles (for example sutures with Vascular Endothelial Growing Factor[81]) with cytokines and growth factors.

Another problem to be solved is how to obtain better SC delivery, survival and function in tissues. All studies except ours have employed cell injections; we thought that biosutures[31-33] could be useful for depositing SCs at the focus of the injury. Other authors have made different modifications to biosutures: Yao *et al*[82] added poly-L-lysine and fibronectin to improve cell adherence, and Horváthy *et al*[83] observed better BM-MSC adherence if the suture was previously covered with albumin and SC survivor in implanted tissues at 5 wk. No evidence exists about the best dose, or at least the minimal "clinically-active" dose. With 1.5×10^6 ASCs, SCs were found to form "clusters" both over the suture and in culture medium, and remained adhered after biosuture usage[64]. Therefore, more studies on suture preparation are needed. Delivered doses could be more controlled by injection, but a similar phenomenon can sometimes be observed; similar clusters appear outside the muscle layer with consequent cellular loss[64]. Injected doses have been very variable in the published literature. To improve survival and function in tissues, the employed strategies have been the use of growth factors and cytokines, as mentioned before; this field will be very interesting in the future.

Related to the mechanism of SC actions, there are many remaining questions. The first to be solved is whether SCs survive, integrate and participate in regeneration. More studies to identify critical pathways that are dysregulated in tissue repair are needed. Studies with myogenic cells have detected the labelling on muscle in acute and subacute phases, but medium- or long-term incorporation, or the differentiation of BM-derived cells, has not been clearly identified, and regeneration is at least doubtful. It is possible that myogenic SCs have a greater role based on differentiation, but MSCs likely base their role much more on immunomodulation, as well as on anti-inflammatory and angiogeneic capabilities. There is growing evidence of the immunomodulation capability of MSCs, which is thought to be largely based on inhibition of T cell and B cell proliferation and dendritic cell maturation[84], as well as on the secretion of a large number of cytokines and growth factors[85]. Németh *et al*[86] observed MSC sepsis attenuation by macrophage reprogramming to increase IL-10, a cytokine that decreases neutrophil migration. Our research team has added contributions to that evidence: Georgiev-Hristov found an early shift from acute to chronic inflammation in the presence of ASCs (neutrophil descent and macrophage increment) after tracheal anastomosis[33], and Riera observed less acute and chronic inflammation during 3 mo, with the increasing fibrosis of the aneurysm sac in pigs[87]. Regeneration is not clearly demonstrated in many studies and is very difficult to observe; it may be that more complex morphometric or molecular analyses are needed to confirm it. Similar studies would also be applied for another mechanism like immunomodulation (studying the amount of different cells and molecules). In fact, there are some remaining barriers to achieve "regeneration" with SCs. We need to teach them how to differentiate in an efficient manner, then, possibly with tissue engineering, we need to integrate them into an appropriate delivery system. Finally, we also need to generate a blood supply and innervation that is sufficient to allow their engraftment and survival.

The last critical question is about safety; although there are other potential side-effects, the most worrisome is possible carcinogenicity. SCs have surpassed preclinical studies on biodistribution and toxicity, but investigations into tumour formation are still ongoing. Some publications have observed that MSCs that are cultured for a long time may develop malignant changes and even promote tumours in mice[88]. However,

subsequent publications, including those from the same authors, attributed those findings to tumour cell cross-contamination[89,90]. Furthermore, other studies did not detect tumourigenesis under extreme culture conditions and it has never been observed *in vivo*. In fact, the relationship between SCs and tumours is contradictory. No direct MSC transformation has been observed, but there is a consensus that MSCs have enhanced tropism toward tumours and have pro-tumour (growing, angiogenesis, participation in the microenvironment, immunomodulation)[91,92] and anti-tumour (apoptosis, proliferation inhibition)[93,94] properties. This relationship depends on a lot of factors, including the type of MSCs, source, type of cancer cell line, *in vivo* or *in vitro* conditions, factors secreted by MSCs, and interactions between MSCs, host immune cells and cancer cells. A possible key factor of these effects is time. When MSCs are administered with an existing tumour, a suppressive effect has been observed[95], but in some studies with co-administration of SCs and tumour cells, tumour growth was higher compared to tumour cells alone[96]. These complex interactions have been studied by several authors and reviewed by Ramdasi *et al*[97]. Tropism to tumours has been exploited to treat tumours in experimental models, as reviewed by Chulpanova *et al*[98]. Moreover, a recent NEJM paper published the first severe adverse event potentially relatable to ASCs. Three women suffering from macular degeneration after undergoing ASC therapies developed complications, including vision loss, detached retinas and bleeding, leaving all with complete blindness (although the ASCs were mixed with blood plasma and large numbers of platelets)[99]. In conclusion, cumulated experience seems to support the oncogenic safety of SCs, but more studies and long-term follow-ups are needed to definitively exclude all the risks.

An in-depth analysis about published literature has been provided at the end of each chapter. The 29 published animal investigations confirm the safety (except one), and generally good morphological and functional results appeared with questions remaining about SCs survival, effect, long-term results, efficacy on chronic conditions, *etc.* In human research, there is one unrelated study[24], six studies involving 55 patients receiving SCs with promising results[68-73] and six ongoing clinical trials. More highly rigorous investigations (related to SC type, dosage, delivery system, adjuvant factors, and safety) are needed before SC therapy for FI becomes a clinical reality.

Related to economy, regenerative strategies use costly culture-expansion procedures that require Good Manufacture Practice laboratories compromising cost-effectiveness, as has been demonstrated in a recent survey of clinicians about SC therapy adoption[100]. It is very difficult to estimate the real potential cost of this kind of therapy for humans because there is no consensus in the type of SC, autologous or allogeneic use, the required dose, *etc*. The real efficacy needs yet to be clarified. If a cure could be achieved, direct and indirect costs mentioned before could disappear, and hospitalization costs might be lower due to less invasive procedures to implant SCs compared with FI surgery. Based on our previous experience in clinical trials for anal fistulae[22-29], approximated costs in Spain are the following: 1500-2500€ (1727.8 to 2879.73 USD) for closed system SVF, 2800-4000€ (3225.48-4607.83$) for $40x10^6$ autologous ASCs and 3500-5000€ (4032.88-5761.26$) for 100×10^6 allogeneic ASCs; the costs for other MSCs are equivalent. It must be taken into account that these costs are for SCs produced and dedicated to research, and not for commercial use (maybe higher at least during the first years). The first allogeneic ASC medicine product for fistula marketing is expected between 2018 and 2019, so we will be able to know the real costs of large-scale production. Moreover, some publications have reported acceptable results with free autologous muscle grafts in FI in children[101] (grafts also contain SCs such as satellite cells, but the processing is easier and cheaper), opening up a new field for study.

CONCLUSION

FI is frequent and the available treatments need to be improved, so alternative treatments are therefore needed. Regenerative therapies have exciting potential to improve patient outcomes, and different strategies have been explored (with or without biomaterials) in preclinical and clinical studies.

In preclinical studies, SCs derived from muscle, bone marrow and adipose tissue have been most intensively investigated. In general, safety seems to be guaranteed and some encouraging results have been observed. Clinical evidence is very limited, but the therapy appears to be safe and may be effective. More data are necessary; to date, no SC-based therapy is yet ready for ordinary clinical use, as both short-term and long-term efficacy and safety have to be firmly established. More knowledge about SC, healing biology, and bioengineering principles is needed before regenerative medicine for FI can become really implemented.

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