microRNA profiles associated with continuous growth of lower incisor in rat model

Sirawit Nipakasem[1*], Waleerat Sukarawan[2,3], and Anjalee Vacharaksa[1,2,4]

[1]Master of Science Program in Geriatric Dentistry and Special Patients Care (International Program), Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand
[2]Research Unit on Oral microbiology and Immunology, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand
[3]Department of Pediatric Dentistry, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand
[4]Department of Microbiology, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand
[*]Corresponding author, E-mail: 6075838732@student.chula.ac.th

Abstract

Rodent incisor is an organ that continuously grows throughout its lifetime to compensate for abrasion. The development process of incisors is a result of delayed root formation and prolonged crown formation, possibly made by stem cell niche, so-called cervical loop, which involves various genes and signaling pathways. However, the growth of the molar discontinues after completing the root formation. MicroRNAs (miRNAs) are small non-coding single-stranded RNAs that involves biological mechanisms of dental development. To study the role of miRNA, the researchers extracted total RNA from the dental pulp tissue collected from the apical tips of incisors and molars and compared the expression of miRNAs. The customized RT-PCR array (Qiagen) demonstrated that the apical part of the incisors expressed miR-720 4-fold higher than that from the molar. Besides, miR-652-33p in the incisors expressed 2-fold higher than that in the molar. On the other hand, miR-338-3p and miR-27a-3p were decreased as compared to the molar group. The results suggested that these miRNAs may have a role in the mineralization process during prolonged crown formation of rodent incisors.

Keywords: *Rodent incisor, Hypselodont, Dentine formation, Continuous growth, microRNA, Cervical loop, Dental Pulp Stem cell*

1. Introduction

The rodent incisor is an organ that continuously grows throughout its lifetime. This growth compensates for abrasion at the distal end of the tooth. The intermediate state during dental development called continuous growth of incisor is believed to be a result of delayed root formation that causes prolonged crown formation (Tummers, 2003). The formation of enamel and dentine in continuous growth is possibly happened by active adult epithelial and mesenchyme stem cells that aggregated from a stem cell niche called cervical loop (Harada et al., 1999; I. Thesleff & Tummers, 2008; Tummers, 2003). The labial cervical loop (laCL) plays a major role in providing the stem cells that can produce ameloblasts that form enamel on the labial side (Seidel et al., 2010). Meanwhile, the mesenchyme stem cells surrounding the cervical loop are differentiated into odontoblast like cells that further form dentinal tubule upright to the surface of enamel (Jiang et al., 2014).

Concerning amelogenesis, several signaling pathways regulate the maintenance and proliferation of the epithelial stem cell niche and their progeny in the laCl to maintain the cervical loop structure and modification of the crown to root transition. The pathways involved in the process are such as fibroblast growth factors (FGFs), WNT signaling, bone morphogenetic protein (BMP), E-cadherin, transforming growth factor-b (TGF-b), and sonic hedgehog (Shh). Fibroblast growth factors (FGFs) regulate dental epithelial stem cells. FGF3 stimulates the proliferation of inner enamel epithelial cells, while FGF7 promotes the proliferation of outer enamel epithelial cells. FGF8 is an autocrine regulator, and FGF10 also maintains the epithelial stem cells in incisors (Jin, Wang, Cheng, Zhao & Li, 2017). For example, the expression of FGF10 in the mesenchyme and components of the Notch signaling pathway in the epithelium was maintained in the continuously growing teeth. In contrast, the disappearance of their expression was correlated with the root formation and loss of the cervical loop stem cell niche in non-continuously growing teeth (Tummers,

