Intermittent hypoxia induces mandibular growth arrest and invokes a different response in condylar and tibial cartilage in infant rats

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ภาวะพร่องออกซิเจนเป็นพักๆ ทำให้เกิดการเจริญเติบโตที่ช้าลงของกระดูกขากรรไกรล่าง และกระตุ้นให้เกิดการตอบสนองที่แตกต่างกันของกระดูกอ่อนบริเวณข้อต่อขากรรไกรและแข้งในหนูแรกเกิด

น.ส.กชกร เล็กวิจิตรธาดา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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กชกร เล็กวิจิตรธาดา : ภาวะพร่องออกซิเจนเป็นพักๆ ทำให้เกิดการเจริญเติบโตที่ช้าลงของกระดูกขากรรไกรล่าง และกระตุ้นให้เกิดการตอบสนองที่แตกต่างกันของกระดูกอ่อนบริเวณข้อต่อขากรรไกรและแข้งในหนูแรกเกิด. (Intermittent hypoxia induces mandibular growth arrest and invokes a different response in condylar and tibial cartilage in infant rats) อ.ที่ปรึกษาหลัก : ผศ. ทพ. ดร.ชิษณุ แจ้งศิริพันธ์

ภาวะพร่องออกซิเจนเป็นพักๆ เกี่ยวข้องกับการเติบโตของโครงกระดูก อย่างไรก็ตาม ยังไม่มีรายงานเกี่ยวกับอิทธิพลของภาวะนี้ต่อการเจริญเติบโตและเมแทบอลิซึมของเซลล์กระดูกอ่อน การศึกษานี้เปรียบเทียบผลของภาวะพร่องออกซิเจนเป็นพักๆ ต่อการเพิ่มจำนวนและการเจริญเติบโตของเซลล์กระดูกอ่อนเส้นใยที่พบในกระดูกขากรรไกรล่างและกระดูกอ่อนชนิดไฮยาลินที่พบในกระดูกแข้งในหนูสายพันธุ์สปราก-ดาวเลย์เพศผู้อายุ 1 สัปดาห์ หนูได้รับอากาศปกติหรืออยู่ในภาวะพร่องออกซิเจนเป็นพักๆ ที่ 20 รอบ / ชม. (ต่ำสุด 4% O2; สูงสุด 21% O2; 0% CO2) เป็นเวลา 8 ชั่วโมงในแต่ละวัน ผลการศึกษาพบว่า ภาวะพร่องออกซิเจนเป็นพักๆ ขัดขวางการเพิ่มของน้ำหนักตัวหนู แต่ไม่มีผลกับการเพิ่มความยาวของกระดูกแข้ง ภาวะนี้ยังทำให้กระดูกเนื้อโปร่งบริเวณข้อต่อขากรรไกรมีความหนาแน่นของกระดูกและความหนาแน่นของกระดูกเชิงปริมาตรเพิ่มขึ้น รวมทั้งส่งผลให้ความหนาของชั้นเซลล์กระดูกอ่อนในระยะปรับตัวลดลง แต่ความหนาของชั้นเซลล์กระดูกอ่อนในระยะไฮเพอร์โทรฟีกลับเพิ่มมากขึ้นในบริเวณส่วนกลางและส่วนท้ายของกระดูกอ่อนข้อต่อขากรรไกร แต่ไม่พบความเปลี่ยนแปลงนี้ในกระดูกแข้ง ผลการศึกษาเนื้อเยื่อแสดงให้เห็นว่าภาวะพร่องออกซิเจนเป็นพักๆ เปลี่ยนแปลงการเพิ่มจำนวนและการเจริญเติบโตของเซลล์กระดูกอ่อนในข้อต่อขากรรไกรล่างไปในทิศทางที่ทำให้เกิดการสร้างกระดูกที่เพิ่มมากขึ้น ผลการทดสอบปฏิกิริยาลูกโซ่พอลิเมอเรสตรวจพบการลดลงของ TGF-β และ SOX9 ในขณะที่ คอลลาเจนชนิด X เพิ่มขึ้นในกระดูกอ่อนบริเวณข้อต่อขากรรไกร แสดงให้เห็นถึงการเปลี่ยนแปลงของเซลล์กระดูกอ่อนไปเป็นเซลล์สร้างกระดูก ซึ่งไม่พบความเปลี่ยนแปลงนี้ในกระดูกแข้ง ผลการทดลองนี้แสดงให้เห็นว่าหนูแรกเกิดที่อยู่ในภาวะพร่องออกซิเจนเป็นพักๆ จะพบการเจริญที่น้อยลงบริเวณส่วนท้ายและส่วนข้อต่อของกระดูกขากรรไกร ร่วมกับตรวจพบการยับยั้งการแสดงออกของยีนที่เกี่ยวข้องกับการสร้างเซลล์กระดูกอ่อนในกระดูกขากรรไกรที่ถูกยับยั้งการเจริญเติบโตนี้

สาขาวิชา ทันตกรรมจัดฟัน ลายมือชื่อนิสิต .....

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KEYWORD: cartilaginous growth, chondrocyte hypertrophy, fibrocartilage, hyaline cartilage, intermittent hypoxia

Kochakorn Lekvijittada : Intermittent hypoxia induces mandibular growth arrest and invokes a different response in condylar and tibial cartilage in infant rats. Advisor: Asst. Prof. CHIDSANU CHANGSIRIPUN, Ph.D.

Intermittent hypoxia (IH) has been associated with skeletal growth. However, the influence of IH on cartilage growth and metabolism is unknown. We compared the effects of IH on chondrocyte proliferation and maturation in the mandibular condyle fibrocartilage and tibial hyaline cartilage of 1-week-old male Sprague-Dawley rats. The rats were exposed to normoxic air (n = 9) or IH at 20 cycles/h (nadir, 4% O_2; peak, 21% O_2; 0% CO_2) (n = 9) for 8 h each day. IH impeded body weight gain, but not tibial elongation. IH also increased cancellous bone mineral and volumetric bone mineral densities in the mandibular condylar head. The mandibular condylar became thinner, but the tibial cartilage did not. IH reduced maturative and increased hypertrophic chondrocytic layers of the middle and posterior mandibular cartilage. PCR showed that IH shifted proliferation and maturation in mandibular condyle fibrocartilage toward hypertrophic differentiation and ossification by downregulating TGF-β and SOX9, and upregulating collagen X. These effects were absent in the tibial growth plate hyaline cartilage. Our results showed that neonatal rats exposed to IH displayed underdeveloped mandibular ramus/condyles, while suppression of chondrogenesis marker expression was detected in the growth-restricted condylar cartilage.

Field of Study: Orthodontics Student's Signature .....

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Chapter I

Background and rationale

Intermittent hypoxia (IH) is one of the preceding symptoms of apnea of prematurity (AOP) and sudden infant death syndrome (SIDS) (1, 2). Infants are susceptible to obstructive sleep apnea (OSA) because of their anatomical and physiological predispositions toward airway obstruction and impeded gas exchange abnormalities; including a superiorly placed larynx, increased chest wall compliance, ventilation–perfusion mismatching, and ventilatory control instability (1). Micromandible, maxillary hypoplasia and macroglossia are the craniofacial characteristics in infants with OSA (3, 4).

