RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

A Mutation in *TRPV4* Results in Altered Chondrocyte Calcium Signaling in Severe Metatropic Dysplasia

Lauren Hurd,[1,2] Susan M. Kirwin,[2] Mary Boggs,[1] William G. Mackenzie,[3] Michael B. Bober,[3,4] Vicky L. Funanage,[1,2] and Randall L. Duncan[1]*

[1]Department of Biological Sciences, University of Delaware, Newark, Delaware

[2]Molecular Diagnostics Laboratory, Nemours/Alfred I. duPont Hospital for Children, Wilmington, Delaware

[3]Department of Orthopaedic Surgery, Nemours/Alfred I. duPont Hospital for Children, Wilmington, Delaware

[4]Division of Genetics, Nemours/Alfred I. duPont Hospital for Children, Wilmington, Delaware

Manuscript Received: 15 January 2015; Manuscript Accepted: 15 May 2015

Transient receptor potential cation channel, subfamily V, member 4 (TRPV4) is a polymodal modulated non-selective cation channel required for normal development and maintenance of bone and cartilage. Heterozygous mutations of this channel cause a variety of channelopathies, including metatropic dysplasia (MD). We analyzed the effect of a novel *TRPV4* mutation c.2398G>A, p.Gly800Asp on intracellular calcium ($[Ca^{2+}]_i$) regulation in chondrocytes and compared this response to chondrocytes with a frequently observed mutation, c.2396C>T, p.Pro799Leu. We observed temperature-dependent $[Ca^{2+}]_i$ oscillations in both intact and MD chondrocytes however, MD mutations exhibited increased peak magnitudes of $[Ca^{2+}]_i$ during oscillations. We also found increased baseline $[Ca^{2+}]_i$ in MD primary cells, as well as increased $[Ca^{2+}]_i$ response to either hypotonic swelling or the TRVP4-specific agonist, GSK1016790A. Oscillations and stimulation responses were blocked with the TRPV4-specific antagonist, GSK205. Analysis of $[Ca^{2+}]_i$ response kinetics showed that MD chondrocytes had increased frequency of temperature-sensitive oscillations, and the magnitude and duration of $[Ca^{2+}]_i$ responses to given stimuli. Duration of the response of the p.Gly800Asp mutation to stimulation was greater than for the p.Pro799Leu mutation. These experiments show that this region of the channel is essential for proper $[Ca^{2+}]_i$ regulation. These studies of primary cells from patients show how both mutant and WT TRPV4 channels regulate cartilage and bone development. © 2015 Wiley Periodicals, Inc.

Key words: metatropic dysplasia; TRPV4; chondrocytes; intracellular calcium signaling

How to Cite this Article:
Hurd L, Kirwin SM, Boggs M, Mackenzie WG, Bober MB, Funanage VL, Duncan RL. 2015. A mutation in *TRPV4* results in altered chondrocyte calcium signaling in severe metatropic dysplasia.
Am J Med Genet Part A 167A:2286–2293.

INTRODUCTION

Intracellular calcium ($[Ca^{2+}]_i$) signaling is a distinct second messenger system critical for homeostasis and response to extracellular stimuli. Changes in calcium (Ca^{2+}) regulate crucial functions in all cell types. The Transient Receptor Potential (TRP) superfamily of non-selective cation channels are important for the regulation of $[Ca^{2+}]_i$. Of these, the transient receptor cation channel, subfamily V, member 4 (TRPV4) channel has been shown to be central to osmo- and mechanosensation [Strotmann et al., 2000; Delany et al., 2001]. This channel is polymodally modulated by a variety of sensory stimuli including temperature [Güler et al., 2002] and a variety of chemical agonists such as endocannabinoids, arachidonic acid metabolites, and phorbol esters [Watanabe et al., 2002a]. In mammals, TRPV4 is widely expressed in the musculoskeletal system [Masuyama et al., 2007; Muramatsu et al., 2007] where responses to mechanical stimulation are crucial to the integrity of bone and cartilage [Ozcivici et al., 2010; Lewis et al., 2011].

The extent of endogenous expression of TRPV4 as well as its breadth of modality supports its role in the normal function of

Grant sponsor: Nemours Foundation Cluster Grant Program; Grant sponsor: Delaware INBRE; Grant number: 5P20GM103446.
*Correspondence to:
Randall L. Duncan, Ph.D., Department of Biological Sciences, University of Delaware, 229 McKinly Hall, Newark, DE 19716.
E-mail: rlduncan@udel.edu
Article first published online in Wiley Online Library (wileyonlinelibrary.com): 6 August 2015
DOI 10.1002/ajmg.a.37182

sensory cellular responses as mutations of this channel cause a diverse array of channelopathies; ranging from hereditary and motor neuropathies to skeletal dysplasias [Rock et al., 2008; Auer-Grumbach et al., 2010]. Overlapping but distinct features are found in TRPV4-associated skeletal dysplasias ranging from mild brachyolmia (BO, OMIM 113500) to the most clinically severe metatropic dysplasia (MD, OMIM 15653) [Krakow et al., 2009]. Primary clinical characteristics of MD include shortened long bones with widened metaphyses, progressive thoracolumbar kyphoscoliosis, and severe platyspondyly [Maroteaux et al., 1966; Miething et al., 1980]. Additional skeletal changes include severe odontoid hypoplasia with reduced cervical spinal stability, halberd proximal femora, joint contractures, and often delayed secondary ossification sites [Hall and Elcioglu, 2004; Leet et al., 2006]. TRPV4 is central to cartilage development and responses to mechanical stimulation in chondrocytes, and thus alteration in channel activity can have a profound effect on cellular phenotype in bone and cartilage. Data have alluded to aberrant chondrogenesis as one of the primary causes of disease in MD and studies in cells transfected with these mutations indicate a gain-of-function [Camacho et al., 2010; Loukin et al., 2011]. However, little is known about channel activity in primary chondrocytes isolated from patients affected by *TRPV4* mutations.