2003). The WNT signaling pathway is the vastly related signaling pathway at all stages of dental development. At the secretory stage, the WNT pathway inhibition shows a positive role in enamel formation (Jin et al., 2017). Besides, the WNT signaling maintains proper odontoblast differentiation, resulting in elongated apically of Hertwig epithelial root sheath (HERS), which is evident in mutants that lack β-catenin, and thus forming the odontoblasts (T. Kim et al., 2013; Zhang et al., 2013). In these mutants, the odontoblasts failed to differentiate and form dentin, and even though HERS elongated, its cellular integrity was perturbed, and roots were not formed. Excessive β-catenin/Wnt signaling also perturbs normal root development, suggesting that correct spatiotemporal regulation of Wnt activity is required for correct odontogenesis and root formation (Bae et al., 2013). Transforming growth factor-b (TGF-b) includes BMPs that play an essential part in early tooth development and the regulation of early tooth morphogenesis and differentiation (Irma Thesleff, 2003), especially in root formation by deletion of SMAD4, which is critical in mediating BMP/TGFβ signaling that resulted in downregulation of Shh and Nfic expression and defects in HERS elongation (Xiaofeng Huang, Xu, Bringas Jr, Hung & Chai, 2010). Also, the removal of BMP receptor type1A (BMPR1A) in the epithelium is the cause of converting crown epithelia into the root lineage due to elevated Wnt signaling (Yang et al., 2013). Furthermore, Sonic hedgehog (Shh), a spontaneous mutant from mice molars which have altered Hh activity by Ptch1 allele, develops smaller roots due to decreased cell proliferation in HERS (Nakatomi, Morita, Eto & Ota, 2006).

MicroRNAs(miRNA) are a new family of a small class of ~21–25-nt non-coding single-stranded RNAs. By two different mechanisms, cleavage and degradation of the target mRNA, or by repressing protein translation (Filipowicz, Bhattacharyya & Sonenberg, 2008), it can inhibit target result in regulating gene function that involved in many ‘‘fine-tuning’’ events of developmental processes of many tissue- and organ-in specific manner and time (He & Hannon, 2004; Mohr & Mott, 2015). Many studies found out that miRNAs are involved in dental development at different sites and stages, as in Table 1 below.

Table 1 List of miRNAs in the customized RT-PCR array

miRNAs		**Target**	**References**
miR-32, miR-885-5p, miR-586	Dentinogenesis	DSPP	(Xin Huang et al., 2011; Jin et al., 2017)
miR-665	Dentinogenesis	Kat6a>>> DSPP, Dlx3	(Heair et al., 2015; Jin et al., 2017)
miR-338-3p	Dentinogenesis	Runx2	(Jin et al., 2017; Q. Sun et al., 2013)
miR-34a	Dentinogenesis	BMP sig, NOTCH	(Jin et al., 2017; F. Sun et al., 2014)
miR-663, miR-27	Dentinogenesis	WNT Sig	(Jin et al., 2017; E. J. Kim et al., 2014; Park et al., 2014)
miR-23a, miR-23b, miR-24	Stemness at maturation stage		(Lacruz et al., 2012)
miR-652, miR-31	Amelogenesis	FGF Sig; Sprouty	(Fan et al., 2015; Jheon, Li, Wen, Michon & Klein, 2011; Jin et al, 2017; Michon, Tummers, Kyyronen, Frilander & Thesleff, 2010; Sehic, Risnes, Khan, Khuu & Osmundsen, 2010)
miR-720	Amelogenesis	FGF Sig; FGF-8 B catenin/pitx2	(Juuri et al., 2012; Michon et al., 2010) (Jin et al., 2017; Michon et al., 2010; Sharp et al., 2014)

Therefore, we aimed to identify miRNAs that could be involved in the tooth mineralization in stem cell niches. We collected dental pulp tissue from the apical tips of incisor and dental pulp from the molar and examined for a specific set of miRNA expression in order to identify candidate miRNA(s) that involve during the mineralization process.

2. Objective

To demonstrate differential microRNA profiles in dental pulp tissue from the apical tips of incisor and dental pulp tissue from molar of the SD rat.

3. Materials and Methods

3.1 Five- to eight-week-old SD rats are euthanized, and the lower jaws were dissected. The mandible separated into two hemi-jaws through a midline incision and separated the hemisection by using a molar reference line, line C located on the mesial part of the first molar, to collect dental pulp at the apical end. For dental pulp tissue from the molar, all molars are extracted and separated by a feather edge blade. Dental pulps from these two sites are collected and immersed in PBS.

