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Establishment of a Stable Cell Line Expressing "Toxic" Transient Receptor Potential A1 Channel

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Abstract Transient receptor potential A1 (TRPA1) is a cold sensitive cation channel, which could also be activated by various pungent compounds. As a transduction channel in a number of sensory modalities, TRPA1 expressing in heterogonous systems serves to provide great convenience in pharmacological analysis and functional investigation. Due to cellular toxicity, establishment of stable TRPA1 cell line has always been challenging. Nevertheless, the first stable human embryonic kidney (HEK-293) cell line with un-controlled expression of TRPA1 was successfully established. It was also confirmed that this stable cell line retained TRPA1 expression for more than 25 passages in culture. The functional analysis of the cell response verified the stability and specificity of this novel recombinant TRPA1 cell line. Altogether, the data indicated this TRPA1-HEK cell line would be a useful tool for functional analysis of TRPA1 and for the development of high throughput screening (HTS) compatible assay in the effort to identify TRPA1 modulators.

Key words transient receptor potential A1 (TRPA1), stable cell line, toxicity

Transient receptor potential (TRP) channels are universal biological sensors that detect changes in the environment[1]. Based on amino acid sequence homology, 7 classes of TRPs have been classified: TRPA (Ankyrin), TRPC (Canonical), TRPM (Melastatin), TRPML (MucoLipin), TRPP (Polycystin), TRPV (Vanilloid) and TRPN (NO-Mechano-Potential) subfamilies[2]. The predicted structure of TRP proteins is a channel-forming structure composed of six putative transmembrane (TM) domains that assemble as tetramers to form cation-permeable pores[3, 4]. Unlike other families of ion channels, the sequence homology of mammalian TRP channels is low and they have a wide variety of modes of activation, regulation, ion selectivity, broad tissue distribution, and physiological functions[5]. There has been tremendous excitement about the newly discovered TRP ion channels both as mediators of sensory signals and, more recently, as novel drug targets[6].

As the single mammalian member of the TRPA subfamily, TRPA1 (ANKTM1 or P120) is distinguished from other TRP channels by the presence of ~14 ankyrin repeats in its N terminus[7]. These ankyrin repeats are thought to play key roles in protein-protein interactions[8, 9]. TRPA1 is expressed in the inner ear and in trigeminal and dorsal root ganglia (DRG) neurons[7, 10]. It also belongs to the ThermoTRP, a subset of TRP ion channels activated by distinct physiological temperatures[11]. In heterologous expression systems, TRPA1 is activated at a broad range of temperatures with an average threshold of noxious cold ~17℃ for humans[7]. It is also activated by various pungent compounds like allicin (the active ingredient in garlic), cinnamaldehyde (the pungent component of cinnamon) and isothiocyanates (the pungent ingredients found in wasabe), etc. Most of these compounds induce acute painful burning or pricking sensation[5, 11]. Despite the facts that TRPA1 has been shown to function as a transduction channel in a number of sensory modalities, including thermal (cold) and mechanical nociception, hearing, and inflammatory pain[12~14], an understanding of the role of TRPA1 in normal physiology is just beginning. Moreover, the progression from mutations in TRPA1 to pathophysiology and disease model remains unclear.

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In addition to the obvious obstacle of lacking of structural information on TRPA1 channel, the very limited specific pharmacological tools have tremendously hindered fast progress in the TRPA1 field.

It is well known that cell-based assays is crucial for understanding the efficiency, specificity, solubility, stability, toxicity and mechanism of compound interaction with target cells. Once a target has been chosen, cell lines stably expressing targets should be established, maintained, and scaled up by cell culture. For the functional analysis of the TRPA1 protein, investigators tried to express large amounts of TRPA1 in cells since TRPA1 was found. However, previous reports have shown that generating stable cell lines expressing TRPA1 is challenging[7, 13, 14]. Jaquemar *et al*.[13] found expression of TRPA1 in eukaryotic cells appeared to interfere with normal growth. Overexpression of the TRPA1 cDNA in normal and transformed cells has not been successful. Even utilization of an ecdysone-inducible system failed to overcome the problem. Therefore, it was concluded that the uncontrolled expression of the TRPA1 protein might be toxic to the transfected cells. To address this issue, a selected domain (rather than the entire protein) was suggested to be a suitable target which might be successfully expressed in eukaryotic cells, experiments performed with certain fragments derived from TRPA1 cDNA might eventually shed light on the function and regulation of TRPA1 protein. A few years later, Story *et al*.[7] also observed cells constitutively expressing TRPA1 appeared unhealthy (membrane blebbing, cytoplasmic granulations) and down-regulated TRPA1 expression after several passages in culture. To circumvent this problem, they successfully generated cell lines under the control of a tetracycline (Tet) -inducible promoter *via* Flp recombinase-mediated recombination. As a result, TRPA1 functional analysis was effectively performed in this Tet-inducible system. After that, investigators continued to construct inducible system to expression TRPA1 for cell-based analysis. In year 2007, Chen *et al*.[14] encountered new problems in the course of generating TRPA1 stable cell lines. They observed extremely slow cell growth (doubling approximately once a week) and unhealthy appearance, cell line degeneration, and loss of functional activity. In an effort to explore alternative strategies, they used large-scale transiently transfected (LSTT) cells. This strategy circumvented the need for developing stable cell lines and thus avoided the associated toxicity. Remarkably, this LSTT approach has been successfully applied for high throughput screening to identify novel TRPA1 antagonists.

