Electroacupuncture Alleviates Functional Dyspepsia by Targeting miR-221-3p to Regulate c-kit/SCF and Raf/Erk Signaling

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Research

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Abstract

Background: Functional dyspepsia (FD) is a gastrointestinal disorder associated with epigastric pain and fullness, with symptoms such as anxiety and depression. Electroacupuncture (EA) has emerged as a potential therapeutic strategy to treat FD via electrical stimulation at specific acupoints of the body and has previously been demonstrated to be effective in promoting gastric motility and attenuating the symptoms of FD. However, the mechanisms underlying the effect of EA remain elusive.

Results: Herein, we constructed an in vivo model of FD in rats and performed EA on FD-induced rats. Preliminary bioinformatic analysis through RNA sequencing revealed that differentially expressed microRNAs exist between non-treated and EA-treated rats subjected to EA. Among them, miR-221-3p was associated with the target gene c-kit, the expression of which is closely linked to interstitial cells of Cajal (ICCs) and gap junction intercellular communication, factors critically implicated in gastrointestinal motility. We further proved that miR-221-3p overexpression aggravated FD, but EA downregulated miR-221-3p to alleviate the symptoms of FD. The mechanisms of action underlying the effect of EA involve an increase in c-kit⁺ ICCs, upregulation of stem cell factor and connexin 43, and enhanced Raf/Erk signaling.

Conclusions: Our findings suggest that EA is an effective method of managing FD by regulating miR-221-3p, the c-kit/stem cell factor axis, and Raf/Erk signaling, reinforcing the strong role of gap junction intercellular communication in gastrointestinal motility.

1 Background

Functional dyspepsia (FD) is a common gastrointestinal disorder that affects 10–30% of the worldwide population [1, 2]. Its main clinical manifestations are epigastric pain and fullness, leading to symptoms such as weight loss and distress [3, 4]. The factors involved in the pathogenesis of FD are manifold and include impedance of gastrointestinal motility, visceral hypersensitivity, abnormalities in the brain-intestinal axis, psychological factors, and gene regulation [5–7]. Because of the complex pathogenesis and differences among individual patients, it is difficult and impractical to apply drug treatment for FD. Thus, non-drug-based therapeutic means, such as acupuncture and moxibustion, have emerged as alternative methods to treat FD.

Acupuncture is a medical practice widely applied in traditional Chinese medicine (TCM), wherein thin needles are inserted into specific points of the human body, known as acupoints, which are stimulated to achieve various effects [8]. Electroacupuncture (EA) is a modified version of acupuncture that induces stimulation by allowing a small current to pass between two needles at different acupoints, thereby inducing acute physiological changes [9]. The theory of TCM proposes that FD is attributed to stomach cramps, contamination, bloating, acid reflux, and vomiting. Though FD is essentially a gastric disorder, it is closely associated with abnormalities and imbalances in the spleen and liver, signs of which include “qi” (air flow) blockage, blood stagnation, and phlegm dampness [10]. EA at the “Zusanli” acupoint (ST36, located on the lower knee at the anterior tibia muscle along the stomach meridian [11] and “Taichong”

acupoint (LV3, located on the foot below the big toe and second toe [12] has previously been demonstrated to alleviate gastrointestinal motility disorders and restore digestive functions in FD [13]. However, the underlying mechanisms associated with the effect of EA remain poorly understood.

In the gastrointestinal tract, interstitial cells of Cajal (ICCs) are formed by synapses and tight gaps between gastrointestinal and smooth muscle cells (SMCs) that act as pacemaker cells for slow-wave activity [14]. A decrease in the number of ICCs, damage to their morphological structure, or abnormalities in the reticular formation of gastrointestinal nerve (GN)/ICCs/SMCs may lead to gastrointestinal motility dysfunction [15]. The structure of ICCs in diabetic gastroparesis with delayed gastric emptying has shown varying degrees of pathological changes, such as reticular damage, mitochondrial degeneration, and organelle reduction [16]. Thus, ICCs have been recognized as essential targets for the treatment of gastrointestinal motility disorders. The GN/ICC/SMC network is mediated by gap junction intercellular communication (GJIC), wherein connexin 43 (CX43) is a crucial protein that co-localizes with c-kit⁺ ICCs [17]. As a specific marker of ICCs, c-kit, also known as stem cell factor (SCF) receptor, binds to its ligand SCF to activate a series of signaling pathways including JAK/STAT and Raf/Erk [18, 19]. We have previously shown that EA enhanced gastrointestinal slow wave activity and upregulated c-kit, CX43, and SCF to varying degrees, effectively promoting gastric emptying and restoring gastric motility [20–22].

