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Altered Ion-responsive Gene Expression in *Mmp20* Null Mice

ABSTRACT

During enamel maturation, hydroxyapatite crystallites expand in volume, releasing protons that acidify the developing enamel. This acidity is neutralized by the buffering activity of carbonic anhydrases and ion transporters. Less hydroxyapatite forms in matrix metalloproteinase-20 null (*Mmp20*$^{-/-}$) mouse incisors, because enamel thickness is reduced by approximately 50%. We therefore asked if ion regulation was altered in *Mmp20*$^{-/-}$ mouse enamel. Staining of wild-type and *Mmp20*$^{-/-}$ incisors with pH indicators demonstrated that wild-type mice had pronounced changes in enamel pH as development progressed. These pH changes were greatly attenuated in *Mmp20*$^{-/-}$ mice. Expression of 4 ion-regulatory genes (*Atp2b4, Slc4a2, Car6, Cftr*) was significantly decreased in enamel organs from *Mmp20*$^{-/-}$ mice. Notably, expression of secreted carbonic anhydrase (*Car6*) was reduced to almost undetectable levels in the null enamel organ. In contrast, *Odam* and *Klk4* expression was unaffected. We concluded that a feedback mechanism regulates ion-responsive gene expression during enamel development.

KEY WORDS: enamel, pH, MMP20, amelogenesis, mineralization.

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INTRODUCTION

Enamel development is stage-specific. The two predominant stages are the secretory and maturation stages. During the secretory stage, a protein scaffold is formed, and the mineral phase appears as long thin ribbons that grow out to form the full thickness of the enamel layer. During the maturation stage, the protein scaffold is removed, while the enamel ribbons grow in width and thickness as the enamel matures into its final hardened form. It is during the maturation stage that the vast majority of hydroxyapatite mineral precipitates, and this precipitation reaction releases between 8 and 14 mol of H^+ ions (depending on the phosphate precursor) for every mol of hydroxyapatite produced (Simmer and Fincham, 1995). Thus, the newly formed enamel becomes acidic, and this acidity is neutralized by the combined activities of carbonic anhydrases and ion transporters. Five of these genes are known to be expressed by ameloblasts during enamel formation. These are carbonic anhydrase II (*Car2*, CAII; intracellular pH regulation), carbonic anhydrase VI (*Car6*, CAVI; extracellular pH regulation), cystic fibrosis transmembrane conductance regulator *(Cftr*; membrane-associated Cl^- channel), solute carrier family 4 anion exchanger member 2 (*Slc4a2*, AE2; Cl^--HCO_3^-exchanger), and solute carrier family 4 sodium bicarbonate co-transporter member 4 (*Scla4a4*, NBCe1; Na^+-coupled HCO_3^- transporter) (Lin *et al*., 1994; Gawenis *et al*., 2001; Sui *et al*., 2003; Smith *et al*., 2006; Lyaruu *et al*., 2008; Paine *et al*., 2008). Models for how these ion transporter genes work cooperatively to regulate pH have been proposed (Lacruz *et al*., 2010).

Additionally, the process of mineralization requires a large volume of Ca^{2+} to be transported by the ameloblasts into the forming enamel matrix. Genes encoding 4 enamel organ calcium transporters have been implicated in this process. They include plasma membrane Ca^{2+} ATPase-1 (*Atp2b1*; PMCA-1), plasma membrane Ca^{2+} ATPase-4 (*Atp2b4*; PMCA-4), and the newly identified solute carrier family 8 member 1 (*Slc8a1*; NCX1; Na^+-Ca^{2+} exchanger) and solute carrier family 8 member 3 (*Slc8a3*; NCX3; Na^+-Ca^{2+} exchanger) (Borke *et al*., 1995; Okumura *et al*., 2010).

Four proteins are secreted into the enamel matrix during the secretory stage of enamel formation. These are: amelogenin, ameloblastin, enamelin, and matrix metalloproteinase-20 (MMP20, enamelysin). Disruption of any one of the genes encoding these proteins in mice causes severe dental enamel defects, and mutations in all but ameloblastin have been demonstrated to cause enamel defects (amelogenesis imperfecta) in humans (reviewed in Hu *et al*., 2007). The full-length proteins are present for only a short time near the mineralizing front of the most recently formed enamel. MMP20 cleaves the secreted enamel proteins, and these cleavage products may form an organic mold that supports the growth of the elongating ribbons.