In this study, we report a novel c-terminal mutation in the TRPV4 channel that results in severe metatropic dysplasia. Using primary chondrocytes isolated from this patient and a patient with a common mutation, we examined how *TRPV4* mutations alter $[Ca^{2+}]_i$ kinetics compared to intact chondrocytes. Determining changes in the Ca^{2+} signaling in these mutations in chondrocytes directly isolated from patients diagnosed with disease allows for a better understanding of how these MD mutations could alter tissue development and function.

MATERIALS AND METHODS

Clinical Information

Patients were recruited from the Skeletal Dysplasia Program at Alfred I. duPont Hospital for Children (AIDHC) in Wilmington, Delaware, under a Nemours IRB-approved protocol. Diagnoses of metatropic dysplasia were based on clinical evaluation and radiographic information.

Mutational Analysis

Genomic DNA was isolated from venous blood. Purified DNA (200 ng) was used to amplify exons 1 through 16 of *TRPV4* using a standard PCR protocol. Bidirectional sequencing was performed using Big Dye Terminator v3.1 and analyzed on an ABI 3130XL DNA sequencer (Applied Biosystems, Grand Island, NY) in the Biomolecular Core Laboratory (supported by COBRE P20GM103464). Sequences were analyzed using MacVector software (MacVector, Inc., Cary, NC) and aligned to an Ensembl *TRPV4* reference sequence (ENST00000261740; Chromosome 12q24.1). Heterozygous changes were compared to control populations and the NCBI SNP database.

Isolation and Culture of Metatropic Dysplasia Primary Chondrocytes

Articular cartilage was received as remnant tissues during surgical procedures from the cervical spine and lumbar spine regions of Patient 1, p.Gly800Asp, and Patient 2, p.Pro799Leu, respectively. Cartilage was placed in a scored 35 mm dish and the tissue was allowed to adhere for several hours in alpha MEM (Modified Eagle Medium media; Gibco Life Technologies) with 15% fetal bovine serum (Gibco, New York, NY) and 100 U/ml penicillin G, and 100 μg/ml streptomycin (Mediatech, Manassas, VA). Cells were maintained in a humidified incubator at 37°C with 5% CO_2, 95% air. Collagenase digestion was not performed as size of the tissue remnant limited the number of cells that could be isolated post digestion. Media was changed every other day for several weeks until primary outgrowths were seen. Cells were passaged at 50% confluence with 0.25% trypsin/2.2M EDTA (Mediatech). Chondrocytes were frozen down at a passage of two. Wild-type articular chondrocytes were purchased from Lonza (Basel, Switzerland) and cultured the same as MD chondrocytes. For analysis of $[Ca^{2+}]_i$, chondrocytes were grown in Chondrocyte Basal Medium (CBM, Lonza) supplemented with Chondrocyte Growth SingleQuots (Lonza) containing R3-IGF-1, bFGF, Transferrin, Insulin, 5% FBS, and 30 mg/ml Gentamicin and 15 μg/ml Amphotericin until 50% confluent, then used for subsequent experiments.

Intracellular Calcium Measurement

Chondrocytes (5,000 cells/cm^2) were plated onto 35 mm MatTek dishes (MatTek Corporation, Ashland, MA) coated with chicken sternal collagen type II (Sigma). $[Ca^{2+}]_i$ measurements were performed using Fluo-4 (Life Technologies, Grand Island, NY) on a Zeiss LSM 510 high speed confocal microscope with a 10× C-Apochromat water immersion lens. Chondrocytes were loaded with 10 μM Fluo-4 for 15 min at 37°C. Cells were washed with HBSS with Ca^{2+} and Mg^{2+} and allowed to equilibrate for 10 min. $[Ca^{2+}]_i$ was visualized with a 488 nm excitation laser and changes in fluorescent intensity were measured over time. Images were gathered over 1–2 min to establish baseline calcium values after which appropriate stimuli were added. For experiments testing the effect of 37°C temperature on cell responses, temperature, and CO_2 were maintained at 37°C and 5% using a temp control 37-2 digital and a CTI controller 3,700 digital, respectively (Zeiss). Images were analyzed using Zeiss Zen Physiology 2012 software. Response to stimulation was measured as change in intensity from average baseline to peak response. The same microscope protocol was used for every experiment with gains, laser intensity, and offsets remaining constant between groups and between experimental runs. Three independent experiments were run with $\geq$10 cells from distinct isolations/passages of chondrocytes for each experiment. Statistical differences were determined with two-way ANOVA and the significance of multiple comparisons was determined using the Tukey-Kramer post hoc test.

