Egr-1 mediates low dose Arecoline induced human oral mucosa fibroblasts proliferation by transaction the expression of Wnt5a

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Abstract

Background

Arecoline is the main carcinogens in Areca nut that induce oral submucous fibrosis to develop into cancer. However, many previous studies have showed that Arecoline may inhibit proliferation and prevent collagen synthesis of fibroblasts.

Results

High dose Arecoline (>32 µg/ml) could inhibit but low dose Arecoline (<16 µg/ml) could promote the proliferation of human oral fibroblasts. Wnt5a was both sufficient and necessary for the promotion of fibroblasts proliferation. Egr-1, but not NF-κB, FOXO1, Smad2 or Smad3, mediated the expression of Wnt5a in fibroblasts. The specific siRNAs of Egr-1, Egr inhibitors or Wnt5a antibodies treatment blocked Arecoline induced Wnt5a upregulation and fibroblasts proliferation.

Conclusions

Egr-1 mediates low dose Arecoline induced human oral mucosa fibroblasts proliferation by transaction the expression of Wnt5a, and Egr inhibitors or Wnt5a antibodies are potential therapeutic drugs of oral submucosal fibrosis and oral cancer.

Background

Arecoline, the main alkaloid of Areca nut, is the fourth most popular consumed psychoactive substance in the world, following only ethanol, nicotine, and caffeine in prevalence [1, 2]. Areca nut has a wide range of prevalence in Asia, such as Hunan, Taiwan and India, and has a trend of expansion [3, 4]. Chewing areca nut is closely related to oral diseases, such as oral submucous fibrosis, oral leukoplakia and oral cancer [4–6]. However, the role of Arecoline in the pathogenesis of disease is still controversial. Many studies have confirmed that Arecoline is to inhibit cell proliferation and migration, stimulate cell differentiation and induce cell apoptosis [7–10], but some studies have also observed that Arecoline can promote the proliferation and migration of certain cells [11, 12].

Wnt family members are secreted glycoproteins that are highly conserved and significantly participates in the regulation of fibroblasts proliferation and tissue fibrosis [13–16]. For example, it has been reported that the pro-fibrotic Wnt1/β-catenin injury response was critically required for preserving cardiac function after acute ischaemic cardiac injury [17]. Other studies have found that Wnt3a induced myofibroblast differentiation by upregulating TGF-β signaling through SMAD2 in a β-catenin-dependent manner [18]. And furthermore, Vuga and Kaminski et al. stated that Wnt5a was a regulator of fibroblast proliferation and resistance to apoptosis [19]. Therefore, Wnt family members may serve as ideal molecular targets in
the control of fibroblasts proliferation. However, the relationship between Arecoline and Wnt, and the transcription factors that control Wnt expression in oral fibroblasts are still unclear.

In this study, we confirmed that low dose Arecoline promoted human oral fibroblasts proliferation, and found that Egr-1 up-regulated Wnt5a expression to mediate the proliferative effect of Arecoline. Collectively, these findings establish Egr-1 and Wnt5a as new potential therapeutic targets for oral submucous fibrosis caused by chewing areca nut.

Results

Low dose Arecoline induces the proliferation of fibroblasts

Previous studies have showed that Arecoline was cytotoxic to oral fibroblasts from the concentration of 50 µg/ml up [8]. However, it is true that the areca nut chewing increases the risk of oral cancer and oral submucous fibrosis. In fact, the salivary Arecoline level during chewing were about 0.1 µg/ml, and increased to about 0.3 µg/ml after chewing [20], which is much lower than the concentration used in most experiments.

In the present study, we intend to test the effect of different dose Arecoline (0.1, 0.5, 1, 4, 8, 16, 32 or 50 µg/ml) on the proliferation of human oral fibroblasts, half of the medium was changed every 24 hours. We confirmed that high dose Arecoline (32 µg/ml and 50 µg/ml) could inhibit the proliferation of fibroblasts (Fig. 1a). Our results also showed that Arecoline promoted the proliferation of fibroblasts at the concentrations from 0.1 µg/ml to 16 µg/ml (Fig. 1b-1f), and the maximum proliferate effect was 8 µg/ml (Fig. 1e). These results showed that low dose Arecoline could promote the proliferation of human oral fibroblasts.