Table. Gene-specific Primers for Quantitative PCR

	GenBank ID #	5′ Primer	3′ Primer	PrimerBank ID	°C
Klk4	NM_019928	CTGGCAGCCGGATGTTAGAGG	AGGAGTGGGGCATTGGGTAGC	n/a	64
Odam	NM_027128	GCTTTTGACAGCTTTGTAGGC	AAGCAGGCTTCCTTCTACTGG	n/a	60
Atp2b1	NM_026482	GCCATCTTCTGCACCATTG	CCGCCAAACTGCACAATTA	n/a	64
Atp2b4	NM_213616	AGATGTCGGGTTTGCTATGG	ATGATGTCTGACGCCTCCTT	n/a	63
Car2	NM_009801	ACTGGAACACCAAATATGGGGA	GCAAGGGTCGAAGTTAGCAAAG	31981657a3	63
Car6	NM_009802	CTAACAACGGACACACAGTATCG	GCCTTTGAGATGAACTCAGTGC	28461317a2	63
Cftr	NM_021050	CCGGTGACAACATGGAACACATAC	CCAGTACGCACCAAATCAGCACTA	n/a	63
Slc4a2	NM_009207	AACTTCGTACCTTAGGTGTGGA	GGCGGTGGTATTCAAAGTCTT	6678021a2	63
Slc4a4	NM_018760	GAAGGTCACCACACGATCTACA	TCCACATCAGATTTGTCGGAGT	9055346a1	64
Ef1α1	NM_010106	ATTCCGGCAAGTCCACCACAA	CATCTCAGCAGCCTCCTTCTCAAAC	n/a	62
Actb	NM_007393	TGACGGCCAGGTCATCACTATT	ACCCAAGAAGGAAGGCTGGAAA	n/a	65
Casc3	NM_138660	GGATCGGAAAAACCCAGCCTACAT	TGTTCCCAGAGACCCTCATCTT	n/a	65
Gapdh	NM_008084	GCAAAGTGGAGATTGTTGCCAT	CCTTGACTGTGCCGTTGAATTT	n/a	64

Primers were designed by analysis of annealing sites by DNAStar Lasergene software and designed to flank intron-exon boundaries or were taken from PrimerBank (Wang and Seed, 2003; Spandidos *et al.*, 2008). n/a = not applicable. °C = annealing temperature.

Interestingly, the enamel from *Mmp20* null mice is severely affected during the maturation stage of development, when MMP20 is no longer expressed (Caterina *et al*., 2002; Bartlett *et al*., 2004). Perhaps this can be attributed to the formation of a defective scaffold during the secretory stage that interferes with the normal growth in width and thickness of the enamel ribbons during the maturation stage. In any case, the enamel from *Mmp20* null incisors has 7-16% higher-than-normal levels of water and protein *per* unit weight than that from wild-type animals, and the enamel mineral content is reduced by approximately 50% (Bartlett *et al*., 2004). Therefore, the quantity of protons released by the precipitating enamel should also be reduced by approximately 50%. This prompted us to ask if expression of ion-responsive genes was reduced in the poorly mineralized enamel.

MATERIALS & METHODS

All animals used in this study were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities. Wild-type and *Mmp20* null C57BL/6 strain mice have been previously described (Caterina *et al*., 2002).

Quantitative Real-time PCR

Expression of odontogenic ameloblast-associated protein precursor (*Odam*; Apin) and kallikrein-4 (*Klk4*) was examined in wild-type and *Mmp20* null enamel organs by qPCR. Five ion-responsive genes (*Car2, Car6, Cftr, Slc4a2*, and *Slc4a4*) and 2 calcium exchange genes (*Atp2b1, Atp2b4*) were also examined. Mouse first molars were harvested from wild-type and *Mmp20*$^{-/-}$ mice at post-natal day 11 (mid-maturation stage, n = 6). The dental papilla was carefully removed, and total RNA was extracted from the enamel organ according to the manufacturer's protocol with TRIzol (Invitrogen, Carlsbad, CA, USA). cDNA was transcribed with the SuperScript III First-Strand Synthesis system (Invitrogen). The enamel organ was subjected to qPCR analysis by iQ SYBR green (Bio-Rad, Hercules, CA, USA). Gene-specific primers (Table) were from PrimerBank (Spandidos *et al*., 2008) or were designed by analysis of annealing sites that flank intron-exon boundaries (DNAStar Lasergene, Madison, WI, USA). Standard curves were generated with each primer set by control cDNA preparations and a 10-fold dilution series ranging from 100 ng/μL to 100 pg/μL. PCR efficiencies and relative expression levels as a function of multiple housekeeping genes [E*ef1α1* (eukaryotic translation elongation factor 1α1 is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome), *Casc3* (cancer susceptibility candidate 3 functions in nonsense-mediated mRNA decay), *Actb* (β-actin), and *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase)] were calculated as previously described (Vandesompele *et al*., 2002). Statistical significance was determined by *t* test (GraphPad Prism 5).