Stimulation of Metatropic Dysplasia Chondrocytes

For hypotonic swelling (HTS), chondrocytes were stimulated by the addition of an equal volume of water for 50% hypotonic stimulation. To determine the response of TRPV4-specific activation, chondrocytes were stimulated with 1 μM GSK1016790A (GSK 101; Sigma) after recording baseline measurements for 1–2 min. Attenuation of TRPV4 activity was determined by pre-treatment of cells for 20 min with 50 μM GSK205 (EMD Millipore, Billerica, MA) and then treatment with various stimulations as described.

CLINICAL REPORTS

Patient 1, female, was born to a G2P0 33-year-old mother and 35-year-old father, with no family history of skeletal dysplasia. Birth weight at term was 2.80 kg; birth length was 48.26 cm. Skeletal survey diagnosed MD on the second day of life and she was discharged home from the NICU at 4 days of age. Body proportions were disproportionate. The limbs were rhizomelic and platyspondyly with mild kyphosis was apparent (Fig. 1A). Severe narrowing of the trunk (Fig. 1B) and protrusion of the anterior sternum was visible. Prominent metaphyses throughout extremities and shortening of long bones were evident (Fig. 1C). The patient's 25-hydroxy vitamin D levels showed a lowest measured level of 25 ng/ml at 2 years 9 months of age, for which the patient was placed on increased supplementation. Dietary calcium intake was adequate and patient was normocalcemic. We have identified a novel deleterious heterozygous mutation of *TRPV4,* using GenBank reference sequence NM_021625.4, c.2398G>A, which predicts p.Gly800Asp that was not observed in control populations or found in NCBI or Ensembl SNP, TGP (1000 Genome Project), or EVS (Exome Variant Server) databases (Supplemental Fig. S1).

Patient 2, female, was selected because the NM_021625.4 (*TRPV4*): c.2396C>T, p.Pro799Leu mutation is one most frequently seen in patients with MD. She was born to a G2P1 27-year-old mother and 26-year-old father, with no family history of skeletal dysplasia. Birth weight at term was 3.45 kg; birth length was 48.26 cm. Skeletal survey diagnosed metatropic dysplasia at 1 day of age. She had cervical instability with progressive kyphoscoliosis (Fig. 1D and E) and shortening of long bones with widened metaphyses (Fig. 1F). Her 25-hydroxy vitamin D levels showed the lowest measured level of 22 ng/ml at 5 years 10 months of age, for which she was placed on increased supplementation. Dietary calcium intake has been adequate and she is normocalcemic.

Chondrocytes Exhibited Spontaneous $[Ca^{2+}]_i$ Oscillations at Physiologic Temperature

TRPV4 channels can be activated by changes in temperature above 25°C [Güler et al., 2002; Watanabe et al., 2002b]. We found that MD chondrocytes had significantly increased ($P < 0.05$) resting baseline $[Ca^{2+}]_i$ compared to WT chondrocytes at both 25°C and 37°C (Fig. 2C). Chondrocytes from Patient 1 (p.Gly800Asp) had significantly increased ($P < 0.001$) basal $[Ca^{2+}]_i$ compared to both WT and p.Pro799Leu chondrocytes. Prior to stimulation, all chondrocytes had spontaneous calcium oscillations at 37°C (Fig. 2A and B) that were not observed at 25°C. Both p.Gly800Asp and p.Pro799Leu chondrocytes had significantly higher oscillation peaks ($P < 0.05$) compared to WT and oscillations were blocked with the TRPV4-specific antagonist, GSK205 (Fig. 2D). The cells with the p.Gly800Asp mutation had an increased time to peak oscillation and time to return to baseline, resulting in increased duration of $[Ca^{2+}]_i$ oscillations when compared to WT and p.Pro799Leu mutated chondrocytes ($P < 0.05$). The p.Pro799Leu chondrocytes had an increased frequency of oscillations compared to WT and p.Gly800Asp chondrocytes ($P < 0.05$) (Table I).

Metatropic Dysplasia Chondrocytes had Altered Calcium Signaling in Response to Channel Activation

To avoid complications with Ca^{2+} oscillations, we stimulated chondrocytes at 25°C with either a 50% hypotonic solution (HTS) or the TRPV4-specific agonist, GSK1016790A (GSK101). The HTS and GSK101 significantly ($P < 0.01$) increased the peak Ca^{2+} response in both MD chondrocyte mutations compared to WT (Fig. 3A–E) that could be significantly reduced ($P < 0.001$) by the TRPV4-specific antagonist GSK205. However p.Gly800Asp chondrocytes had significantly ($P < 0.001$) enhanced responses to both stimuli compared to WT and p.Pro799Leu chondrocytes.