Recently, microRNAs (miRNAs) have been shown to regulate ICC activity, gastrointestinal function, and SCF/c-kit-related signaling. miRNAs are short (~ 22 nucleotides) non-coding RNAs that are critically implicated in physiological processes of cell cycle progression, growth, metabolism, and apoptosis. In the context of gastrointestinal function, gastric muscle-specific miR-1 and miR-133 were observed to be downregulated in mice undergoing accelerated gastric emptying [23]. In addition, studies have revealed that miR-193, miR-221, and miR-222 play potential roles in the regulation of SCF/c-kit signaling [24]. We hypothesized that EA stimulation causes specific changes in one or more miRNA(s), of which c-kit is a target gene. We propose that EA ameliorates FD by regulating the behaviors of ICCs to strengthen GJIC in the GN/ICC/SMC network, in turn restoring gastrointestinal motility via c-kit-mediated SCF signaling. In this study, we verified the miRNA(s) and pathways participating in this process through bioinformatics analysis and established an in vivo model of FD to further elucidate the underlying mechanisms of EA. The findings contribute to the understanding of the therapeutic potential of EA in FD and other gastrointestinal disorders.

2 Results

2.1 Construction of the FD model

We first validated the construction of the FD model by monitoring the behavior of the experimental animals over a 14-day period (FD induction and/or EA with/without miR-221-3p agomir/antagomir intervention). FD led to a significant decrease in rat weight over 14 days (Fig. 1A), possibly due to indigestion and reduced water and food intake (Fig. 1B and 1C, respectively). This signifies that the induction of FD using our modeling method was successfully carried out. However, rats subjected to EA

showed a clear increase in body weight compared to FD-induced rats without EA treatment, along with improved food and water intake. In particular, by the end of 14 days, water intake was restored to levels close to those of control rats.

We proceeded to observe the morphology of and integrity of ICCs before and after rats were subjected to FD (Fig. 1D). Using transmission electron microscopy (TEM), we were able to visualize the distinct morphology of typical ICCs [25], which is characterized by densely packed euchromatin (denoted by “Eu”) and the presence of well-defined mitochondria (denoted by “M”) with clearly structured cristae. In FD-induced rats, morphological changes were noted in the ICCs. The euchromatin was denser than that in normal ICCs, and an abundance of rough endoplasmic reticulum (denoted by “rER”) and vesicles (denoted as “V”) were observed. In particular, the mitochondria of FD-induced ICCs showed distinct differences as normal mitochondria, as they lost the clear ultrastructure observed in normal ICCs, especially the cristae. This may indicate that mitochondria were severely damaged during the induction of FD.

2.2 Identification of differentially expressed miRNA after EA

To identify differentially expressed miRNAs in the gastric antrum of EA-treated rats after FD induction, we performed miRNA sequencing and mapped the clean reads to the rat genome. More than 500 mature miRNAs were detected in each sample, with 260 to 320 novel miRNAs (Table 1). Comparing FD to FD + EA, differentially expressed miRNAs were screened using the following criteria: fold change > 2 ($|\log_2| > 1$) and p-value < 0.05. Compared to FD, 21 differentially expressed miRNAs were identified after EA treatment, wherein 13 were downregulated and 8 were upregulated (Table 2). Using TargetScan [26], we found that among the differentially expressed miRNAs, miR-221-3p was the only one that exhibited a targeting relationship with the c-kit gene (Supplementary Figure S1). Thus, miR-221-3p was chosen as the main subject of this study.

Table 1
miRNA sequencing in the gastric antrum of EA-treated rats.

	Raw reads	Clean reads (≥ 18 nt)	Detected miRNAs	Novel miRNAs
FD-1	12410808	11628070	551	263
FD-2	9976159	9218966	539	252
FD-3	11777521	10818022	551	275
FD + EA-1	12344236	11448824	539	315
FD + EA-2	12767677	12016131	558	309
FD + EA-3	11734560	10735591	518	266

Table 2
Differentially expressed (downregulated or upregulated) miRNAs comparing between FD + EA and FD.