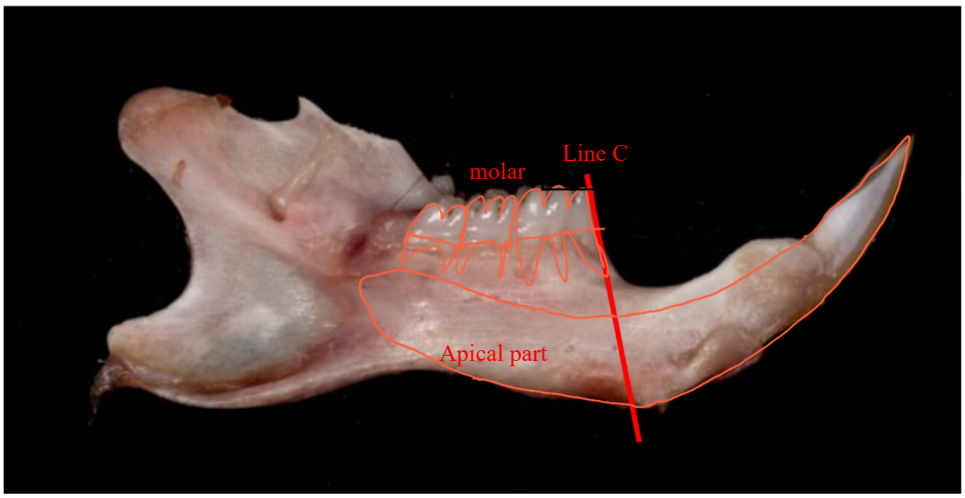

Figure 1 Left hemi-jaws and a reference line C, extending from the mesial surface of the first molar upright to the labial surface of the mandible. Bar = 1 mm. ~ 4.2

3.2 After collecting the sample, total miRNAs were isolated by miReasy mini kit (Qiagen, Valencia, CA, USA). The concentration, purity, and amount of total RNAs were quantified using the Nano-Drop® ND-1000 ultraviolet spectrophotometer (Thermo Scientific, Wilmington, DE, USA). For the qualification of total RNA, the A260/A280 ratio should be at least 1.8. For cDNA, 500 ng of total RNA sample was converted to cDNA by using the miScript II RT Kit (Qiagen, Hilden, Germany) on thermal cycler, following the manufacturer's instructions. For miRNA profiling studies, miScript miRNA PCR Array (Qiagen, Valencia, CA, USA) containing selective miRNAs in dental development pathway was used in combination with the miScript SYBR® Green PCR Kit (Qiagen, Valencia, CA, USA).

3.3 After collected data from the CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), raw quantification cycle values (Ct) of each well will be collected, Ct values > 35 were considered to be undetectable. Therefore, only the miRNAs with a Ct ≤ 35 were included in the analyses, then Ct will be normalized by using the housekeeping gene (miR-16-5p, miR-423-3p) as normalizers for relative quantification of dental pulp from the apical part of incisors and molars. The relative of each miRNA in the apical group (ΔCt_{target}) compared with the amount detected in the molar as a control group ($\Delta Ct_{control}$) was determined by the relative formulas (relative miRNA=2^-ΔΔCt=2^ [- (Ct_{target} -$Ct_{housekeeping\ gene}$) - ($Ct_{control}$ – $Ct_{housekeeping\ gene}$)]).

Proceedings of RSU International Research Conference (2020)

4. Results and Discussion

Table 2 Relative Expression of miRNAs in apical dental pulp from rat incisors to those in dental pulp from rat molars

	Rat 1	Rat 2	Rat3
miR-32-5p	NT	NT	NT
miR-885-5p	1.8107	31.848	1.1128
miR-586	NT	NT	NT
miR-665	3.3719	NT	1.1989
miR-338-3p	0.7612	0.1169	0.0326
miR-34a-5p	1.2988	0.8195	0.5437
miR-663a	0.2418	1.1302	0.4375
miR-27a-3p	0.4199	0.5015	0.2436
miR-23a-3p	2.227	1.0792	1.1341
miR-23b-3p	1.8261	0.3691	1.8296
miR-24-3p	0.9757	0.5281	0.6761
miR-652-3p	3.3694	1.3269	2.7708
miR-720	4.222	3.3068	4.838
miR-200a-3p	1.5795	0.3514	0.1123