Kinetic Parameters of $[Ca^{2+}]_i$ With Stimulation are Altered in Metatropic Dysplasia Chondrocytes

There was no significant difference in response time to peak or return to baseline in p.Pro799Leu chondrocytes compared to WT; however, p.Gly800Asp chondrocytes had a significantly ($P < 0.01$) increased time to peak response and return to baseline response compared to both WT and p.Pro799Leu chondrocytes when stimulated with HTS (Table II). With TRPV4 specific stimulation via GSK101, both p.Gly800Asp and p.Pro799Leu cells had an increased slope in return to baseline compared to WT ($P < 0.05$). Upon GSK101 stimulation, WT chondrocytes showed a sharp increase in peak magnitude; however, the rate of return to baseline was much slower compared to MD chondrocytes. This was not observed in MD chondrocytes; where the response profile was similar to that seen in HTS, with a sharp increase in slope to peak and then a rapid return back to baseline (Table II). With GSK101 stimulation, both p.Gly800Asp and p.Pro799Leu chondrocytes had an increased ($P < 0.05$) slope to peak response as seen with HTS as well, suggesting an increased sensitivity or "priming" of the mutant channel.

DISCUSSION

Chondrocytes are responsible for the development of cartilage and subsequent endochondral bone formation and constantly experience changing states of ionic, osmotic, and compressive environments. Intracellular Ca^{2+} signaling is central to both cartilage development and the response to these environmental changes and influences many facets of cell behavior, including proliferation,

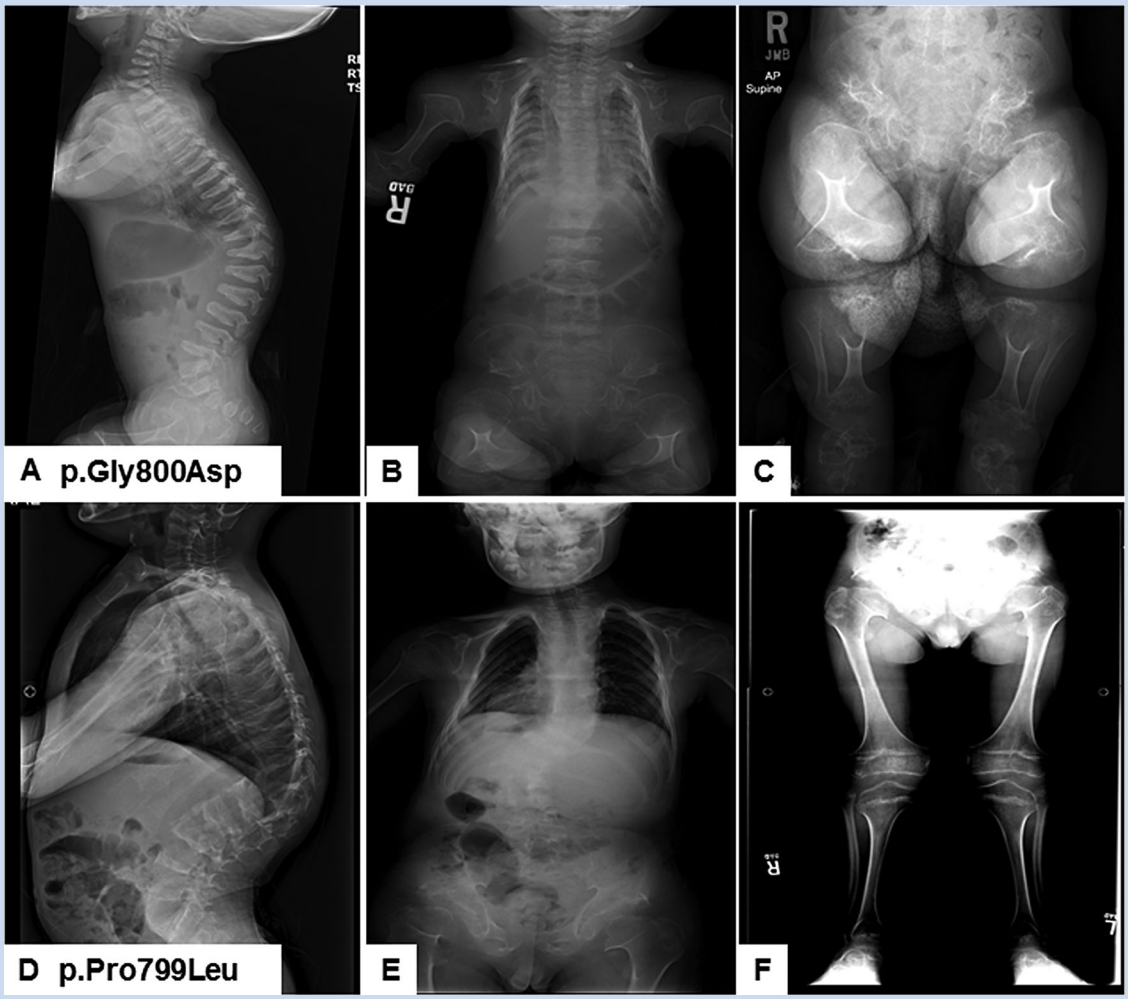

FIG. 1. Patient 1, with p.Gly800Asp mutation presented with severe infantile metatropic dysplasia (2 years). A, B: Severe platyspondyly and kyphosis with narrowing of the thorax. C: Prominent metaphysis throughout extremities and shortening of long bones is evident. Due to continued cervical stenosis and instability, at 12 months of age, the patient underwent an occiput C1 decompression and occiput to C2 fusion, with halo. D–F: Patient 2 with common p.Pro799Leu mutation presents with classic metatropic dysplasia (5 years) D, E: Progressive platyspondyly and kyphoscoliosis due to the progression of kyphoscoliosis at 4 years 9 months of age, the patient underwent an anterior release with halo traction, and vertical expandable prosthetic titanium rib (VEPTR) F: Shortened stature with mild widening of metaphysis of long bones.

gene regulation, cell motility, and differentiation. Central to this signaling in chondrocytes is the TRPV4 channel. Mutations in this channel result in skeletal dysplasias of variable severity. Here we show that two mutations, a novel p.Gly800Asp and a more typical p.Pro799Leu, increase $[Ca^{2+}]_i$ at basal levels and when channels are stimulated by either a specific agonist or by hypotonic challenge. This aberrant intracellular Ca^{2+} signaling may significantly alter cell activity that would result in abnormal skeletal development.

