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Arch Oral Biol. Author manuscript; available in PMC 2015 June 01.

Published in final edited form as:
Arch Oral Biol. 2014 June ; 59(6): 601–607. doi:10.1016/j.archoralbio.2014.03.005.

Molecular Motor *MYO1C*, Acetyltransferase *KAT6B* and Osteogenetic Transcription Factor *RUNX2* Expression in Human Masseter Muscle Contributes to Development of Malocclusion

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Author Contributions;

1. **Heather Desh,** while a graduate orthodontic resident helped collect MYO1C and MYO1H data
2. **S Lauren Gray,** while a dental student at Temple University Dental School conducted MYO1C gene expression experiments as part of a dental student research project
3. **Michael J Horton** oversaw all RT-PCR experiments in the Sciote Laboratory at Temple University
4. **Gwenael Raoul and Joel Ferri** collected all subject samples for the study at the University of Lille
5. **Anthea M Rowlerson** performed the fiber type histologic analysis and helped prepare the manuscript for publication
6. **Alexandre R Vieira** is the group's human genetics expert. He was also the first to identify type I myosin contributions to malocclusion. He also provided statistical consultation for the project
7. **James J Sciote** was the principal investigator for the study and prepared the manuscript for publication

Conflict of Interest Statement: The authors have no conflict of interest, either financial or personal which could influence the development and presentation of this work.

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Abstract

Objective—Type I myosins are molecular motors necessary for glucose transport in the cytoplasm and initiation of transcription in the nucleus. Two of these, *MYO1H* and *MYO1C*, are paralogs which may be important in the development of malocclusion. The objective of this study was to investigate their gene expression in the masseter muscle of malocclusion subjects. Two functionally related proteins known to contribute to malocclusion were also investigated: *KAT6B* (a chromatin remodeling epigenetic enzyme which is activated by *MYO1C*) and *RUNX2* (a transcription factor regulating osteogenesis which is activated by *KAT6B*).

Design—Masseter muscle samples and malocclusion classifications were obtained from orthognathic surgery subjects. Muscle was sectioned and immunostained to determine fiber type properties. RNA was isolated from the remaining sample to determine expression levels for the four genes by TaqMan® RT-PCR. Fiber type properties, gene expression quantities and malocclusion classification were compared.

Results—There were very significant associations ($P<0.0000001$) between *MYO1C* and *KAT6B* expressions. There were also significant associations ($P<0.005$) between *RUNX2* expression and masseter muscle type II fiber properties. Very few significant associations were identified between *MYO1C* and masseter muscle fiber type properties.

Conclusions—The relationship between MYO1C and KAT6B suggests that the two are interacting in chromatin remodeling for gene expression. This is the nuclear myosin1 (NM1) function of MYO1C. A surprising finding is the relationship between RUNX2 and type II masseter muscle fibers, since RUNX2 expression in mature muscle was previously unknown. Further investigations are necessary to elucidate the role of RUNX2 in adult masseter muscle.

Keywords

MYO1C; KAT6B; RUNX2; malocclusion; masseter muscle

1. Introduction

Jaw growth imbalances often require interdisciplinary orthodontic treatment and orthognathic surgery to restore function and esthetics to the entire masticatory unit. While much is known about the behavioral and environmental contributions to these dysplasias, the genetic etiology of skeletal growth and adaptation of facial growth patterns remains substantially unknown.[1] A key environmental influence on jaw deformation during growth is masticatory muscle strength, which is determined by the size and proportion of muscle fiber types that associate with vertical growth.[2,3] Sagittal jaw bone length deformations are more closely influenced by genetic variations associated with skeletal tissue growth. This is especially true for mandibular prognathism, which has an autosomal dominant inheritance with incomplete penetrance.[4] Since general heritability estimates for muscle strength and bone length traits in adolescents are at >80%[5,6], genetic contributions to growth of both jaw

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bones and muscles are important determining factors and the mechanisms by which deviations in craniofacial morphology develop are complex and require further investigation.