FD + EA vs. FD only	
Downregulated miRNAs	Upregulated miRNAs
rno-miR-708-3p	rno-miR-370-3p
rno-miR-122-3p	rno-miR-3572
rno-miR-203a-3p	rno-miR-423-3p
rno-miR-122-5p	rno-miR-129-5p
rno-miR-293-5p	rno-let-7b-3p
rno-miR-708-5p	rno-miR-326-3p
rno-miR-205	rno-miR-667-3p
rno-miR-221-5p	rno-miR-674-5p
rno-miR-135a-3p	
rno-miR-221-3p	
rno-miR-369-3p	
rno-miR-29c-3p	
rno-miR-615	

To verify the results of bioinformatic screening in vivo, we evaluated the expression of miR-221-3p in the gastric antrum of FD-induced rats with and without EA treatment. Compared to control (non-FD) rats, miR-221-3p was significantly upregulated by FD, but upon EA treatment, the expression of miR-221-3p declined drastically (Fig. 1E). Notably, the mRNA expression of c-kit exhibited the opposite trend, wherein FD induced a significant decrease but EA elevated the mRNA level of c-kit (Fig. 1F). We also performed a western blot to confirm these results and showed that the protein expression of c-kit was consistent with the mRNA expression (Fig. 1G), further validating the correlation between miR-221-3p and c-kit. We then performed a dual luciferase activity assay to verify the binding between miR-221-3p and c-kit (Fig. 1H). We validated this targeting relationship by confirming that miR-221-3p mimics bound to the WT 3' untranslated region (UTR) of the c-kit gene, but there was no binding between miR-221-3p and the MUT 3'UTR of c-kit (Fig. 1I), consistent with the preliminary prediction.

2.3 Characterization of gastric antrum morphology

The effect of EA intervention on FD in association with miR-221-3p was further investigated by administering inhibitors (miR-i) and mimics (miR) of miR-221-3p in conjunction with EA. First, the morphology and pathological features of the gastric antrum were observed after rats were subjected to

the indicated treatments (Fig. 2). No abnormal features or lesions were found in control rats, whereas in FD-induced rats, the gastric mucosa was thinner and notable inflammatory infiltration was observed. When the FD-induced rats were treated with EA, the gastrointestinal mucosa and the epithelial cell structure were restored to a relatively normal state. In addition, miR-i alleviated the symptoms of FD by contributing to the recovery of gastric antrum morphology to a normal state. However, the administration of miR accentuated the pathological features in the gastric antrum. In all cases, the NC did not exert any noticeable effect on gastric antrum morphology.

2.4 Effect of EA and miR-221-p on GJIC and c-kit-associated activity in ICCs

We then examined the effect of EA and/or miR-221-3p overexpression or inhibition on FD at the mRNA level, in terms of c-kit/SCF and Raf/Erk signaling (Fig. 3). After the FD model was induced in rats, CX43, SCF, c-kit, Raf, and Erk were downregulated in gastric antrum tissues whereas the administration of miR-221-3p antagomirs (miR-i) or agomirs (miR) enhanced and suppressed their expression, respectively ($p < 0.05$). This suggests that inhibition of miR-221-3p counteracted whereas miR-221-3p overexpression accentuated the effect of FD at the mRNA level. Furthermore, EA treatment showed mixed results and exhibited the greatest effect when applied in conjunction with miR-i (significant difference in mRNA expression of CX43, c-kit, Raf, and Erk compared to FD + miR-i only). In all cases, NC did not exert any significant difference compared to FD (with or without EA). These findings imply that EA did not exert a prominent effect on the gene expression of relevant factors of interest.

