A comparison of microRNA profiles between rat incisors and molars

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การเปรียบเทียบรูปแบบไมโครอาร์เอ็นเอระหว่างฟันหน้าและฟันหลังในหนู

นายสิรวิชญ์ นิภาเกษม

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สิรวิชญ์ นิภาเกษม : การเปรียบเทียบรูปแบบไมโครอาร์เอ็นเอระหว่างฟันหน้าและฟันหลังในหนู. (A comparison of microRNA profiles between rat incisors and molars) อ.ที่ปรึกษาหลัก : ผศ. ทญ. ดร.อัญชลี วัชรักษะ, อ.ที่ปรึกษาร่วม : ผศ. ทญ. ดร.วลีรัตน์ ศุกรวรรณ

ฟันหน้าของสัตว์ฟันแทะนั้นเป็นอวัยวะที่มีคุณสมบัติในการเจริญเติบโตในบริเวณปลายฟันได้อย่างต่อเนื่องซึ่งแตกต่างจากฟันกราม การเจริญเติบโตอย่างต่อเนื่องนั้นเป็นผลจากการที่ยับยั้งการสร้างของรากฟันและเพิ่มการสร้างของตัวฟันอันเกิดจาก stem cell niche เรียกว่า cervical loop ซึ่งกระบวนการเหล่านี้เกี่ยวข้องกับยีนส์และวิถีการส่งสัญญาณต่างๆมากมาย จากการศึกษาที่ผ่านมาพบว่า อาร์เอ็นเอเกลียวเดี่ยวไม่มีการแปลรหัสขนาดเล็กที่เรียกว่าไมโครอาร์เอ็นเอนั้น มีความเกี่ยวข้องในกระบวนการหลายๆอย่างที่สำคัญของเซลล์ซึ่งรวมไปถึงกระบวนการพัฒนาของฟัน

เพื่อที่จะศึกษาบทบาทของไมโครอาร์เอ็นเอในกระบวนการดังกล่าว ผู้วิจัยได้ทำการสกัดและเปรียบเทียบการแสดงออกของไมโครอาร์เอ็นเอจากเนื้อเยื่อโพรงประสาทฟันจากบริเวณปลายรากและปลายฟันในฟันหน้ากับฟันกรามของหนูแร็ท จากผล RT-PCR array พบว่าการแสดงออกของไมโครอาร์เอ็นเอ 6 ชนิดจากบริเวณปลายรากของฟันหน้า (miR-32-5p, 885-5p, 665, 338-3p, 663a, 200a-3p) และไมโครอาร์เอ็นเอ 4 ชนิดจากบริเวณปลายฟันของฟันหน้า (miR-32-5p, 665, 338-3p, 663a) มีระดับการแสดงออกที่น้อยกว่าฟันกรามอย่างมีนัยสำคัญ ในขณะที่การแสดงออกของไมโครอาร์เอ็นเอชนิดเดียว (miR-23a-3p) จากทั้งปลายรากและปลายฟันของฟันหน้ามีระดับการแสดงออกที่มากกว่าฟันกรามอย่างมีนัยสำคัญ จากการศึกษาที่ผ่านมาพบว่าไมโครอาร์เอ็นเอเหล่านี้ มีส่วนเกี่ยวข้องกับยีนส์และวิถีการส่งสัญญาณต่างๆที่สำคัญในกระบวนการสร้างทั้งชั้นเคลือบฟันและชั้นเนื้อฟันได้แก่ DSPP, RUNX2, Wnt/β-catenin, E-cadherin และ Sprouty2 เป็นต้น ดังนั้นจากผลการศึกษาอาจกล่าวได้ว่าไมโครอาร์เอ็นเอเหล่านี้มีบทบาทสำคัญในกระบวนการสร้างฟันอย่างต่อเนื่องในหนู

สาขาวิชา ทันตกรรมผู้สูงอายุและการดูแลผู้ป่วยพิเศษ ลายมือชื่อนิสิต .....

ปีการศึกษา 2562 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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Sirawit Nipakasem : A comparison of microRNA profiles between rat incisors and molars. Advisor: Asst. Prof. ANJALEE VACHARAKSA, D.D.S., Ph.D Co-advisor: Asst. Prof. WALEERAT SUKARAWAN, D.D.S., Ph.D

Unlike the molar, rodent incisor is a distinct organ that have continuously growth at distal end of tooth throughout its lifetime to compensate for abrasion. The key of continuous growth is the result of delayed root formation and prolonged crown formation that possibly made by stem cell niche, so-called cervical loop, which involve various genes and signaling pathways. MicroRNAs (miRNAs) are small non-coding single stranded RNAs that involved in biological mechanisms of dental development. To study the role of miRNA, we extracted total RNA from the dental pulp tissue collected from the apical part, coronal part of incisors and molars, and compared the expression of miRNAs. The customized RT-PCR array (Qiagen) demonstrated that 6 miRNAs (miR-32-5p, 885-5p, 665, 338-3p, 663a, 200a-3p) in apical part and 4 miRNAs (miR-32-5p, 665, 338-3p, 663a) from coronal part are significantly lower than molar group whereas miR-23a-3p is the only one that has significantly increase in both apical and coronal part compare to the molars group. Previous studies also found role of these miRNAs that involved in both amelogenesis and dentinogenesis included DSPP, RUNX2, Wnt/β-catenin, E-cadherin and Sprouty2 genes. The results suggested that these miRNAs may have a role in mineralization process during prolonged crown formation of rodent incisors.

Field of Study:	Geriatric Dentistry and Special Patients Care	Student's Signature .....
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		Co-advisor's Signature .....

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Sirawit Nipakasem

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Chapter 1

Introduction

The rodent incisor is an organ that grow continuously throughout its lifetime. Continuous growth of the incisor is an evolutionary adaptation to compensate for abrasion at the distal end of the tooth. During tooth formation, growth of tooth is regulated by the crosstalk between the epithelium and neural crest-derived mesenchyme, which involves numerous signals and their pathway modification. MicroRNAs (miRNAs), a class of small non-coding RNA species, have been relatively well studied recently that may contribute in amelogenesis, morphogenesis, odontoblast differentiation, dentin formation and tooth eruption. MiRNAs functional activity and its targets, as well as the communication of different miRNAs have been characterized in different pathways. Some studies reported the role of miRNAs in mouse incisor, a well-accepted model for tooth development, however the reported miRNAs remain inconsistent across the studies. Therefore, identification of miRNAs during continuously growing teeth in rat model will be beneficial for potential biological functions. Future studies can be continued to use as a novel approach in dental pulp therapy.

Chapter 2
Review literature

2.1 Rodent tooth

There is a variety among animals in terms of the number and shape of teeth. Rats and mice are commonly used as a model for investigation of tooth development or odontogenesis. Unlike human dentition, rodent show a unique dentition consists of 4 incisors and 12 molars, which are separated by a toothless area called the diastema. Rodent dentition have no replacement(1), however, rodent incisors can grow continuously throughout lifetime.

Development of rodent tooth originates from neural crest (NC) cells, a group of cells that separate from the neural tube and migrate away from their parental epithelium to reaggregate with other cells. In the developing embryo, almost all organs including craniofacial skeleton, cornea, tooth and dentin, thyroid gland, thymus, cardiac septa, adrenal gland, melanocyte, autonomic nerve, sensory nerve, and Schwann cells, composed of these neural crest cells (2-4). Neural crest-derived mesenchyme also develops many dental tissues, such as dentin, pulp, and periodontal ligaments, whereas the oral ectoderm derived dental epithelium primarily produces the ameloblasts and their supporting cells (5-7).

Tooth development begins with a localized condensing or placodes within the primary epithelial bands that formed after approximately 37 days. In a subdivision of the primary epithelial band, the dental lamina, localized proliferative activity leads epithelial growths into the ectomesenchyme. Because the underlying ectomesenchyme is more active than the epithelial cells, these ecto-mesenchyme cells accumulate the epithelial growth afterwards (8).

At the bud stage (Embryonic day (ED) 11–13.5), the epithelium begins to invaginate into the underlying mesenchyme, which reacts by condensing to form the dental papilla. The internal part of the tooth bud contains star-like shaped, glycosaminoglycan synthesizing stellate reticulum cells. Some cells within the stellate reticulum in mice have been identified as putative stem cells (9).

Odontogenic potential is shifted from the epithelium to ectomesenchyme throughout the bud stage. When in the bud stage, it seem like many ectodermal organs such as exocrine glands, hair follicles, and teeth share morphological similarity, but these ectodermal organs become specific different from the beginning of bud-to-cap transition.

The tooth bud transforms into a cap (ED 12-16) by differential proliferation and folding in of the dental epithelium (10). As the epithelial bud cells proliferate, ecto-mesenchyme cells condense and structural differences between tooth germs begin during the cap stage. At ED 12, the enamel knot, transient signaling center characterized by expression of several secreted factors, will come to have a role by regulated in size and shape of the tooth crown without cell proliferation activity. After their transient organizing role is complete, they become apoptosis at the end of the bell stage (ED 16) (11). Afterwards, in the late of cap stage and to the next bell stage the cells of the crown ameloblasts and odontoblasts are differentiated. To begin with the epithelial growth is commonly referred as enamel organ, the condensed ecto-mesenchyme cells are referred as dental papilla which the dental follicle are covers the outside of these two substances. These three organs (The enamel organ, dental papilla, and dental follicle) are constitute to the tooth germ. During the late cap stage, major morphologic differences between the developing incisor and molar appear, as the developing of the incisor begins to parallelly growth to the long axis of the jaw.