Staining of Incisors with pH Indicators

Hemimandibles and hemimaxillae from adult wild-type and *Mmp20* null mice were removed, immersed in liquid nitrogen, and freeze-dried at –55°C for 48 hrs. The bone and enamel organs covering the incisors were removed, and the exposed enamel surfaces were gently wiped clean of cellular debris. Incisors were then dipped into their respective pH indicator (methyl red, bromophenol red, or resazruin) and photographed (Sasaki *et al*., 1991).

RESULTS

Loss of MMP20 Expression Does Not Affect the Expression of ODAM or Kallikrein-4

Enamel from *Mmp20* null incisors has higher-than-normal levels of protein than does that from wild-type animals. We therefore sought to determine if the loss of MMP20 expression caused a compensatory up-regulation of *Klk4* in an attempt to clear the excess protein. At the mRNA level, we found no difference in

Klk4 expression between wild-type and *Mmp20* null mouse enamel organs (Fig. 1A). Similarly, the expression of the maturation-stage-specific gene *Odam* was not altered (Fig. 1B).

Staining of Mouse Incisors with pH Indicators Demonstrates that *Mmp20* Null Enamel has an Altered Banding Pattern Indicative of Altered Ion Control

In *Mmp20* null incisors, the enamel mineral content is reduced by approximately 50%, which reduces the quantity of protons released by hydroxyapatite growth. We therefore examined the pH of *Mmp20* null enamel during development. A comparison of wild-type rat and mouse incisor banding patterns was made by use of methyl red staining (Fig. 2, top panels). Methyl red stains areas of acidity red, and neutral areas remain unstained. Note that for mandibular incisors, the rat has at least one more band of acidity than does the mouse. The 4 bottom panels of Fig. 2 show incisors from wild-type and *Mmp20* null mice. For each of these panels, the top incisor is stained with methyl red, the middle incisor is stained with bromophenol red (neutral areas stain a shade of pink, and acid areas stain brownish-red), and the bottom incisor is stained with resazurin (neutral areas stain light blue, and acid areas stain a shade of brown). The staining pattern of the *Mmp20* null incisors is distinctly different from that of the wild-type control. The areas of acidity are greatly reduced in the null mouse enamel when compared with controls. This prompted us to ask if ion exchange or bicarbonate regulation was altered in the null mouse enamel organ.

Expression of Selected Ion-responsive Genes are Significantly Reduced in *Mmp20* Null Mouse First Molar Enamel Organs Compared with Enamel Organs from Wild-type Controls

Unerupted first molars from day 11 mice will contain predominantly mid-maturation-stage ameloblasts. Compared with wild-type enamel organ, the *Mmp20*$^{-/-}$ mouse enamel organ had significant reductions in maturation-stage gene expression for *Atp2b4, Cftr*, and solute carrier *Slc4a2* (Figs. 3B, 3E-3F). No significant change in expression was seen for *Atp2b1*, *Car2*, or *Slc4a4* (Figs. 3A, 3C, 3G). *Car6* showed the most dramatic change in gene expression between wild-type and null enamel organs, since it was decreased to almost undetectable expression levels in the *Mmp20* null mouse (Fig. 3D). This suggests that while enamel matrix genes are not sensitive to feedback inhibition, ion-responsive genes do sense and adjust to external ion concentrations.

DISCUSSION

Here we show that loss of MMP20 causes decreased expression of several ion-responsive genes in mouse enamel organ. We attribute this to a feedback mechanism that senses altered pH during enamel development. During the maturation stage of enamel formation, the mass precipitation of hydroxyapatite normally causes the extracellular matrix to become mildly acidic, with pH as low as 6.2 (Smith *et al*., 1996). Mice lacking *Mmp20* expression contain approximately 50% less bulk mineral, so fewer H^+ ions are produced during the maturation of *Mmp20*$^{-/-}$ enamel, resulting in less acidity. Examination of incisors dipped in various pH indicators clearly demonstrated that the pH of developing enamel from *Mmp20*$^{-/-}$ mice is different from wild-type mice. The *Mmp20* null mouse enamel is less acidic.