In the current study, we successfully established a stable cell line expressing TRPA1. Although it is in contradiction with previous reports, we found uncontrolled expression of TRPA1 compatible with normal growth of the parental HEK-293 cells and the absence of gradual loss of TRPA1 expression and activity following several passages in culture. The successful establishment of this stable cell line expressing “toxic” TRPA1 will be a useful tool for functional analysis of the TRPA1 protein. Most important of all, it will act to enable rapid establishment of cell-based assays suitable for high throughput screening of TRPA1 modulators and become an important pillar of the preclinical drug discovery process.

1 Materials and methods

1.1 Molecular biology

The Homo sapiens transient receptor potential cation channel, subfamily A, member 1 (TRPA1) cDNA at pCMV6-XL4 vector was purchased from ORIGENE. This TRPA1 sequence data has been matched to the accession number NM_007332. It should be noted that the complete sequence of this molecular clone differs from the sequence published for this corresponding accession number, by a single nucleotide polymorphism (SNP) and 3 synonymous mutations. The reference ID of this SNP is rs7819749. It variably biased resulting in a variable amino acid (Lys to Asn) at this location, where the bias is dependent on the population.

1.2 Stable TRPA1 cell line development

Human embryonic kidney (HEK-293) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum (Hyclone) in a humidified atmosphere of 5% CO_2 at 37℃. Plate HEK-293 cells in 6-well dishes at 50% density for stable transfection. The pCMV6-XL4/TRPA1 and pcDNA3.1/Hygro (+) (Invitrogen) plasmid was co-transfected into HEK-293 cells by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. 24 h later, cells were split at the dilutions of 1∶20, 1∶30 and 1∶40 into 10 cm dishes. Selective DMEM medium (Hygromycin, final concentration 100 mg/L) was added the following day.

Selective medium was changed every other day for about 2 weeks' selection. Once all cells have died in the dish of negative control (non-transfected HEK-293), cell colonies were picked from the plates. Totally 21 HEK-293/TRPA1 single colonies were isolated and transferred to 96-well plate. After the clones were grown up, agonist was used to induce a calcium response for positive clones screening. Those clones with significant agonist inductions (designated as P0) were picked and amplified for further characterization and validation. When the cells reach 90% confluence, it is normally split at 1∶8 to 1∶10 dilution twice weekly without any antibiotic. Using the same procedure, we also generated stable human TRPV3-HEK, TRPV4-HEK and TRPM8-HEK cell lines.

1.3 Reverse transcription-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) as the lysis buffer. Complementary DNA (cDNA) was synthesized using oligo (dT) 18 primers and M-MLV RT (Promega) followed by 30 PCR amplification cycles (denaturation at 95℃ for 20 s, annealing at 58℃ for 30 s, and extension at 72℃ for 40 s). Reverse transcription-PCR was performed with oligonucleotide primers in Table 1. β-Actin was used as a reaction standard. Each PCR product was analyzed by 1% agarose gel electrophoresis.