The bell stage starts with terminated the differentiation of ameloblasts from IDE and odontoblasts from mesenchymal cells of dental papilla, included formation of the two main hard tissues of the tooth such as enamel and dentin which is regulated by interactions between the epithelium and mesenchyme(8). While dental papilla is the origin of the dental pulp in the future, dental follicles also give rise to cementoblasts, osteoblasts, and fibroblasts. During the bell stage, the final tooth shape and the tooth specific cell types become apparently seen. The primary enamel knot are replaced by secondary enamel knot in the molars, which relate to numbers and morphologic of upcoming tooth cusps (12). In conclusion, neural crest

cells give rise to dentin-producing cells knowing as odontoblasts, cementoblasts which produce cementum to covering the root dentin, osteoblasts which participate in the formation of dental alveolar bone and fibroblasts which synthesize collagen for periodontal ligaments.

As above, the development of the rodent incisor progresses through the same stages and also have similarly molecular regulating as the well-studied molar (13), with a bit differences by being the proximal-distal rotation at the bell stage, as well as the absence of secondary enamel knot in the incisor (14). The process is characterized by reciprocal epithelial-mesenchymal communications that are under regulated by several key-signaling pathways, including sonic hedgehog (SHH), bone morphogenetic protein (BMP), fibroblast growth factor (FGF), WNT, NOTCH etc. (15)

2.2 Continuous growth

Interestingly, rodent species have incisors that have potential to growth throughout their lifetime, and this growth is compensated by continuous wear at the incisal end. The continuous growth of incisor, called hypsodont, is thought to be the result of delayed root formation, leading to prolonged crown formation (16). It is hypothesized that hypsodonty is a halfway between brachydonty and hypselodonty during development, which the tooth still have crown formation without undergoing root genesis (16).

The continuous growth of enamel and dentin are possibly made by the presence of active adult epithelial and mesenchymal stem cells. During the bud stage, the dental epithelium separated into two histologically apparent cell lineages, the peripheral basal cells contacting the basement membrane, and centrally located loosely arranged cells, called the stellate reticulum, which are derived from the supra basal cell layers of the surface ectoderm. These two tissue layers will form the epithelial components of the stem cell niche in the continuously growing teeth that called cervical loop (16-18).The basal epithelial cell layer of the loop adjacent to the dental papilla is known as the inner enamel epithelium and the part facing the dental follicle is known as the outer enamel epithelium. Labial cervical loop (laCL)

induced by stellate reticulum (SR) and outer enamel epithelium (OEE) to produce ameloblasts that form a crown analogue at the labial side (19), besides, dental mesenchyme stem cells surrounding the cervical loop that self-renewal to form colonies and differentiate into a native lineage of odontoblast cells (20). Both epithelium and mesenchyme cells are stimulated and formed in two integrated layers of highly intense enamel like tissue and dentine like tissue which turned into further dentinal tubule like tissue perpendiculated to the surface of enamel like tissue (20). Meanwhile, the liCL which is smaller in size compared to the laCL, has fewer and does not contain proliferating cells that usually form ameloblasts. For this reason, the enamel is enlargement asymmetrically, as it is only be seen on the labial surface of the tooth, in contrast to the softer dentin, which covers around the entire incisor. This could be the reason that why they are a bit difference in hardness between the outer and inner surfaces of the tooth that allows to abrasion through mastication on the inner side which mostly be dentin component and primarily recontouring to be a sharp tip of incisors (14). So, in all continuously growing teeth, this cervical loop structure has given a supply of stem cells through regulation of different aspects of the regulatory network, which is shown in the variations of the development process and morphology of these teeth (17, 21).

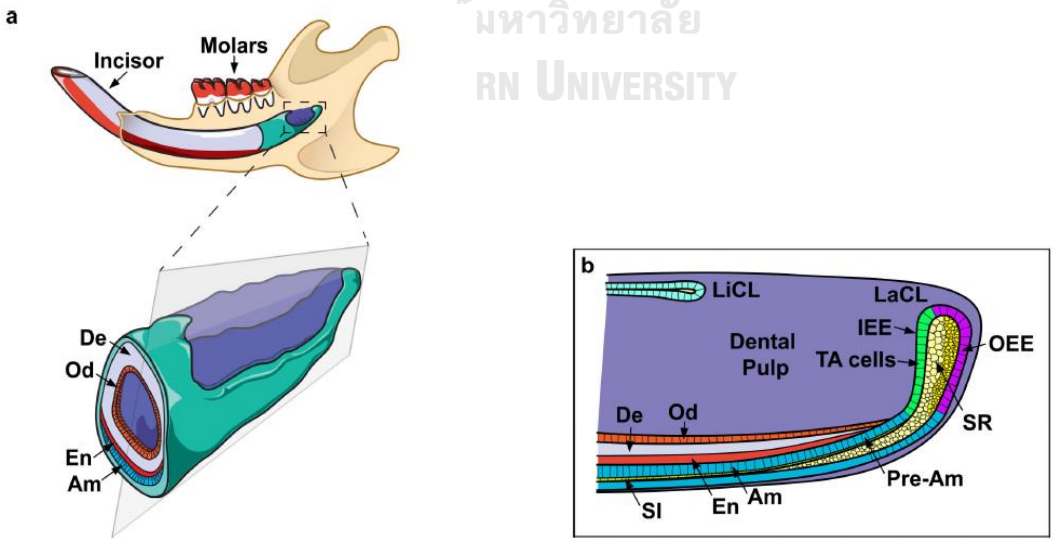


Figure 1. *The morphology of rodent mandible and cervical loops; cite from Kuang-Hsien Hu, J,2014 (14))*

(a) Anatomy of rodent lower incisor; Tooth is mostly embedded into the jawbone. At the apical end, is the compartment of stem cell niche that continually supply ameloblasts (Am) and odontoblasts (Od) for substituting the worn enamel (En) and dentin (De).

(b) Sagittal section of the apical end of incisor; The epithelial stem cells are in the outer enamel epithelium (OEE) and stellate reticulum (SR) of the labial cervical loop (laCL). These cells give rise transit-amplifying (TA) cells in the inner enamel epithelium (IEE) that will rise to pre-ameloblasts (Pre-Am) that differentiated to ameloblasts, as well as cells in the stratum intermedium (Si). Two structural distinctly seen in the SR, one adjoining to the OEE and the other adjoining to the TA cells. Compared to the laCL, the lingual cervical loop (liCL) is lesser and does not have potential to produce ameloblasts. Finally, mesenchymal stem cells that generate odontoblasts are believed to reside in the proximal dental pulp (14).

One important point for the derivation of hypselodonty is the maintenance of the cervical loop structure. Fibroblast growth factor (FGFs), bone morphogenetic protein (BMP), E-cadherin, transforming growth factor-b (TGF-β), and sonic hedgehog (Shh) are the signaling pathways that have potential to maintaining and proliferating of the epithelial stem cell niche and their progeniture in the laCL (13) (19).By comparing gene expression in continuously growth mouse and volve incisors and their molars, it found the expression of Fgf10 in the mesenchyme and components of the Notch signaling pathway in the epithelium was maintained in the continuously growing teeth, while the absent of these signaling pathways were associated with root formation and loss of the cervical loop stem cell niche in non-continuous growth teeth (16). Interestingly, underwent of root formation are found when cultured under kidney capsules in Fgf10 null mouse incisors. Conversely, overexpression of Fg10 in the mouse molar dental papilla resulted in the formation of cervical loop like structures, although it is unclear whether these were functionally corresponding to the incisor apical buds (22). Therefore, it is probable that throughout the evolution, the preserved expression of Fgf10 and other genes may be

the key step to maintained the existence of the cervical loop during development, which first led to the development of the long crown teeth and terminated in the transition to hypselodonty.

Another key step in the evolution of continuous growth teeth is the alteration of the crown to root transition, which is currently unclearly understood. Even though, there still have some basic knowledge about root formation in mouse molars that provided from previous mouse genetic and in vitro culturing studies. For example, previous study found spontaneous mutant Ptch1 allele from mice molars have potential to modified Hh activity result to develop smaller roots due to decreased cell proliferation in HERS (23). Insulin-like growth factor-I and vasoactive intestinal peptide also demonstrate proliferative events that can promote root sheath elongation (24, 25), and the initiation of HERS formation is aided by the cessation of EGF expression, which otherwise the presence of the cervical loop (26). Furthermore, maintenance of HERS elongates apically is depends on proper odontoblast differentiation, which is regulated by canonical Wnt signaling. Previous study demonstrated the mutants that lack β-catenin, and thus Wnt signaling, in the forming odontoblasts. Disturbing of these cellular integrity was result to failed odontoblasts differentiation and produce dentin, and also HERS elongated result in lack of roots formation. (27) (28). On the contrary, excessive of β-catenin/Wnt signaling also disturbs typical root development, suggesting that proper comprehensive regulation of Wnt activity is required for proper odontogenesis and root formation (29). Furthermore, evidence base also shown that Bmp and TGF-β signaling may play important roles in root formation as seen by deletion of SMAD4, which is consequence in moderated BMP/TGF-β signaling, result in downregulation of Shh and Nfic expression and also defects in HERS elongation(30). In addition, deletion of BMP receptor type1A (BMPR1A) in the epithelium are the cause of converting crown epithelium into the root lineage due to raise of Wnt signaling. For those reason may suggested that BMP and TGF- β signaling may regulate in different aspects of crown to root transition(31).