IH causes multi-factorial morbidities in infants: neurobehavioral impairment, retardation of growth and development, and immature physiological responses in sudden infant death syndrome (5-8). Clinical investigations suggest that treatments of upper airway obstruction by adenotonsillectomy and continuous positive airway pressure (CPAP) rescue children with OSA from oro-facial growth retardation (9, 10). Consistently, animal studies in the prepubertal rodents exposed to IH have recapitulated the craniofacial growth impairment; nasal bone hypoplasia with a reduced nasal cavity volume (11), mandibular growth retardation with an increased bone mineral density (11-13) and the underdevelopment of the mandibular ramus/condyle unit (14). However, there has been no report to demonstrate the

effect of IH on the craniofacial growth during infancy, despite craniofacial growth reaches its highest rate during the first 3-5 years of postnatal life in humans (15) and the first 3 weeks of postnatal life in rodents (16).

Temporomandibular joint cartilage is the fibrocartilage derived from embryonic secondary cartilage cells, and epiphyseal cartilage of mandibular condylar head is the major site of mandibular growth and remodeling (17). It has different developmental characteristics from the limb growth plates of hyaline cartilage, primary cartilaginous joint in cellular composition and organization, extracellular matrix components, and embryonic origin (18). Of interest, there are different responses to growth factors between fibrocartilage and hyaline cartilage, such as TGF-β (19, 20). Chondrocytes isolated from the temporomandibular joint and the limb express chondrogenic markers, such as type II and X collagens, SOX9, cartilage oligomeric matrix protein (COMP). Collagen type X proteins is highly expressed in the hypertrophic chondrocytes (21, 22), and its expression apparently reflects endochondral ossification in the both types of cartilage (23, 24). Chondrogenic differentiation is triggered by transforming growth factor beta (TGF-β) / Smad signaling pathway through SOX9 (25), which upregulates collagen type II and reciprocally down-regulates collagen type X in chondrocytes (26). In addition, Sox9 keeps Runx2 expression and β-catenin signaling related to osteogenesis, which reduces the conversion of hypertrophic chondrocytes into osteoblasts and osteocytes in the cartilage-to-bone transition during the endochondral bone formation (27).

Previously, animal studies showed that IH affected a longitudinal growth of the rat mandible, compared to the tibia (11, 13). There might be specific metabolic responses to IH between the two exemplified types of cartilage: the fibrocartilage of the mandibular condyle and the hyaline cartilage of the tibial growth plate. Based on these lines of evidence, we speculated that there was a different response of cartilage or bone metabolism between the fibrous cartilage of the mandibular joint and the hyaline cartilage of the limb growth plate to IH, and to thus investigate temporal chondrogenesis marker expression.

1.1 Research questions

- Does IH disturb the growth of craniofacial bones in neonatal rats?
- Does IH differently affect growth in the cartilages of the mandibular condylar (fibrocartilage) and tibial growth plate cartilage (hyaline cartilage) in neonatal rats?
- Does IH cause temporal changes in chondrogenesis marker expression in neonatal rats?

1.2 Research null hypotheses

- There is no difference in growth of the craniofacial bones between experimental and control group.

- There is no difference in growth of the condylar and tibial cartilage between experimental and control group.
- There is no difference in chondrogenesis marker expression between experimental and control group.

1.3 Objectives of the study

Objective 1: To inspect whether IH disturb the growth of craniofacial bones in neonatal rats.

Objective 2: To investigate the effects of IH on mandibular condylar and tibial cartilage growth in neonatal rats.

Objective 3: To investigate the effects of IH on chondrogenesis marker expression in rats at early stage of growth.

1.4 Benefits of this study

This study will help us to provide a clearer picture of the response of mandibular condylar cartilage and tibial cartilage to IH in a neonatal in vivo model, which will fill up the gap in our knowledge about effects of this condition on different types of cartilage, which is important in craniofacial growth and growth of the tibia during early growing stage.

1.5 Keywords

Cartilaginous growth, chondrocyte hypertrophy, fibrocartilage, hyaline cartilage, intermittent hypoxia

Chapter II

Literature Review

Intermittent Hypoxia

Intermittent hypoxia (IH) has been an issue of interests in many fields of research in the past decades. It has been widely discussed in both positive and negative consequences of IH in many physiological systems. Currently, there is no universal definition of IH, still it is broadly defined as the condition that oxygen pressure is episodically decreased, lead to oxygen deficiency within the blood for a short period of time, combined with the period of normal oxygen level (28-30). These phenomena resulted in disturbances in normal physiological activities.

According to previous study, IH has been reported to be the most frequent form of hypoxia occurring in the developing mammal. Since the maturational process of many systems in the body, such as neural, mechanical, pulmonary and sleep-state dependent factors, prefer the existence of IH during early postnatal life (31).

Episodes of IH can be found in neonatal and early infancy, because of immature respiratory control. The incidence is increased in preterm and low birth weight infants, especially in the 2-8 weeks of postnatal life (Figure 1) (32, 33).

Respiratory pausing, a cessation of breathing lasting a short period of time, can occurs in neonatal and early infancy, which considered normal in both term and preterm infants (34-36). The length of the pause in breathing air flow >15 s or >20 s

defined as apnea, and the clinically significant will be increased if associated with changes in perfusion, heart rate or oxygen desaturation (37-40). The pause of breathing > 20s or shorter duration but accompanied by bradycardia; heart rate <2/3 of baseline for ≥4 s, and/or oxygen desaturation; SpO2 ≤80% for ≥4 s, in an infant younger than 1 year 37 weeks' gestational age will be defined as apnea of prematurity (AOP) (Figure2) (41, 42). AOP is the developmental disorder that occurs in preterm infants, and apnea can be resolved by term gestation, except those who were born less than 28 weeks gestation (43-45). Although the short respiratory pauses or apnea with adequate oxygen level maintained may be of minimal consequences, they can be problematic if accompanied by intermittent hypoxemia (Figure3).

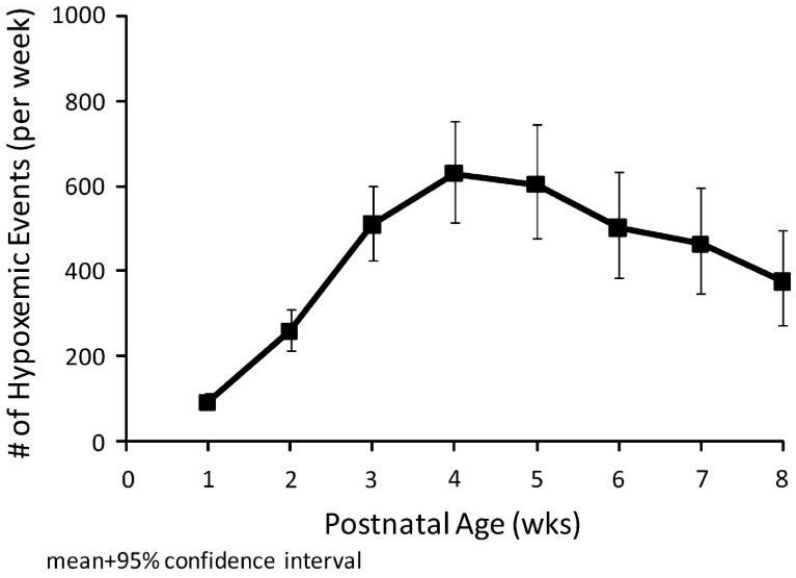

Figure 1. Number of desaturation episodes in preterm infants.
There were very few desaturation episodes during the first week, followed by a progressive increase over the 2-4 weeks, and then a decrease in weeks 6–8 in preterm infants over the first 8 weeks of life (32).