Taking a look at protein expression, we revealed that FD significantly downregulated CX43, SCF, and c-kit relative to the control (Fig. 4A) in gastric antrum tissues. Correspondingly, the administration of miR-i or miR enhanced or suppressed the expression of these proteins after FD, respectively. In all cases, EA induced an upregulation of the expression of these proteins relative to that in the absence of EA treatment, and NC did not exert any significant difference compared to FD (with or without EA). In terms of Raf/Erk signaling, the trend was more or less the same as that of the protein expression of CX43, SCF, and c-kit, with a few exceptions (Fig. 4B). Notably, EA did not exert any difference in the ratio of p-Raf/Raf after FD-induced rats were treated with miR-i. Additionally, the changes in p-Erk/Erk seemed to be minimal, wherein only miR administration showed a difference compared to the other groups, with or without EA. Compared to the absence of EA, p-Erk/Erk was not affected significantly by EA. We also evaluated the serum levels of the abovementioned factors (Fig. 5) and observed that FD reduced the secretion of CX43, SCF, c-kit, Raf-1, and Erk in the serum ($p < 0.05$). This reduction was counteracted by miR-i but accentuated by miR in all cases ($p < 0.05$), and NC did not exert any effect as anticipated. In all cases, EA increased the level of each protein compared to that in the absence of EA ($p < 0.05$).

3 Discussion

Acupuncture is a traditional technique in TCM that has been practiced for thousands of years in China and other Asian countries. Although it is an ancient medical approach, its therapeutic efficacy has been

well proven in recent years through evidence-based studies related to conditions such as postherpetic neuralgia [27] and FD [28]. EA is a modified form of acupuncture where a slight electrical stimulation is applied to the needles at a constant frequency. Previously, it has been demonstrated that EA influenced the tactile and thermal sensitivity of resiniferatoxin-treated rats in an in vivo model of postherpetic neuralgia, and the effect of EA was exerted via the regulation of a variety of miRNAs showing differential expression [27]. EA also reportedly improved cerebral blood flow and alleviated neurological deficits by modulating the expression of cell proliferation-associated miRNAs in a rat model of stroke [29]. These studies show the strong correlation between EA and miRNAs in neurological conditions and thus, it is reasonable to propose that EA may also affect FD via miRNAs because of the implications of the brain-intestinal axis in gastrointestinal motility. In fact, EA has been shown to relieve dyspeptic symptoms in patients with refractory FD [28], which could be associated with changes in the levels of serum ghrelin, calcitonin gene-related peptide, and glucagon-like peptide 1 [30]. Whether one or more miRNA(s) is/are involved in the anti-FD effect of EA and whether these effects are related to changes in ICCs and GJIC remain elusive and form the basis of our study.

The nerve fiber/ICC/SMC network mediates the production and regulation of neurotransmitters and forms the basis of gastrointestinal motility [14, 31]. Therein, ICCs are identified by the expression of its marker gene c-kit (or SCF receptor), the activation of which by SCF is critical for the function of ICCs [32]. ICCs play a critical role as pacemaker cells that produce electrical stimulus in the form of slow waves in the gastrointestinal tract. These slow waves are transmitted to adjacent SMCs through gap junctions, resulting in gastrointestinal contractions [33]. Accordingly, the proper function of gap junction proteins is necessary for the maintenance of gastrointestinal motility. A decrease in CX43 expression can cause changes in the junctional structure, affecting the signaling within the GN/ICC/SMC networks (the ICCs are electrically coupled to the excitation and inhibition signals) and leading to gastrointestinal dysfunction [34, 35].

FD is associated with abnormal slow wave function and loss of ICCs [36, 37]. Given the intrinsic electrical properties of ICCs, it is conceivable to propose that EA, which principally functions by electrical stimulation, may be able to exert similar effects as those of ICCs on surrounding cells or ICCs themselves and alleviate FD. Our results are consistent with a report by Zhang et al. that demonstrated the effect of EA in FD-induced rats, particularly that while FD caused damage to ICCs and reduced CX43 levels, EA led to recovery from these changes [38]. Similarly, other studies have shown that EA, particularly at the ST36 (Zusanli) acupoint, had protective effects on ICCs [39, 40]. We note interestingly that our results of quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot seemed to be complementary. That is to say, the expression of CX43, SCF, c-kit, and Raf was minimally affected by EA at the mRNA level, but significant changes were induced at the protein level. Conversely, the mRNA expression of Erk was altered, whereas the protein expression of p-Erk/Erk remained unchanged after EA. This finding suggests that EA affects ICCs and GJIC at both the gene and protein level, albeit this depends on the specific signaling factors involved. We hypothesize that EA altered CX43, SCF, c-kit, and Raf only at the translational or post-translational level by protein degradation or modification (e.g.

phosphorylation in the case of Raf). However, for Erk, no post-transcriptional alterations are exerted by EA. This hypothesis requires validation in future investigations.