There are a lot of genes and transcription factor that involve maintaining cervical loop and inhibit of HERS formation. The first marker, previous study found the sonic hedgehog (Shh) responsive to Gli1 gene that expressing to give rise to differentiated ameloblasts and cells in the stratum intermedium (SI) (19),The next two markers are Bmi1 and Sox2. Bmi1-expressing cells also generate progeny that populate the differentiated ameloblasts, that plays a critical role in promoting proliferation and self-renewal by suppressing the expression of Ink4a/Arf. Besides, Bmi1 also clearly prevents improper differentiation by inhibiting the expression of Hox genes. As a result, lack of Bmi1 found decreased in stem cell self-renewal result in gradually loss of enamel over time (32). Opposed to Gli1 and Bmi1, SOX2 expression provide to all epithelium lineages in the incisor and may do so by migrating from the SR directly to the IEE and TA regions, which appear to regulate the OEE. Sox2 expression is induced by FGF8 signaling and also fine-tuned by microRNAs (33). FGF regulators is encoded by the Sprouty genes (Spry1, 2, and 4), the intracellular antagonists which expressed in both the lingual and labial epithelium as well as the adjacent mesenchyme(34). When Sprouty genes are erased and the inhibitory signal is eliminated, cells in both lingual and labial epithelium and mesenchyme show increased sensitivity to FGF signaling. This take the lead to ectopic mesenchymal Fgf3 and Fgf10 expression and the formation of lingual ameloblasts (34). Furthermore, the transcription factors nuclear factor I-C (NFI-C) and MSX2 is also play important tasks in root formation. Mice with mutations in NFI-C lack completely roots formation (35), whereas Msx2 null mice have abnormal root morphogenesis (36).

2.3 MicroRNA

MicroRNAs(miRNA) are a new family of small class of ~21–25-nt non-coding single-stranded RNAs, that regulate gene function post-transcriptionally (37) (38). It has been expected that more than a third of the protein-coding genes are controlled by these microRNAs (39). MicroRNAs are small RNAs with stem-loop structures called pri-miRNA; these are converted to pre-miRNA through the cleavage activity of the Drosha enzyme. Drosha crops the flanking regions of pri-miRNA to liberate the 60- to 70-nt premiRNA (40). The pre-miRNA is exported to the cytoplasm by Exportin-5. The

pre-miRNA is processed into 18-22 nucleotide miR duplexes by Dicer and, in humans, its partner TRBP. One strand of the duplex is degraded, and the other strand cumulates as a mature miRNA. Animal miRNAs are imperfectly paired to the 3′UTR of target mRNA and inhibit protein production.

miRNA can inhibit target by two different mechanisms; by cleavage and degradation of the target mRNA or by suppressing protein translation (41). The mechanism that occurs is dependent on binding complementation of the miRNA to the mRNA. Perfect complementation leads to degradation of the transcript, and imperfect complementation leads to inhibition of translation (41). Because of miRNA can attach either perfectly or imperfectly which means one miRNA can regulate many different targets and allowing in both specific and large-scale regulation process. So, miRNAs are involved in many developmental processes, such as cell proliferation, apoptosis, development and metabolism of many tissues and organs at specific place and time, especially during development process when their expression is regulated in a time variant. It is potential that miRNAs may be participated in specific fine tuning events. (37) furthermore, it is not surprising that they are strictly regulated and play a part in many disease states. Due to their capability to regulate numerous different of proteins, gene and the pathways. By the way, they also be good as a candidates for therapeutics. (42)

Many research found out that several miRNAs involved in regulated in dental development at different development stages, for example, conditional deletion of Dicer1 in mice incisor, which can altered from pre-miRNAs into mature miRNAs phase resulted in phenotypes fluctuating from supernumerary incisors formation to ectopic budding of the cervical loops, depending on the specific time only when removal occurred(21, 43). Therefore, miRNAs profiling of developing oral tissues is essential for understanding the role of miRNA function. Nowadays, considerable by their enhancing or inhibitory functions, many researchers display miRNAs function in tooth morphogenesis and epithelial cell differentiation in the development of both molars and incisors by targeting related signaling pathways and transcription factors as table and picture below (44).

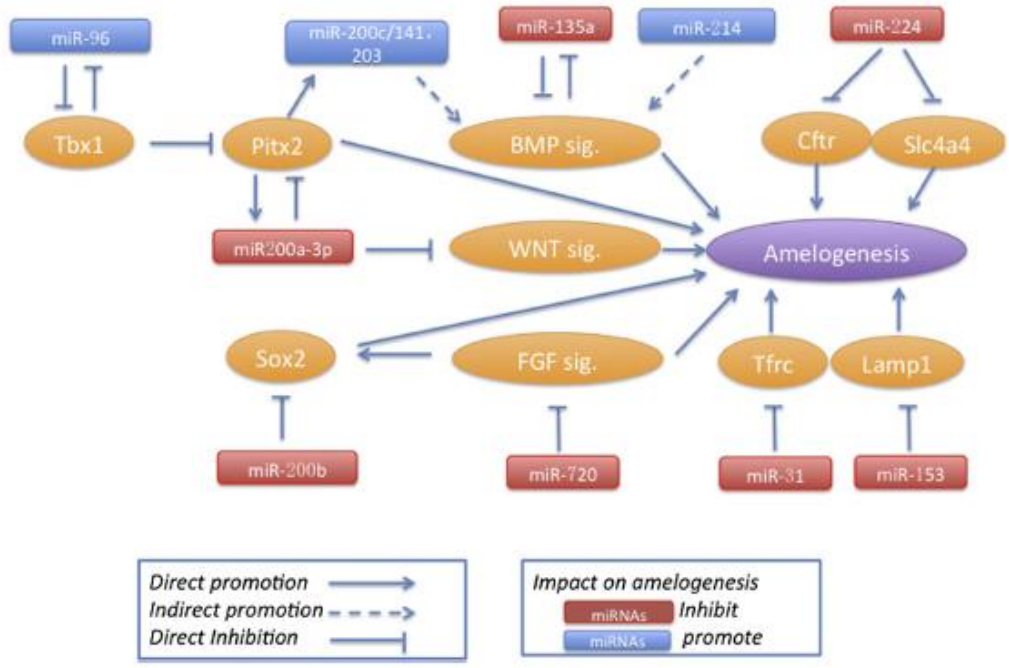


Figure 2. *Schematic of miRNA regulation on amelogenesis*

miRNA regulation of ameloblast differentiation and morphogenesis by targeting transcriptional factors and signaling pathway proteins (44).

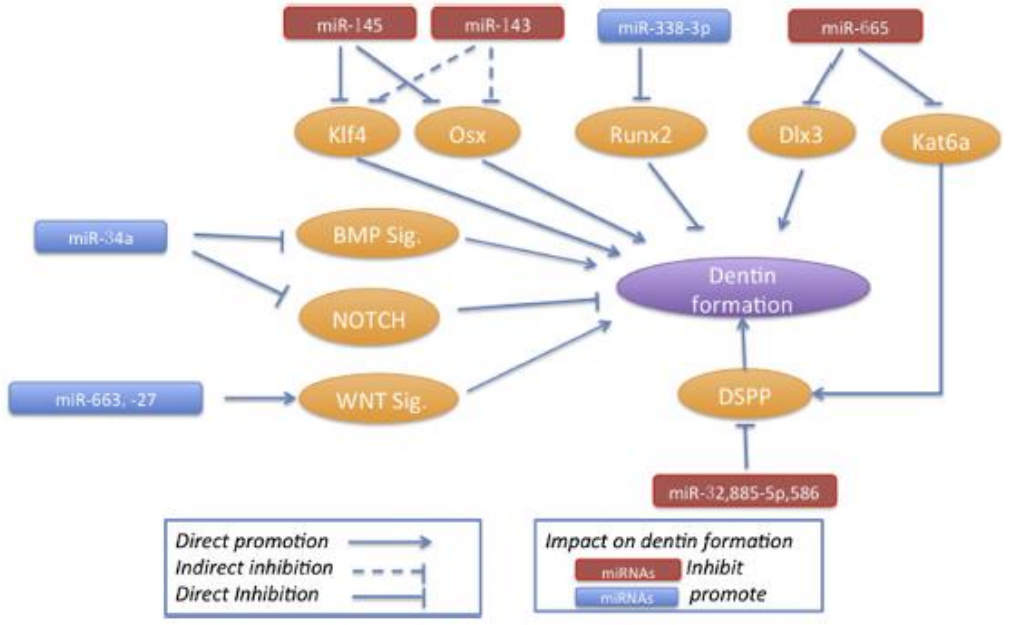


Figure 3. *Schematic of miRNA regulation of dentinogenesis*

miRNA regulation of odontoblast differentiation and dentin formation.by targeting transcriptional factors, signaling pathway proteins (44).

Table 1. miRNAs in tooth development (44)

Location	MicroRNAs	Potential target	Sample source	Origin	Function	Ref.
Dental epithelium	miR-214	TGF-β1/Clusterin	Molar tooth germ	newborn mice	Ameologenesis, tooth eruption ↑	(Khan et al., 2013; Sehic et al., 2011)
	miR200a-3p	β-catenin/Pitx2	LS-8 cell line	mice	Epithelial dif. ↓	(Sharp et al., 2014)
	miR-203	Bmper	LS-8 cell line	mice	Epithelial dif. ↑	(Cao et al., 2013)
	miR-200c/141	Noggin	LS-8 cell line	mice	Epithelial dif. ↑	(Cao et al., 2013)
	miR-720	Fgf8	P2 incisor	mice	Epithelial dif. ↓	(Juuri et al., 2012)
	miR-200b	Sox2	P2 incisor	mice	Epithelial dif. ↓	(Juuri et al., 2012)
	miR-135a	Bmpr-Ia and Bmpr-Ib	Molar tooth germ/E14	mice	Tooth calcification ↓	(Kim et al., 2014)
	miR-153	Lamp1	LS-8 cell line	mice	Enamel formation ↓	
	miR-31	Tfrc	LS-8 cell line	mice	Enamel formation ↓	
	miR- 224	Slc4a4 and Cftr	hOEs	human	Enamel mineralization ↓	(Fan et al., 2015)
	miR96-5p	Tbx1	LS-8 cell line	mice	Epithelial dif. ↑	(Gao et al., 2015)
Dental papilla	miR-32,885-5p,586	Dspp	hDPPC/impacted third molars	human	Odontoblast dif. ↑	(Huang et al., 2011)
	miR-34a,	Bmp7/Hes1/Notch1,2	hFDPC/bell stage tooth buds	human	Dental papilla cell dif. ↑	(Sun et al., 2014
	miR-145	Osx/Klf4	mDPC/E18.5 molars	mice	Odontoblast dif. ↓	(Liu et al., 2013)
	miR-338-3p	Runx2	mDPC6T	mice	Odontoblast dif. ↑	(Sun et al., 2013)
	miR-665	Kat6a/Dlx3	mDPC-23/OD-21/M06-G3	mice	Dentin formation ↓	(Heair et al., 2015)
	miR-27,663	Apc	mDPC-23	mice	Odontoblast dif. ↑	(Kim et al., 2014; Park et al., 2014)
Dental follicle	miR-146a	Egfr	hDFC	human	Tooth eruption ↓	(Chen et al., 2014)

LS-8 cell line, murine ameloblast-derived cells; hOEs, human fetal oral buccal mucosal epithelial cells; hDPPC, human dental pulp cells; hFDPC, human fetal dental papilla cells; mDPC, mouse dental papilla cells; hDFC, human dental follicle cells; OD-21, rat odontoblast-like cells; M06-G3, mouse odontoblast-like cells. Red and down arrow indicates inhibition effect, and blue and up arrows indicates promotion effect. (For interpretation of the references to colour in the table, the reader is referred to the web version of this article.)