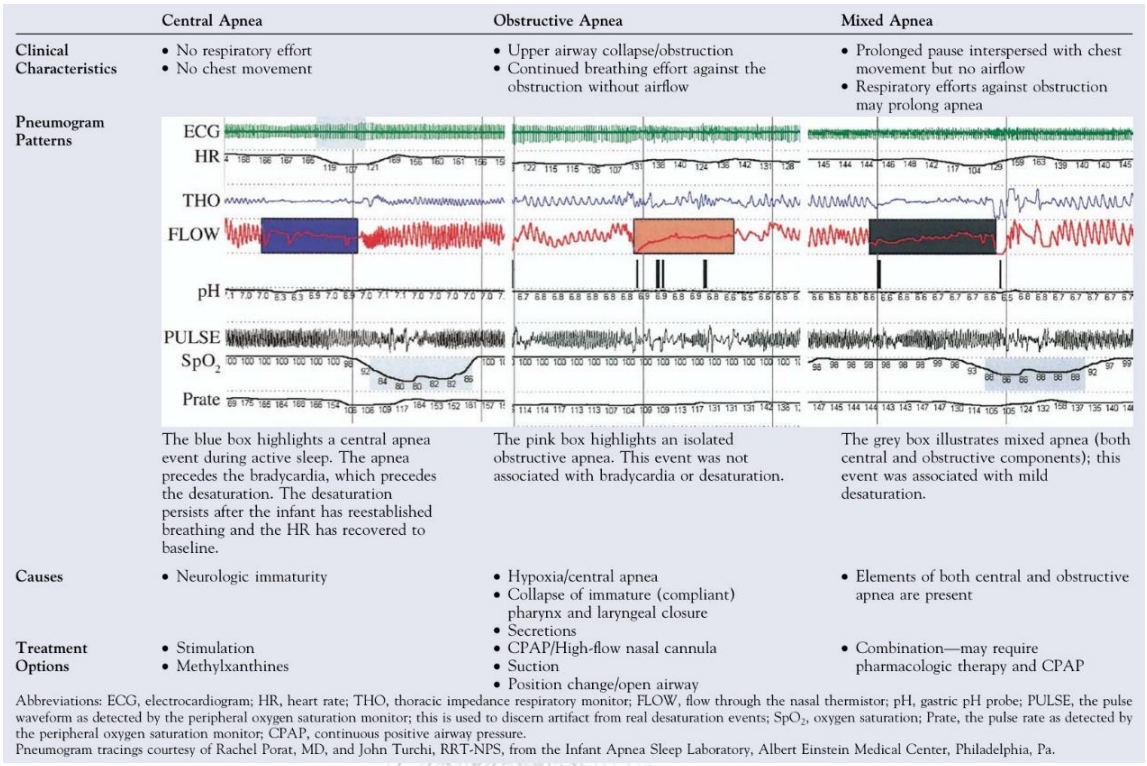

Figure 2. Subtypes of apnea of prematurity.

Subtypes of apnea of prematurity, based on the pathophysiologic mechanisms. Central apnea; complete cessation of both chest movement and airflow. Obstructive apnea; an apnea with or without respiratory effort but without airflow. Mixed apnea; components of both central and obstructive apnea (40).

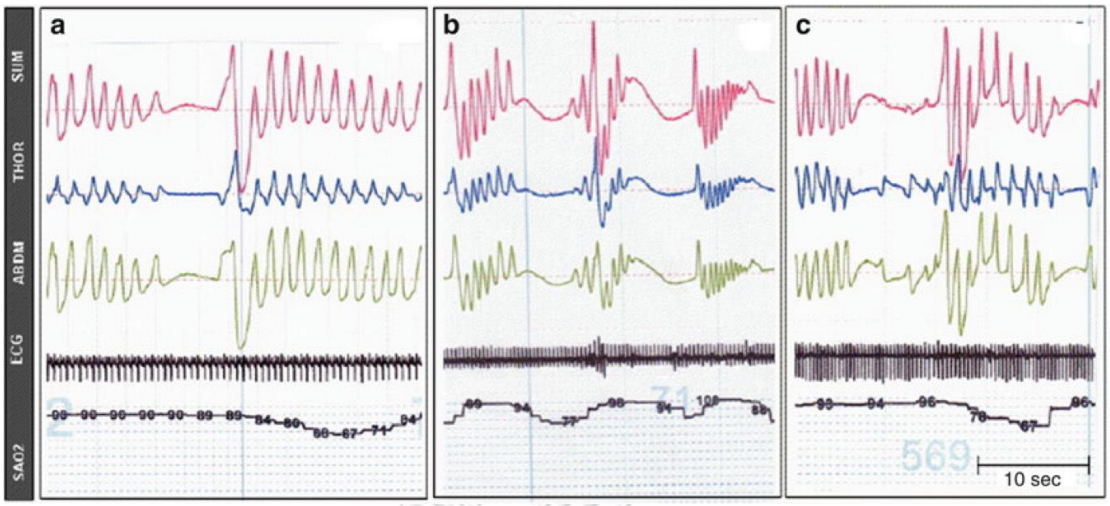

Figure 3. Desaturation in preterm infants secondary to apnea or respiratory pauses. The examples of desaturation in preterm infants secondary to apnea or respiratory pauses. (a) An isolated central apnea. (b) The periodic breathing with repetitive IH. (c) A mixed apnea with obstructed inspiratory efforts (33).

The frequency and severity of AOP has been related to various unfavorable outcomes including abnormal myelin, synaptic connections, and mental development (46, 47).

Chronic intermittent hypoxia (CIH) regularly occurs in preterm infants, associated with high frequency of apnea, as reported by Di Fiore et al in 2010, these group of infants have greater incidence of retinopathy of prematurity and higher risk of neural development disturbances (32, 48, 49).

Exposure to intermittent hypoxia results in multi-system morbidity in adult: cardiorespiratory instability, arterial hypertension, and neurobehavioral impairment (6-8, 50). In contrast to adult, the side-effects of intermittent hypoxia in neonatal and infants often relates to growth and the development.

IH effects

In the past decades, it has been more and more evident that hypoxia, even when short duration, can alter subsequent systemic responses and stimulate or inhibit variety of genes expressions, which lead to persistence of the consequences for much longer time than the hypoxic duration itself. There are many reports about several pathophysiologic problems, which supposed to be consequences of IH exposure in neonatal and early infancy (Figure 4).

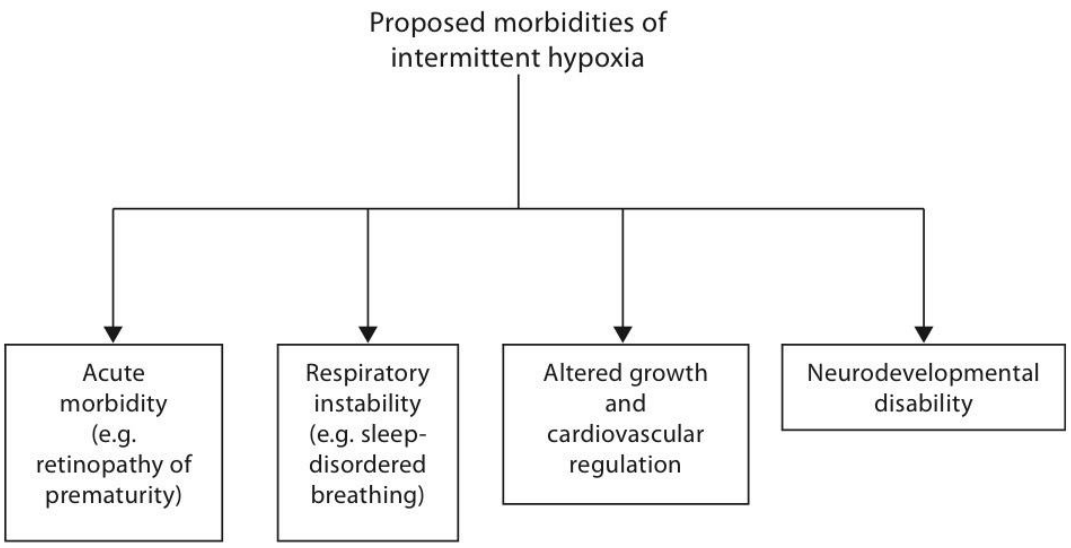

Figure 4. Consequence of intermittent hypoxia in early life
Various of pathophysiologic problems that might be a consequence of intermittent hypoxia in early life (48).

