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Detection of Murine TRPA1 Transcripts in Keratinocytes

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Abstract

Background: Transient receptor potential ankyrin 1 (TRPA1) is expressed by nociceptive neurons of the dorsal root ganglia (DRGs) and trigeminal ganglia, but its expression in other cell types is largely unexplored. TRPA1 contributes to chemical, mechanical and cold nociception and expression of TRPA1 in keratinocytes may extend the detection of chemical and physical stimuli from the nerve endings to the surface of the skin.
Results: Comparison of TRPA1 genes from multiple inbred mouse lines revealed a large number of genetic variations in the TRPA1 locus. These genetic variations may affect annealing of primers to detect TRPA1 transcript when using nucleic acid-based methods. To consistently detect TRPA1 in genetically diverse mouse strains and in different tissue types, an optimized qPCR protocol for TRPA1 detection was created.
Conclusions: TRPA1 transcripts are consistently detected in murine keratinocytes.

Keywords: TRPA1, Transient Receptor Potential A1, Ion Channel, Keratinocytes, Skin, Sensory Neurons, qPCR

1. Background

The peripheral endings of sensory neurons in the outer most edge of skin relay environmental signals to the spinal cord where it is processed. In some specialized cases such as Merkel cell endings, intricate microstructures are present to detect sensory stimuli before the information is transmitted to the nerve ending (1). Keratinocytes that surround sensory endings do not show any specialization, so these cells are often thought of as a protective barrier despite their well described involvement in different sensory modalities (1-3). Since there are no chemical or electrical synapses between the sensory endings and keratinocytes, there is the possibility of paracrine signaling between the cell types due to their close spatial juxtaposition. ATP signaling between keratinocytes and sensory endings of dorsal root ganglion neurons (DRG) has been proposed for transmitting sensory information (4).

Of the molecules involved in cutaneous sensation, the TRP superfamily of ion channels has been implicated in multiple sensory modalities (5). Many of the TRP channels have been reportedly expressed in the epidermis. TRPA1 is a multimodal noxious sensor that includes mustard oil, extreme cold (< 17°C) and mechanical pain (6-8). TRPA1 is expressed in both the DRGs and keratinocytes of the skin (9-13). Of the TRP channels in keratinocytes, expression of TRPA1 was reported in specific regions of human and mouse skin. TRPA1 expression in human scalp is differentially expressed and localized to epidermal keratinocytes, melanocytes, and fibroblasts (14). Similarly, using a sensitive human placental alkaline phosphatase reporter placed under the endogenous TRPA1 promoter, TRPA1 message was observed in DRG and keratinocytes obtained from adult murine glabrous or hairy skin taken from the hind paw (10). Together, these studies suggest the expression of TRPA1 in a subpopulation of cells in skin. In addition to the expression of TRPA1 in skin, topical application of TRPA1 chemical agonist mustard oil or cinnamaldehyde leads to cellular changes in keratinocytes (15). These studies support a role of TRPA1 in keratinocyte function.

2. Objectives

The objective of the study is to design a robust quantitative PCR (qPCR) assay to detect TRPA1 transcript in different mouse strains and different tissue types. Using keratinocytes, an optimized qPCR assay was used to consistently detect TRPA1 message.

3. Materials and Methods

3.1. Animals

All animals used in this study were obtained from Jackson Labs (Bar Harbor, ME, USA). TRPA1 heterozygote knockout animals (B6; 129P-Trpa1tm1Kykw/J, JAX 006401) were mated to obtain wild-type and TRPA1 knockout animals as littermate controls. In this study, 7 wild-type and 6 TRPA1 knockout littermates were used for the experiments. All animal work conducted was approved by the IACUC at Rutgers university.

3.2. Harvesting Keratinocytes from Mouse Skin

Skin was obtained from the lower hind quarter and hindpaws of early post-natal pups (P3-P6), placed in cold 0.25% trypsin with EDTA at 4°C until the epidermis could be peeled away from the dermal layer (16). These cells were rinsed with cold 1X PBS and immediately collected for RNA extraction.