In a cross sectional study, we identified a genetic variation in Myosin 1H (*MYO1H*), which contributes to class III malocclusion due to mandibular prognathism.[7] Unlike Class II myosin heavy chains (MHCs), which are responsible for muscle contraction and are the basis for classification of skeletal muscle fiber types, Class I is an unconventional myosin group of single-headed monomers involved in cellular signaling mechanisms that regulate membrane dynamics, intracellular vesicle transport and auditory mechanotransduction.[8] Eight Class I myosins, designated *MYO1A* to *MYO1H*, are found in humans; some function as tension-sensors that respond to load changes by altering their ATPase activity and mechanical properties, but others have as of yet no known function.[9] Among the Class I myosins, *MYO1C* and *MYO1H* are vertebrate-specific sister paralogs which evolved from a gene duplication event.[10] The molecular functions of *MYO1H* are not known, but there is extensive information for *MYO1C* since it codes for the first single-headed myosin identified in mammals.[11]

The *MYO1C* gene produces three protein isoforms through alternative splicing. Isoforms 1 and 2 can be found in both nuclear and cytoplasmic locations, but isoform 3 is restricted to nuclear functioning only. Isoforms 1 and 2 have been given the name cytoplasmic *MYO1C* protein and isoform 3 the name nuclear myosin 1 (NM1). The proteins have redundancy since *MYO1C* can replace NM1 functioning in the nucleus when NM1 is knocked out in animal experiments.[12] Isoforms 1 and 2 of *MYO1C* regulate glucose uptake via facilitated glucose transporter 4 (*GLUT 4*) in skeletal muscle by acting as a motor for movement of *GLUT4*- stored vesicles to plasma membranes after stimulation with insulin and contraction.[13-15] In micro-array experiments we recently found that expression of *GLUT4* is nearly 3× higher in masseter from open-bite compared to deep bite patients.[16] *GLUT4* is expressed at higher levels in Type I, slow contracting fibers in human vastus lateralis muscle, but not in soleus or triceps brachii muscles.[17,18] It is possible that both *MYO1C* and *GLUT4* expression in masseter muscle may be increased in skeletal open bite due to elevated levels of Type I fibers, or hybrid fibers which express some Type I myosin in addition to other myosin heavy chain isoforms.

NM1 performs a separate and critical role in activation of transcription in the nucleus.[19] NM1 participates in the formation of the multi-protein assembly B-WICH, which is comprised of the William syndrome transcription factor complex (WSTF), Cockayne syndrome group B protein (CSB) and NM1, that is necessary for chromatin remodeling.[20] In this process nucleosomes are repositioned which leads to their binding with histone acetyltransfeases (HATs). The HATs confer transcriptional specificity since they function as active gene promoters.[21] Using RT-PCR we recently found that expression of a HAT, K(lysine) acetyltransferase 6B (*KAT6B*), positively correlates with mandibular prognathism, and with Class II myosin heavy chain (MHCs type IIA and IIX) expression in masseter muscle.[16] Increased expression of these fast-twitch type II MHC isoforms and type II skeletal muscle fibers enhances masticatory strength, which contributes to the development of deep bite malocclusion by decreasing vertical growth of the face.[2-3] The association of

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KAT6B with mandibular prognathism could be related to its activation of the osteogenic transcription factor *RUNX2*[22] which is required for mandibular condylar cartilage growth.[23]

Given the importance of these genetic and epigenetic influences on sagittal and vertical jaw growth, we compared gene expression of *MYO1H*, *MYO1C*, *KAT6B* and *RUNX2* in masseter muscle to malocclusion classification and muscle fiber type distribution.

2. Materials and Methods

2.1. Patient population and surgical procedure

Recruitment was from orthodontic patients undergoing orthognathic surgery at the *Hôpital Roger Salengro, Service de Chirurgie Maxillo-Faciale et Stomatologie at the Centre Hospitalier Universitaire de Lille* in Northern France. Subject participation was in accordance with the research ethics committee's approval at Temple University and at the University of Lille. Masseter muscle samples were obtained from 28 females and 21 males (average age 22 yrs) undergoing the sagittal split procedure. The surgeries were performed by two surgeons, the Department Head and the Graduate Program Director for Maxillofacial Surgery. Surgical procedures for all subjects in this study included at least a mandibular bilateral sagittal split osteotomy using Epker's technique. This osteotomy separates the ascending branch of the mandible from the dental arch and mandibular body. The Epker technique uses structural elasticity to split the bone through the bony channel of the inferior alveolar nerve. The technique is advantageous since during the split, the inferior alveolar nerve and blood vessels are visualized and protected to avoid damage, which would affect chin and lip sensation. The bony separation is performed with a Tessier distractor in order to drive the split by using bone flexibility, which assures a more accurate and consistent sagittal split. At separation the deep portion of the masseter muscle is exposed, and some muscle fibers are lacerated in the middle of the split. The muscle samples used for this study were taken from this point, in the deep part of the anterior portion of the superficial masseter, just in front of the limit between the mandibular angle and horizontal branch. Malocclusion classification was based on the surgical treatment plan and pre-surgical orthodontic diagnosis. Subjects were grouped into one of 6 malocclusion classifications, based on sagittal and vertical dysplasia, that include either skeletal Class II or III and either open, normal or deep bite (Table 1). Samples were de-identified with a unique coding number and stored at -80°C in the Federation de Recherche Clinique (Clinical Research Center) until processing.