Table 1 Primer sequences used for reverse transcription PCR assays

Gene	Size	Primer sequences
TRPA1	487 bp	Forward 5′ CCCCTCTGCATTGTGCTGTAG 3′
		Reverse 5′ CCATTGTCCAGGCACATTTTG 3′
β-Actin	564 bp	Forward 5′ CTGGGACGACATGGAGAAAA 3′
		Reverse 5′ AAGGAAGGCTGGAAGAGTGC 3′

1.4 Real-time quantitative PCR

After the reverse-transcription reaction, cDNA templates were amplified by quantitative real-time PCR. The reaction was performed using the 7300 real-time PCR system (AB Applied Biosystems) with SYBR Green Ⅱ (CAMBREX). Human TRPA1 primers were designed using Vector NTI 8 software (InforMax, Inc., Table 2). Human GAPDH primers were from Primer Bank and used as an internal control. The PCR mixture included 2 μl of 10× SYBR Green, 0.2 μl of ROX Reference Dye (Invitrogen), 1 μl of cDNA(50 ng), 2 μl of each 2 μmol/L primer, 0.75 U Taq DNA polymerase, 2 μl of 10× amplification buffer (Mg^{2+} free), 2 μl of 25 mmol/L $MgCl_2$ and 1.5 μl of 2.5 mmol/L solution of four dNTP in a final volume of 20 μl. The conditions of amplification cycles were as follows: 40 cycles consisting of denaturation at 95℃ for 20 s, annealing at 55℃ for 25 s, and extension at 72℃ for 30 s. The 7300 Sequence Detection software was used for instrument control, automated data collection and data analysis. The data was analyzed for relative quantity or relative expression as described by Livak, *et al*.[15].

Table 2 Primer sequences used for real-time PCR assays

Gene	Size	Primer sequences
TRPA1	188 bp	Forward 5′ GACATAGTCCTGAACAAGCAGC 3′
		Reverse 5′ TTCATGCATTCAGGGAGG 3′

1.5 Western blot

A polyclonal antibody of TRPA1 (NP_015628) was purchased from Abnova Corporation, which corresponds to amino acids 1 033 ～ 1 118 (C terminus). β-Tubulin antibody (Cell Signaling) was used for loading control. Cells were collected and treated with ice-cold RIPA buffer with freshly added proteinase inhibitors. Cell lysates were centrifuged to remove debris, and protein was quantified. Equal amounts of protein were loaded and resolved by 6% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The blot was developed with TRPA1 or β-tubulin antibody as primary antibody and horseradish peroxidase-linked IgG as secondary antibody (Santa Cruz). Immunoreactive proteins were detected on the blot with the ECL system.

1.6 Ca^{2+} influx assay

Ca^{2+} influx assay was analyzed using FLEX Station instruments (Molecular Devices) with calcium indicator Fluo-3/AM (Molecular Probes). Cells are seeded at a density of 3×10^4/well in a Matrigel (BD Bioscience) coated 96-well black wall/clear bottom plate (Costar) and incubated over night at 37℃ in a 5% CO_2 incubator. On the day of the assay, remove the medium carefully, and add 100 μl per well 1× dye-loading solution into 96-well plates. Incubate the plate at 37℃ in dark for at least 60 min. Test agonist cinnamaldehyde (Sigma) or trans-2-pentenal (Sigma) were prepared as 5× stock of desired concentration and

25 μl per well was added to the cells at a delivery rate of 26 μl/s. More than 120 s readout duration with one time of compound transfer from compound plate to assay plate at 18 s. A double addition protocol was used to evaluate antagonist activity. Add 25 μl 5× antagonist Ruthenium Red (Tocris) per well and incubate the plate at 37℃ for 10 min then followed the second addition of 25 μl 6× agonist after FLEX Station monitored baseline fluorescence for 18 s and read the fluorescence for an additional 120 s.

1.7 Membrane potential assay

Membrane potential assay was analyzed using FLEX Station instruments with Membrane Potential Assay Kit (Molecular Devices). Cells are seeded at a density of 3×10^4/well in a Matrigel coated 96-well black wall/clear bottom plate and incubated over night at 37℃ in a 5% CO_2 incubator. On the day of the assay, remove the medium carefully, and add 100 μl per well 1× Membrane Potential Reagent Component A into 96-well plates. Incubate the plate at 37℃ in dark for 40 min. Test agonist cinnamaldehyde was prepared as 5 × stock of desired concentration and 25 μl per well was added to the cells at a delivery rate of 26 μl/s. More than 300 s readout duration with one time of compound transfer from compound plate to assay plate at 18 s.

2 Results

2.1 TRPA1 gene maintained in stable cell line

Human TRPA1 cDNA in pCMV6-XL4 vector co-transfected with pcDNA3.1/Hygro(+) into HEK-293 cells for mammalian expression and antibiotic screening. Among 21 single colonies, 16 were isolated with positive agonist inductions (designated as Passage 0). There were 3 clones with highest signal and best conditions. They were confirmed and sub-cultured twice passages a week without any antibiotic up 25 passages. Cell lines were healthy. It is compatible with the normal growth of human embryonic kidney (HEK-293) cells. These observations were contradictory with previous reports that uncontrolled expression of TRPA1 cell line grew very slowly and appeared unhealthy in culture[7, 13, 14].