Calcium oscillations are one mechanism observed in many non-excitable cells that establish temporal and spatial signals to alter this cell behavior [Xu et al., 2003; Ye 2010]. We show temperature sensitive $[Ca^{2+}]_i$ oscillations in both WT and MD chondrocytes that could be blocked with the specific TRPV4 antagonist, GSK205. The $[Ca^{2+}]_i$ oscillations were observed at 37°C in both WT and mutant MD chondrocytes; however both p.Pro799Leu and p. Gly800Asp chondrocytes had significantly higher oscillation peaks compared to WT. If these oscillations are representative of spontaneous activity occurring *in vivo*, these changes in $[Ca^{2+}]_i$ would be much greater than in cells with intact TRPV4 channels. This is demonstrated by the area under the curve in Table I that shows that the p.Gly800Asp mutation would allow approximately twice as much calcium into the cells as would occur in WT cells. This increase in $[Ca^{2+}]_i$ could be detrimental to the normal growth and development of cartilage. In addition, at 25°C MD chondrocytes exhibited an overall increased basal level of $[Ca^{2+}]_i$ and increased $[Ca^{2+}]_i$ response when stimulated with HTS and GSK101. These data support the possibility that MD chondrocytes experience deleteriously elevated overall levels of $[Ca^{2+}]_i$. Numerous studies

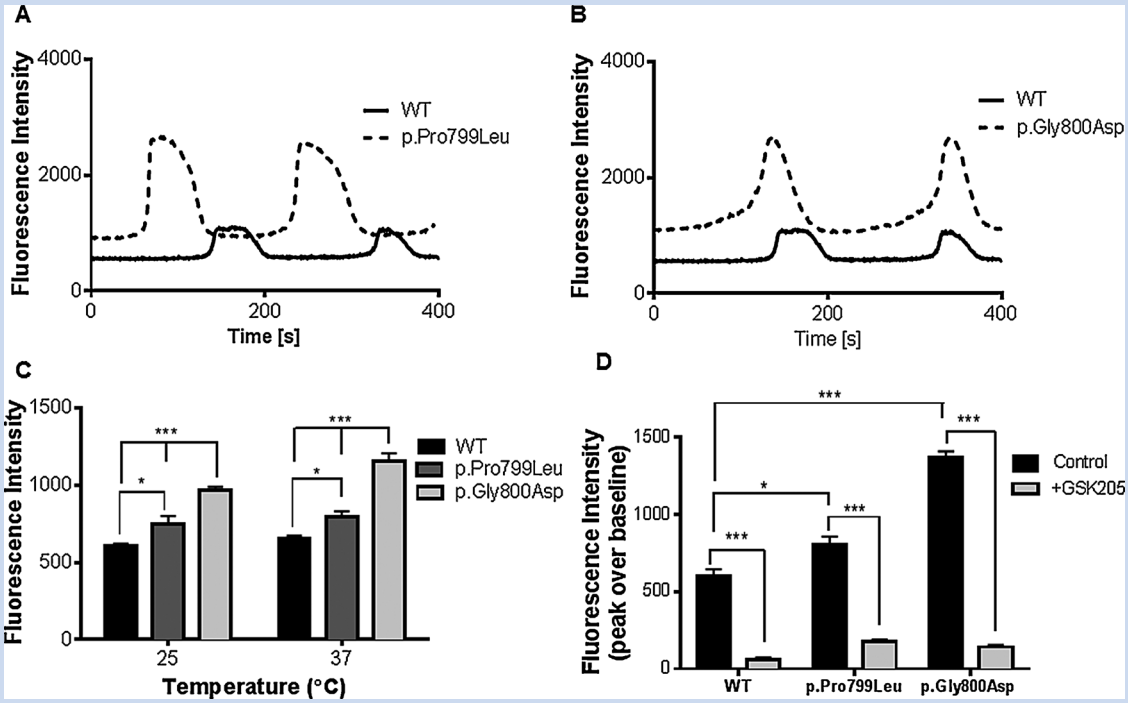

FIG. 2. Chondrocytes exhibit calcium oscillations at physiologic temperature, with metatropic dysplasia mutations having altered oscillatory patterns. A–B: Representative oscillatory traces of WT, p.Pro799Leu and p.Gly800Asp chondrocytes, respectively. C: Mean basal $[Ca^{2+}]_i$ levels measured at 25°C and 37°C. D: Mean peak oscillations during non-stimulated conditions without and with 50 μM GSK205 pretreatment. $^{*}P < 0.05$, $^{***}P < 0.001$. Error bars represent SEM.

have observed the effect of $[Ca^{2+}]_i$ levels on chondrogenic differentiation. Studies using high density cultures of mesenchymal stem cells (MSCs) have shown that an increase in $[Ca^{2+}]_i$ corresponds temporally with chondrogenic differentiation. When Ca^{2+} influx was significantly increased with addition of an ionophore, there was a dual effect on chondrogenesis. Increased $[Ca^{2+}]_i$ caused chondrogenic differentiation, but a continuous increase abolished cartilage formation [Matta et al., 2008]. It has also been shown that basal cytosolic Ca^{2+} levels can greatly influence the regulation and maturation of growth plate chondrocytes through the regulation of differentiation through parathyroid-related protein (PTH-rP) and other mediators [Zuscik et al., 2002].