Arecoline Promotes Fibroblasts Proliferation By Inducing Wnt5a Expression

Earlier reports have shown that activation of Wnt/β-catenin signaling may promote the proliferation of fibroblasts by regulating the expression level of Wnt1, Wnt2, Wnt3a or Wnt5a [19]. To further study whether other Wnt isoforms take part in accommodation of human oral fibroblasts proliferation, the mRNA expression levels of all 19 Wnt gene family members in human oral fibroblasts exposed to 8 µg/ml Arecoline for 24 h were analyzed by RT-PCR. As illustrated in Table 1, Arecoline altered the transcription of ten Wnts (Wnt1, 2, 3a, 5a, 5b, 8b, 10a, 10b, 11 and 16). Of those, Wnt3a, Wnt5b, Wnt8b, Wnt10a, Wnt10b, Wnt11 and Wnt16 were expressed only at very low levels in both control and treatment group, whereas Wnt1, Wnt2 and Wnt5a expressed at higher levels. We next detected the protein expression level of Wnt1, Wnt2 and Wnt5a in 8 µg/ml Arecoline time course experiment. As expected, Arecoline significantly promoted the expression of Wnt1, Wnt2 and Wnt5a on the protein level (Fig. 2a).
Table 1
Normalized mRNA expression of Wnts

| Gene Name | Control | Arecoline 8 µg/ml for 24 h |
|-----------|---------|--------------------------|
|           | Relative Fold | CT value | Relative Fold | CT value |
| Wnt1* | 1 | 27.53 ± 0.86 | 1.92 ± 0.18 | 26.71 ± 0.69 |
| Wnt2* | 1 | 29.26 ± 0.29 | 2.25 ± 0.21 | 28.12 ± 0.54 |
| Wnt2b | 1 | 33.09 ± 1.37 | 1.12 ± 0.33 | 32.84 ± 1.71 |
| Wnt3 | 1 | 30.41 ± 0.84 | 0.92 ± 0.22 | 31.38 ± 1.25 |
| Wnt3a* | 1 | 36.17 ± 1.26 | 1.42 ± 0.27 | 35.67 ± 0.85 |
| Wnt4 | 1 | 21.71 ± 0.42 | 0.87 ± 0.21 | 22.32 ± 0.62 |
| Wnt5a* | 1 | 23.59 ± 0.31 | 2.57 ± 0.64 | 21.89 ± 0.78 |
| Wnt5b* | 1 | 34.12 ± 1.73 | 1.63 ± 0.22 | 32.25 ± 1.47 |
| Wnt6 | 1 | 29.35 ± 0.68 | 0.94 ± 0.15 | 28.46 ± 0.33 |
| Wnt7a | 1 | 37.31 ± 1.79 | 1.07 ± 0.26 | 36.77 ± 1.36 |
| Wnt7b | 1 | 31.64 ± 0.52 | 0.95 ± 0.25 | 32.53 ± 0.90 |
| Wnt8a | 1 | 28.03 ± 1.15 | 1.17 ± 0.39 | 27.41 ± 1.17 |
| Wnt8b* | 1 | 35.52 ± 1.27 | 1.78 ± 0.32 | 34.93 ± 0.83 |
| Wnt9a | 1 | 26.47 ± 1.07 | 0.89 ± 0.17 | 27.05 ± 0.79 |
| Wnt9b | 1 | 28.02 ± 1.33 | 0.96 ± 0.11 | 28.62 ± 0.41 |
| Wnt10a* | 1 | 35.74 ± 2.28 | 1.44 ± 0.27 | 34.62 ± 1.49 |
| Wnt10b* | 1 | 36.17 ± 1.41 | 1.75 ± 0.48 | 34.91 ± 1.58 |
| Wnt11* | 1 | 34.86 ± 1.73 | 2.63 ± 1.39 | 33.17 ± 1.37 |
| Wnt16* | 1 | 36.77 ± 1.03 | 1.81 ± 0.42 | 35.82 ± 0.73 |

The values represent the mean ± S.E. of three independent experiments. *P< 0.05

To determine if Wnt1, Wnt2 or Wnt5a was required for Arecoline to promote the proliferation of fibroblasts, recombinant Wnt1, Wnt2 or Wnt5a protein and Wnt1, Wnt2 or Wnt5a antibody were used to treat human oral fibroblasts. Results showed that the proliferation of fibroblasts was not affected by Wnt1, Wnt2 protein or their antibody (Fig. 2b and 2c); recombinant Wnt5a protein could increase the proliferation of fibroblasts (Fig. 2d); and the Arecoline-induced cell proliferation was inhibited by Wnt5a.
antibody (Fig. 2d). Furthermore, we found the specific siRANs Wnt5a inhibited the Arecoline-induced cell proliferation, but the proliferation of fibroblasts was not affected by Wnt1 or Wnt2 siRNAs (Fig. 2e). Together, these results demonstrated that Wnt5a mediated the Arecoline-induced fibroblasts proliferation.