It was reported that expression of TRPA1 mRNA is selectively turned off in transfected cells [7, 13]. To confirm TRPA1 mRNA is maintained in stable cell line. Total RNA was extracted from stable TRPA1-HEK cell line at passages 3 and 27 (P3 and P27) and untransfected HEK-293 cells. Reverse transcription PCR of RNA isolated from stable TRPA1-HEK cells and parental HEK-293 cells using primers specific for the TRPA1 sequence (Table 1) yielded a specific band of appropriate size (487 bp) in the stable TRPA1-HEK cells only (Figure 1a). It demonstrated that TRPA1 mRNA exist in stable TRPA1-HEK cell line. RT-PCR with β-actin-specific primers showed same bands (564 bp) confirmed equal total RNA loading from all cells (Figure 1b). Two strong bands of TRPA1-HEK cell line passage 3 and passage 27 showed similar brightness indicating that TRPA1 gene stable maintained in TRPA1-HEK cell line.

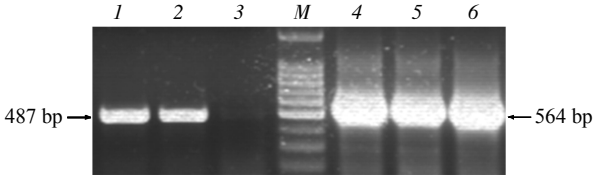

Fig. 1 Demonstration of TRPA1 mRNA in the stable TRPA1-HEK cell line

RT-PCR with TRPA1-specific primers was performed on total RNA from stable TRPA1-HEK cell line at passage 3 (lane *1*), passage 27 (lane *2*) and parental HEK-293 (lane *3*); RT-PCR with β-actin-specific primers was performed on total RNA from stable TRPA1-HEK cell line at passage3 (lane *4*), passage 27 (lane *5*) and parental HEK-293 (lane *6*); Marker lane (100 bp DNA Ladder) is indicated as *M*.

The relative expression of TRPA1 gene of stable TRPA1-HEK cell line at different passages was also determined using real-time quantitative PCR (Figure 2).

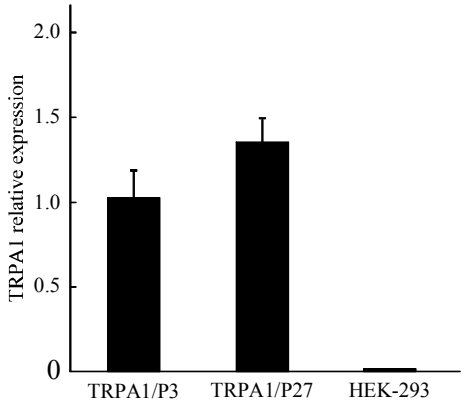

Fig. 2 TRPA1 gene expression level by real-time quantitative PCR

TRPA1 relative expression levels were detected in different passages of TRPA1 stable expression cell lines (TRPA1/P3: TRPA1-HEK passage 3; TRPA1/P27: TRPA1-HEK passage 27) and parental HEK-293 cell line. The expression levels have been normalized with the average expression of the endogenous control gene GAPDH. The TRPA1 average relative expression level of the TRPA1-HEK cell line passage 3 has been set to 1. Assays were performed in triplicate. Data points represent $\bar{x} \pm s$.

Interestingly, TRPA1 expression level slightly increased in TRPA1-HEK cell at passage 27 compared to passage 3. This phenomenon was also observed at other stable TRPA1-HEK clones we generated (data not show). In contrast, TRPA1 gene was undetectable in parental HEK-293 cells. These studies confirmed that stable TRPA1-HEK cell lines reliably retained TRPA1 gene.

2.2 TRPA1 protein stable expression in cell line

To analyze TRPA1 protein expression in TRPA1-HEK cell line, total cellular protein was isolated from TRPA1-HEK at passages 1 and 25 (P1 and P25) and untransfected HEK-293 cells. Expression of TRPA1 was then analyzed by Western blot (Figure 3a). Two TRPA1 specific immunoreactive bands were detected in TRPA1-HEK but not in untransfected HEK-293 cells. One band of ~127 ku was the predicted size of TRPA1 protein, another band of ~150 ku possibly represented posttranslational modified protein[14], Western blot with β-tubulin antibody (55 ku) confirmed total protein loading was equivalent (Figure 3b). Two strong bands of TRPA1 existed in both TRPA1-HEK at passages 1 and 25. There was no significant reduction of TRPA1 expression, indicating that robust protein expression of TRPA1 was maintained after passage 25.