NT=Not detectable

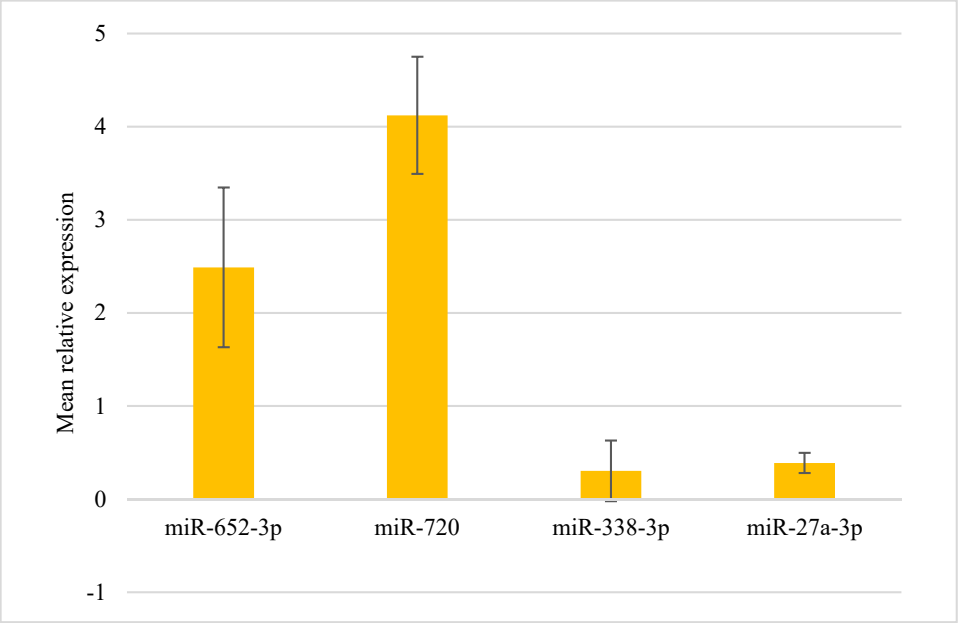

Figure 2 Differential expression of miRNAs in apical dental pulp from rat incisors compared to those in dental pulp from rat molars. Mean relative expression of miR-652-3p, miR-720, miR-338-3p, miR-27a-3p in apical dental pulp from rat incisors compared to those in dental pulp from rat molars; standard deviation as error bars

miRNAs are involved in many stages in the dental development process as important regulators. Many studies have identified an important role of miRNAs during the mineralization of dental pulp tissue. However, the number of studies remains limited, and the complex regulatory mechanisms during developmental processes require elucidation. In contrast to rodent molars, the incisors, especially rat incisors, continuously grow throughout their lifetime. In this study, we focus on the apical part of incisors, which contain abundant potential

stem cells that can differentiate into ameloblasts and odontoblasts, potential cells that later initiate enamel and dentin formation.

Quantitative RT-PCR demonstrates the expression profile of miRNAs in dental pulp from the apical part of rat incisors and molars. Sixteen miRNAs (Table 1) were analyzed for miRNA candidates contributing to mineralization during crown formation in rat incisors. Eight miRNAs, including miR-32-5p, miR-885-5p, miR-586, miR-665, miR-338-3p, miR-34a-5p, miR-663a, miR-27a-3p, were reported to involve in dentinogenesis, miR-23a-3p, miR-23b-3p, miR-24-3p were found in stem cell at maturation stage of amelogenesis, and miR-652-3p, miR-720, miR-200a-3p involved in amelogenesis. In addition, miR-16-5p and miR-423-3p, the stably expressed reference genes (Schwarzenbach, Da Silva, Calin & Pantel, 2015), were added for housekeeping control for data normalization. Among these candidates, the expression of miR-32-5p and miR-586, both target Dentin sialo-phosphoprotein (DSPP) mRNA, were below the detection limit as they were detected at the average Ct values more than 35. However, other miRNAs in the customized array were detectable in all dental pulp samples, and these miRNAs were then calculated for means relative expression in the apical part of rat incisors as compared to rat molars. MiR-16-5p was selected for data normalization, while the data of miR-423-3p was discarded because the Ct value of miR-16-5p is relatively constant in most samples throughout this study.