To examine the significance of miRNAs function in fine tuning of signal pathway activities during incisor renewal, many researchers investigated miRNAs expression profiling in many aspects. In 2010, Michon et al find out that miR-720, expressed in the ameloblast cell lineage from the progenitors to secretory ameloblasts at the tip of the E18 incisor, the miR-200 family (miR-429, miR-200a, miR-200b and miR-200c) and miR-31t that mainly expressed the labial cervical loop whereas the total basal part (containing mainly mesenchymal cells), expressed miR-652 and miR-365 (43) . Predicting gene targets from miRTooth1.0 database (http://bite-it.helsinki.fi/miRNA.htm) revealed Sprouty2 (Spry2), an antagonist of FGF signaling (34) , as a target of miR-720 and miR-652, and Noggin as a target of miR-200b, miR-200c and miR-429 (43).

Sehic A, et al, identified 79 miRNAs in the incisor tooth germ of the mouse(P0 and P10) (45), in eleven of these miRNAs (mmu-let-7a, mmu-let-7b,mmu-let-7c, mmu-let-7d, mmu-let-7f, mmu-miR-145,mmu-miR-210, mmu-miR-214, mmu-miR-30b, mmu-miR-320, and mmu-miR-92) were among the 76 miRNAs that were found to be expressed in molar tooth germ (46). plus, there are different levels of expression

between incisal tip and adjacent segment area in development time (higher levels of expression in the incisal tip segment compared with the adjacent segment at P0: mmu-miR-199a-3p, mmu-miR-500, mmu-miR-190b, mmu-miR-652, mmu-miR-376b, and mmu-miR-296-3p, at P10: mmu-miR-210 and mmu-miR-500)(45).

Jheon et al, found differential expression of miRNAs in the liCL, laCL and the ameloblasts. In the adult mouse incisor, miR-31 showed higher expression in the laCL region specifically in the region of the T-A cells compared to the liCL and miR-138 showed higher expression in ameloblasts compared to the laCL (47). According to miRTooth1.0 database (http://bite-it.helsinki.fi/miRNA.htm), the miRNAs enriched in each region (laCL, liCL or ameloblasts) were predicted to target distinct sets of genes such as 67% of the miRNAs in ameloblasts, compared to 9% of miRNAs in the laCL, target components of the FGF pathway. Spry2 is a potential target of miRNAs enriched in the laCL from all 3 databases. Second, laCL and liCL populations, but not ameloblasts, are enriched for miRNAs that target amelogenin and ameloblastin, two essential matrix proteins in enamel development in mice and humans (48, 49).

RODRIGO S. LACRUZ et al (50), identified genes of enamel mineralization between secretory and mid-late maturation stages findings the regulation of differentially expressed genes (DEGs) is controlled by miRNAs.Three miRNA targets were identified as being important in maturation stage relative to secretory stage including miR-23a, miR-23b which included (Atp6v1b2, Ptk2b, Slc1a1, Sec14l1, Klf5, Lamp1, Slc6a14, Slc6a14, Slc6a14, and Pkp4), miR- 24 included (Sema4a, Arhgef5, Sesn1, Gpx3, and Rab5c) and miR-19a, miR-19b which included (Atp6v1b2, Ptk2b, Slc6a8, Slc24a4, Sdc1, Ptp4a1, Tnfrsf12a, and Tp53inp1). Two miRNAs were identified as down-regulated in maturation including miR-199a (Bmp2, Cd24, Plod2, Fnbp1l, and Plekhh1) and miR-524 (Prickle1, Slc17a6, Col14a1, Ndel1, Olfm1, P4ha2, Fnbp1l, and Atp2b1) (50).

Juuri et al, identified that SOX2 expression, specific marker for stem cells to the labial cervical loop during tooth morphogenesis, and renewal of enamel-producing ameloblasts, in dental epithelial stem cells is possibly regulated by miR-200b and FGF8 expression, specific miRNAs, suggesting a fine-tuning to maintain homeostasis of the dental epithelium, is possibly regulated by miRNAs-720(33).

Cao et al, identified miR-200c/141 contributes to a mechanism related to upregulation of E-cadherin amelogenin and downregulation of noggin, an antagonist of BMP signaling (48). Evidence indicates that miR-200c directly repressed noggin expression by binding on LS-8 murine dental epithelium-like cells. In addition, miR-203 targeted and repressed Bmper, BMP antagonist to regulate cytodifferentiation of dental epithelium-like cells (51).

Sharp et al. have identified that miR-200a-3p has an important role in Pitx2: β-catenin regulatory pathway that involved in epithelial cell differentiation and conversion of mesenchymal cells to amelogenin expressing epithelial cell. Interestingly, miR-200a-3p that regulated by PITX2 is directly targeted the β-catenin 30UTR and repressed β-catenin expression result in upregulation of E-cadherin and amelogenin(52). Moreover, Kim et al shows an important role of MiR-135a expression that can down regulating enamel formation by targeting BMPR-IA and BMPR-IB in BMP signaling(53).

In addition to the well-known signaling factor, according to enamel mineralization, Fan et al reported that miR-224 can bind to the 30UTRs of Slc4a4 and cystic fibrosis transmembrane conductance regulator mRNAs, which are highly expressed during ameloblast differentiation as ion transporters to maintain pH homeostasis and support enamel mineralization(54).

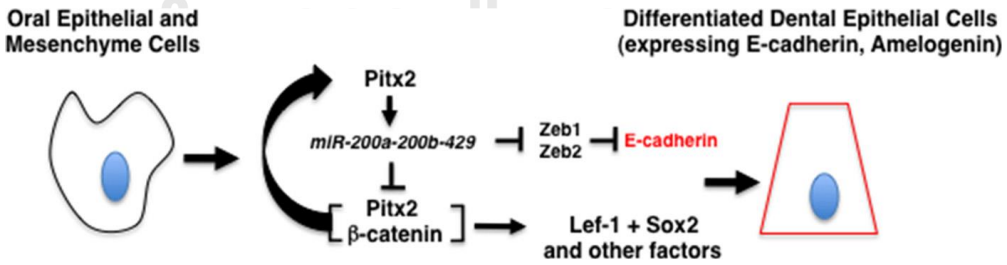


Figure 4*. Role of Pitx2 and* miR-200a.

oral epithelial or dental mesenchymal cells are regulated with Pitx2 and *miR-200a* by inhibits *Zeb1* and *Zeb2*, which repress E-cadherin expression result cells to differentiated to dental epithelium cells. Furthermore, Pitx2 activates

its own expression in loop to fine tune both Pitx2 and *miR-200a* expression. (52))

About dentinogenesis, Huang et al, identified that the Dentin sialophosphoprotein (DSPP), an important marker (55), expression of dental pulp cells is regulated post-transcriptionally by miR-32, miR-885-5p, and miR-586 during odontoblast differentiation, although the complex regulatory network between miRNA and mRNAs remains to be determined (56). In additional studies, Heair et al, also found that miR-665 as a potential repressor of odontoblast maturation by contribute to the alteration in the chromatin status of DSPP and Dmp1 by targeting Kat6a expression and also directly targeted Dlx3 mRNA and decreased DLX3 expression and its downstream targets (Runx2,Osx,DSPP) in mouse odontoblasts (57).

Sun et al 2013, identified that miR-338-3p can promote odontoblast differentiation by suppressing Runx2 expression, a transcription factor associated with osteoblasts and odontoblast differentiation (58). Furthermore, they found that miR-34a plays an important role in dentinogenesis by direct inhibiting NOTCH and BMP signaling pathway that can fine-tune the odontoblast differentiation in stem cells (59).