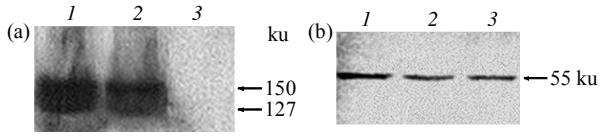

Fig. 3 TRPA1 protein expression in TRPA1-HEK cell line

(a) Western blot with TRPA1 antibody was performed on total cellular protein from stable TRPA1-HEK cell line at passage1 (lane *1*), passage 25 (lane *2*) and parental HEK-293 (lane *3*). (b) Western blot with β-tubulin antibody was performed on total cellular protein from stable TRPA1-HEK cell line at passage1 (lane *1*), passage 25 (lane *2*) and parental HEK-293 (lane *3*).

2.3 Functional characterization of the TRPA1-expressing cell line

TRPA1 is a calcium permeable cation channel that responds to cold and pungent natural compounds present in cinnamon oil, mustard oil, such as cinnamaldehyde and trans-2-pentenal, and is blocked by ruthenium red[5, 7, 11]. This property can be used to develop a Ca^{2+} influx assay to record Ca^{2+} influx mediated by TRPA1 channel opening using a cell line stably expressing TRPA1. We reported here the establishment of a TRPA1-expressing cell line and validation of TRPA1 responsiveness to corresponding agonists and blockers (Figure 4). Cinnamaldehyde and trans-2-pentenal induced concentration-dependent fluorescence increases in cells transfected with TRPA1 but not in untransfected cells (Figure 4a,b). The EC_{50} of cinnamaldehyde in TRPA1-expressing cells was 9.77 μmol/L. The EC_{50} of trans-2-pentenal was 25.57 μmol/L. The inhibitory effect of ruthenium red on agonist-induced Ca^{2+} influx was determined using 60 μmol/L cinnamaldehyde (Figure 4c). Ruthenium red inhibited agonist-induced fluorescence change in a concentration-dependent manner, with an IC_{50} of 0.82 μmol/L. The results showed that the potency was compatible with reference data[7, 16~18]. It validated TRPA1-HEK cell line expressing functional TRPA1 cation channel.

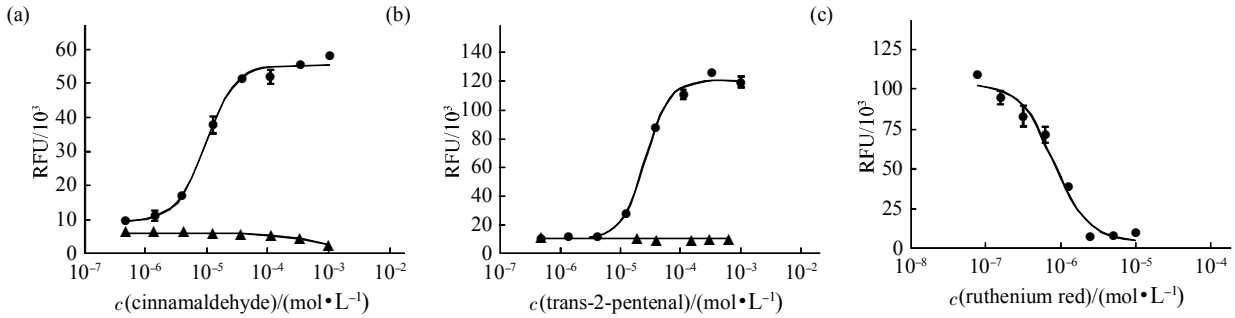

Fig. 4 Concentration-effect curves of TRPA1 agonist and antagonist in the Ca^{2+} influx assay using stable TRPA1-HEK and parental HEK-293 cell lines

Changes in relative fluorescence units (RFU) are shown in: (a) Concentration-effect relationship of fluorescence change elicited by cinnamaldehyde. The EC_{50} of cinnamaldehyde was 9.77 μmol/L. (b) Concentration-effect relationship of fluorescence change elicited by trans-2-pentenal. The EC_{50} of trans-2-pentenal was 25.57 μmol/L. (c) Concentration-effect relationship of inhibition by ruthenium red. Agonist cinnamaldehyde (60 μmol/L) was used to induce a calcium influx. The IC_{50} for RR block was 0.82 μmol/L. Assays were performed in duplicate. Data points represent $\bar{x} \pm s$. EC_{50} and IC_{50} values were determined using GraphPad Prism 5 software. ●—●: TRPA1; ▲—▲: HEK-293.