2.2. Tissue processing for immunohistochemistry and fiber typing

Muscle specimens, weighing between 60-120mg and about 0.5cm^2 in size were placed onto gauze saturated with sterile saline on ice and taken away for freezing. Each masseter sample was mounted onto a cork disc in a perpendicular orientation using Tissue-Tek© OCT (Optimum Cutting Temperature) and snap-frozen in isopentane cooled by liquid nitrogen (-196°C) within minutes of excision.

Frozen muscle was sectioned at 10μm on a Bright OFT cryostat and sections were stored at -800C until immunohistochemical staining and morphometric analysis of fiber types was performed. Serial muscle sections were immunoreacted with anti-MHC antibodies in order

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to classify skeletal fibers as detailed previously.[24] The MHC specific antibodies were: type I (BA-F8), all type II (MY-32), type IIA only (SC-71), neonatal (a polyclonal prepared by Dr. Anthea Rowlerson) and α-cardiac (MAS 366). Immunostaining permitted identification of 8 fiber types, which were organized into 4 groups as follows: type I, containing only type I MHC; type II, containing only type IIA and/or IIX MHC; type I/II hybrid fibers, containing both type I and II isoforms; and type neonatal/atrial fibers, containing neonatal or α-atrial MHC in combination with type I or type II isoforms.[24] The cross-sectional area of identified fibers was measured with image-analysis software by displaying each digital image and tracing its outer border with a VIDS-V image-analysis system (Ai, Cambridge, United Kingdom) linked to a Nikon Labophot microscope (Nikon, Tokyo, Japan). Fibers with adequate staining and morphology for analysis were obtained from all samples. Mean fiber areas (μm^2) and total fiber number were used to calculate the percent tissue area occupancy (Fiber Percent Occupancy) for each of the 4 fiber groups.[2,3] Tests for measurement error included intra-rater reliability in determination of fiber area (by repeating morphometric tracing of all fibers areas in one biopsy by one examiner), which resulted in an R^2 value of 0.9452, and inter-rater reliability in determination of fiber area error, which resulted in an R^2 value of 0.9752.

2.3 RNA isolation and quantitative RT-PCR

RNA was isolated from the remainder of muscle samples with TRIzol™ reagent (Invitrogen, Carlsbad, CA), digested with DNase I, re-isolated with RNAqueous® and quantified by absorbance at A_{260}.[25] Target RNA was quantified by triplicate assays of TaqMan® (Applied Biosystems, Foster City, CA) RT-PCR using RNA-to-C_T™ 1-Step reagent and an Applied Biosystems Step One Plus instrument. Reactions included specific primer-probe sets for *MYO1H, MYO1C, KAT6B, RUNX2* and for the endogenous control gene hypoxanthine phosphoribosyltransferase-1 (*HPRT1*). Amplifications were designed to span exon junctions to detect only spliced mRNA products. Commercially prepared RNAs from human skeletal muscle and thymus (Ambion) were used as positive tissue controls and reference standards for comparison with biopsied muscle.[26] With the exception of *MYO1H*, 15-30ng of masseter muscle RNA was used for each assay. Because of its low copy number, *MYO1H* analyses required approximately 10 fold greater amounts (100-150ng) of RNA. The *MYO1C* gene probe was complimentary to a sequence common to all 3 protein isoforms. After tests to establish assay conditions, a 25ng amount of skeletal muscle standard was selected as a reference calibrator and relative quantities were determined by the comparative threshold cycle (C_T) method (C_T)[27] that measures fold-difference between normalized amounts of target in test samples and in an internal reference when both genes are amplified at approximately the same efficiency.

2.4. Statistical Comparisons

Descriptive statistics, including mean and standard deviation, were calculated and used to compare data according to sex, age and vertical and sagittal malocclusion groups in independent and combined analyses. Using a 2-way ANOVA with interaction, a model was created consisting of the variables: sex, age, vertical malocclusion groups (deep, normal, open), sagittal malocclusion classes (II, III) and vertical by sagittal to predict gene expression. An unpaired *t*-test was used to evaluate significant differences in gene

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expression between class II and class III in sagittal dimension malocclusion. A third statistical analysis consisted of creating correlations using the Pearson comparison. In both analyses, P values less than 0.05 were considered significant.