3.3. Primers for TRPA1 Amplification

TRPA1 specific primers for reverse transcriptase were designed to anneal to regions devoid of known SNPs or In/Dels. These primers anneal to the coding sequence and 3' untranslated region of the TRPA1 transcript. For the reverse transcriptase, three gene specific primers were used for cDNA synthesis: mmTRPA1 GS RT1 5'- GTGCCTGGGTCTATTTGGATAC-3', mmTRPA1 GS RT2 5'-CCACTCCCATAAGACACAGAAC-3' and mmTRPA1 GS RT3 5'- GAGGTCCTTCAGCCGATATTT-3'. For qPCR, the mmTRPA1F 5'-TGACCTTTGGGCAGCTTATT-3' and mmTRPA1R 5'-CTTCTGGACCTCAGCAATGT-3' were used to amplify the coding sequence of murine TRPA1. GAPDHF 5'- AGGTCGGTGTGAACGGATTTG-3' and GAPDHR 5'- TGTAGACCATGTAGTTGAGGTCA-3' were used to detect GAPDH. All primers were ordered from Integrated DNA Technologies (Coralville, IA, USA).

3.4. qPCR Analysis of Murine TRPA1

Total RNA was extracted using Trizol reagent (life technologies, Carlsbad, CA, USA) according to manufacturer instructions, resuspended in 10 mM Hepes pH 7.4 for storage and diluted to 0.5 μg/μL in water when used. 1 μg of total RNA was used to make cDNA using the qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA) according to manufacturer instructions except for the primers used. Oligo dT and the three gene specific TRPA1 primers were combined together with total RNA and adjusted a 10 μL volume for a final concentration of 1.25 μM of each primer. RNA and primers were incubated at 65°C for 5 minutes and then quickly placed on ice for 1 minute to denature RNA secondary structures and facilitate annealing. To the RNA-primer mix, reaction buffer, enzyme and the appropriate amount of water was added to adjust the reaction volume to 20 μL (Appendix 1). The reaction was place in thermocycler at 25°C for 10 minutes, 50°C for 50 minutes and 85°C for 5 minutes. After the reaction was completed, samples were stored at 4°C. For the qPCR reaction, 2 μL of the cDNA mix was added to SYBR green master mix Perfecta SYBR green fast mix ROX master mix (Quanta biosciences, Gaithersburg, MD, USA) along with 0.3 μM final concentration of each qPCR primer. Each reaction was adjusted to a 20 μL reaction volume (Appendix 2). An initial denaturation step at 95°C for 3 minutes was followed by a two-step protocol of denaturation at 95°C for 15 seconds and an annealing and extension step at 60°C for 1 minute. The two-step protocol was repeated for a total of 45 cycles. Thermocycling was performed on a StepOnePlus real-time PCR machine (life technologies, Carlsbad, CA, USA). Analysis was done the $\Delta\Delta$Ct method with GAPDH as the internal control and ROX as a passive reference dye. The ratio of SYBR green and ROX fluorescence was used to normalize the SYBR green fluorescence intensity and was referred to as Rn in the study. qPCR results were analyzed using conventional methods (17).

3.5. Statistical Analysis

A bootstrapping technique was employed to provide a 95% confidence interval for the normalized expression levels using REST 2009 software. Hypothesis testing determined whether the results were statistically significant within the confidence interval (17).

4. Results

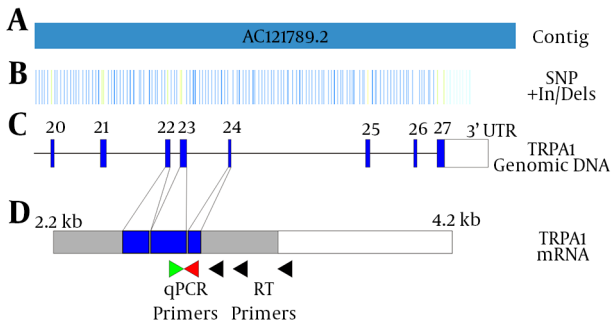
A survey of TRPA1 tissue distribution was done using the mouse gene expression database (GXD) (http://www.informatics.jax.org) (18, 19). Regions of the developing embryo, organ system and tissues revealed differing levels of TRPA1 expression in different tissue types. Despite previous reports of TRPA1 in skin (10, 14, 15), no annotated results were reported for developing embryonic or adult skin. To validate and detect TRPA1 in skin, qPCR was performed.