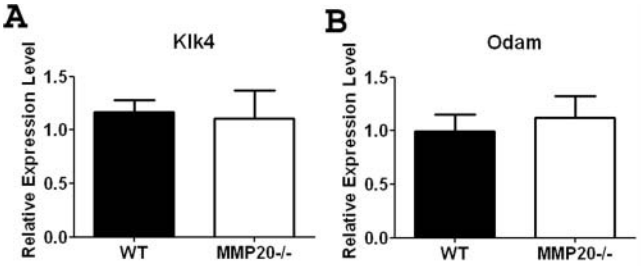

Figure 1. Expression of *Odam* and *Klk4* in the maturation-stage mouse enamel organ. Expression of *Klk4* **(A)** and *Odam* **(B)** was not significantly altered in the *Mmp20* null mouse. Data are presented as mean ± SEM and represent measurements of 6 individual mice, with duplicate measurements for each mouse (n = 6). Results are presented as relative gene expression normalized to the geometric mean of *Eef1α1, Gapdh, β-actin,* and *Casc3* mRNA expression. Statistical analysis was determined by *t* test.

The regulation of ion concentration and pH during enamel development is vital for proper enamel formation. Acidotic and alkalotic rats and dogs have disturbed enamel mineralization (Angmar-Mansson and Whitford, 1990; Whitford and Angmar-Mansson, 1995). Individuals with mutations and mice that are null for *Slc4a2, Slc4a4*, or *Cftr* have malformed dental enamel, among other abnormalities, thus highlighting the critical role of these genes in enamel formation (Wright *et al*., 1996a,b; Sui *et al*., 2003; Demirci *et al*., 2006; Gawenis *et al*., 2007; Lyaruu *et al*., 2008). Additionally, 2 enzymes generating bicarbonate (CAII and CAVI) are expressed in the enamel organ. Our results show that the expression of these genes, with the exception of *Car2* (CAII), was decreased during enamel formation in the *Mmp20*$^{-/-}$ mouse enamel organ. The most dramatic change was the decrease to almost undetectable *Car6* (CAVI) expression in the *Mmp20* null mouse.

CAVI is a secreted zinc metalloenzyme that catalyzes the reversible hydration reaction of carbon dioxide with water to produce carbonic acid [$CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$]. CAVI has been identified in numerous fluids and tissues, including serum, saliva, milk, salivary and mammary glands, and liver (Nishita *et al*., 2007). It has been implicated in the protection of surface epithelial cells from gastric ulcers (Parkkila *et al*., 1997), and decreased secretion of CAVI is associated with distortion and loss of taste and smell (Henkin *et al*., 1999). There is no null mouse for *Car6* to elucidate the function of CAVI in enamel formation. However, it was postulated that CAVI may aid with local buffering by providing bicarbonate ions or recycling excess carbonic acid (Smith *et al*., 2006).

Since we did not detect any differences in expression of *Odam* or *Klk4* in the knockout mouse, we assume that the