1. Retinopathy of prematurity (ROP)

Retinopathy of the prematurity (ROP) is a disorder that can produced significant loss of vision resulted from perturbations in retinal vascular development. A swing in oxygen levels is one of the important factors in ROP development. The

first phase of ROP begin with a delayed retinal vascular growth from hyperoxia, which suppressed vascular endothelial growth factor (VEGF), resulting in normal vascularlization inhibition. The second phase involves with the raising of the hypoxia-induced VEGF level, bring about the vascular overproliferation, which later turns into a pathological retinal neovascularization. A previous cohort study in neonates of 24-28 weeks' gestation showed the association between IH episodes and the development of severe ROP; which required laser treatment. They demonstrated that an increased frequency of IH episodes during the first 8 weeks of life is associated with ROP severity (Figure 5) (32).

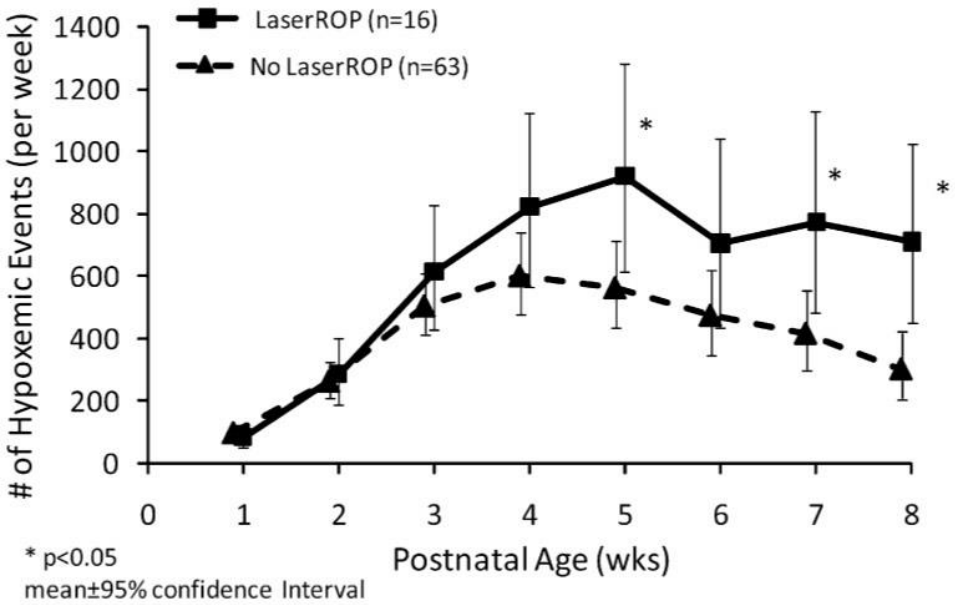

Figure 5. Intermittent hypoxemic events in the preterm infants.

The model-based of approximated intermittent hypoxemic events in the preterm infants. The overall incidence was higher in the LaserROP infants (32).

The ROP severity is involved with not only the higher incidence of IH, but also the pattern of IH episodes. The studies in rodent model and in human infants showed that severe ROP was associated with higher incidence of IH events of longer, more variable, and less predictable duration (51, 52).

2. Respiratory instability: sleep-disordered breathing

It has become evident that early postnatal exposure to oxygen perturbations in neonates resulted in marked changes in the respiratory functions. As discussed in previous animal studies, postnatal hyperoxic/hypoxemic exposure diminished peripheral chemoreceptors functions, and hypoxic ventilatory responses. Such an effects in early life contribute to lifelong alteration in respiratory control (53-56). Intermittent hypoxia is more likely to occur postnatally, especially in preterms infants. There are two studies published that these groups of infants are at greater risk for sleep disordered breathing in childhood and young children (57, 58). However, the hidden mechanisms of these relationship are still unidentified. There is suggestion from prior study, which reported that neonates exposed to xanthines had an increased risk of sleep-disordered breathing (SDB), that AOP and its resultant IH that occurred in early life could be a factor of risk in developing perturbation and SDB later on (59). Another study in neonatal rat pups demonstrated that sustained exposure to IH lead to increased sensory activity of the carotid body; a primary chemoreceptors for arterial blood oxygen detection, activation (60). Such an effect

has been reported in human preterm infants' studies that a raise in carotid body activation in response to intermittent hypoxia led to unstable breathing (61, 62). Reactive oxygen species have been suggested to be involved with the aforementioned situation (48).

3. Cardiovascular consequences

Since IH episodes are common among preterm infants, exposure to IH at early postnatal age may lead to alterations in cardiovascular control, as for example the impaired in baroreflex responses (63). Reeves et al. 2006 reported CIH exposure of rat pups in first 30 days postnatal resulted in structural changes within brainstem nuclei that control cardio-respiratory functions (64). More recent studies found that exposure to IH, of different patterns, during the early postnatal life lead to sustained decreased in heart rate, higher systolic blood pressure, increased in heart weight, and increased size of cardiomyocytes (5, 63, 65). It has been suggested that repetitive IH exposure during developmental period contributed to prolonged changes in cardiovascular dysfunction later in the adulthood life (65, 66). There are epidemiological studies that have identified the strong relationship between prematurity and greater risk for developing cardiovascular problems in adulthood. They suggested that events occurred during early postnatal life, such as hypoxic episodes, may lead to alteration in normal development of cardiovascular systems and such effects may prolonged into later life (67-70).

4. Neurodevelopmental outcome

IH in early infancy may result in inflammation, brain injury, which later exhibits as cognitive and executive function deficits. In previous studies, neonatal exposed to IH exhibited increased in apoptosis in the cortex and hippocampus, impaired hippocampal neural function and reduced dopamic signaling and myelination in the corpus callosum and white matter (71-75). Clinical manifestations of neonatal IH, which had been detected in animal model, are long-term behavioral alterations, memory and learning impairments, and locomotor hyperactivity (76, 77). However, the degree of IH in those studies are not the same, depended on the protocol used in each laboratory. There are two studies stated the degree of IH applied in their model. The study by Darnall et al 2017 reported that neonatal rat exposed to mild IH, as might usually found in preterm infants, displayed decreased in white matter integrity, changes in metabolic and proinflammatory responses (78). In contrast with other studies, Bouslama et al 2015 reported that moderate IH offers neuroprotection against brain lesions and cognitive dysfunctions, and they suggested that AOP may have favorable effects in some preterm infants (79).