Practical mouse genetics requires generating genetically heterogeneous cohort of animals. These animals may harbor diverse genetic variation in TRPA1 that affect primer based detection methods such as qPCR. To compare the genetic variation in murine TRPA1, sequence from a reference contig was compared to sequence from mouse inbred lines from the Ensembl genome assembly (GRCm38.p4) (Figure 1A), 1,928 single nucleotide polymorphisms (SNP) as well as small insertions and deletions (In/Dels) were identified along the entire murine TRPA1 gene locus (Figure 1B). Within the coding sequence, there are 61 SNPs and In/Dels (20). To avoid inefficient detection of TRPA1 transcript by qPCR, gene specific primers (mmTRPA1 GS RT1-3) located in regions devoid of SNPs or In/Dels were used with an oligo dT for reverse transcriptase reaction. After cDNA synthesis, primers (mmTRPA1F and mmTRPA1R) that annealed to exons 22-24 that encode for the putative pore domain of TRPA1 were used for qPCR (Figure 1C). Ablation of these exons in the TRPA1 knockout animals allowed use of tissues from TRPA1 knockout animals as negative controls (8). To

harvest keratinocytes for TRPA1 detection, wild-type and TRPA1 knockout animals were generated from TRPA1 heterozygous mouse matings. The epidermis where the keratinocytes reside was peeled off from the dermis and harvested. Wild-type and TRPA1 knockout keratinocytes were lysed and used to generate total RNA. cDNA was generated from the total RNA samples and qPCR was performed using the aforementioned primers (Figure 1D).

Quantitative PCR (qPCR) using SYBR green dye was performed on individual cDNA samples from wild-type and TRPA1 knockout keratinocytes. Technical triplicates were done for each cDNA sample to ensure consistency. As an internal positive control for the integrity of the cDNA, the housekeeping gene GAPDH was amplified. Plotting the normalized fluorescence intensity (Rn) as a function of amplicon abundance, GAPDH was detected in all samples (n = 13) (Figure 2A). Using cDNA from wild-type animals (n = 7), TRPA1 transcripts was consistently detected from keratinocytes (Figure 2A). Using cDNA generated from TRPA1 knockout keratinocytes (n = 6) and reactions lacking cDNA as negative controls, no appreciable TRPA1 amplification was observed above the threshold (dotted line) even after 45 cycles (Figure 2A). These results indicated that TRPA1 transcripts are present and readily detected in wild-type keratinocytes. To display the representative cycle threshold (CT) as an indicator of amplicon abundance, technical triplicates from 2 independent wild-type and 2 independent TRPA1 knockout qPCR reactions were plotted. The average CT of GAPDH in both wild-type and knockout samples was 10.5. The average CT of TRPA1 in wild-type was 26.3, while the CT in knockout samples was set at 45, the cycle number corresponding to the end of the thermocycling profile (Figure 2B). To quantify the relative amount of TRPA1 transcript in the samples, the $\Delta\Delta$CT method was employed (17). Samples were first normalized to GAPDH before comparing TRPA1 levels relative to wild-type samples (Figure 2C). Wild-type keratinocyte samples showed detectable TRPA1 message (n = 7), while knockout animals (n = 6) had no detectable (N.D.) transcripts. A statistically significant difference in TRPA1 transcript levels between the wild-type and mutant cohorts was observed (P = 0) (17). Finally to ensure that the amplified PCR product corresponded to a single amplicon, a high resolution melting curve analysis was done for both the TRPA1 and GAPDH. The derivative of fluorescence was graphed relative to the melting temperature. Individual peaks in these graphs corresponded to a specific species of PCR product. For TRPA1, a major peak was observed at 80°C (dotted line) with a minor peak at 84.2°C. For GAPDH, a single peak was observed for at a melting temperature of 83.7°C (dotted line) (Figure 2D). These results suggest a single major amplicon was generated by qPCR for both TRPA1 and GAPDH.