Table 2. Target miRNAs and target genes

miRNA	**Species**	**miRNA Expression**	**Target gene**	**Ref**
miR-720	Mice	Secretory ameloblast at incisal tip	Sprouty2	Michon et al ,2010 (43)
miR-652	Mice	Basal part of incisor	Sprouty2	Michon et al ,2010 (43)

miR-429 miR-200a miR-200b miR-200c	Mice	laCL	noggin	Michon et al ,2010 (43)
miR-199a-3p miR-500 miR-190b miR-652 miR-376b miR-296-3p	Mice	Incisal tip at P0	-	Sehic A, et al,2010 (45)
miR-210 miR-500	Mice	Incisal tip at P10	-	Sehic A, et al,2010 (45)
miR-31	mice	laCL	Sprouty2	Jheon et al,2011 (47)
miR-138	mice	ameloblast	(Aldh) -1a2 and versican	Jheon et al,2011 (47)
miR-23a miR-23b	Wistar rat	Maturation stage	Atp6v1b2, Ptk2b, Slc1a1, Sec14l1, Klf5, Lamp1, Slc6a14, Slc6a14, Slc6a14, and Pkp4	RODRIGO S. LACRUZ et al ,2012 (50)

miR-24	Wistar rat	Maturation stage	Sema4a, Arhgef5, Sesn1, Gpx3, and Rab5c	RODRIGO S. LACRUZ et al ,2012 (50)
miR-19a miR-19b	Wistar rat	Maturation stage	(Atp6v1b2, Ptk2b, Slc6a8, Slc24a4, Sdc1, Ptp4a1, Tnfrsf12a, and Tp53inp1	RODRIGO S. LACRUZ et al ,2012 (50)
miR-199a	Wistar rat	Maturation stage	Bmp2, Cd24, Plod2, Fnbp1l, and Plekhh1	RODRIGO S. LACRUZ et al ,2012 (50)
miR-52a	Wistar rat	Maturation stage	Prickle1, Slc17a6, Col14a1, Ndel1, Olfm1, P4ha2, Fnbp1l, and Atp2b1	RODRIGO S. LACRUZ et al ,2012 (50)
miR-200b	Mice	Incisor at P2	SOX2	Juuri et al,2012 (33)
miR-720	Mice	Incisor at P2	FGF8	Juuri et al,2012 (33)
miR-200c/141	Mice	LS-8 dental epithelium-like cells	E-cadherin amelogenin Noggin	Cao et al,2013 (51)
miR-203	Mice	LS-8 mice dental epithelium-like cells	Bmper	Cao et al,2013 (51)
miR-200a-3p	Mice	LS-8 mice dental epithelium-like cells	b-catenin/Pitx2	Sharp et al,2014 (52)

miR-135a	Mice	Molar tooth germ/E14	Bmpr-Ia and Bmpr-Ib	Kim et al,2014 (53)
miR-224	Human	hOEs	30UTRs of Slc4a4 cystic fibrosis transmembrane conductance regulator mRNAs	Fan et al,2015(54)
miR-32 miR-885-5p miR-586	Human	hDPPC/impacted third molars	DSPP	Huang et al,2011 (56)
miR-665	Mice	mDPC-23/OD-21/M06-G3	Kat6a/Dlx3	Heair et al,2015(57)
miR-338-3p	Mice	mDPC6T	Runx2	Sun et al,2013 (58)
miR-34a	Human	hFDPC/bell stage tooth buds	Bmp7/Hes1/Notch1,2	Sun et al,2014 (59)
miR-663a miR-27	Mice	mDPC-23	APC	Kim&Park et al, 2014(60, 61)

2.4 Summary

In conclusion, we aimed to identify miRNAs that could be involved in the tooth mineralization in stem cell niches. We collected three parts of dental pulp tissue, one from the apical region of the incisors, one from the coronal region of the incisors, and the other was the molar dental pulp tissue. Expression level of the selected miRNAs were investigated by using quantitative PCR and compared among the parts.

Keyword(s)

Rodent tooth, Hypselodont, Dentine formation, Continuous growth, microRNA, Cervical loop, Stem cell niche

2.5 Research question

Does miRNA(s) expressed in the rodent dental pulp tissue play a role to support continuous growth in the incisors, but limit growth in the molars.

2.6 Research hypothesis

Differential miRNA profile in the incisors and molars may relate to their role in continuous growth

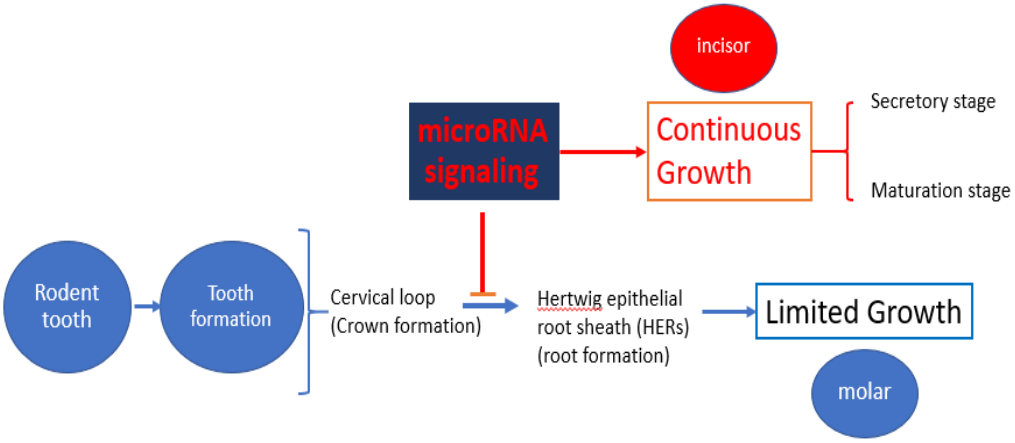
2.7 Research objective

To demonstrate differential microRNA profiles in rodent incisor and molar of the Sprague–Dawley rats

2.8 Study design

A Descriptive cross-sectional study

2.9 Research Conceptual framework



Chapter 3
Material&Method

3.1 The Sprague–Dawley rats model and dental pulp sample collection

Figure 5. *Sprague* Dawley *rats*

5-8 weeks old Sprague *Dawley* rats derived from Chulalongkorn University Laboratory Animal Center (CULAC).

Five- to eight-week-old Sprague–*Dawley* rats, weighed between 100-150 gram (62) were derived from Chulalongkorn University Laboratory Animal Center (CULAC), the Department of Veterinary, Chulalongkorn University, and were handled with the principles and procedure of the guide for the care and use of laboratory animals. All procedures were approved by Chulalongkorn University Animals Care and Use Committee before the use of animals. The animals were prepared and euthanized for a control group in the other study (Protocol number 1973005), and the excess teeth (N=9) that matched our criteria were collected for analysis in this study. Animals were euthanized by carbon dioxide, and the lower jaw were dissected. The mandibles were separated into two hemi-jaws by through a midline incision between the lower incisors. To compare between apical end and coronal end , a molar reference line, line C located on mesial part of 1st molar (62, 63), were used to isolate the lower incisor. After dissection of the tooth, the pulps were released from the root wall by using endodontic hand file (0.15-mm diameter tip, 2% taper, 21 mm) (64) and immersed in PBS.

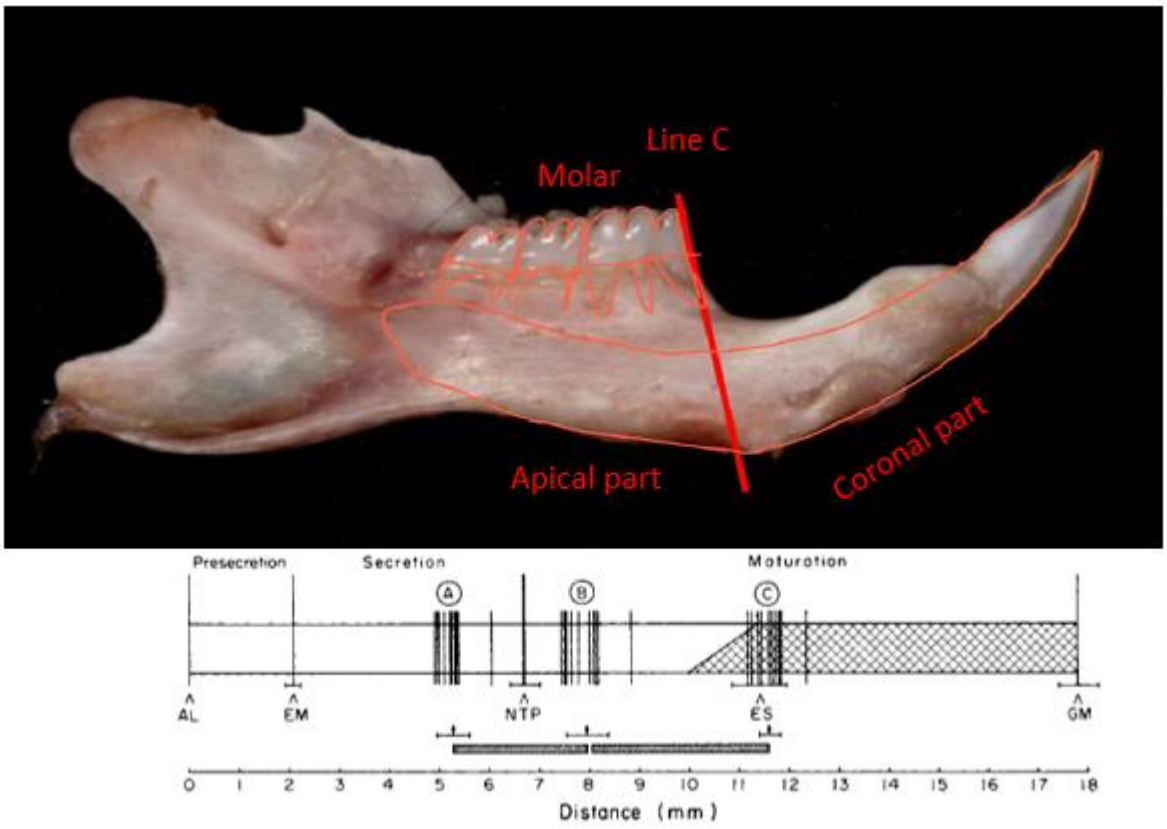


Figure 6 . *Left hemi-jaws and a reference line c*

A reference line C extending from mesial surface of 1st molar upright to labial surface of mandible. Bar = 1 mm. ~ 4.2 (62)

For dental pulp tissue from molars, all molars are gently extracted and separated cross-sectional and longitudinal cut by feather edge blade. After dissection of the tooth, the pulps were released from the pulp chamber and immersed in PBS.

3.2 miRNA isolation and miRNA PCR Array analysis

After collected samples, total RNAs were isolated with miRNeasy 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, A260/A280 ratio should be at least 1.7.