Importantly, our study is the first to show that miR-221-3p is critically implicated in the effect of EA on FD through the regulation of its target gene c-kit. miR-221-3p reportedly contributes to the development of metastatic cervical squamous cell carcinoma [41] and major depressive disorder [42] and mediates cellular processes such as inflammation [43] and lipid metabolism [44]. However, whether it plays a role in gastrointestinal disorders has not been studied. Through bioinformatic analysis, we screened for miRNAs that were differentially expressed in FD-induced rats after EA treatment. Among the identified miRNAs, miR-221-3p was the only one with a target gene that is strongly associated with gastrointestinal motility, namely c-kit. A regulatory correlation between miR-221 and CX43 has also been demonstrated in a previous study, wherein GJIC was disrupted by miR-221/22, which targeted and downregulated CX43 [45]. We additionally expand on the role of the Raf/Erk signaling pathway and its indirect involvement in FD. Among the various properties of Raf/Erk, its relationship with CX43 and regulation of gap junction activity has been well characterized [46]. Stains and Civitelli proposed that second messengers in the cell traverse gap junctions, which are mediated by CX43, to activate Raf/Erk signaling. Activated Erk is then translocated into the nucleus for downstream gene transcriptional regulation [47]. While studies have proved that EA enhanced the expression of Raf [48] and the phosphorylation of Erk1/2 [48, 49], the relationship between miR-221-3p and Raf/Erk signaling has not been reported and will be the focus of future studies.

4 Conclusions

Collectively, our findings suggest that EA has the capability to alleviate FD by targeting miR-221-3p. The downregulation of miR-221-3p in turn results in the upregulation of the c-kit/SCF axis, improved GJIC via the regulation of CX43 and Raf/Erk signaling, and the recovery of ICC function. These findings provide evidence of the utility of EA in FD treatment, forming the basis for the extension of this technique to other gastrointestinal disorders.

5 Materials And Methods

5.1 Establishment of FD model

All animal experiments were performed at the Model Animal Research Institute at Wuhan Myhalic Biotechnology Co., Ltd. (Wuhan, China). The procedures conformed to the Guidelines for Animal Care and Use and have been approved by the institutional review board (approval number HLK-20180420-01). To establish the in vivo model of FD, male Sprague-Dawley rats (specific-pathogen-free, 6-week-old, purchased from China Three Gorges University) were subjected to multi-factor stress intervention to mimic the symptoms of FD using a method described by Guo et al. [50]. This method applies a tail-clamping approach to elicit stress in the experimental animals, resulting in behaviors of FD such as anxiety, mood swing, and depression [51]. Five rats were kept in each cage, and the rats were enraged

using a Foerster clamp to clip the distal end of their tail. This action resulted in pain and discomfort, provoking violent fights among the rats in the same cage. This procedure was performed twice a day for 14 days, each fight lasting 30 min. During the 14 days, the rats were given normal access to drinking water but made to fast every other day. The rats were also subjected to intraperitoneal administration of iced physiological saline (2 mL, 4°C, 0.9% NaCl) twice a day. During the 14-day intervention period, rat weight, water intake, and food intake were monitored every other day. Successful induction of FD was indicated by weight loss, dry and yellow hair, loose stool, reduced food and water intake, inactivity, slow response, and irritability and depression due to mood changes.

5.2 EA and/or miR-221-3p intervention

During the 14-day period, FD-modeled rats were subjected to EA and/or injection of miR-221-3p agomirs or antagomirs. For EA, acupuncture needles were inserted at the Zusanli (ST36) and Taichong (LV3) acupoints on both sides of each rat. The needles were connected to an acupoint stimulator (SDZ-II, Hwato, Suzhou, China) and a continuous wave was applied at a frequency of 10 Hz and an intensity of 1 mA to stimulate both acupoints simultaneously for 30 min. Sham operation was performed without electric stimulation. The entire EA procedure was performed once a day. After acupoint stimulation, 5 nmol of miR-221-3p agomirs (miR40000890-4-5, Ribobio, Guangzhou, China) or antagomirs (miR20000890-1-5, Ribobio) in physiological saline were administered via tail vein injection, once every five days. Injection of vehicle only (physiological saline) was treated as a negative control (NC). At the end of day 14, the rats were sacrificed by an overdose of pentobarbital sodium via intraperitoneal injection. The gastric antrum and serum were preserved at -80°C for further analysis.