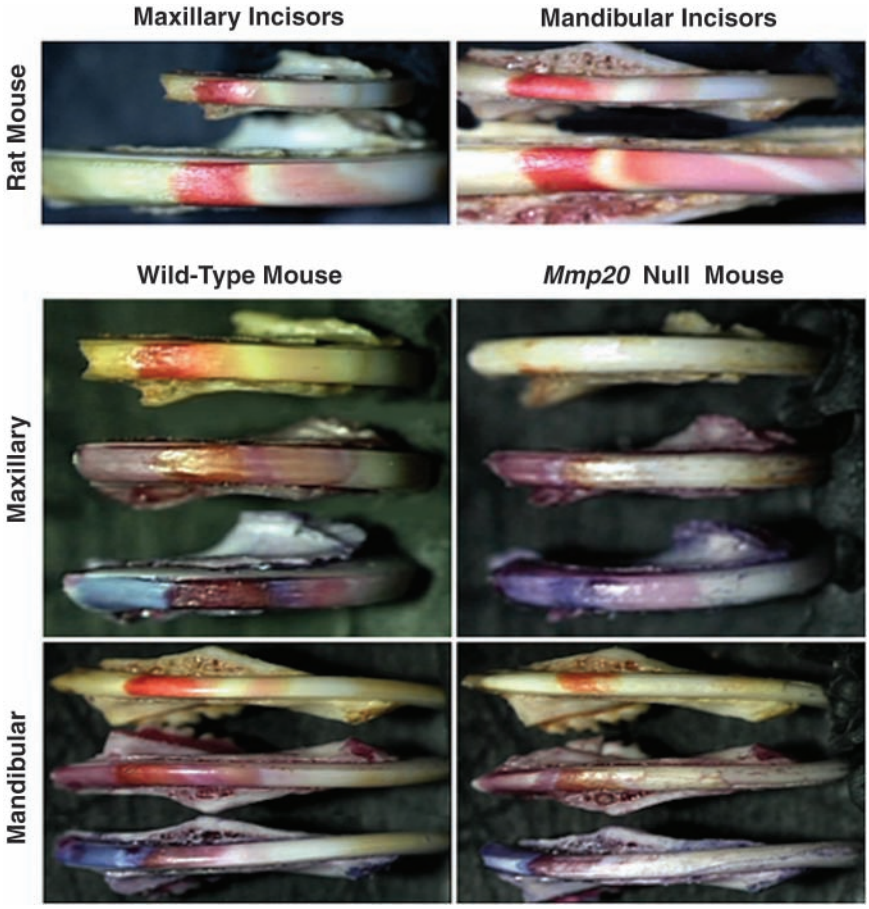

Figure 2. Staining of incisors with pH indicators. A comparison of wild-type rat and mouse incisor banding patterns was made by use of methyl red staining (**top panels**). Note that for mandibular incisors, the rat has at least one more band of acidity than does the mouse. The 4 **bottom panels** show incisors from wild-type and *Mmp20* null mice. For each of these panels, the top incisor is stained with methyl red, the middle incisor is stained with bromophenol red, and the bottom incisor is stained with resazurin. The staining pattern of the *Mmp20* null incisors is distinctly different from that of the wild-type control, and the areas of acidity are greatly reduced in the null mouse enamel.

decreased expression of the ion-responsive genes is directly correlated with altered pH. Although the mechanisms of regulation of these ion transporter genes are still being elucidated, it is evident that their expression is affected by pH, and that they regulate one another. *Car2* expression is up-regulated in fish exposed to acidic conditions (Hirata *et al*., 2003), and Paine *et al*. found that *Slc4a2* and *Slc4a4* transcripts increased when the pH fell below 7.0 in LS8 ameloblast-like cells (Paine *et al*., 2008). In contrast, *Slc4a2* is up-regulated in the kidney cortical collecting duct under alkaline load and is decreased during metabolic acidosis (Fejes-Toth *et al*., 1998). Furthermore, *Slc4a2* is activated by alkaline pH in transiently transfected CHOP and 293 cells (reviewed in Alper *et al*., 2002). *Slc4a2* may be regulated by *Car2* (Alper *et al*., 2002), and *Cftr* is regulated by *Slc26a6*, which encodes a chloride, oxalate, sulfate, and bicarbonate transporter (Wang *et al*., 2006). CFTR in turn is an ion channel regulator which, through feedback mechanisms, controls the activity of SLC26a6 and other transporters, including the Na^+/H^+ exchanger (NHE), HCO_3^-/Cl^- exchanger and co-transporters, intermediate conductance outwardly rectifying (ICOR) Cl^- channels, and Ca^{2+}- and volume-activated Cl^- channels (reviewed in Steward *et al*., 2005; Linsdell, 2006). Therefore, although the ion-responsive genes influence each other's activity, it is not known specifically how this occurs or which gene is the master regulator.