5. Growth

In spite of the fact that exposure to chromic IH appears to have less impact on physical growth in animals and humans compare to that of chronic constant hypoxia, some researchers reported severe IH can also contribute to negative

influences on growth and development. In 2008, Farahani et al have shown that neonatal exposure to hypoxia in mice resulted in slower weight gain and developmental delay in many organs (5). Similarly, Pozo et al in 2012 demonstrated that rat pups exposed to repetitive IH had a significant effect on body weight and contribute to growth restriction. However, the acceleration of growth can catch-up to normoxic controls by 4 weeks of age (65). The disturbance of hypoxia with growth and development depends on the severity and duration of hypoxia and age at the time of exposure (5). Despite several reports on neonatal IH and body growth, none of them have mentioned the growth in craniofacial area.

Condylar and Tibial Cartilage growth

During postnatal growth period, it is classically said that mandibular condylar cartilage act as an important mandibular growth site responsible for an increasing mandibular height, length and width, which is related to anteriorly and inferiorly displacement of the mandible (80). While another explanation introduces that downward growth of the mandible is resulted from surrounding tissues development (81, 82). There are studies attempted to clarify the role of mandibular cartilage on mandibular growth and found out that even though condylar cartilage did not responsible for the entire growth of the mandible, it is still necessary as a part in normal mandibular growth, especially in increasing ramus height (83-87).

Endochondral ossification and intramembranous ossification are the processes of bone growth responsible for normal development of the mandible (88-90). It has been reported that disturbance in endochondral ossification at mandibular condylar cartilage resulted in reduction of ramus height, without changing anteroposterior mandibular length (87). Thus, any interruption in endochondral ossification processes may affect the ramus vertical growth.

Since bones are formed through two processes and most of the skeleton, including long bones and mandibular condyle, forms by endochondral ossification; a process which required a cartilage intermediate, the cartilage formation and factors involved in its growing process is also considered to be the important in the body growth. Of the three types of cartilage found in the human body, hyaline cartilage and fibrocartilage are the most frequently occurring types of cartilage. These two types of cartilage differ in cellular organization and composition, extracellular matrix components and also in their embryonic origin (20). The main extracellular components of hyaline cartilage are type II collagen and proteoglycans. Fibrocartilage, in contrast, contains type I and type II collagen together with proteoglycans, with type I collagen being the predominant matrix constituent (91). Previous ex vivo studies compared these two types of cartilage and a higher content of glycosaminoglycans (GAG) was found in hyaline cartilage compared to fibrocartilage (92, 93). So, next to differences in expression of different types of collagen, other

extracellular matrix components appear to differ also. These differences might be related to the specific functional demands laid upon the different cartilage types.

Not only the extracellular matrix but also the cells within the two types of cartilage are different. Several studies evaluated the responses of fibrocartilage and hyaline cartilage to certain growth factors, such as basic fibroblast growth factors (bFGFs), insulin-like growth factor-l (IGF-1) and transforming growth factor beta (TGF-β) (19, 94). These authors found that the factors stimulated a higher cell proliferation in fibrocartilage than in hyaline cartilage. They also found that fibroblast growth factor-2 (FGF-2) had a stronger effect on fibrocartilage cell metabolism compared to hyaline cartilage. These findings indicate differences in sensitivity of different populations of chondrocytes to growth factors may related to a cell type specific sensitivity of these cells.

Cartilage that covers the mandibular condyle is considered as fibrocartilage while the cartilage in tibial growth plate is hyaline cartilage. The differences as well as the similarities of these two cartilages make it of interest to investigate the responses of these two types of cartilage to IH.

Mandibular condylar cartilage differs from tibial growth plate cartilage primarily in its superficial layers: perichondrium (fibrous layer), in which the prechondroblastic (chondroprogenitor / mesenchymal) cells secretes the type I collagen rather than type II collagen secreted by in active chondrocytes in resting

zone of the growth plate (Figure 6) (95-98). These prechondroblastic cells in the perichondrium later proliferate and mature into chondrocytes by the regulation of SRY-Box Transcription Factor 9 (Sox9). Unlike in the growth plate, the cells that proliferate are chondrocytes (96). Sox9 has been found to be an important regulator in cartilage formation due to its function in multi-step of the chondrogenesis pathway and regulating type II collagen expression in chondrocytes (Figure 7) (99, 100).

Chondrocytes start producing type II collagen, undergo hypertrophy and express type X collagen, which later leads to onset of endochondral ossification (93, 101). This process is similar to the hypertrophic chondrocytes in the growth plate cartilage (Figure 8).

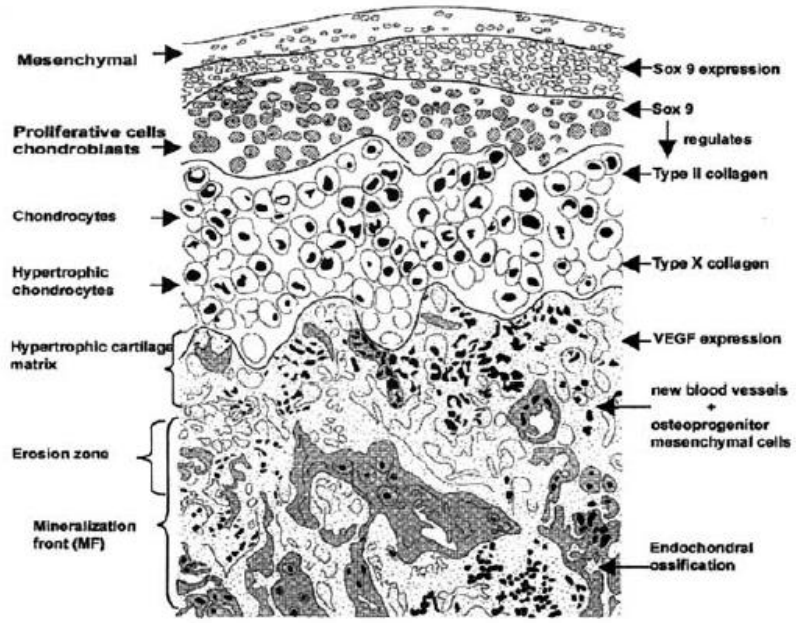

Figure 6. The schematic of cellular differentiation in mandibular condyle.
The pattern of cellular differentiation, blood vessel invasion, and bone formation in mandibular condyle (100).

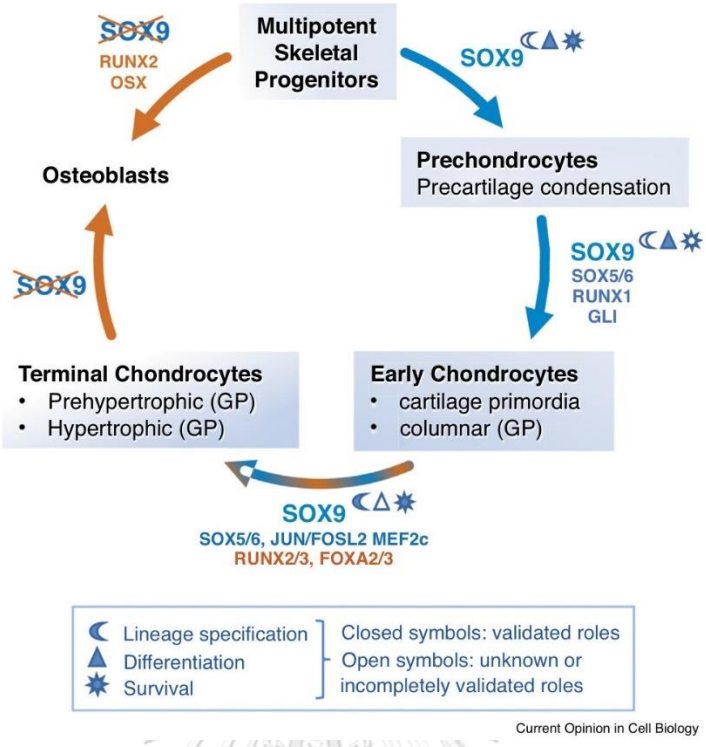

Figure 7. SOX9's roles in skeletal progenitors, chondrocytes and osteoblasts. SOX9 has been shown to be involved and expressed in chondrogenesis, from lineage specification until hypertrophic differentiation. The repression of SOX9 is required for osteoblast differentiation from terminal chondrocytes later on (99).