Among the miRNAs candidates, upregulation levels of miR-652-3p and miR-720 are observed in the apical part of incisors when compared to molars, while miR-338-3p, miR-27a-3p are down-regulated. The comparison between molars and incisors demonstrates that the expression of miR-720, and miR-652-33p, from the apical part of incisors, was 4-fold, and 2-fold, higher than molars, respectively.

Previous studies demonstrated various locations where miR-652-3p and miR-720 expressed in the dental pulp of rodent incisors. Michon et al. suggested that miR-720 expressed in ameloblast progenitor cells at the tip, but miR-652 expressed in mesenchymal cells at the basal part of the E18 incisor (Michon et al., 2010). The study by Sehic A et al. reported high levels of miR-652 expressed in the incisal tip segment where prism-free enamel is formed in P0 mouse (Sehic et al., 2010). In addition, the study by Juuri et al. (2012) showed the expression of miR-720 in the pre-odontoblasts in sagittal sections of P2 wild-type incisor (Juuri et al., 2012). The fibroblast growth factor (FGF) family plays a role in the process of dentinogenesis and odontoblast differentiation. According to the miRtooth1.0 database, miR-720 and miR-652 share the same target, which is the Sprouty2 gene (Spry2), an antagonist of FGF signaling (Klein et al., 2008). Also, miR-720 expressed in the main population of human dental pulp to promote stem cell differentiation by repressing NANOG, the stem cell marker (Hara et al., 2013). Furthermore, FGF-8, involving in the homeostasis of the dental epithelium, is possibly regulated by miRNAs-720 (Juuri et al., 2012). In conclusion, the upregulation of miR-652-3p and miR-720 in rat incisors, but not molars, may contribute to pulp cell proliferation and growth of crown. Further study is needed to understand the underlying mechanism of these miRNAs.

In contrast, down-regulation of miR-338-3p and miR-27a-3p suggests that these miRNAs may be expressed in molar pulp tissue higher than incisor. As reported by Sun et al., miR-338-3p may suppress Runx2, a key transcription factor, to promote odontoblast differentiation (Q. Sun et al., 2013). Besides, another study demonstrated that miR-27 suppressed APC in the WNT signaling pathway and activated Wnt/b-catenin signaling through the accumulation of b-catenin in the nucleus resulting in the differentiation of MDPC-23 cells to odontoblasts (Park et al., 2014). Therefore, these two miRNAs may be essential for odontoblast differentiation and tooth mineralization during active growth in rat incisors.

5. Conclusion

This study shows the differential expression of miRNAs in the apical pulp tissue of incisors and molars. Among candidate miRNAs, the expression level of miR-720 and miR-652-33p was up-regulated in the apical pulp tissue of the incisors as compared to the molars. Conversely, miR-338-3p and miR-27a-3p decreased. The results suggested that these miRNAs may have a role in the mineralization process of dental pulp tissue in the rodent incisors. Due to the limitation of this study, an increased number of samples may improve our understanding of these miRNAs in the rat dental pulp tissue. Although many animal studies have shown the key role of miRNAs in enamel and dentine mineralization, correlation in humans remains ambiguous, and further studies are necessary.

6. Acknowledgements

This study was supported by members of the Department of Microbiology and Department of Oral biology for laboratory and technical support. Also, thanks to Chulalongkorn University Laboratory Animal Center (CULAC) for experiment facility and veterinary supports.

7. References

Bae, C., Lee, J., Kim, T., Baek, J., Lee, J., Yang, X., Cho, E. (2013). Excessive Wnt/β-catenin signaling disturbs tooth-root formation. *Journal of periodontal research, 48*(4), 405-410.