5.3 TEM

The morphology of ICCs in the gastric antrum was observed using TEM. Gastric antrum tissues were cut into 1-mm³ pieces and fixed in 2.5% glutaraldehyde at 4°C for 30 min. The fixed tissues were washed three times with 0.1 M phosphate-buffered saline (PBS) for 10 min each and fixed again in 1% osmium acid for 1 h. After three washed in 0.1 M PBS for 10 min each, the tissue specimens were dehydrated using a graded concentration series of ethanol (5 min at 50%, 70%, 80%, 90%, 90%; then 4 min at 100% twice). The specimens were then half-immersed in a 1:1 mixture of acetone:epoxy resin 812 at 40°C for 6 h and fully immersed in pure epoxy resin at 40°C for 4 h. After fixing, the specimens were embedded in a polymerization box for 4 h at 40°C, 2 h at 50°C, and 12 h at 90°C. The embedded specimens were cut into ultrathin sections (60 nm in thickness) using an ultramicrotome and subjected to double-staining. The sections were first dyed in uranium acetate (Xi'an Ding Tian Chemical Co., Ltd., Xi'an, China) in the dark for 20 min, washed three times with double-steamed water, and dried. The sections were then dyed with lead citrate (Boliante, Xi'an, China) for 15 min, and excess lead solution was washed with double-distilled water. Thereafter, the specimens were subjected to TEM analysis using a Hitachi HT770 apparatus.

5.4 Identification and analysis of differentially expressed miRNAs

After FD induction, EA treatment, and/or miR-221-3p agomir/antagomir intervention, RNA from was extracted from the gastric antrum of the experimental rats. Small RNA libraries construction and deep sequencing were carried out using the TruSeq Small RNA Sample Prep Kit (Illumina) according to the manufacturer's instructions on an Illumina Hiseq system. The raw reads were filtered by removing poor-quality reads, 5' adapter reads, reads without 3' adapters, reads containing poly (A) tails, rRNA, or tRNA, and reads < 18-nt in length to obtain clean reads. Known miRNAs were determined from the clean reads using miRbase and their expression levels were determined by the number of reads per million clean tags. Differentially expressed miRNAs were analyzed using DEGseq using the following criteria: $p \leq 0.05$ and fold change ≥ 2 . Target genes of the differentially expressed miRNAs were predicted using TargetScan.

5.5 qRT-PCR

RNA was isolated from gastric antrum tissue samples using TRIzol (15596026, Ambion, Inc., Foster City, CA) and reverse-transcribed into complementary DNA using the Advantage RT-for-PCR Kit (639505, TaKaRa, Dalian, China). qRT-PCR was performed using the SYBR Green PCR kit (KM4101, KAPA Biosystems, Wilmington, MA) with the primers listed in Supplementary Table S1. The experiment proceeded as follows: initial denaturation at 95°C for 3 min; 39 cycles of denaturation at 95°C for 5 s, annealing at 56°C for 10 s, and extension at 72°C for 25 s; and final extension at 65°C for 5 s and 95°C for 50 s. Data were acquired using qbase plus software and analyzed with the $2^{-\Delta\Delta C_t}$ method.