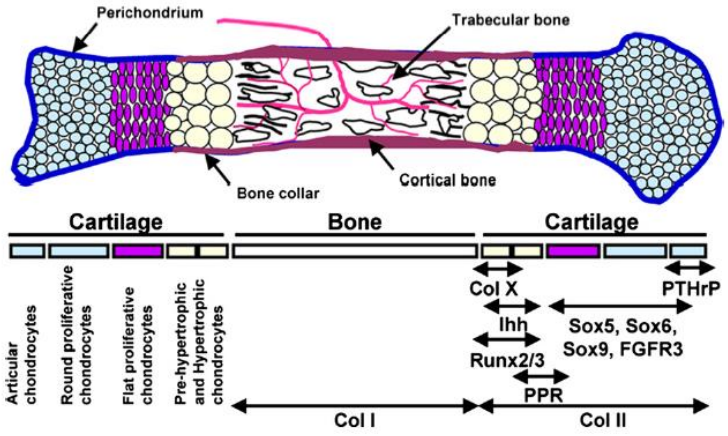

Figure 8. Schematic of endochondral bone formation.
Endochondral bone formation of a mouse tibia at the late stage of fetal development (97).

Type X collagen formation is proposed to facilitate the bone replacement, due to its unique short chain characteristic of this collagen which make it easy to resorb when compared to type II collagen (100, 102). Cartilage oligomeric matrix protein (COMP) is another crucial non-collagenous protein found in cartilage extracellular matrix (103). COMP is localized not only in the cartilage, but also in the ligament, tendon and meniscus (104, 105). The important roles of COMP are binding extracellular matrix to collagens and chondrocytes, storage and delivery hydrophobic hormones, a calcium-binding protein and a maker of cartilage turn over in joint disease (106-108). The mutations in COMP associated with multiple epiphyseal dysplasia and pseudochondroplasia (109-112). COMP and collagen X expression are highly expressed by hypertrophic chondrocytes (21, 22, 111) and collagen type X expression appears to be related to the level of endochondral ossification (23).

Apart from the cartilage growth, bone remodeling process are also an important part in endochondral ossification. Osteoprotegerin (OPG) and the receptor activator of nuclear factor-kB ligand (RANKL) are the genes known to be related with osteoclasts function and bone resorption, which also help maintaining normal bone mass (113). OPG suppressed osteoclasts survival while RANKL plays essential role in osteoclast differentiation and bone resorption (114, 115). The previous studies showed that animals overexpressing OPG exhibited increased bone density and a notable decreased in osteoclast number (116). Another study in mice lack of OPG also found osteopenia resulting from higher osteoclast activity (117). On the other

hand, studies in RANK and RANKL deficient mice revealed that these mice developed osteopetrosis in consequence of absence of osteoclasts (118-120).

Hypoxic condition effects on cartilage and bone development

During past decades, it has become widely acknowledged that oxygen is one of the important regulatory signals that controls the expression of specific genes involved in various tissues development, including cartilage and bone. Reduced oxygen availability is crucial in normal development, via activation of transcriptional responses (121). All embryonic tissues encounter a low oxygen tension environment, i.e., hypoxia, during developmental process, when the circulatory system was not formed yet. The vascularization of the placenta and embryo is activated by this physiological hypoxia (122). The transcriptional factors involved in adaptive response of cells to hypoxia are the Hypoxia Inducible Transcription Factors (HIFs), whose expression and function are mostly post-translationally controlled by hydroxylation reactions (123). HIF-1 is a heterodimer of two proteins, HIF-1α and HIF-1β. The stability of HIF-1α protein is hypoxia-sensitive. Under normal oxygen environment, HIF-1α can only sustained in the cytoplasm for a very short period of time (<5 min). Since it contains specific hydroxylated proline that are detected by the Von Hippel-Lindau protein (pVHL-containing E3 ubiquitin ligase complex), it can be easily targeted and brought to degradation via the proteasome (124, 125). On the other

way round, when oxygen drops below 5%, hydroxylation is reduced and HIF-1α subunit is free to migrate into the nucleus, binds with the β-subunit, then act as a transcription factor on the target genes. The β subunit is specifically identified and bind to the 5'-RCGTG-3' sequence of the hypoxia responsible element (HRE) in DNA of the target genes (Figure 9) (124). More than 10 target genes have found to be regulated by HIF, such as erythropoietin, glucose transporter protein and vascular endothelial growth factor (VEGF) (Figure 10) (126-128).

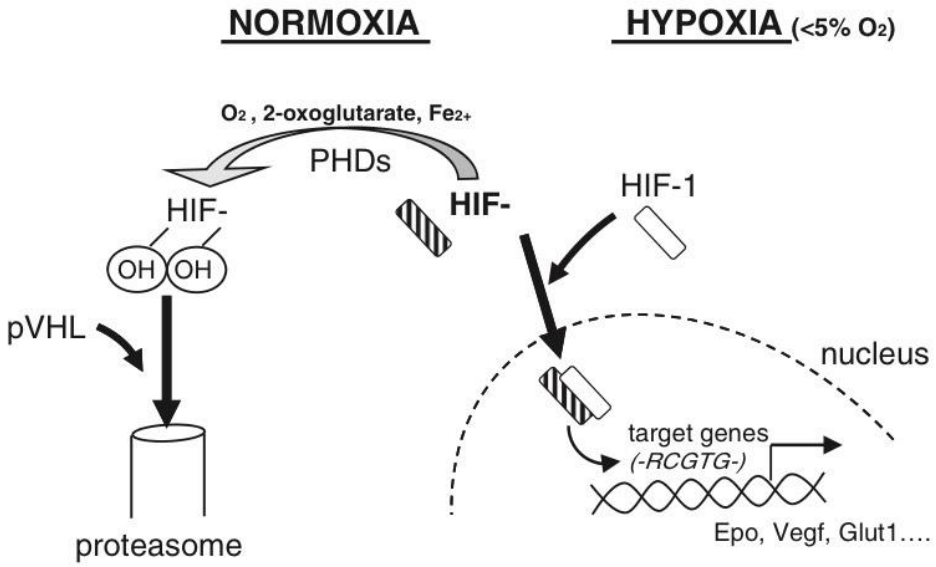

Figure 9. The schematic of HIFs regulation and signaling.
In low oxygen environment prolylhydroxylases (PHDs) is activated, lead to inhibition of HIF-1α hydroxylation. Conversely, the non-hydroxylated HIF-1α can dimerize with its β-subunit and migrates into the nucleus in normal oxygen environment (129).