3. Results

3.1. Expression of MYO1C, RUNX2, KAT6B and MYO1H in masseter muscle compared to malocclusion class

The four genes of interest were differentially expressed between malocclusion groups (Table 2), but significance was determined only for *KAT6B* values between sagittal classes. By ANOVA comparisons, there was no effect of age and sex on expression values. Although relative quantities of *MYO1C* and *KAT6B* were greatest in muscle from skeletal open bite subjects, differences between vertical and combined vertical and sagittal malocclusion groups were not statistically significant by an ANOVA test. In sagittal malocclusion, as previously reported[16], an unpaired *t*-test of data showed significance for *KAT6B* RNA that was greater in muscle of Class III than in Class II subjects ($P = 0.007$). No significant expression differences between vertical and sagittal dimension classes were detected for either the transcription factor *RUNX2* or *MYO1H*. *MYO1H* RNA was expressed at least 100 fold less than *MYO1C* and was not detected in most samples, but quantities were sufficient for analysis in 10 of the masseter muscles (Table 2).

3.2. Correlation of masseter muscle fiber type % occupancy with MYO1C gene expression

Fiber type occupancy values of the 4 major fiber types in masseter muscles were examined for a correlation with *MYO1C* quantities for each malocclusion classification (Table 3). A significant positive correlation was found for hybrid type (I/II) fiber percent occupancy in the vertical dimension normal bite group, but no other correlations reached significance

3.3. Correlation of masseter muscle fiber type % occupancy with RUNX2 gene expression

RUNX2 gene expression had highly significant positive correlations with the percent occupancy of type II fibers in masseter muscle (Table 4). Strong positive correlations in sagittal Class III and vertical open bite greatly contributed to the overall significance seen here. The only negative correlation in type II fibers occurred in normal bite subjects. Significant negative correlations in all subjects and in vertical deep bite were found with the hybrid I/II fibers that co-express both slow type I myosin and fast type IIA and IIX. Other correlations were non-significant.

3.4. Correlation between the expression of MYO1C with KAT6B and RUNX2

Analysis of *RUNX2* expression indicated a significant correlation with *MYO1C* only in the vertical normal bite group (Table 5). Analysis of expression levels of *KAT6B* in a subset of samples showed a highly significant positive correlation with *MYO1C* for all subjects regardless of malocclusion. When considered by malocclusion class, significance was high for both sagittal groups. For the vertical classifications, significance was high for both normal and open bites. Expression correlations between *KAT6B* and *RUNX2* showed no relationship (data not shown). The relative abundance of *KAT6B* and *RUNX2* were

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calculated from the efficiency of amplification and difference in Ct (cycle threshold) values. *KAT6B* RNA was approximately 50 (i.e. 53.4) fold greater than *RUNX2*.

4. Discussion

Malocclusion is a common disorder that requires identification of its underlying causes in order to improve diagnosis and prevention. Numerous studies have confirmed the association between skeletal form and muscle function, with muscle fiber composition being a significant factor in affecting skeletal development.[24] Also, muscle fiber type properties and fiber percent occupancy have been shown to have robust statistical relationships to facial growth in the vertical dimension.[3,28-29] These reports from different research groups agree that as masticatory muscle type II fiber populations increase, there is an associated decrease in vertical facial dimension. Conversely, when type I fibers predominate; there is an increase in clockwise mandibular rotation and development of skeletal open bite malocclusion. These observations suggest that an appropriate distribution between fast contracting type II and slow contracting type I muscle fibers is necessary to ensure balanced muscle function during vertical skeletal growth of the face and eruption of teeth. These studies also suggest that muscle function has limited effects on growth in length of jaw bones, and therefore the sagittal dimension. Genetic investigations on growth and anthropometric traits indicate that heritability has very strong influence in development of skeletal muscle size, strength and human height.[5,6] Since occlusal phenotypes encompass muscle and bone, sets of genes that act independently in each tissue and genes with common effects in both tissues should be considered in the development of malocclusion.[2-3,7] We selected gene expression of *MYO1H*, *MYO1C*, *KAT6B* and *RUNX2* in masseter muscle for further study because they have known associations with malocclusions and masseter muscle fiber type composition.