Fan, Y., Zhou, Y., Zhou, X., Sun, F., Gao, B., Wan, M., Zheng, L. (2015). MicroRNA 224 Regulates Ion Transporter Expression in Ameloblasts To Coordinate Enamel Mineralization. *Mol Cell Biol, 35*(16), 2875-2890. doi:10.1128/MCB.01266-14

Filipowicz, W., Bhattacharyya, S. N., & Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nature Reviews Genetics, 9*(2), 102.

Hara, E. S., Ono, M., Eguchi, T., Kubota, S., Pham, H. T., Sonoyama, W., Kuboki, T. (2013). miRNA-720 controls stem cell phenotype, proliferation and differentiation of human dental pulp cells. *PLoS One, 8*(12).

Harada, H., Kettunen, P., Jung, H.-S., Mustonen, T., Wang, Y. A., & Thesleff, I. (1999). Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. *The Journal of cell biology, 147*(1), 105-120.

He, L., & Hannon, G. J. (2004). MicroRNAs: small RNAs with a big role in gene regulation. *Nature Reviews Genetics, 5*(7), 522.

Heair, H. M., Kemper, A. G., Roy, B., Lopes, H. B., Rashid, H., Clarke, J. C., Hassan, M. Q. (2015). MicroRNA 665 Regulates Dentinogenesis through MicroRNA-Mediated Silencing and Epigenetic Mechanisms. *Mol Cell Biol, 35*(18), 3116-3130. doi:10.1128/MCB.00093-15

Huang, X., Xu, S., Gao, J., Liu, F., Yue, J., Chen, T., & Wu, B. (2011). miRNA expression profiling identifies DSPP regulators in cultured dental pulp cells. *International journal of molecular medicine, 28*(4), 659-667.

Huang, X., Xu, X., Bringas Jr, P., Hung, Y. P., & Chai, Y. (2010). Smad4-Shh-Nfic signaling cascade–mediated epithelial-mesenchymal interaction is crucial in regulating tooth root development. *Journal of Bone and Mineral Research, 25*(5), 1167-1178.

Jheon, A. H., Li, C. Y., Wen, T., Michon, F., & Klein, O. D. (2011). Expression of microRNAs in the stem cell niche of the adult mouse incisor. *PLoS One, 6*(9), e24536. doi:10.1371/journal.pone.0024536

Jiang, N., Zhou, J., Chen, M., Schiff, M. D., Lee, C. H., Kong, K., Mao, J. J. (2014). Postnatal epithelium and mesenchyme stem/progenitor cells in bioengineered amelogenesis and dentinogenesis. *Biomaterials, 35*(7), 2172-2180. doi:10.1016/j.biomaterials.2013.11.061

Jin, Y., Wang, C., Cheng, S., Zhao, Z., & Li, J. (2017). MicroRNA control of tooth formation and eruption. *Arch Oral Biol, 73*, 302-310. doi:10.1016/j.archoralbio.2016.08.026

Juuri, E., Saito, K., Ahtiainen, L., Seidel, K., Tummers, M., Hochedlinger, K., Michon, F. (2012). Sox2+ stem cells contribute to all epithelial lineages of the tooth via Sfrp5+ progenitors. *Dev Cell, 23*(2), 317-328. doi:10.1016/j.devcel.2012.05.012

Kim, E. J., Lee, M. J., Li, L., Yoon, K. S., Kim, K. S., & Jung, H. S. (2014). Failure of Tooth Formation Mediated by miR-135a Overexpression via BMP Signaling. *J Dent Res, 93*(6), 571-575. doi:10.1177/0022034514529303

Kim, T., Bae, C., Lee, J., Ko, S., Yang, X., Jiang, R., & Cho, E. (2013). β-catenin is required in odontoblasts for tooth root formation. *Journal of Dental Research, 92*(3), 215-221.

Klein, O. D., Lyons, D. B., Balooch, G., Marshall, G. W., Basson, M. A., Peterka, M., . . . Martin, G. R. (2008). An FGF signaling loop sustains the generation of differentiated progeny from stem cells in mouse incisors. *Development, 135*(2), 377-385.