The super family of mammalian transient receptor potential (TRP) ion channels is composed of six subfamilies. TRPA1, as well as TRPV1, TRPV2, TRPV3, TRPV4 and TRPM8 have been identified as temperature sensors. They are usually grouped as thermo-TRPs[11]. To further validate TRPA1-HEK cell line specific expressing TRPA1 channel, we tested TRPA1 agonist cinnamaldehyde dose response curve on TRPA1-HEK and stable TRPV3-HEK, TRPV4-HEK, TRPM8-HEK cell lines side by side (Figure 5). Cinnamaldehyde specifically induced TRPA1-expressing cell line calcium signal as expected but not other TRP ion channels expressing cell lines. The EC_{50} of cinnamaldehyde in TRPA1-expressing cells was 10.18 μmol/L, suggesting that this TRPA1-HEK cell line was expressing specific functional TRPA1.

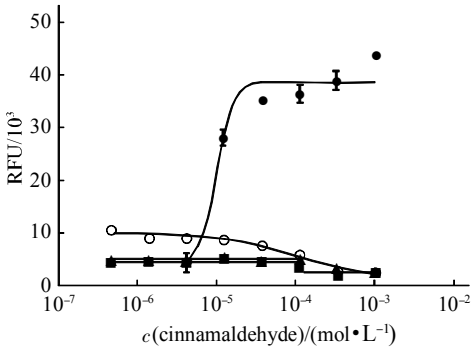

Fig. 5 TRPA1 agonist specificity test in the Ca^{2+} influx assay using stable TRPA1-HEK, TRPV3-HEK, TRPV4-HEK and TRPM8-HEK cell lines

The y-axis represents change in relative fluorescence units (RFU). Concentration-effect relationship of fluorescence change elicited by cinnamaldehyde. The EC_{50} of cinnamaldehyde on stable TRPA1-HEK cell line was 10.18 μmol/L. Assays were performed in duplicate. Data points represent $\bar{x} \pm s$. EC_{50} value was determined using GraphPad Prism 5 software. ●—●: TRPA1; ▲—▲: TRPV3; ■—■: TRPV4; ○—○: TRPM8.

2.4 TRPA1 protein functionally retained in stable cell line

The former study demonstrated that clonal TRPA1 cell line during passage often resulted in loss of protein expression and target activity[7, 13, 14]. To determine whether our TRPA1-HEK cell line has stably retained TRPA1 activity, its pharmacological responses to agonist and antagonist were evaluated at different passages by calcium assay (Figure 6). Upon the agonist induction, no significant signal window drop was observed for the cell line, and the calculated EC_{50} were comparable among the selected passages (Figure 6a). The EC_{50} values of agonist cinnamaldehyde were 17.55, 14.07, 14.35, 16.55 and 17.72 μmol/L for passage 5 cells, passage 10 cells, passage 15 cells, passage 20 cells and passage 25 cells respectively (Table 3). The IC_{50} values of ruthenium red inhibition were also quite similar at every 5 passages (0.84, 0.96, 0.75, 0.66, and 0.54 μmol/L, respectively; Figure 6b; Table 3). The potency was compatible with the data published before[7, 16~18]. Taken together, these studies demonstrated that TRPA1-HEK cell line passaging for long periods retained robust signals and displayed similar pharmacological property. The apparently similar potencies obtained from different passages highly suggested relatively same level of overall protein expression and ion channel activity.