**Egr-1 Is Necessary For The Expression Of Wnt5a**

Previous studies have identified many transcription factor binding sites in the human Wnt5a promoter, such as NF-κB, FOXO1, Smad2, Smad3 and Egr-1 [21, 22]. To identify signaling mechanisms regulating Wnt5a expression, specific siRNAs for NF-κB p65, FOXO1, Smad2, Smad3 or Egr-1 were used. The results revealed that NF-κB p65, FOXO1, Smad2 or Smad3 siRNAs could not affect the promoter activity and the expression of Wnt5a (Fig. 3a and 3b), demonstrating an NF-κB p65, FOXO1, Smad2 or Smad3-independent manner of regulating Wnt5a expression.

To confirm if Egr-1 is involved in Wnt5a regulation, fibroblasts were transfected with Egr-1 siRNAs. Arecoline induced Wnt5a expression was effectively blocked by siEgr-1 (Fig. 3a and 3c), demonstrating that Egr-1 is involved in Wnt5a regulation. In support of this result, Egr-1 siRNAs significantly suppressed Wnt5a protein expression (Fig. 3c). Therefore, we concluded that Egr-1 is essential for transcriptional induction of Wnt5a in human oral fibroblasts.

**Inhibition Of Egr Activity Prevents Arecoline Induced Fibroblast Proliferation**

We next assessed the roles of Egr-1 in fibroblasts proliferation. Our results showed that Egr-1 knockdown inhibited the Arecoline-induced proliferation of fibroblasts (Fig. 4a). Furthermore, Mithramycin A (MMA) and Chromomycin A3 (CHA) were used to treat cells (MMA and CHA repress transcription by selectively displacing GC-rich DNA binding transcription factors, such as Egr-1 [23, 24]). The results showed that MMA or CHA treatment blocked Arecoline induced Wnt5a upregulation and fibroblasts proliferation (Fig. 4b, 4c and 4d). These results indicated that the expression and activity of Egr-1 were required for Arecoline induced fibroblasts proliferation.

**Discussion**

Oral submucosal fibrosis (OSF) is one of the precancerous lesions of oral squamous cell carcinoma, which is related to the frequent chewing of Areca nut. OSF used to be reported mainly in Southeast Asia, but it is now found in the Asian immigrant populations of Britain and America and has become a global health problem [25]. The main pathological manifestation of OSF is abnormal accumulation of collagen in the lamina propria under the oral mucosa [26]. With the development of OSF, 3%-19% of patients may have cancer, and this probability increases year by year [27]. The habit of chewing areca nut is considered to be the most likely factor for the occurrence and malignant progress of OSF [28]. In 2003, the World Health Organization listed areca nut as a primary carcinogen.
Areca nut contains many substances, such as alkaloids, polyphenols and nitrosamines. Among them, the alkaloids are Arecoline, Tetrahydronicotinic acid, etc. [29]. Arecoline is the main carcinogens in Areca nut that induce OSF to develop into cancer by their toxic effects directly. However, many studies showed that Arecoline inhibited cell growth, proliferation and collagen synthesis in human oral fibroblasts in a dose dependent manner [7, 8, 30]. Chang et al. found that Arecoline was cytotoxic to human oral fibroblasts at a concentration higher than 50 µg/ml by depleting intracellular thiols and inhibiting mitochondrial activity [8, 30]; Jeng et al. found Arecoline inhibited the migration, attachment, spreading, growth and collagen synthesis of human oral fibroblasts at concentrations of 0.4 mM (62 µg/ml) and 1 mM (155 µg/ml) [7]. In fact, Venkatesh et al. found that the salivary Arecoline level were about 0.1 µg/ml to about 0.3 µg/ml after chewing commercially available areca nut [20], which is much lower than the concentration used in most experiments. In this study, we found that low dose Arecoline could promote the proliferation of human oral fibroblasts. In according with our findings, Xia et al. found a relative low dose (20 µg/ml) Arecoline treatment could increase the collagen production of oral fibroblasts [31]. Combined with these situations, we suggest that more studies should focus on the effect of low dose Arecoline on oral diseases.