We have shown that MD chondrocytes had an increased Ca^{2+} response compared to WT chondrocytes upon stimulation with

TABLE I. Oscillation Ca^{2+} Kinetics at 37°C

	WT	p.Pro799Leu	p.Gly800Asp
Time to peak (sec)	36.5 ± 1.45	47.4 ± 1.49[a]	47.0 ± 1.39[a]
Time return to baseline (sec)	55.6 ± 1.52	59.9 ± 2.6	66.2 ± 1.82[a]
Time base to base (sec)	49.5 ± 3.58	29.8 ± 7.19[a]	69.4 ± 4.00[a,b]
Time peak to peak (sec)	137.8 ± 4.52	136.9 ± 10.24	182.4 ± 5.72[a,b]
Period of peak (sec)	90.4 ± 2.15	107.2 ± 3.06[a]	115.6 ± 2.12[a]
Slope to peak	17.2 ± 1.20	22.2 ± 1.75	20.9 ± 1.39
Slope from peak	−14.0 ± 0.83	−16.2 ± 1.30	−24.4 ± 1.44[a,b]
Area under curve	344.61 ± 21.55	531.46 ± 30.14[a]	792.26 ± 49.28[a,b]
Frequency	0.004	0.008[a]	0.004
Percent oscillation	0.72	0.87	0.89

Measure of various kinetic elements of calcium kinetics in response to 37°C physiologic conditions.
Mean values ± SEM, [a] indicates significant difference compared to WT, [b] indicates significant difference compared to p.Pro799Leu.

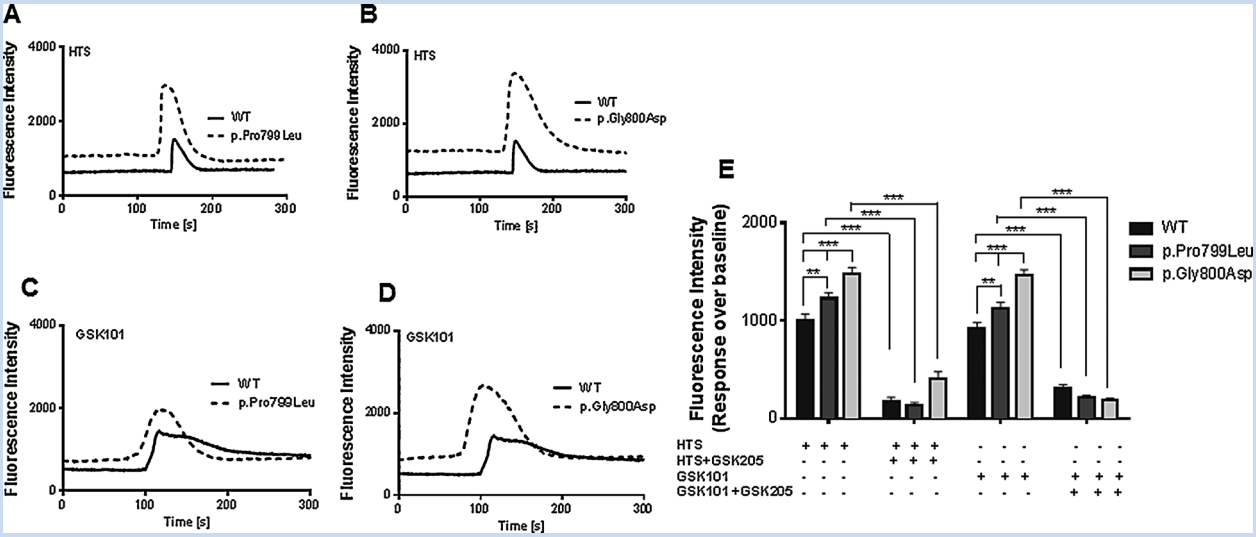

FIG. 3. Metatropic dysplasia chondrocytes have increased basal and stimulated calcium levels. A, B: Representative hypotonic swelling traces of WT, p.Pro799Leu and p.Gly800Asp chondrocytes, respectively. C, D: Representative calcium traces observed with 1 μM GSK1016709A treatment. E: Quantification of average response to both HTS and GSK101, along with inhibition of given stimuli with 50 μM GSK205. $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars represent SEM.