Expression of MYO1H was found to be extremely low in the masseter muscle samples, from which we conclude that it does not contribute to variability in muscle composition in adult subjects. *MYO1C* motor function promotes the translocation of *GLUT4* vesicles to the plasmalemma in response to insulin stimulation[15] and *GLUT4* expression in masseter does show a difference between open and deep bite subjects.[16] However, although we found that *MYO1C* was abundantly expressed, it showed little evidence of any significant correlation with fiber type composition, and also has not been identified as a gene responsive to endurance exercise programs in animal studies.[30] A more interesting possibility is that the significant results for *MYO1C* expression lies with its nuclear function, not the cytoplasmic one.

NM1 (*MYO1C* isoform 3) participates in chromatin remodeling[20], which leads to activation of histone acetyltransfeases (HATs). The HATs confer transcriptional specificity since they function as active gene promoters.[21] Using RT-PCR we recently found that expression of a HAT, K(lysine) acetyltransferase 6B (*KAT6B*), positively correlates with mandibular prognathism, and with Class II myosin heavy chain (MHCs type IIA and IIX) in masseter muscle.[16] In this study we found a highly significant association between *MYO1C* and *KAT6B* for all malocclusion groups (Table 5). As this correlation was always positive, it is probably related to the expression of *MYO1C* isoform 3, i.e. NM1, and supports the role of

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MYO1C in HAT activation in skeletal muscle. The *MYO1C* gene probe used for RT-PCR did not distinguish between the *MYO1C* gene transcripts, therefore further study of the relationship between *NM1* and *KAT6B* is necessary.

The association of *KAT6B* with mandibular prognathism could be related to its activation of the osteogenic transcription factor RUNX2, which is essential to bone growth and maintenance.[31] Given that *KAT6B* is a potent regulator of RUNX2 expression in skeletal tissues[22], we investigated the relationship of *RUNX2* with *MYO1C*. Contrary to expectation, although *RUNX2* was expressed in the muscle samples, it showed little overall relationship with *MYO1C* (Table 5) or *KAT6B*. However, very surprisingly, it showed a very significant correlation with type II fiber occupancy (Table 4). Apart from a small and non-significant negative correlation in normal bite subjects, the correlation with type II occupancy was large, positive and significant in all other malocclusions. These correlations have significant implications for facial form in both the sagittal and vertical dimensions. In the sagittal dimension, the mechanism by which *RUNX2* might operate is through its effect on condylar growth[23], and periosteal activation of osteoblast gene expression.[32] In the vertical dimension, we know that variation in type II fiber occupancy is important, but there is as yet no information about the mechanism by which *RUNX2* could affect muscle composition in the adult.

Here, we demonstrate that a Class I myosin associates with *KAT6B* (a histone acetyltransferase, active in promotion of gene expression) which is known to influence muscle fiber type properties and mandibular condylar growth. Although the association of *MYO1H* with mandibular prognathism suggests there may be an important link between class-I myosins and malocclusion, we found it was expressed at extremely low levels in the muscle, which suggests its effects are more likely exerted during growth, rather than in the mature muscle. By contrast, its paralog *MYO1C* is highly correlated with *KAT6B* across all malocclusion classes, suggesting an important function of the nuclear isoform. The surprising and highly significant correlation of *RUNX2* expression with masseter muscle type II fiber occupancy remains to be explained. Future studies investigating its expression at the cellular level may throw some light on this observation. Vertical and sagittal jaw deformation is difficult to treat [33], in part because the underlying mechanisms which produce them are not well understood and may lead to relapse after treatment. Genetic and epigenetic studies offer an opportunity to identify new factors which will lead to discovery of the molecular pathways involved in the etiology and severity of malocclusion, with the potential for enhanced diagnosis and clinical treatments, including long-term stability.

Acknowledgments

This work is supported by the National Institute of Dental & Craniofacial research through a grant to Dr. Sciote; Musculoskeletal Heritable Influences on Malocclusion - R21DE022427. The previous report on MYO1H (Tassopoulou-Fishell et al. 2012) was supported by an Orthodontic Faculty Development Fellowship Award from American Association of Orthodontists Foundation.

A portion of this work was supported by a S. Eugene Coben Endowed Scholarship to Dr. Desh for her Master for Science in Oral Biology Thesis for the Temple University Graduate School.

We gratefully acknowledge Professor Eugene Whitaker, DMD, PhD, Department of Restorative Dentistry and Jeffrey H. Godel DDS, Department of Orthodontics, Kornberg School of Dental Medicine, Temple University.