It has been suggested that ions will likely affect calcium transport. External Na^+ concentration would be expected to affect Na^+-Ca^{2+} exchangers, whereas external H^+ would likely affect plasma membrane Ca^{2+}-ATPase expression (Hubbard, 2000). We found the expression of 2 Na^+-Ca^{2+} exchangers, *Slc8a1* (NCX1) and S*lc8a3* (NCX3), to be at very low levels in mouse enamel organ and were therefore not included in our present analysis. We were able to examine the effect of loss of MMP20 on *Atp2b4* (PMCA-4) and *Atp2b1* (PMCA-1) expression. Expression of *Atp2b4* was significantly decreased in null mice, whereas *Atp2b1* expression was unaltered. These 2 ubiquitously expressed Ca^{2+}-ATPases were originally proposed to serve as housekeeping genes, but recent studies suggest that they are functionally independent. PMCA-1 is proposed to be required for the maintenance of intracellular Ca^{2+}, and PMCA-4 may play a primary role in Ca^{2+} transport and efflux (Magosci *et al*., 1992; Borke *et al*., 1995). We propose that the decreased H^+ production in the null mouse down-regulates *Atp2b4* expression, thereby reducing Ca^{2+} extrusion and causing further reduction of hydroxyapatite formation. Interestingly, 2 of the 3 ion-regulating genes whose expression was not altered are the proposed intracellular-regulating genes *Car2* and *Atp2b1*.

We conclude that a feedback mechanism regulates ion-responsive gene expression during enamel development. Current models depict CAII, CAVI, CFTR, AE2, and NBCe1 working cooperatively to regulate ion concentrations and pH in ameloblasts and the enamel matrix (Lacruz *et al*., 2010). pH regulation is critical for proper enamel formation and involves an integrated network of carbonic anhydrases and ion transporters.

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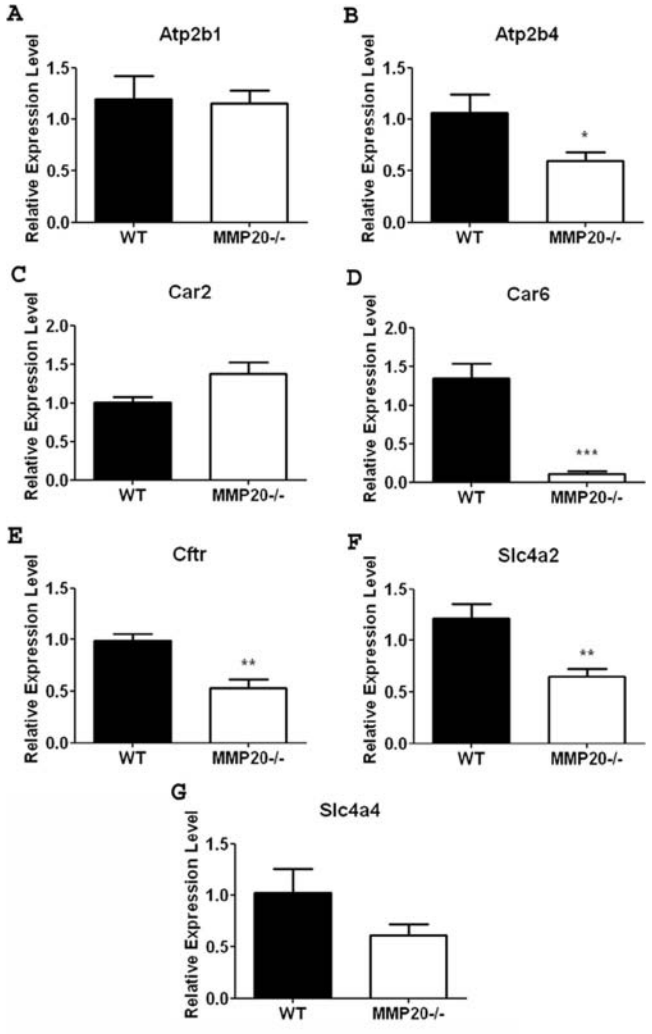

Figure 3. Ion transporter gene expression in maturation-stage mouse enamel organ. *Atp2b4* **(B)**, *Car6* **(D)**, *Cftr* **(E)**, and *Slc4a2* **(F)** all showed a significant decrease in mRNA expression in the absence of MMP20. Expression of *Atp2b1* **(A)** and *Car2* **(C)** was not significantly altered in the *Mmp20* null mouse. Although a trend existed, the data for *Slc4a4* **(G)** was not significant due to variability within the data sets. Data are presented as mean ± SEM and represent measurements of 6 individual mice, with duplicate measurements for each mouse (n = 6). Results are presented as relative gene expression normalized to the geometric mean of *Eef1α1*, *Gapdh*, *β-actin*, and *Casc3* mRNA expression. Statistical analysis was determined by *t* test (*p < 0.05, **p < 0.01, ***p < 0.001).

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