HTS. However it is not clear how over-activity of TRPV4 in patients with MD can lead to aberrant chondrogenesis. TRPV4 is the primary mediator of hypo-osmotic stimulation in porcine chondrocytes and Ca^{2+} signaling in these cells, and subsequent regulatory volume decrease (RVD), could be abolished by blocking TRPV4 [Phan et al., 2009]. During endochondral ossification, hypertrophic chondrocytes undergo an increase in volume through HTS and subsequent RVD. If TRPV4 mutations increase the $[Ca^{2+}]_i$ response to HTS, progression of chondrocytes to hypertrophy and the final stages of endochondral ossification could be disrupted. Histologic analyses of growth plates from lethal variants of MD exhibit a reduced hypertrophic region and disorganized endochondral ossification, supporting this postulate [Camacho et al., 2010].

When analyzing individual Ca^{2+} kinetics in MD chondrocytes, time to response and recovery response time were comparable to WT when p.Pro799Leu chondrocytes were stimulated with HTS. However upon TRPV4 specific stimulation, mutant chondrocytes responded and recovered much more rapidly compared to WT suggesting a decreased "reset" time. These results suggest that when

TABLE II. Stimulation Ca^{2+} Kinetics at 25°C

	WT	p.Pro799Leu	p.Gly800Asp
A. Hypotonic Swelling			
Time to peak (sec)	28.9 ± 1.2	25.7 ± 1.1	40.3 ± 1.9[a,b]
Time return to baseline (sec)	69.7 ± 4.2	67.7 ± 4.7	86.4 ± 3.6[a,b]
Slope to peak	33.5 ± 2.6	53.4 ± 3.9[a]	61.4 ± 2.2[a]
Slope from peak	−17.1 ± 1.3	−25.5 ± 2.0[a]	−21.5 ± 1.0[a]
Area under curve	433.7 ± 42.1	622.4 ± 54.9[a]	1245.7 ± 73.0[a,b]
Percent response (>50%)	0.91	0.93	0.94
B. GSK1016790A			
Time to peak (sec)	28.6 ± 1.59	38.8 ± 2.53[a]	37.1 ± 1.45[a]
Time return to baseline (sec)	131.9 ± 8.57	64.8 ± 2.41[a]	89.9 ± 5.44[a,b]
Slope to peak	40.6 ± 2.66	46.9 ± 3.09	55.7 ± 2.56[a]
Slope from peak	−4.5 ± 0.38	−18.4 ± 0.88[a]	−17.6 ± 0.977[a]
Area under curve	1040.8 ± 41.4	660.3 ± 54.5[a]	1297.6 ± 95.5[a,b]
Percent response (>50%)	0.92	0.94	0.91

Kinetic measurements for stimulation to A. hypotonic swelling responses and B. GSK1016790A stimulation.
Mean values ± SEM, [a]indicates significant difference compared to WT, [b]indicates significant difference compared to p.Pro799Leu.

TRPV4 is specifically activated in vivo, there may be a reduced refractory period that allows mutant chondrocytes to be stimulated again more quickly that is not seen in WT chondrocytes. p.Gly800Asp chondrocytes are from a patient with severe MD, whereas, the p.Pro799Leu chondrocytes were derived from a less severely affected patient, a trend that is observed in the calcium phenotype of these cells. Whereas the p.Pro799Leu chondrocytes had an increased baseline and response to stimulation compared to WT, the p.Gly800Asp chondrocytes had significantly increased calcium kinetics over both WT and p.Pro799Leu. Furthermore, while the Ca^{2+} oscillation dynamics were not different in the p.Pro799Leu and WT chondrocytes, p.Gly800Asp chondrocytes had significantly higher peak magnitudes and increased duration of the transient.

To date, studies of *TRPV4* mutations and the calcium signaling associated with these mutations have used transformed, transfected and re-programmed cells in which there are often a lack of consistent cell calcium kinetics or even contradicting activity based on cell and mutation type. We used chondrocytes directly isolated from affected patients to ascertain channel activity in MD as it is known that aberrant chondrogenesis is responsible for some of the clinical symptoms of disease. Once intact TRPV4 channel behavior is elucidated, insight can be gained into the effects of aberrant Ca^{2+} signaling and downstream signaling pathways in skeletal disease.

ACKNOWLEDGMENTS

We thank the patients and their families for participating in the study. This work would not be possible without their participation and cooperation. This work was supported by the Nemours Foundation Cluster grant program, the Delaware INBRE Grant 5P20GM103446 and a private donation from Dr. Jeremie Axe.

REFERENCES

Auer-Grumbach M, Olschewski A, Papić L, Kremer H, McEntagart ME, Uhrig S, Fischer C, Fröhlich E, Bálint Z, Tang B, Strohmaier H, Lochmüller H, Schlotter-Weigel B, Senderek J, Krebs A, Dick KJ, Petty R, Longman C, Anderson NE, Padberg GW, Schelhaas HJ, van Ravenswaaij-Arts CM, Pieber TR, Crosby AH, Guelly C. 2010. Alterations in the ankyrin domain of TRPV4 cause congenital distal SMA, scapuloperoneal SMA and HMSN2C. Nat Genet 42:160–164.

Camacho N, Krakow D, Johnykutty S, Katzman PJ, Pepkowitz S, Vriens J, Nilius B, Boyce BF, Cohn DH. 2010. Dominant TRPV4 mutations in nonlethal and lethal metatropic dysplasia. Am J Med Genet A 152A:1169–1177.