They participated as Thesis Committee members for Dr. Desh's Masters of Science degree in Oral Biology from Temple University Graduate School.

Statistical support was provided by Patrick Hardigan from Nova Southeastern University, College of Dental Medicine.

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Table I

Distribution of malocclusion types among subjects

Gender	Subjects (n)	Mean Age (yr)	Occlusal Dimension			
			Vertical	**(n)**	**Sagittal**	**(n)**
			Normal Bite	9		
					Class-II	17
Female	28	21.7	Open Bite	12		
					Class-III	11
			Deep Bite	7		
			Normal Bite	6		
					Class-II	10
Male	21	21.9	Open Bite	7		
					Class-III	11
			Deep Bite	8		

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Table 2

Expression of genes of interest in masseter muscle from malocclusion patients undergoing orthognathic surgery.[a]

	Vertical Dimension			Sagittal Dimension	
Gene	Normal	Open	Deep	Class II	Class III
MYO1C	0.880 ± 0.42 (n = 15)	1.03 ± 0.46 (n = 19)	0.83 ± 0.32 (n = 15)	0.89 ± 0.38 (n =27)	0.96 ± 0.45 (n = 22)
RUNX2	0.731 ± 0.71 (n = 15)	0.87 ± 0.63 (n = 19)	0.88 ± 0.64 (n = 15)	0.89 ± 0.67 (n = 27)	0.75 ± 0.62 (n = 22)
KAT6B	3.348 ± 1.31 (n = 11)	4.08 ± 1.50 (n = 16)	3.68 ± 1.48 (n = 8)	3.32 ± 1.09[b] (n = 21)	4.41 ± 1.09[b] (n = 14)
MYO1H	1.414 ± 0.33 (n = 3)	1.41 ± 1.39 (n = 5)	0.81 ± 0.04 (n = 2)	1.23 ± 0.70 (n = 6)	1.38 ± 1.42 (n = 4)

[a] Values are relative quantities of RNA ± SD

[b] Significant difference in expression, P<0.01

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Table 3

Correlations between *MYO1C* gene expression and fiber % occupancy in masseter muscles from subjects with malocclusion.

				Fiber Type		
		n	I	Hybrid I/II	II	Neo/Atrial
All Subjects		49	-0.11	0.12	-0.03	0.01
Occlusal Dimension						
Sagittal	Class II	27	-0.04	-0.13	0.13	0.05
	Class III	22	-0.15	0.34	-0.20	-0.03
Vertical	Normal Bite	15	-0.48	0.66[a]	-0.12	-0.13
	Open Bite	19	0	-0.05	0.01	0.10
	Deep Bite	15	-0.33	-0.08	0.38	-0.12

[a] $P<0.01$

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Table 4

Correlations between RUNX2 gene expression and fiber % occupancy in masseter muscles from subjects with malocclusion.

			Fiber Type			
		n	I	Hybrid I/II	II	Neo/Atrial
All Subjects		49	-0.07	-0.28[a]	0.43[c]	-0.16
Occlusal Dimension						
Sagittal	Class II	27	-0.09	-0.20	0.39[a]	-0.18
	Class III	22	-0.11	-0.36	0.54[b]	-0.15
Vertical	Normal Bite	15	-0.03	0.14	-0.15	-0.07
	Open Bite	19	-0.34	-0.31	0.86[d]	-0.18
	Deep Bite	15	0.30	-0.59[a]	0.51[a]	-0.48

[a] $P<0.05$;

[b] $P<0.01$;

[c] $P<0.005$,

[d] $P<0.000005$

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Table 5

Correlations of gene expression for MYO1C with KAT6B and RUNX2 in masseter muscles from subjects with malocclusion.

		KAT6B	RUNX2
All Subjects		0.78^{d} (N = 35)	0.04 (N = 49)
Occlusal Dimension			
Sagittal	Class II	0.71^{c} (N = 21)	0.22 (N = 27)
	Class III	0.84^{b} (N = 14)	-0.14 (N = 22)
Vertical	Normal Bite	0.81^{b} (N = 11)	0.52^{a} (N = 15)
	Open Bite	0.79^{c} (N = 16)	-0.24 (N = 19)
	Deep Bite	0.68 (N = 8)	-0.16 (N = 15)

[a] P<0.05;

[b] P<0.005;

[c] P<0.0005;

[d] P<0.0000001