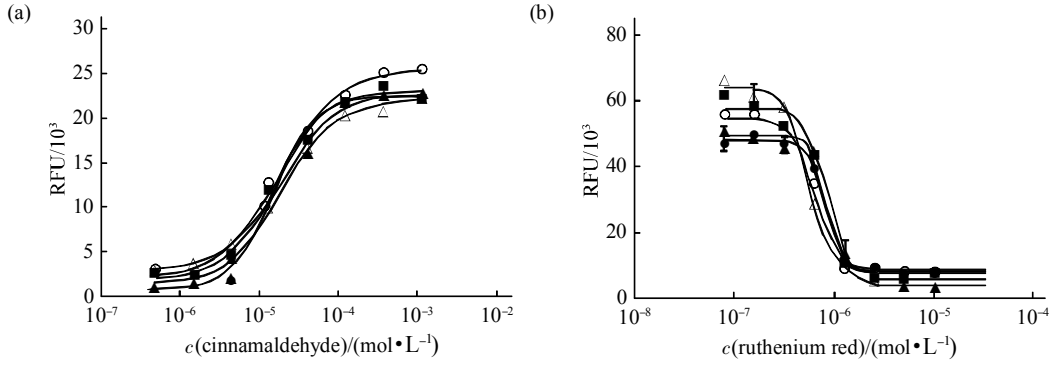

Fig. 6 Stability test of TRPA1-HEK cell line in the Ca^{2+} influx assay using every 5 passages (P5, P10, P15, P20 and P25)

Changes in relative fluorescence units (RFU) are shown in: (a) Concentration-effect relationship of fluorescence change elicited by cinnamaldehyde. (b) Concentration-effect relationship of inhibition by ruthenium red. Agonist cinnamaldehyde (60 μmol/L) was used to induce a calcium influx. Assays were performed in duplicate. Data points represent $\bar{x} \pm s$. EC_{50} value of cinnamaldehyde and IC_{50} value of RR on every 5 passages were determined using GraphPad Prism 5 software (**Table 3**).●—●: P5; ▲—▲: P10; ■—■: P15; ○—○: P20; △—△: P25.

Table 3 Comparison of TRPA1 activities at different TRPA1-HEK cell passages in the Ca^{2+} Influx assays

	Passage 5	Passage 10	Passage 15	Passage 20	Passage 25
EC_{50} /(μmol•L^{-1})	17.55	14.07	14.35	16.55	17.72
IC_{50} /(μmol•L^{-1})	0.84	0.96	0.75	0.66	0.54

IC_{50} for block of cinnamaldehyde-induced Ca^{2+} influx by ruthenium red (RR) was determined using 60 μmol/L cinnamaldehyde. EC_{50} value of cinnamaldehyde and IC_{50} value of RR on every 5 passages were determined using GraphPad Prism 5 software.

To confirm the stable activity of exogenous TRPA1 expression, TRPA1-HEK cell line was further evaluated by measuring membrane potential. Membrane potential assay was carried out using FLEX Station instruments with Membrane Potential Assay Kit, which has good correlation with manual patch clamping data for assessing ion channel activity and pharmacology. Agonist cinnamaldehyde activates the TRPA1 ion channel opening, positive calcium ion enter and accumulate inside the cell, the membrane becomes depolarized, and a resultant increase in fluorescence intensity is read. The functional properties of the TRPA1 channel were compared between passage 9 cells and passage 32 cells (Figure 7). TRPA1 specific agonist cinnamaldehyde evoked a similar intensity increase of fluorescence in both passages; however no change was observed in untransfected HEK-293 cells. Thus, the expression level and functional characteristic of TRPA1 channel expressed in TRPA1-HEK cell line were affirmatively similar between different passages, indicating this TRPA1 expression cell line was stable.

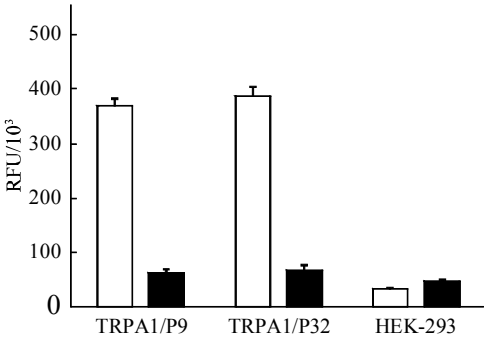

Fig. 7 Stability test of TRPA1-HEK cell line in the membrane potential assay using different passages of TRPA1 stable expression cell lines (TRPA1/P9: TRPA1-HEK passage 9; TRPA1/P32: TRPA1/HEK passage 32) and parental HEK-293 cell line

The *y*-axis represents change in relative fluorescence units (RFU). Average fluorescence increase elicited by cinnamaldehyde (100 μmol/L) and buffer. Assays were performed in quadruplicate. Data points represent $\bar{x} \pm s$. □: Cinnamaldehyde; ■: Buffer.