Recently, the role of the Wnt/β-catenin pathway is proving to be central to mechanisms of pulmonary, hepatic, renal and cardiac fibrosis [16, 32–34]. Wnt family has a large number of members, among which Wnt5a is closely related to fibrosis. Vuga et al. found Wnt5a played a role in fibroblast expansion and survival characteristics of idiopathic pulmonary fibrosis and other fibrotic interstitial lung diseases that exhibit usual interstitial pneumonia histological patterns [19]; Villar et al. suggested that the Wnt/β-catenin signaling pathway is activated very early in sepsis-induced acute respiratory distress syndrome and could play an important role in lung repair and fibrosis [35]; Abraityte et al. found Wnt5a is elevated in serum and myocardium of heart failure (HF) patients and promoted myocardial inflammation and fibrosis [36]; Martin-Medina et al. found Wnt5a was secreted on extracellular vesicles in lung fibrosis and induced by TGF-β in primary human lung fibroblasts [37]. We screened the expression of Wnt family members in fibroblasts after Arecoline stimulation and further determined their basic functions. Our results confirmed that Wnt5a also played a proliferation role in low dose Arecoline stimulated human oral fibroblasts. This result suggests that the treatment of oral submucous fibrosis may also refer to the treatment of other organ fibrosis.

Although many transcription factors have been reported to regulate Wnt5a expression [21, 22], we finally confirmed that Egr-1 regulated Wnt5a expression. Interestingly, Egr-1 is a typical immediate early gene (IEG) [38]. IEGs are genes which are activated transiently and rapidly in response to a wide variety of cellular stimuli. These characteristics of Egr-1 are consistent with the wavy change of Arecoline concentration in areca nut chewer's saliva [20]. And in this study, we also produced an Arecoline concentration fluctuation by replacing half of the cell culture medium every 24 hours. These phenomena are consistent with our results, which further confirms our results. Meaningfully, our results showed that MMA or CHA treatment blocked Arecoline induced fibroblasts proliferation. MMA is a U.S. Food and Drug Administration-approved drug that may against fibrosis, cancer and neurodegenerative diseases [39–41].
Therefore, additional exploration of its mechanisms in disease models may identify MMA as a promising drug candidate for the treatment of OSF and oral cancer.

**Conclusions**

The study demonstrated that high dose Arecoline could inhibit but low dose Arecoline could promote the proliferation of human oral fibroblasts. Egr-1 mediates low dose Arecoline induced human oral mucosa fibroblasts proliferation by transaction the expression of Wnt5a, and Egr inhibitors or Wnt5a antibodies are potential therapeutic drugs of OSF and oral cancer.

**Methods**

**Reagents**

Arecoline (#S2614, Selleck Chemicals), Recombinant human Wnt1 protein (#ab84080, Abcam plc.), Recombinant human Wnt2 protein (#H00007472-P01, Bio-Technne China Co. Ltd.), Recombinant human Wnt5a protein (#645-WN-010, R&D system, Wiesbaden-Nordenstadt, Germany), Mithramycin A (Sigma), Chromomycin A3 (Sigma) and antibodies were used at the indicated concentrations and time points. Lipofectamine LTX (#15338100, Invitrogen) and Lipofectamine RNAiMAX (#13778150, Invitrogen) were used for transient gene or siRNA transfection of cells. The following primary antibodies were used: Wnt1 (#ab15251), Wnt2 (#ab109222), Wnt5a (#ab179824) and GAPDH (#ab181602) were from Abcam plc..

**Cell Culture And Treatment**

Human oral fibroblasts (ATCC® PCS-201-018™) were cultured in fibroblast basal medium (ATCC PCS201030) containing 2% heated-inactivated fetal bovine serum supplemented with 5 ng/ml rh FGF-b, 7.5 mM L-glutamine, 50 µg/ml Ascorbic acid, 1 µg/ml Hydrocortisone Hemisuccinate, 5 µg/ml rh Insulin, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 10 Units/ml penicillin, 25 µg/ml Amphotericin B and 10 µg/ml streptomycin.

**DNA Growth Assay**

Following treatment of cells, the media was discarded, cells were solubilized for 30 min at 37 °C in 0.1% SDS and the amount of DNA was estimated using a Hoechst 33258 microassay.