Table 3. The concentration and purity of samples (n=9)

Sample	Part	Nucleic Acid Conc. (ng/µl)	A 260/280
1	Apical	509.3	2.10
	Coronal	69.4	2.09
	Molar	103.9	2.08
2	Apical	162.5	2.13
	Coronal	45.8	1.97
	Molar	44.7	1.73
3	Apical	936.5	2.10
	Coronal	138.1	2.09
	Molar	152	1.83
4	Apical	434.0	2.05
	Coronal	47.1	2.20
	Molar	76.5	2.22
5	Apical	544.9	2.06
	Coronal	66.2	1.82
	Molar	54.5	1.80
6	Apical	527.1	2.07
	Coronal	59.8	1.77
	Molar	54.7	1.79
7	Apical	497.3	2.01
	Coronal	134.6	1.93
	Molar	96.7	1.88
8	Apical	300.3	2.08
	Coronal	41.8	2.25
	Molar	63.5	2.07
9	Apical	281.3	2.12
	Coronal	99.4	2.13
	Molar	51.3	2.18

For cDNA, 500 ng of total RNA sample were converted to cDNA by using 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) were used in combination with the miScript SYBR® Green PCR Kit (Qiagen, Valencia, CA, USA). The customized PCR Array were selected miRNAs from developmental pathway that identified in rat tooth development, as well as appropriate housekeeping genes for miRNAs normalization and quality controls.

Figure 7. *Rt-PCR array material and instrument*

customized miScript miRNA PCR Array with SYBR® Green PCR Kit (Qiagen, Valencia, CA, USA) were using with CFX connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA)

Table ***4. Interested miRNAs and target genes for miRNA profiling with miScript miRNA PCR Array (Qiagen, Valencia, CA, USA)***

	target	miRNA	ref
Dentinogenesis	DSPP	miR-32, miR-885-5p, miR-586	Huang et al ,2011 (56)
	Kat6a>>> DSPP Dlx3	miR-665	Heair et al,2015 (57)
	Runx2	miR-338-3p	Sun et al,2013(58)
	BMP sig, NOTCH	miR-34a	Sun et al,2014(59)
	WNT Sig	miR-663, miR-27	Kim et al,2014& Park et al,2014 (60, 61)
Stemness at maturation stage		miR-23a, miR-23b, miR-24,	LACRUZ et al ,2012(50)

Amelogenesis	FGF Sig; Sprouty	miR-652	Michon et al ,2010 (43)
	FGF Sig; FGF-8	miR-720	Michon et al ,2010 (43) Juuri et al,2012 (33)
	B catenin/pitx2	miR-200a-3p	Michon et al ,2010 (43) Sharp et al,2014 (52)

After collected data from CFX connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), raw quantification cycle values (Ct) of each well were collected, Ct values= 0 and Ct values > 35 were undetectable and were excluded from our study. Therefore, only the miRNAs with a Ct ≤ 35 were included in the analyses, then Ct were normalized by using housekeeping gene (miR-16-5p, miR-423-3p) as normalizers for relative quantification of dental pulp from apical part of incisors, coronal part of incisors and molars.

For statistical analysis by SPSS 22.0 statistic software, the ΔΔCt model were used to determine the difference of each miRNA in the apical group and coronal group (ΔCt target=Ct target -Ct housekeeping gene) compared with the means values in each miRNAs in the molar as a control group (ΔCt control= (Ct control – Ct housekeeping gene). In this study, the difference of each miRNA (the ΔΔCt data) of the apical group as a target and the means values in each miRNAs in the molar as a control group molars (ΔΔCt Apical: Molar control) and the coronal group as a target and the means values in each miRNAs in the molar as a control group (ΔΔCt Coronal: Molar control) were collected and test for normal distribution by using Shapiro-Wilk for normality test. As test above found that some data are not normal distribution. In this case, non-parametric test should be more appropriate for analysis. As a non-parametric alternative to the t-test, one sample Wilcoxon Sign rank test were used to analyze the ΔΔCt values of each miRNAs in apical group to the molar group (ΔΔCt Apical :Molar) and coronal group to the molar group (ΔΔCt Coronal : Molar) by null hypothesis=0. After we found differences between target and control group, the relative formulas (relative miRNA=2^- ΔΔCt =2^-(ΔCt target-ΔCt control)=2^ [- (Ct target -Ct housekeeping gene) - (Ct control – Ct housekeeping

gene)]) was used to determine the relative of each miRNA that are significantly different from one sample Wilcoxon Sign rank test. In this study, the relative expression of apical group as a target and the means values in each miRNAs in the molar as a control group molars (ΔΔCt Apical: Molar control) and relative expression of the coronal group as a target and the means values in each miRNAs in the molar as a control group (ΔΔCt Coronal: Molar control) were presented by fold change compared to the control group.

Chapter 3
Result

Quantitative RT-PCR demonstrates the expression profile of miRNAs in dental pulp from the apical part, coronal part of rat incisors and molars. Fourteen miRNAs (Table 3) were analyzed for miRNA candidates contributing to mineralization during crown formation in rat incisors. Among these candidates, the expression of miR-586 were below detection limit as they were detected at the average Ct values more than 35 in all three groups. However, other miRNAs in the customized array (miR-32-5p, miR-885-5p, miR-665, miR-338-3p, miR-34a-5p, miR-663a, miR-27a-3p, miR-23a-3p, miR-23b-3p, miR-24-3p, miR-652-3p, miR-720, miR-200a-3p) included 2 housekeeping gene (miR-16-5p, miR-423-3p) were detectable in all dental pulp samples. In this study, miR-16-5p was selected for data normalization and calculated for ΔCt values of each miRNAs, because Ct value of miR-16-5p is relatively constant in most samples throughout this study.

From statistical analyses by SPSS 22.0 statistic software, the difference of each miRNA (the ΔΔCt data) of the apical group as a target and the means values in each miRNAs in the molar as a control group, the coronal group as a target and the means values in each miRNAs in the molar as a control group were collected and test for normal distribution. In this study, Shapiro-Wilk test was used to analyze the normal distribution of the ΔΔCt (Table 5). Only some ΔΔCt values demonstrated normal distribution (n=9). In this case, non-parametric test should be more proper for further analysis.

Table ***5. The test of normal distribution of ΔΔCt obtained from selected miRNAs expressed in apical and coronal part of rat incisor dental pulp***

	Apical: Molar control			Coronal: Molar control		
ΔΔCt	Statistic	df	Sig.	Statistic	df	Sig.
ΔΔCt miR325p	.781	7	.027	.881	7	.230

ΔΔCtmiR8855p	.834	7	.088	.889	7	.272
ΔΔCtmiR665	.801	7	.042	.976	7	.938
ΔΔCtmiR3383p	.970	7	.897	.951	7	.738
ΔΔCtmiR34a5p	.842	7	.104	.903	7	.351
ΔΔCtmiR663a	.865	7	.166	.896	7	.306
ΔΔCtmiR27a3p	.564	7	.000	.743	7	.011
ΔΔCtmiR23a3p	.949	7	.716	.980	7	.961
ΔΔCtmiR23b3p	.903	7	.351	.738	7	.009
ΔΔCtmiR243p	.902	7	.341	.829	7	.078
ΔΔCtmiR6523p	.988	7	.988	.891	7	.280
ΔΔCtmir7203p	.942	7	.656	.961	7	.824
ΔΔCtmiR200a3p	.974	7	.928	.581	7	.000

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

As a non-parametric alternative to the t-test, one sample Wilcoxon Sign rank test were used to analyze the ΔΔCt values of each miRNAs in apical group to the molar group (ΔΔCt Apical : Molar) and coronal group to the molar group (ΔΔCt Coronal : Molar). Among the miRNAs candidates, The results showed that miR-32-5p, 885-5p, 665, 338-3p, 663a, 23a-3p, 200a-3p expressed in apical tissue were significantly different from tissue in molars, whereas only miR-32-5p, 665, 338-3p, 663a, 23a-3p in coronal part were significantly different from tissue in molars.

Table **6. One sample Wilcoxon Sign rank test of ΔΔCt of selected miRNAs in apical dental pulp from rat incisors to dental pulp from rat molars (ΔΔCt Apical: Molar) and coronal part of dental pulp from rat incisors to dental pulp from rat molars (ΔΔCt Coronal: Molar)**

	ΔΔCt Apical : Molar		ΔΔCt Coronal : Molar	
	Observed Median ± SD	Asymptotic Sig. (2-sided test*)	Observed Median ± SD	Asymptotic Sig. (2-sided test*)
miR-32-5p	4.30 ± 1.05	0.018	4.34 ± 2.63	0.018
miR-885-5p	0.67 ± 1.67	0.028	0.90 ± 1.21	0.176

miR-665	1.77 ± 1.51	0.012	2.04 ± 0.98	0.018
miR-338-3p	2.43 ± 1.48	0.008	1.36 ± 1.07	0.043
miR-34a-5p	0.38 ± 2.56	0.051	0.11 ± 0.78	0.735
miR-663a	0.85 ± 1.63	0.008	0.96 ± 1.24	0.028
miR-27a-3p	1.20 ± 4.01	0.173	0.27 ± 1.28	0.499
miR-23a-3p	-1.43 ± 1.38	0.011	-2.50 ± 2.59	0.043
miR-23b-3p	0.01 ± 1.39	0.953	-0.53 ± 1.77	0.063
miR-24-3p	1.07 ± 1.28	0.051	0.09 ± 1.56	0.612
miR-652-3p	-0.61 ± 1.55	0.441	-0.64 ± 1.87	0.128
miR-720	-1.09 ± 1.59	0.086	-1.02 ± 1.56	0.128
miR-200a-3p	3.04 ± 1.87	0.011	4.20 ± 13.58	0.237

For the relative expression, significant upregulation of miR-23a-3p are observed in both apical (P=0.11) and coronal (P=0.043) tissue of incisors when compared to molar (Figure 9), while miR-32-5p, miR-665, miR-338-3p, miR-663a are significantly down-regulated in both apical part and coronal part of incisors when compared to molar (Table 7, Figure 9). In contrast, miR-885-5p, miR-200a-3p are found significantly down regulated only in apical part compare to those in molar. Interestingly, expression of miR-23a-3p from apical part of incisors is 5.7-fold higher compare to the molars and miR-23a-3p from coronal part of incisors also 6-fold higher compare to the molars.