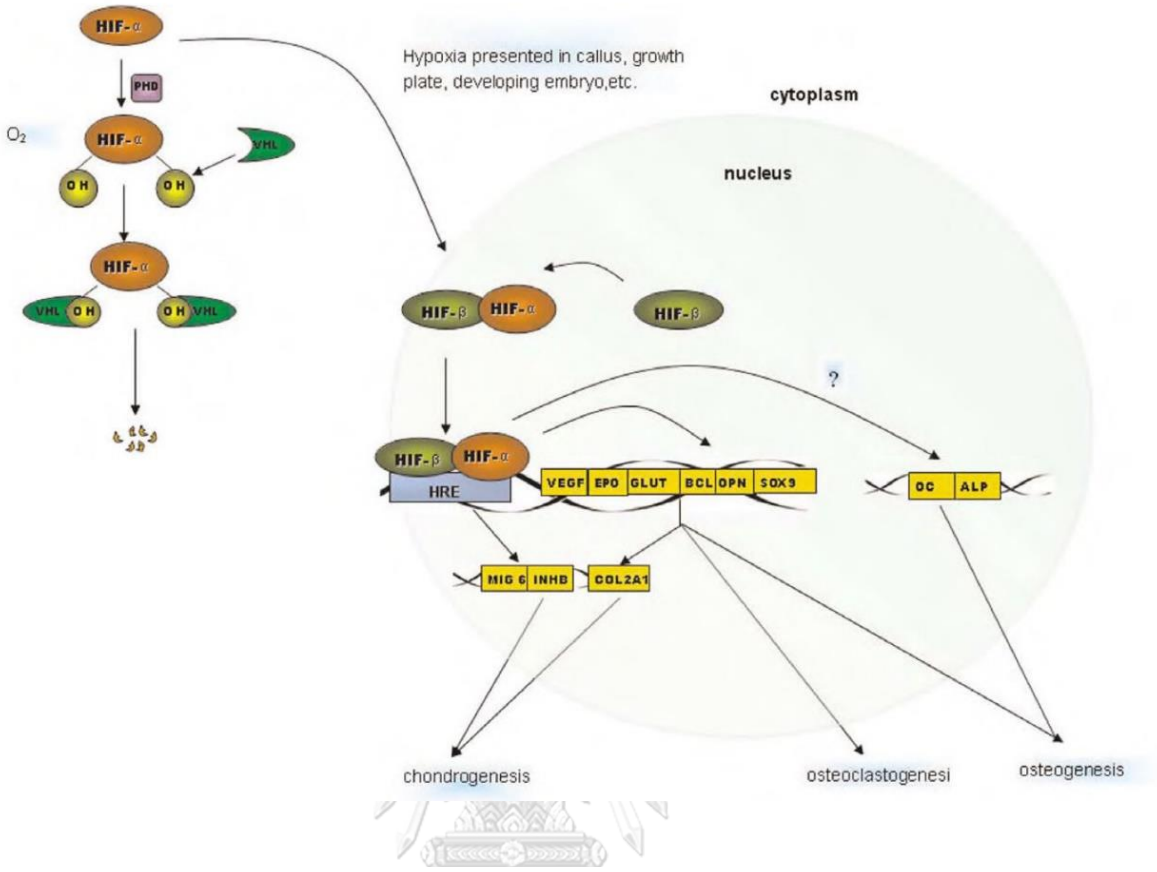

Figure 10. HIF downstream genes.
HIF alpha-beta dimers bind to HRE in the region of HIF target genes, allowing the transcription of the HIF downstream genes; which impact osteogenesis and chondrogenesis (130).

Other isoforms of HIF-**α** have been recently discovered: HIF-2**α** and HIF-3**α**. Though the HIF-2**α** is similar to HIF-1**α** as it is also regulated by oxygen level and shared some common targets, their biological function and tissue localization are not the same (131-133). It has been reported that mice lacking HIF-2**α** could still survived postnatally, while those lacking HIF-1**α** could not (134). In contrast, the biological role of HIF-3**α** is not well understood, it has been suggested that it could act as a dominant negative function (135).

HIF can be regulated not only by oxygen level, but also by some metal ions; Mg2+, Ni2+, Co2+ and Cu2+, physical factors; ultrasonic waves, some cytokines; interleukin-1 (IL1), tumor necrosis factor (TNF) and fibroblast growth factor (FGF), multiple mechanisms; transcription, translation, nuclear transport, and downstream genes feedback loops, and various signaling pathway; phosphoinositide 3-kinase (PI-3K/AKT), P38, extracellular signal-regulating kinase (ERK) and mitogen-activated protein kinase (MAPK) pathways (130).

Hypoxia plays an important role in controlling endochondral bone development by activating genes that regulate the process. The fetal growth plate chondrocytes are highly proliferative; produce collagen type ll, and line up to form a columnar layer. They later undergo terminal maturation, differentiate into hypertrophic chondrocytes; produce collagen type X, and mineralize their surrounding matrix before being replaced by bone. This process requires blood vessels invasion. As the growth plate is avascular for most of its length, it is apparently contained hypoxic regions (Figure 11).

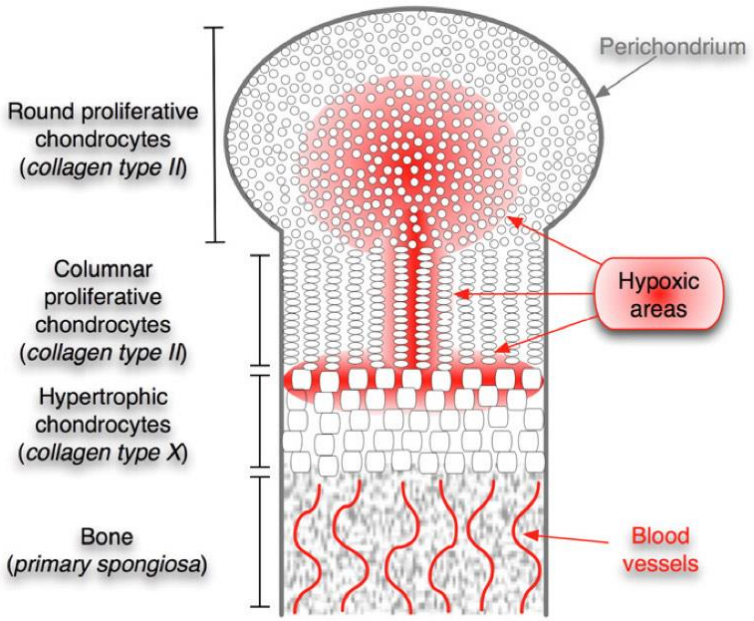

Figure 11. The fetal growth plate.
The more hypoxic areas are found near the joint space, while the hypertrophic chondrocytes at the border with the primary spongiosa are not hypoxic (136).