**Real-time Polymerase Chain Reaction (real-time PCR)**

Total RNA was extracted using the EastepTM Universal RNA Extraction Kit (Promega, Madison, WI) and transversely transcribed with the GoScriptTM Reverse Transcription System (Promega, Madison, WI) according to the manufacturer’s instruction. Real-time PCR was performed in triplicate with GoTaq®
qPCR Master Mix (Promega, Madison, WI) and run on the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Framingham, MA). The mRNA level of the housekeeping gene \( \beta\text{-actin} \) was used as a control. The following primer pairs were used: Wnt1 forward (5′- GAA ATG CCC CCA TTC TCC CA -3′) and reverse (5′- CGT GGC TCT GTA TCC ACG TT -3′); Wnt2 forward (5′- GGA TGA CCA AGT GTG GGT GT -3′) and reverse (5′- GGT CAT GTA GCG GTT GTC CA -3′); Wnt2b forward (5′- GAC GGC AGT ACC TGG CAT AC -3′) and reverse (5′- TGT CAC AGA TCA CTC GTG CC -3′); Wnt3 forward (5′- ACT TTT GTG AGC CCA ACC CA -3′) and reverse (5′- TTC TCC GTC CTC GTG TTG TG -3′); Wnt3a forward (5′- AGC AGG ACT CCC ACC TAA AC -3′) and reverse (5′- AGA GGA GAC ACT AGC TCC AGG – 3′); Wnt4 forward (5′- CAT GAG TCC CCG CTC GTG-3′) and reverse (5′- CCA GGT ACA GCC AGT TGC TC -3′); Wnt4 forward (5′- CAT GAG TCC CCG CTC GTG-3′) and reverse (5′- CCA GGT ACA GCC AGT TGC TC -3′); Wnt5a forward (5′- CTC CAT TCC TGG GCG CAT C-3′) and reverse (5′- GCA GTG AAC CGG AGC TGA AG -3′); Wnt5b forward (5′- AGC CAC AGT GAC CAT TAG CAG-3′) and reverse (5′- AGT AGG GTT CCC TCT GTC ACC – 3′); Wnt6 forward (5′- TGG CCT CTA GGA GGA AAC AGT-3′) and reverse (5′- ATT GAT ACT AAC CTC ACC CAC C -3′); Wnt7a forward (5′- ACG GCC TGT GCT TCT TA-3′) and reverse (5′- GCC CAC TTG GCA AAC AGA AC -3′); Wnt7b forward (5′- AAG TGC GGA CAC ATT GCC – 3′) and reverse (5′- ACC TCG AAG CCC GGT TGA – 3′); Wnt8a forward (5′- AAG AGC TGC TGA TTT CCT CCC – 3′) and reverse (5′- AGG GCC AAG TCC AGA GAA GGT -3′); Wnt8b forward (5′- ACA GCT GGT CGG TGA ACA AT -3′) and reverse (5′- CTG CCA CAC TGC TGG AGT AA -3′); Wnt9a forward (5′- GGC AAG ATG CTG GAT GGG T -3′) and reverse (5′- GGT CGC AGG CCT TGT AGT G -3′); Wnt9b forward (5′- GAG ATG CTA GAG GGC GCA G -3′) and reverse (5′- CAG TGC CCA ATC CTG GGA AG -3′); Wnt10a forward (5′- CTG GGT GCT CCT GCT CC -3′) and reverse (5′- TTA GGC ACA CTG TGT TGG CA -3′); Wnt10b forward (5′- CTG ACA AGG GGA GAC AAC CC -3′) and reverse (5′- CAG GAC CTC CAG TGG TTG GG -3′); Wnt11 forward (5′- GGG GTG GCA CTT CTC AAT TC -3′) and reverse (5′- TGC CGA GTT CAC TTG ACG -3′); Wnt16 forward (5′- TAC AGC TCC CTG CAA ACG AG -3′) and reverse (5′- CCA AGT TAT CCC TCG CCC TC -3′); GAPDH forward (5′- GAC AGT CAG CCG CAT CTT CT -3′) and reverse (5′- GCG CCC AAT AGC ACC AAA TC -3′).

**Western Blotting**

Protein from cells (30 µg) were separated by SDS-PAGE and transferred onto PVDF membranes. Then the members were blotted with primary antibodies at 4 °C overnight. Blots were incubated with HRP-conjugated secondary antibody for 1 h. The proteins were visualized using the ECL Plus WB detection system (Pierce, Rockford, IL).