Figure 1. Genetic Variations in Murine TRPA1



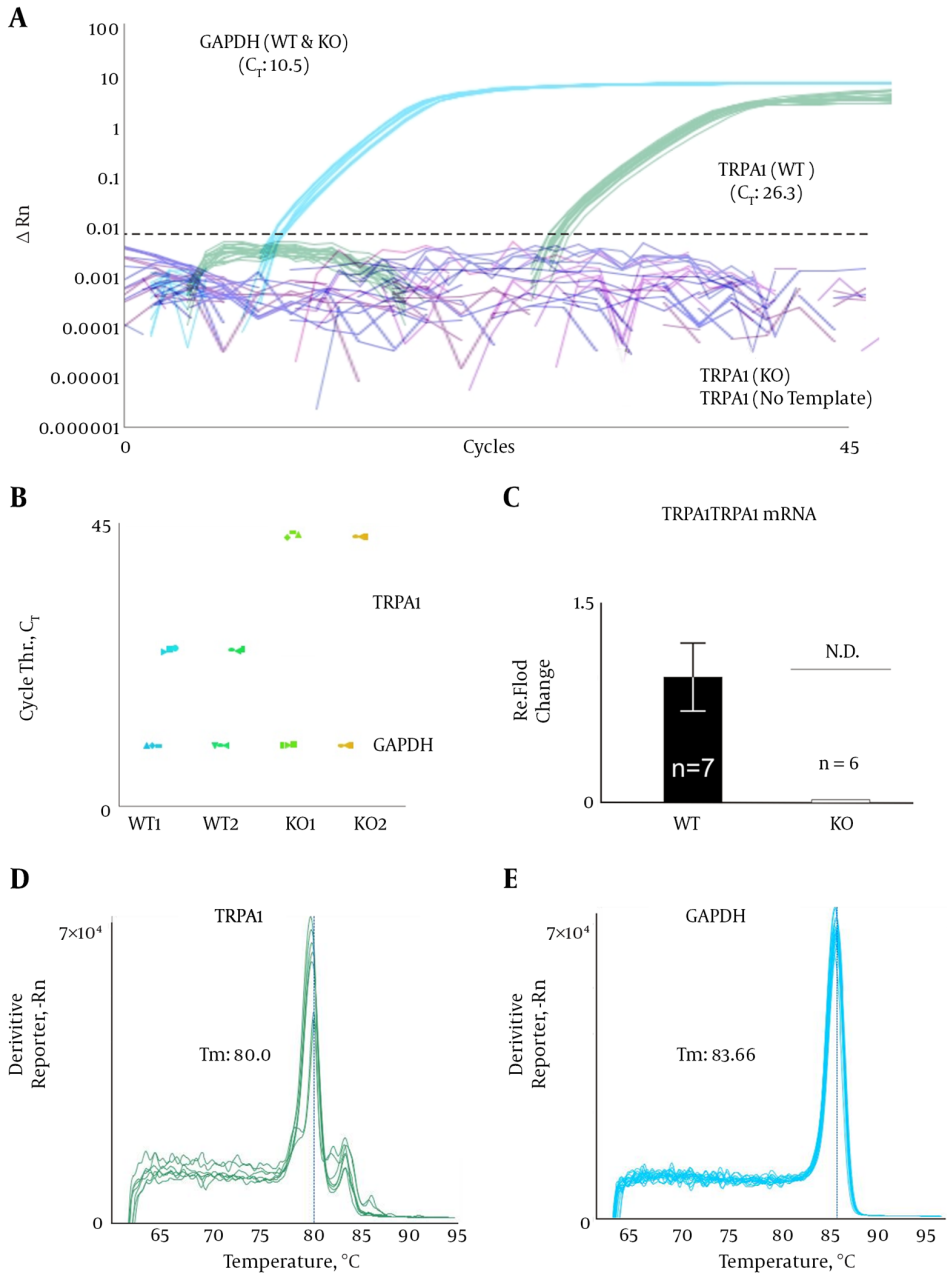
Mapping of TRPA1 annotated genetic variations from different inbred mouse strains. A, TRPA1 genomic sequence from contig AC1217982 was used as a reference; B, single nucleotide polymorphisms (SNPs) as well as insertions and deletions (In/Dels) are represented as tick marks. Blue ticks represent variations in the intronic regions. Green and yellow marks denote synonymous and missense variants in the coding sequence. Light blue marks are variants in the 3' untranslated region (UTR); C, A portion of the TRPA1 genomic region of interest is shown. Blue and white rectangles denote pertinent exons and the 3' UTR respectively; D, a portion of the TRPA1 mRNA from 2.2 - 4.2 kb including coding sequence from exons 22 - 24. Grey rectangles represent the other regions of the TRPA1 mRNA. Approximate location of qPCR primer pair is denoted by green and red triangles. Approximate location of primers used for RT-PCR that lie downstream of the qPCR primers.