3 Discussion

The results of this study showed that it was possible to generate HEK-293 cell line stably expressing uncontrolled. To our knowledge, this was the first stable cell line constitutively expressing TRPA1. Our study sufficiently proved the feasibility of maintaining stable expression of TRPA1 in HEK-293 as well as the existence of functional TRPA1 protein in the stable cell line. This TRPA1-HEK stable cell line has been successfully applied to HTS in our laboratory.

Noticeably, other investigators have previously claimed difficulty in generating stable cell lines expressing TRPA1[7, 13, 14], mainly due to cellular toxicity caused by the target. Cells constitutively expressing TRPA1 were unhealthy and gradually lost TRPA1 expression and activity, possibly caused by a sustained Ca^{2+} influx mediated by TRPA1 channel opening. Others reported expression of the TRPA1 mRNA was selectively turned off in transfected cells, either by specific removal of the TRPA1 cDNA or by selective inactivation of the incorporated gene. They argued that stable expression of TRPA1 caused cell toxicity was due to the direct or indirect role of TRPA1 in signal transduction and growth control. To overcome problems associated with stable TRPA1 cell line generation, scientist performed TRPA1 functional analyses in the inducible expression system or transient transfected cells[7, 14].

A careful analysis of our transfection experiments indicated that the difference between the present study and previous reports was human TRPA1 cDNA sequence differed from the sequence published (NM_007332) by a SNP (rs7819749). A single nucleotide switched from A to C, resulting in an alteration of amino acid from Lys (K) to Asn (N). The position of the variant amino acid was 186. The length of the TRPA1 protein was 1 119; from 1 to 719 was N-terminal located in cytoplasm. TRPA1 was characterized by the presence of a large number of

ankyrin repeat motifs located on the cytosolic amino N-terminal domain (TRPAnkyrin)[7, 10]. Multiple ankyrin repeats in TRPA1 have been proposed to function as a mechanical spring directly linking TRPA1 gating to cytoskeletal proteins and to play key roles in protein-protein interactions[4, 8, 9]. Amino acids from 164 to 193 were the position of the fourth ankyrin repeats. The SNP variant was located at this area. The amino acid of position 186 in human TRPA1 was not conserved across the TRP channel family; it was not homologous between human and rat or mouse TRPA1 either. This SNP bias was dependent on the population. For example, the amino acid Lys (K) at this location more often occurred in Europe population and Asn (N) was apt to occur in East Asia, West Africa and North America. Because of the lack of structural information, the molecular basis for TRPA1 function remains unknown. Whether this large size and basic Lys (K) changed to medium size and polar Asn (N) truly affected TRPA1 toxic character needs to be further investigated.

Knowledge of TRPA1 involvement in health and disease is in its infancy, the function of the novel protein remains obscure. Therefore, in an effort to identify TRPA1 modulators and analyze TRPA1 function, it is necessary to employ a cell line stably expressing TRPA1. Moreover, cell-based functional assays have been increasingly used in high-throughput screening (HTS) and lead optimization[19, 20]. Compared to inducible expression system or transient transfected cells used before, this TRPA1 stable cell line offers many advantages including simplification of the induction process prior to experiment, elimination of the potential effect of revulsant and consistency of assay performance which is an important factor in long-lasting compound screening campaigns and lead development.

In conclusion, we have established a stable cell line expressing “toxic” TRPA1. The cell model responds to known TRPA1 modulators and its sensitivity is similar to those observed in other systems. Because of its stable TRPA1 expression, increased flexibility and consistency, this cell line is useful for pharmacological analysis, functional investigation and screening for TRPA1 modulators.

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稳定表达“毒性”瞬时受体势 A1 通道细胞系的建立

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摘要 瞬时受体势 A1 (TRPA1) 是一种对低温敏感的离子通道，除响应温度外，也可被各种刺激性化合物激活，是许多感觉模型的转导通道．建立 TRPA1 异源表达系统将为药理分析及功能研究提供很大的便利，但是 TRPA1 的表达会引发细胞毒性，因此构建 TRPA1 稳定细胞系一直面临着挑战．在人胚肾细胞(HEK-293)中非调控的表达 TRPA1 稳定细胞系被成功建立．实验证实，培养至 25 代以上，该细胞系仍持续表达 TRPA1，且细胞的功能检测也进一步验证了该重组 TRPA1 细胞系的稳定性及特异性．TRPA1-HEK 细胞系不但是 TRPA1 功能性分析的便利工具，而且可应用于高通量药物筛选系统，鉴定 TRPA1 特异性调节剂．

关键词 瞬时受体势 A1 (TRPA1)，稳定细胞系，毒性

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