**Constructs**

The Human Wnt5a-luciferase (pGL3-Wnt5a, containing nucleotides from −2152 to +275 of the Human Wnt5a gene (Gene ID: 7474) reporter were cloned into the pGL3-basic vector. Constructs were transfected into cells using Lipofectamine LTX.
Dual-luciferase Reporter Assays

Constructs were transfected into cells using Lipofectamine LTX. For the dual-luciferase reporter assays, cells were transfected with 1 µg of a luciferase reporter plasmid and 200 ng of the pRL-CMV Renilla luciferase reporter plasmid (Promega). After transfection, cells were kept in conditioned media for 12 or 24 h and then transferred to treatment media for 12 h. Firefly luciferase activity was normalized to Renilla luciferase activity according to the protocol.

siRNA Interference

Wnt1, Wnt2, Wnt5a, NF-κB p65, Smad2, Smad3 or Egr-1 specific siRNAs were from Dharmacon (ONTARGETplus SMARTpool, named as si-1) and Santacruz (named as si-2); the negative control (NC) siRNA (no silencing small RNA fragment) was synthesized by GenChem Co. (Shanghai, China). siRNAs were transfected into cells using Lipofectamine RNAiMAX transfection reagent.

Statistical analysis

Data are presented as mean ± SEM. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) using ANOVA followed by post hoc tests as appropriate. Statistical significance was declared when p < 0.05. The experimenters were not blind to group assignment and no data were omitted.

Abbreviations

Wnt5a
Wingless-type family member 5a

Egr-1
early growth response protein 1

NF-κB
nuclear factor kappa-B

FOXO1
Forkhead box O1

Smad
Sma and Mad proteins

TGF
transforming growth factor

OSF
oral submucosal fibrosis

Declarations
• **Ethics approval and consent to participate** Not applicable

• **Consent for publication** Not applicable

• **Availability of data and materials**

The datasets analyzed during the current study are available from the corresponding author on request.

• **Competing interests**

The authors declare no conflict of interest.

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• **Authors' contributions**

QC, JYJ, SJH, XLL and ZC contributed to the design, experiments and manuscript preparation of the study. YYW, ZHM and JR were involved in the acquisition and analyzation of study data. All Authors had access to the final article and have final responsibility for the decision to submit and approved the submitted version.

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Figures
Low dose Arecoline induces the proliferation of fibroblasts. Human oral fibroblasts were treated with different dose Arecoline for indicated times, then cell proliferation rate was quantified. * denotes $p < 0.05$. 
Figure 2

Arecoline promotes fibroblasts proliferation by inducing Wnt5a expression. a. Human oral fibroblasts were treated with or without 8 μg/ml Arecoline for indicated times, then cell lysates were analyzed by Western blotting using indicated antibodies. b-d. Human oral fibroblasts were treated with 8 μg/ml Arecoline, recombinant Wnt1, Wnt2 or Wnt5a protein and Wnt1, Wnt2 or Wnt5a antibody for 48 h, then cell proliferation rate was quantified. e. Human oral fibroblasts were treated with 8 μg/ml Arecoline, Wnt1, Wnt2 or Wnt5a sepecific siRNAs for 48 h, then cell proliferation rate was quantified. * denotes p < 0.05.
Egr-1 is necessary for the expression of Wnt5a. a. Human oral fibroblasts were transfected with Wnt5a promoter luciferase reporter plasmids, treated with 8 μg/ml Arecoline and p65, Smad2, Smad3 or Egr-1 siRNAs for 48 h, then luciferase reporter assays were performed to detect the activity of Wnt5a promoter. b. Human oral fibroblasts were treated with 8 μg/ml Arecoline and p65, Smad2, Smad3 or Egr-1 siRNAs for 48 h, then RT-PCR assays were performed to detect the mRNA levels of Wnt5a. c. Human oral fibroblasts were treated with 8 μg/ml Arecoline and Egr-1 siRNAs for 24 h, then cell lysates were analyzed by Western blotting using indicated antibodies. * denotes p < 0.05.
Figure 4

Inhibition of Egr activity prevents Arecoline induced fibroblast proliferation. a. Human oral fibroblasts were treated with 8 μg/ml Arecoline, Wnt1, Wnt2 or Wnt5a specific siRNAs for indicated times, then cell proliferation rate was quantified. b. Human oral fibroblasts were transfected with Wnt5a promoter luciferase reporter plasmids, treated with 8 μg/ml Arecoline, 2 μM Mithramycin A (MMA) or 1 μM Chromomycin A3 (CHA) for indicated times, then luciferase reporter assays were performed to detect the activity of Wnt5a promoter. c-d. Human oral fibroblasts were treated with 8 μg/ml Arecoline, 2 μM MMA or
1 μM CHA for indicated times, then RT-PCR assays were performed to detect the mRNA levels of Wnt5a (c) or cell proliferation rate was quantified (d). * denotes p < 0.05.

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