5.6 Dual luciferase activity assay

Based on preliminary screening, miR-221-3p was identified to bind to the 3'UTR of c-kit at a seven-base binding site. pmirGLO reporter plasmids (Addgene, Watertown, MA) were constructed and wild-type (WT) and mutant (MUT) 3'UTR segments were prepared as follows: c-kit WT forward, 5'-CTAGCGTAAATATTGAAATGTAGCAAUAATGTCTTTTGAATATTCCTGAGCCCCATGAGTCCCTGAAAAT-3', WT reverse, 5'-CTAGATTTTCAGGGACTCATGGGGCTCAGGAATATTCAAAGACATTTTGCTACATTTCAATATTTAC-3'; MUT forward, 5'-CTAGCGTAAATATTGAATAGGCATAUAATGTCTTTTGAATATTCCTGAGCCCCATGAGTCCCTGAAAAT-3', reverse, 5'-CTAGATTTTCAGGGACTCATGGGGCTCAGGAATATTCAAAGACATTTTATGCCTATTCAATATTTAC-3'. For the dual luciferase assay, miR-221-3p mimics (or negative control/NC) were co-transfected with c-kit 3'UTR-WT or c-kit 3'UTR-MUT in HEK293T cells using Lipofectamine 2000 according to the manufacturer's instructions. Firefly luciferase and renilla luciferase signals were detected using a LucPair™ Duo-Luciferase Assay Kit (LF001, GeneCopoeia, Rockville, MD) with a Glo-MAX 20/20 analyzer.

5.7 Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed using kits from Bioswamp (CX43: RA20769; Raf: RA21040; Erk: RA21301; SCF: RA20600; SCF receptor: RA21199) with all required reagents provided. Prior to the assay, the wells of a 96-well plate were coated with respective antibodies. Rat serum (~ 40 μ L) collected after treatment were added to each well and incubated, and 10 μ L of biotinylated antibodies against the respective protein

was added to each well. Then, 50 μ L of horseradish peroxidase-conjugated reagent was added to each well and incubated for 30 min at 37°C. For color detection, chromogen was added to each well and incubated for 10 min at 37°C. After stop solution was added, the absorbance of the wells was evaluated using a plate reader (Labsystems Multiskan MS) at 450 nm.

5.8 Western blot

Tissue samples were lysed using radioimmunoprecipitation assay buffer supplemented containing protease and phosphate inhibitors. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using 20 μ g of protein in denaturing conditions, and the separated proteins were transferred onto polyvinylidene fluoride membranes (IPVH00010, Millipore, Burlington, MA). After 2 h of blocking in 5% skim milk at room temperature, the membranes were incubated at 4°C overnight with rabbit primary antibodies against CX43 (1:8000; ab11370, abcam, Cambridge, UK), SCF (1:1000; ab64677, abcam), c-kit (1:500; ab5505, abcam), p-Raf (1:5000; ab173539, abcam), Raf (1:5000; ab137435, abcam), p-Erk (1:1000; ab201015, abcam), Erk (1:1000; ab17942, abcam), and GAPDH (1:2000; PAB36264, Bioswamp). Thereafter, the membranes were incubated at 4°C for 1 h with goat anti-rabbit IgG (1:10000, PAB160011, Bioswamp) secondary antibodies in the absence of light. The membranes were immersed in an enhanced chemiluminescence reagent (WBKLS0010, Millipore) for color detection. Protein bands were visualized using an automatic analyzer (Tanon-5200, Tanon, Shanghai, China) and gray values were generated using Tanon GIS software.

5.9 Hematoxylin & eosin (HE) staining

Gastric antrum tissues embedded in paraffin, sliced into 4- μ m sections using a microtome, and fixed on microscopic slides. For HE staining, tissue sections were dehydrated and washed with water for 1 min. Hematoxylin staining (PAB180015, Bioswamp) was performed for 3 min, following by 5 min of washing under running water. The sections were differentiated in 1% hydrochloric alcohol for 1 min, washed with water for 3 min, and immersed in bluing solution for 1 min. After washing in water for 3 min, the sections were stained in 0.5% eosin solution (PAB180016, Bioswamp) for 3 min and rinsed with distilled water for 10 s. The sections were then washed with 80% ethanol for 15 s, 95% ethanol for 15 s, and anhydrous ethanol for 3 min. Thereafter, the sections were immersed twice in xylene for 5 min each and sealed with neutral balsam.