Table ***7. Relative expression of*** **selected miRNAs in apical dental pulp from rat incisors to dental pulp from rat molars *(Apical: Molar control)* and coronal part of dental pulp from rat incisors to dental pulp from rat molars (*Coronal: Molar Control*)**

	Apical: Molar control		Coronal: Molar Control	
	Relative expression (fold change)	Asymptotic Sig. (2-sided test*)	Relative expression (fold change)	Asymptotic Sig. (2-sided test*)
miR-32-5p	.0822	0.018	.2440	0.018
miR-885-5p	.7421	0.028	.9809	0.176
miR-665	.3744	0.012	.2837	0.018
miR-338-3p	.2454	0.008	.5689	0.043
miR-663a	.5120	0.008	.5331	0.028
miR-23a-3p	5.6955	0.011	5.9795	0.043
miR-200a-3p	.2874	0.011	.1543	0.237

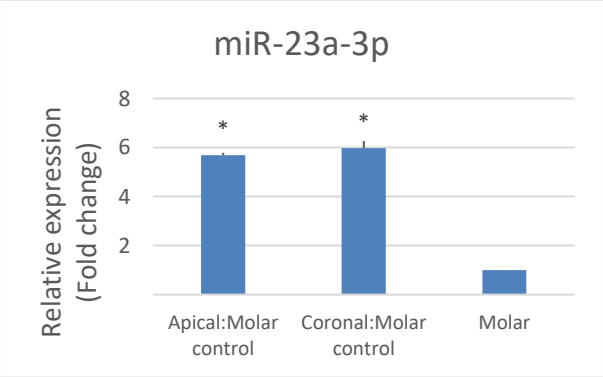


Figure 8. *Relative expression of miRNAs-23a-3p.*

Upregulation of miRNAs-23a-3p in apical dental pulp and coronal dental pulp of incisors are 5.7-fold and 6 fold higher compared to the dental pulp from rat molars

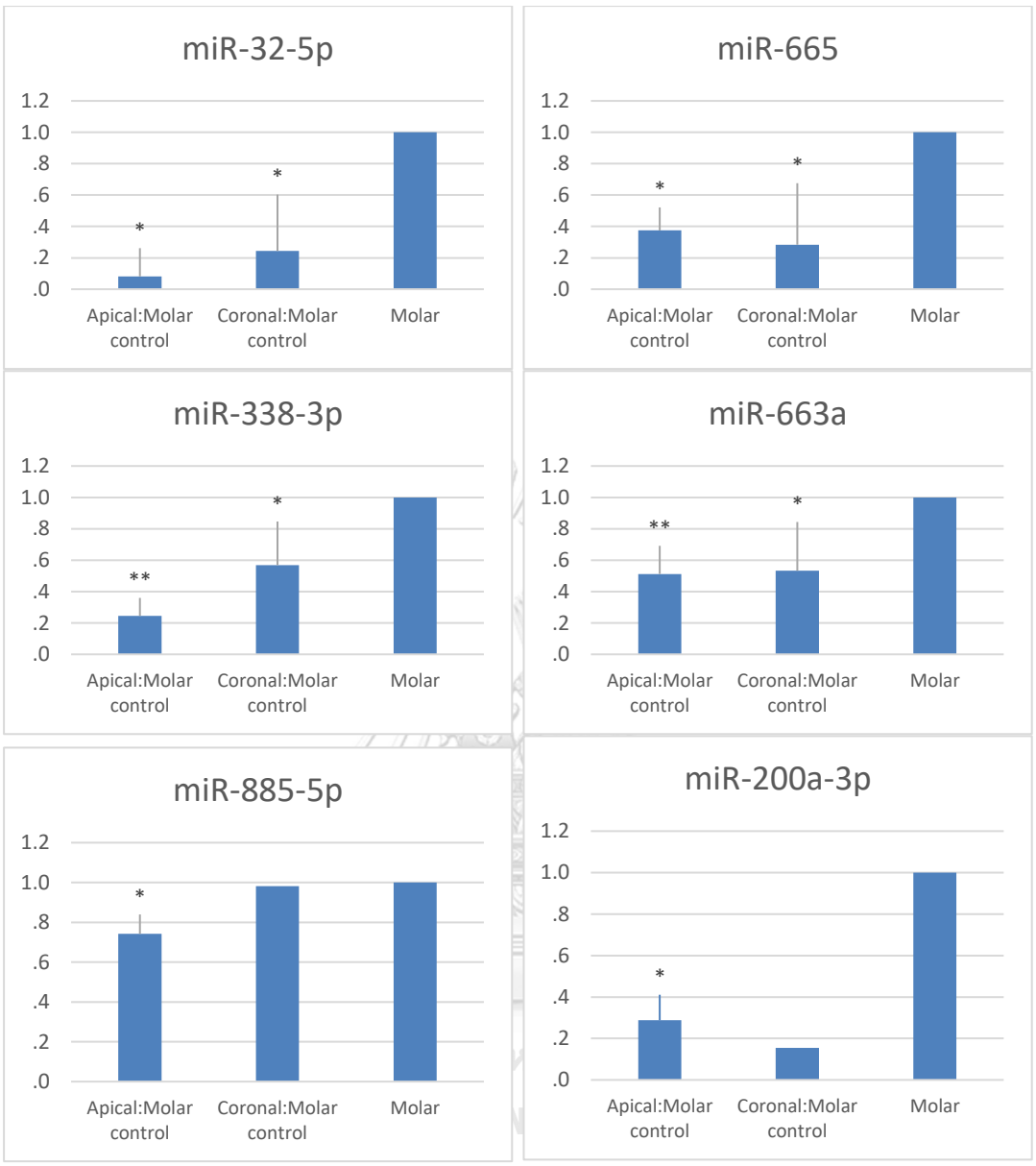


Figure 9. *Mean relative expression of miRNAs miR-32-5p, 885-5p, 665, 338-3p, 663a, 200a-3p*

Mean relative expression of miRNAs in apical dental pulp and coronal dental pulp from rat incisors compared to those in dental pulp from rat molars. Without miR-23a-3p, the other miRNAs are down-regulated compared to dental pulp from rat molars

Chapter 4

Discussion

miRNAs are involved in many stages in the dental development process as the important regulators. Many studies have identified an important role of miRNAs during 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, mainly in secretory stage of amelogenesis which secrete structural enamel matrix proteins (EMP) and containing abundant potential stem cells that can differentiate into ameloblasts and odontoblasts. Furthermore, we focus on coronal part of incisors which mostly on maturation stage of incisors development which ameloblast remove EMP and start for mineralization (62).

Quantitative RT-PCR demonstrates the expression profile of miRNAs in dental pulp from the apical part of rat incisors and molars. Fourteen miRNAs (Table 1) were analyzed for miRNA candidates contributing in 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 (65), were added for housekeeping control for data normalization. In this study, miR-16-5p was selected for data normalization because of Ct value were relatively constant in most samples throughout this study.

From our result, the expression of miR-586 were below detection limit in all groups as they were detected at the average Ct values more than 35 whereas other miRNAs in the customized array were detectable in all dental pulp samples. This corresponds to recent reports indicated this miRNA become lowest level compare to the other miRNAs (mir-32, mir-885-5p), when DSPP was the peak level. Moreover, the undetected

level might cause by this candidate miRNA derived from study that has experiment on human dental pulp cells (56).

Thirteen detected miRNAs were calculated ΔΔCt for statistical analysis, 7 miRNAs from apical part of incisors (miR-32-5p, 885-5p, 665, 338-3p, 663a, 23a-3p, 200a-3p) are significantly different from dental pulp from molar whereas 5 miRNAs from coronal part of incisors (miR-32-5p, 665, 338-3p, 663a, 23a-3p) are significantly different from those in molar in molar. After that, those significances miRNAs were calculated for means relative expression in the apical part of rat incisors as compared to rat molars and coronal part compared to rat molars. 6 miRNAs in apical part (miR-32-5p, 885-5p, 665, 338-3p, 663a, 200a-3p) and 4 miRNAs from coronal part (miR-32-5p, 665, 338-3p, 663a) that have down regulated compared to molars group suggests that these miRNAs may be expressed in molar pulp tissue higher than incisors. Only 1 miRNA from apical and coronal part are up regulated(miR-23a-3p) compared to the molars group.

For these down regulated miRNAs, 5 of them are involve in Dentin formation. Three of them (miR-32, miR-885-5p, miR-665) have inhibit effect to Dentin sialo-phosphoprotein (DSPP) mRNA, the odontoblast specific marker that expressed in odontoblast and momentarily in pre-ameloblasts which involve in odontoblast differentiation and mineralization (55). From previous study, expression levels of three miRNAs (miR-586, miR-32, miR-885-5p) were subsequently low especially miR-586 whereas DSPP were relatively high (56). In Addition, recent study also found miR-665 targeting Kat6 and DLX3 mRNA result to decreased their expression that effect to down regulated DSPP activity (57). That's means from our result, down regulated of these 3 miRNAs included miR-32-5p and miR-665 in both apical part and coronal part of incisors and miR-885-5p in apical part of incisors would be effect to uprising DSPP activity in incisors and may explained that why in rodent incisors steadily have increased function in dentinogenesis compare to the molars.