Lacruz, R. S., Smith, C. E., Bringas, P., Jr., Chen, Y. B., Smith, S. M., Snead, M. L., . . . Paine, M. L. (2012). Identification of novel candidate genes involved in mineralization of dental enamel by genome-wide transcript profiling. *J Cell Physiol, 227*(5), 2264-2275. doi:10.1002/jcp.22965

Michon, F., Tummers, M., Kyyronen, M., Frilander, M. J., & Thesleff, I. (2010). Tooth morphogenesis and ameloblast differentiation are regulated by micro-RNAs. *Dev Biol, 340*(2), 355-368. doi:10.1016/j.ydbio.2010.01.019

Mohr, A. M., & Mott, J. L. (2015). Overview of microRNA biology. *Semin Liver Dis, 35*(1), 3-11. doi:10.1055/s-0034-1397344

Nakatomi, M., Morita, I., Eto, K., & Ota, M. (2006). Sonic hedgehog signaling is important in tooth root development. *Journal of Dental Research, 85*(5), 427-431.

Park, M.-G., Kim, J.-S., Park, S.-Y., Lee, S. A., Kim, H.-J., Kim, C. S., Kim, D. K. (2014). MicroRNA-27 promotes the differentiation of odontoblastic cell by targeting APC and activating Wnt/β-catenin signaling. *Gene, 538*(2), 266-272.

Schwarzenbach, H., Da Silva, A. M., Calin, G., & Pantel, K. (2015). Data normalization strategies for microRNA quantification. *Clinical chemistry, 61*(11), 1333-1342.

Sehic, A., Risnes, S., Khan, Q. E., Khuu, C., & Osmundsen, H. (2010). Gene expression and dental enamel structure in developing mouse incisor. *European journal of oral sciences, 118*(2), 118-130.

Seidel, K., Ahn, C. P., Lyons, D., Nee, A., Ting, K., Brownell, I., Schober, M. (2010). Hedgehog signaling regulates the generation of ameloblast progenitors in the continuously growing mouse incisor. *Development, 137*(22), 3753-3761.

Sharp, T., Wang, J., Li, X., Cao, H., Gao, S., Moreno, M., & Amendt, B. A. (2014). A pituitary homeobox 2 (Pitx2):microRNA-200a-3p:beta-catenin pathway converts mesenchymal cells to amelogenin-expressing dental epithelial cells. *J Biol Chem, 289*(39), 27327-27341. doi:10.1074/jbc.M114.575654

Sun, F., Wan, M., Xu, X., Gao, B., Zhou, Y., Sun, J., Zheng, L. (2014). Crosstalk between miR-34a and Notch Signaling Promotes Differentiation in Apical Papilla Stem Cells (SCAPs). *J Dent Res, 93*(6), 589-595. doi:10.1177/0022034514531146

Sun, Q., Liu, H., Lin, H., Yuan, G., Zhang, L., & Chen, Z. (2013). MicroRNA-338-3p promotes differentiation of mDPC6T into odontoblast-like cells by targeting Runx2. *Mol Cell Biochem, 377*(1-2), 143-149. doi:10.1007/s11010-013-1580-3

Thesleff, I. (2003). Epithelial-mesenchymal signalling regulating tooth morphogenesis. *Journal of cell science, 116*(9), 1647-1648.

Thesleff, I., & Tummers, M. (2008). Tooth organogenesis and regeneration. In *StemBook*. Cambridge (MA).

Tummers, M. (2003). Root or crown: a developmental choice orchestrated by the differential regulation of the epithelial stem cell niche in the tooth of two rodent species. *Development, 130*(6), 1049-1057. doi:10.1242/dev.00332

Yang, Z., Hai, B., Qin, L., Ti, X., Shangguan, L., Zhao, Y., . . . Chang, J. Y. F. (2013). Cessation of epithelial Bmp signaling switches the differentiation of crown epithelia to the root lineage in a β-catenin-dependent manner. *Molecular and Cellular Biology, 33*(23), 4732-4744.

Zhang, R., Yang, G., Wu, X., Xie, J., Yang, X., & Li, T. (2013). Disruption of Wnt/β-catenin signaling in odontoblasts and cementoblasts arrests tooth root development in postnatal mouse teeth. *International journal of biological sciences, 9*(3), 228.