5.10 Statistical analysis

The data are presented as the mean $\pm$ standard deviation (SD). The means among more than two groups was compared using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. $p < 0.05$ indicates a statistically significant difference

List Of Abbreviations

FD	Functional dyspepsia
EA	Electroacupuncture
ICCs	Interstitial Cells of Cajal
TCM	Traditional Chinese Medicine
SMCs	Smooth Muscle Cells
GN	Gastrointestinal Nerve
GJIC	Gap Junction Intercellular Communication
CX43	Connexin 43
SCF	Stem Cell Factor

Declarations

Ethics approval and consent to participate

All animal experiments were performed at the Model Animal Research Institute at Wuhan Myhalic Biotechnology Co., Ltd. (Wuhan, China). The procedures conformed to the Guidelines for Animal Care and Use and have been approved by the institutional review board (approval number HLK-20180420-01).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

PX, YD, and HZ conceptualized and designed the study. LZ performed constructed the FD model and performed EA experiments. PX and LZ performed the bioinformatics analysis, PCR, ELISA, western blot, dual luciferase assay, and HE staining. ZK, JF, and XP assisted in the in vivo experiments. XZ, JL, YC, and HH assisted in the biological assays. PX and LZ analyzed all of the data and prepared the manuscript. HZ assisted in the interpretation of bioinformatics data. HZ and YD revised the manuscript. All authors have read and approved the final version of this manuscript.

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Figures

Figure 1

Construction of the FD model. (A) Rat body weight during the 14-day FD modeling period, with or without EA treatment. Daily (B) food and (C) water intake during the 14-day FD modeling period, with or without EA treatment. (D) TEM characterization of the ultrastructure of ICCs in the gastric antrum of control rats, FD-induced rats, and FD-induced rats subjected to EA. Eu: euchromatin; M: mitochondria; rER: rough endoplasmic reticulum; V: vesicle. (E) Relative expression of miR-221-3p in the gastric antrum of control and FD-induced rats with or without EA treatment. (F) Relative mRNA expression of c-kit in the gastric antrum of control and FD-induced rats with or without EA treatment. (G) Relative protein expression of c-kit in the gastric antrum of control and FD-induced rats with or without EA treatment. All numerical data are expressed as the mean $\pm$ SD using ANOVA ($n = 6$); $*p < 0.05$.

Figure 2

Verification of targeting relationship between miR-221-3p and c-kit. (A) Construction of WT and MT c-kit 3'UTR and binding sites with miR-221-3p. (B) Dual luciferase activity assay of the binding between miR-221-3p and the 3'UTR of c-kit. Relative expression of (C) miR-221-3p and (D) c-kit mRNA in GES-1 cells treated with miR-221-3p agomirs (miR), antagomirs (miR-i), or their corresponding negative control (NC). (E) Relative protein expression of c-kit in GES-1 cells treated with miR, miR-i, or their corresponding NC. All numerical data are expressed as the mean $\pm$ SD using ANOVA (n = 6); *p < 0.05.

Figure 3

HE staining of gastric antrum. Rats were subjected to FD and/or treated with miR-221-3p antagonomirs (miR-i) or agomirs (miR), with or without EA stimulation. NC: negative control. Scale bar = 100 μ m.

Figure 4

Gene expression of c-kit-related factors and signaling components. qRT-PCR of the relative mRNA expression of CX43, SCF, c-kit, Raf, and Erk in the gastric antrum of rats subjected to FD and/or NC/miR-i/miR, with or without EA treatment. The data are expressed as the mean $\pm$ SD using ANOVA (n = 6); #p < 0.05, *p < 0.05 compared to the same group without EA.

Figure 5

Protein expression of c-kit-related factors and signaling components. (A) Western blot and relative protein expression of CX43, SCF, and c-kit in the gastric antrum of rats subjected to FD and/or NC/miR-i/miR, with or without EA treatment. GAPDH was used as an internal control. (B) Western blot and protein expression of p-Raf and p-Erk relative to respective total protein content in the gastric antrum of rats subjected to FD and/or NC/miR-i/miR, with or without EA treatment. The data are expressed as the mean $\pm$ SD using ANOVA ($n = 6$); $\times$ indicates non-significance compared to Control, $\#p < 0.05$, $*p < 0.05$ compared to the same group without EA.

Figure 6

Serum secretion of c-kit-related factors. ELISA of the relative secretion of CX43, SCF, c-kit, Raf, and Erk in the serum of rats subjected to FD and/or NC/miR-i/miR, with or without EA treatment. The data are expressed as the mean $\pm$ SD using ANOVA ($n = 6$); # $p < 0.05$, * $p < 0.05$ compared to the same group without EA.

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