5. Discussion

In this study, TRPA1 transcripts were detected in keratinocytes by qPCR. One potential issue that prevents TRPA1 detection are the large number of annotated SNPs and In/Dels in the TRPA1 locus (20). These genetic variations can potentially affect the TRPA1 function resulting in different sensitivities to noxious chemical and physical stimuli in inbred mouse lines. Genetic TRPA1 variations in DRG or keratinocytes could underlie the differences in pain behavior observed in inbred mice (21). The diversity in genetic variation of TRPA1 may potentially be attributed to different levels of pain sensitivity in humans.

Genetic variations within the TRPA1 locus make detection difficult when genetically heterogeneous mouse strains are employed. SNPs as well as In/Dels in the TRPA1 locus could affect primer binding and subsequently the efficiency of the qPCR reaction. By using multiple primers that bind specifically to different regions on the TRPA1 transcript during the reverse transcriptase reaction, potential issues for primer binding can be overcome. When the amplicons are generated, presence of SNPs and In/Dels could alter melting temperature and amplicon size. The additional smaller peak in the high resolution melting curve for the TRPA1 amplicon could be due to the presence of unannotated SNPs or a small insertion in the mouse strains used. These genetic variations would produce a different species of TRPA1 amplicons, alter the melting temperature

Figure 2. Quantitative PCR Detection of TRPA1 in Keratinocytes



A, Detection of SYBR green signal as an indicator of transcript amplification. GAPDH transcript from wild-type (WT) (n = 7) and TRPA1 knockout (KO) samples (n = 6) were detected above threshold (dotted line). TRPA1 transcripts were observed in WT samples only (n = 7). TRPA1 amplicon from TRPA1 knockout keratinocytes and no template controls were below the threshold and served as negative controls; B, plotting of cycle threshold (CT) values for TRPA1 and GAPDH qPCR reactions from two WT and two KO keratinocyte samples. Technical triplicates for each reaction are shown; C, quantification of relative TRPA1 transcript from WT and KO keratinocytes after normalization against GAPDH. TRPA1 was not detectable (N.D.) in KO animals. High resolution melting curves for D, TRPA1 and E, GAPDH.

and result in an additional peak during high resolution melting curve analysis.

Cells from different tissue types and different developmental stages may contain different levels of TRPA1 transcript. Having a qPCR assay that allows detection of varying amounts of transcript will be useful for initial compar-

ison of TRPA1 transcript in various tissue types during development. Using an optimized cDNA synthesis protocol with gene specific primers that avoided known SNPs and In/Dels in common genetic mouse strains, TRPA1 transcript from post-natal keratinocyte samples were consistently detected by qPCR. This method will allow for determination of dynamic TRPA1 levels during development and maturation of keratinocytes.

Supplementary Material

Supplementary material(s) is available here.

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Footnote

Conflict of Interest: The author declares that there is no conflict of interest.

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