Delany NS, Hurle M, Facer P, Alnadaf T, Plumpton C, Kinghorn I, See CG, Costigan M, Anand P, Woolf CJ, Crowther D, Sanseau P, Tate SN. 2001. Identification and characterization of a novel human vanilloid receptor-like protein , VRL-2. Physiol Genomics 4:165–174.

Güler AD, Lee H, Iida T, Shimizu I, Tominaga M, Caterina M. 2002. Heat-evoked activation of the ion channel, TR P V4. J Neurosci 22:6408–6414.

Hall CM, Elcioglu NH. 2004. Metatropic dysplasia lethal variants. Pediatr Radiol 34:66–74.

Krakow D, Vriens J, Camacho N, Luong P, Deixler H, Funari TL, Bacino CA, Irons MB, Holm IA, Sadler L, Okenfuss EB, Janssens A, Voets T, Rimoin DL, Lachman RS, Nilius B, Cohn DH. 2009. Mutations in the gene encoding the calcium-permeable ion channel TRPV4 produce spondylometaphyseal dysplasia, Kozlowski type and metatropic dysplasia. Am J Hum Genet 84:307–315.

Leet AI, Sampath JS, Scott CI, MacKenzie WG. 2006. Cervical spinal stenosis in metatropic dysplasia. J Pediatr Orthop 26:347–352.

Lewis R, Feetham CH, Barrett-Jolley R. 2011. Cell volume regulation in chondrocytes. Cell Physiol Biochem 28:1111–1122.

Loukin S, Su Z, Kung C. 2011. Increased basal activity is a key determinant in the severity of human skeletal dysplasia caused by TRPV4 mutations. PLoS One 6:e19533.

Maroteaux P, Spranger J, Wiedemann HR. 1966. Metatrophic dwarfism. Arch Kinderheilkd 173:211–226.

Masuyama R, Vriens J, Torrekens S, Moermans K, Bosch AV, Bouillon R, Nilius B, Carmeliet G. 2007. TRPV4 affects bone remodeling by regulating calcium signaling required for osteoclast activity. J Bone Miner Res 22:S30–S30.

Matta C, Fodor J, Szíjgyártó Z, Juhász T, Gergely P, Csernoch L, Zákány R. 2008. Cytosolic free Ca2+ concentration exhibits a characteristic temporal pattern during in vitro cartilage differentiation: A possible regulatory role of calcineurin in Ca-signalling of chondrogenic cells. Cell Calcium 44:310–323.

Miething R, Stover B, Noeske H. 1980. Metatropic dysplasia—Rare skeletal anomaly. Monschr Kinderheilkd 128:153–156.

Muramatsu S, Wakabayashi M, Ohno T, Amano K, Ooishi R, Sugahara T, Shiojiri S, Tashiro K, Suzuki Y, Nishimura R, Kuhara S, Sugano S, Yoneda T, Matsuda A. 2007. Functional gene screening system identified TRPV4 as a regulator of chondrogenic differentiation. J Biol Chem 282:32158–32167.

Ozcivici E, Luu YK, Adler B, Qin YX, Rubin J, Judex S, Rubin CT. 2010. Mechanical signals as anabolic agents in bone. Nat Rev Rheumatol 6:50–59.

Phan MN, Leddy HA, Votta BJ, Kumar S, Levy DS, Lipshutz DB, Lee SH, Liedtke W, Guilak F. 2009. Functional characterization of TRPV4 as an osmotically sensitive ion channel in porcine articular chondrocytes. Arthritis Rheum 60:3028–3037.

Rock MJ, Prenen J, Funari VA, Funari TL, Merriman B, Nelson SF, Lachman RS, Wilcox WR, Reyno S, Quadrelli R, Vaglio A, Owsianik G, Janssens A, Voets T, Ikegawa S, Nagai T, Rimoin DL, Nilius B, Cohn DH. 2008. Gain-of-function mutations in TRPV4 cause autosomal dominant brachyolmia. Nat Genet 40:999–1003.

Strotmann R, Harteneck C, Nunnenmacher K, Schultz G, Plant TD. 2000. OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. Nat Cell Biol 2:695–702.

Watanabe H, Davis JB, Smart D, Jerman JC, Smith GD, Hayes P, Vriens J, Cairns W, Wissenbach U, Prenen J, Flockerzi V, Droogmans G, Benham CD, Nilius B. 2002a. Activation of TRPV4 channels (hVRL-2/mTRP12) by phorbol derivatives. J Biol Chem 277:13569–13577.

Watanabe H, Vriens J, Suh SH, Benham CD, Droogmans G, Nilius B. 2002b. Heat-evoked activation of TRPV4 channels in a HEK293 cell expression system and in native mouse aorta endothelial cells. J Biol Chem 277:47044–47051.

Xu F, Satoh E, Iijima T. 2003. Protein kinase C-mediated Ca^{2+} entry in HEK 293 cells transiently expressing human TRPV4. Br J Pharmacol 140:413–421.

Ye B 2010. Ca^{2+} oscillations and its transporters in mesenchymal stem cells. Physiol Res 59:323–329.

Zuscik MJ, D'Souza M, Ionescu AM, Gunter KK, Gunter TE, O'Keefe RJ, Schwarz EM, Puzas JE, Rosier RN. 2002. Growth plate chondrocyte maturation is regulated by basal intracellular calcium. Exp Cell Res 276:310–319.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.