For the others 2 miRNAs (miR-338-3p, miR-663a), previous study reported miR-338-3p has promoting effect on odontoblast differentiation by directly suppressing RUNX2 expression. Runx2 is down-regulated in differentiated odontoblasts during cytodifferentiation and up-regulated in the condensed dental mesenchyme (58) which means from our result, lower expression level of miR-338-3p in apical part and coronal

of incisor might be able to get uprising of RunX2 that may result to suppressed differentiation of odontoblast but also up regulated the condensed dental mesenchyme. As mentioned above may explained that why incisor they still have gradually increased of dental mesenchyme compare to the molars. Furthermore, expression of miR-663a is suppressing Adenomatous polyposis coli (APC) gene, which can activated Wnt/β-catenin signaling pathway, one of important pathway in both amelogenesis and odontogenesis which may involve in root odontogenesis in mouse and also result in odontogenic differentiation without inducing cell proliferation (60). So, due to our result, higher expression of miR-663a in molars compare to the incisors may explain why in molars still have odontogenic differentiation but also decreased in cell proliferation and may explain why in molars have a root formation that opposing to rodent incisors that don't have this activity.

About amelogenesis, our result found that miR-200a-3p from apical part of incisors is down regulated compared to the molars group. According to previous study, miR-200a-3p is directly targeted and suppressed the β-catenin expression, regulator that involve in enamel formation by regulates incisor epithelial cell proliferation and differentiation. In addition, miR-200a-3p also suppressed PITX2, a gene that control dental progenitor cell proliferation and differentiation. PITX2 is activated and reciprocally suppressed by miR-200a-3p. Amalgamation of PITX2 and miR-200a-3p can reprograms mesenchyme cells to dental epithelial cells, which involved by upregulated of the stem cell marker SOX2, E-cadherin, and amelogenin and decreased expression of mesenchyme markers (52). So, higher expression level of miR-200a-3p in molars compare to the incisors may explain why incisors still have continual amelogenesis activity compare to the molars by this model.

There are only 1 upregulated miRNA (miR-23a-3p) in both 2 groups compare to the molar. Our data shown the expression of miR-23a-3p from apical part of incisors is 5.7-fold higher compare to the molars and miR-23a-3p from coronal part of incisors also 6-fold higher compare to the molars. This candidate miRNA we selected from previous study that found this miRNAs in maturation stage of the lower incisor of Wistar rat (50) but in the other studied involved dental subjects only found this

miRNAs in other part such as in periodontitis tissues (66), especially in periodontal mesenchymal stem cells and gingival crevicular fluid that involve in osteogenesis (67). On the other hand, in the other aspect also found the role of this miRNA in angiogenesis and choroidal neovascularization by targeting Sprouty2 and Sema6A proteins, which exert antiangiogenic activity result to enhance angiogenesis (68). As we known Sprouty2 gene also a gene that regulated FGF, one of the main signaling pathway that regulated and promote proliferation dental epithelial stem cell which involved in amelogenesis (34). Even though recent reported shown the role of this miRNA in different location, but in this case may suspected that it possibly have the same potential involve dental development too which mean it may have the linked between this miRNA in rodent incisors and this miRNA may have potential in up regulatory module in amelogenesis or dentinogenesis. On the other way, unlike the rodent molar that have completed root formation, the rodent incisors does not have root formation which mean on the apical tip are opening and should have abundance of blood supply compare to the molars that may be the reason why we found the miR-23a-3p higher in the apical part and coronal part.

Table ***8. Summaries of significance different miRNAs expression between incisors and molar with their mechanism, (*)= Not detectable in any dental pulp tissue (52, 56-58, 60, 68)***

miRNA	Function	Apical	Coronal	Function of miRNAs
miR-200a-3p	Amelogenesis	↓	--	Repress E-cadherin expression
miR-32-5p	Dentinogenesis	↓	↓	Down-regulated DSPP activity
miR-885-5p		↓	--	Down-regulated DSPP activity
miR-586*		--	--	Down-regulated DSPP activity
miR-665		↓	↓	Down-regulated DSPP activity
miR-338-3p		↓	↓	Suppressing RUNX2 expression
miR-663a		↓	↓	Activated Wnt/β-catenin signaling pathway

miR-23a-3p	Stemness	↑	↑	Targeting Sprouty2 in Choroidal neovascularization

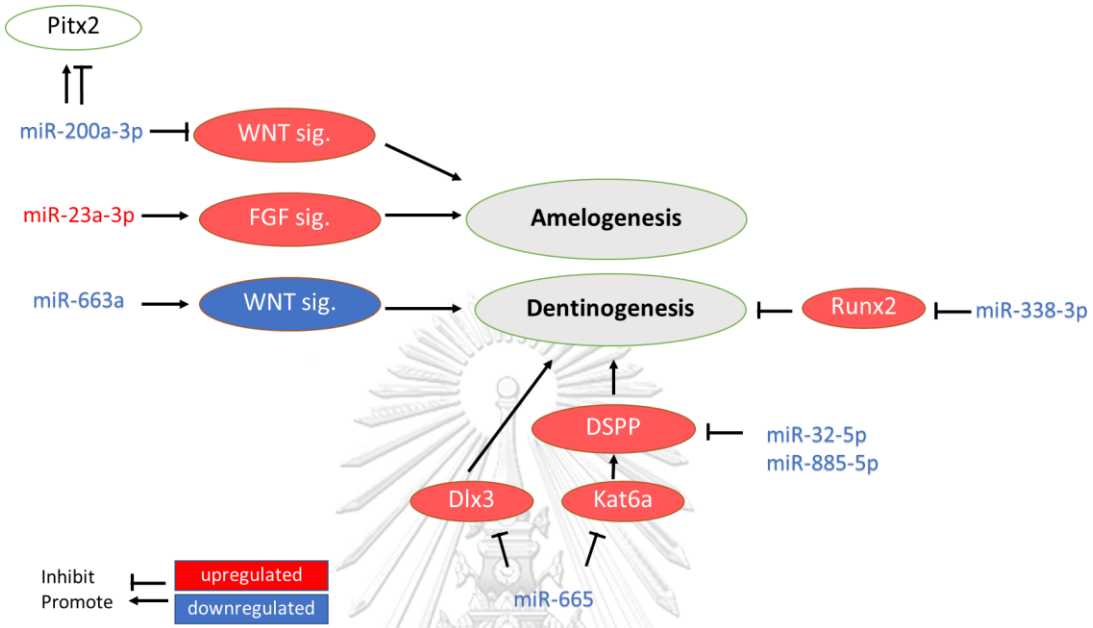


Figure 10. *Schematic of miRNAs expression in apical part of incisor compare to the molars.*

These 7 miRNAs may have potential to promote and inhibit gene, transcription factor and signaling pathways especially DSPP which result upregulated amelogenesis and dentinogenesis.

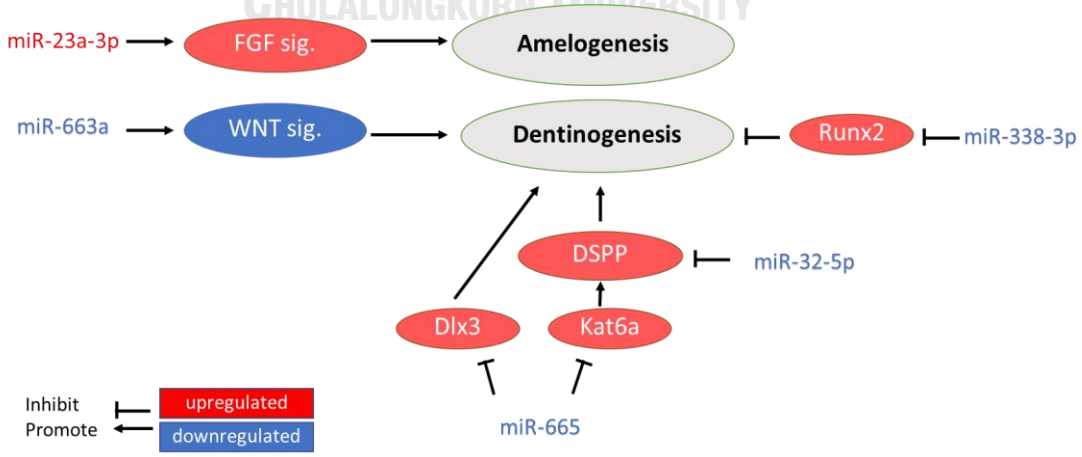


Figure 11. *Schematic of miRNAs expression in coronal part of incisor compare to the molars.*

These 5 miRNAs may have potential to promote and inhibit gene, transcription factor and signaling pathways especially DSPP which result upregulated amelogenesis and dentinogenesis.

Limitation

However, Due to the complexity of dental development mechanism, many of cells, transcription factor, growth factor, gene and signaling pathways would have more or less impact factor on the development process compare to these miRNAs. Furthermore, there are a few knowledges and controversial about specific miRNAs that could involve in mineralization of tooth in previous studies. Although many animal studies have shown the key role of miRNAs in enamel and dentine mineralization, correlation in humans remains ambiguous. Moreover, due to ethical issue in this study, our protocol strictly limits of miRNA candidate in customize tray, increased number of candidate miRNAs from previous study may improve our understanding of these miRNAs in the rat dental pulp tissue and further studies are necessary.

Chapter 5
Conclusion

This study shows the differential expression of miRNAs in the apical pulp tissue of incisors and molars. In conclusion, we conclude that 6 miRNAs (miR-32-5p, 885-5p, 665, 338-3p, 663a, 200a-3p) in apical part and 4 miRNAs (miR-32-5p, 665, 338-3p, 663a) from coronal part are significantly lower than molar group whereas miR-23a-3p is the only one that has significantly increase in both apical and coronal part compare to the molar group. The results suggested that these miRNAs may have a role in mineralization process during prolonged crown formation of rodent incisors. Due to the limitation of this study, increased number of samples and further studies are necessary.

Resource

This study was supported by Faculty of Dentistry, Chulalongkorn University

Ethical issue

The study protocol was reviewed and approved by Chulalongkorn University Animals Care and Use Committee. Faculty of Veterinary, Chulalongkorn University, Bangkok, Thailand (Protocol number 1973005).

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