There were numerous studies conducted to clarify the role of hypoxia and its subsequent genetic control that modulate endochondral bone formation. HIF-1α is essential for chondrogenesis and chondrocyte's ability to survive and differentiate in the hypoxic environment. Numerous downstream mediators of HIF-1α are essential for chondrocyte survival: Phophoglycerate kinase (PGK-1; enzyme for anaerobic glycolysis), VEGF-A (responsible for angiogenesis) (137-139). HIF-1α also regulates chondrocyte proliferation and differentiation. Previous studies addressed that HIF-1α deficiency led to strikingly increase in chondrocyte proliferation rate, while mice lacking VHL (which results in HIF-1α and HIF-2α overexpression) exhibit decreased

proliferation rate but increased matrix deposition (137, 138). HIFs not only take part in chondrocytes differentiation but also in its function. Hypoxia was shown to increase type ll collagen, Aggrecan and Sox9 (140). Lafont et al. conducted the selective depletion of HIF-1α and HIF-2α in chondrocytes placed in hypoxia, and found that HIF-2α, not HIF-1α, is critical for Sox9 stimulation; which later bring about collagen ll expression (141). The later study from the same researcher, analyzed the 101 chondrocyte-specific and hypoxia responsive genes response of chondrocytes to hypoxia: comparing in 20% with 1% oxygen. They revealed that all the chondrocytes' markers, eg. Sox9, collagen ll and aggrecan, are positively regulated by hypoxia. Thus, low oxygen level is assumed to have positive effect on chondrocytes (142). As hypertrophic chondrocytes undergo apoptosis, blood vessels start invasion, and the bone formation begins. Osteogenesis and angiogenesis are crucial in bone formation, so the genetic program modulated by hypoxia involved with these two processes considered the key factor in this mechanism. HIFs function is closely related to osteogenesis due to its activation of angiogenic factor: VEGF. Activation of VEGF increases blood and oxygen supply to osteoblasts. The expression of VEGF has been reported to be mostly associated with HIF-1α and HIF-2α, which is activated under hypoxic condition (126, 143). Even though osteoblasts and osoteocytes have an adaptation ability in hypoxic condition, hypoxia still has adverse effects on osteogenesis. Osteoblasts exposed to hypoxic environment resulted in inhibition of the formation of mineralized nodules, reduction of alkaline phosphatase (ALP) and

osteocalcin (OCN) mRNA level, and low quality of collagen production; resulted in weakened the bone properties (144, 145). Hypoxia also led to down-regulation of Runx-2, which later resulted in collagen type l and OCN suppression (146). Conversely, hypoxia and its subsequence induction of HIF: hepatic growth factor (HGF), macrophage colony stimulating factor (M-CSF), osteopontic (OPN), Bcl-2, BNIP3, Glut-1, VEGF, stimulate osteoclast function (147, 148).

Previous related studies

Previous studies showed that hypoxic conditions in children resulted in underdevelopment of the posterior facial height (10, 149). (Figure12) In line with these findings are animal studies that confirm an effect of IH on craniofacial growth at prepubertal age. These effects include the reduction of nasal cavity volume, an increased bone density in the mandible, and a decreased size of mandibular bones (11-13). There is, however, no study which addresses the question whether during the early postnatal growth period condylar and tibial cartilage are affected similarly under IH conditions. The present study will be undertaken to investigate this in detail.

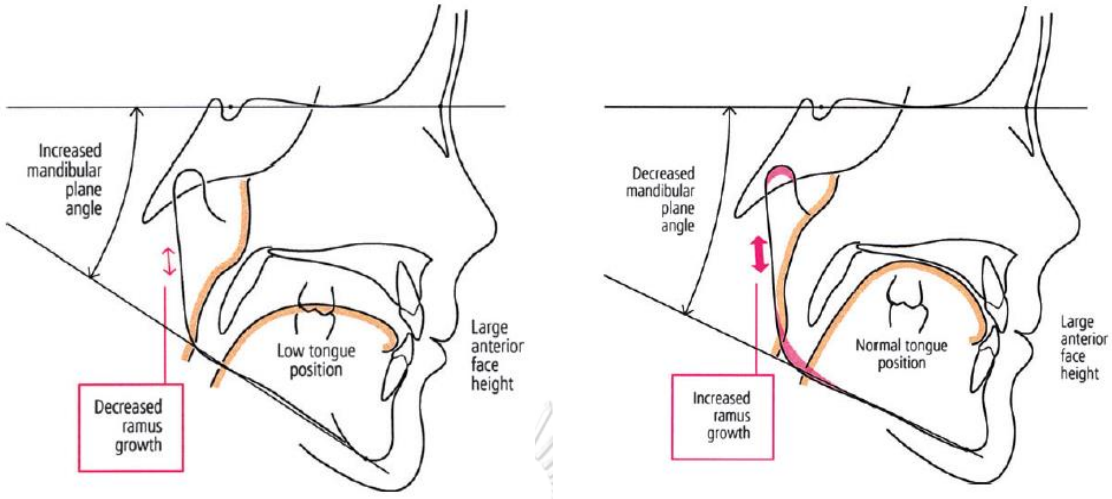

Figure 12. Tracing of a child with large adenoids before and after adenotonsillectomy. (Left) A child with large adenoids showed high anterior face height and increased mandibular plane angle cephalometrically. (Right) After adenotonsillectomy, the mandibular plane angle has been found to decrease, mandibular growth was increased with intensive growth in the condylar cartilage and in the lower border of the mandible (149).

There are several studies using the IH-exposed rodent model to evaluate the effects of IH in many systems of the body (150-152). However, the oxygen fraction used to induce hypoxic conditions in each study differs among investigators, depended on their specific field of study and perspective. Most of the studies adjusted the severity of hypoxia range from 2%-16% of inspired oxygen, the duration of hypoxic episodes varied from 15-30 s to 12 h, 3 to 2,400 cycles per day and the cumulative IH duration ranges from <1 h to between 2 and 90 days (28, 29, 153).

However, there is no exact protocol of IH settings in terms of:

1. The severity of hypoxia. e.g., level of hypoxemia
2. The duration of hypoxia exposure within episodes.
3. The numbers of hypoxia/reoxygenation episodes per day.
4. The total duration of exposure.
5. The pattern of presentation.
6. Other relevant variables e.g., arterial carbon dioxide level. (28, 154)

Since there is no actual standard for IH settings reported at this time, researchers have designed the rates of hypoxic events according to the sample's age and severity of hypoxia. The previous studies mentioned craniofacial growth in prepubertal rats (11-13), demonstrated the effects of IH exposure: O2 nadir ranging from 4-21%, through the protocol that mimicked severe hypoxia.

Chapter III

Research methodology

Experimental model

The protocol was approved by the Institutional Animal Care and Use Committee of Tokyo Medical University, Japan (approval no. H29-0021 and H30-0022). All experiments complied with relevant guidelines and regulations. Eighteen 5-day-old male Sprague-Dawley rats and their mothers were housed in computer-controlled environmental chambers, where a moment-to-moment desired oxygen concentration of the chamber was programmed and adjusted automatically. The oxygen concentration was monitored and readjusted every day to maintain normoxic and hypoxic conditions, by using the O2 analyzer. The level of hypoxia in the chamber was controlled by an automated system, which was reported by Nagai and colleagues. They established the original hypoxic chamber used in this study (155). (Figure 13)

From 7 days of age, pups and mother of the IH group were exposed to IH condition, in the rate of 20 cycles/hr (3 min interval, nadir, 4% Oxygen to peak 21% Oxygen with 0% Carbondioxide), for 8 hour each day during the light-on period (11-13). The pups of the normoxic group (N group) were exposed to room air with their mother in the same computer-controlled environmental chambers throughout the experiment. Animals were fed with normal chow diet and were housed in a controlled environment with 12-hour light-dark cycles (8:00 a.m. to 8:00 p.m.) in

constant temperature (24±0.2 ℃) with *ad libitum* access to food and water. Body weight of each rat was recorded daily from the start until the end of experimental period. All pups were euthanized at the age of 2 weeks under anesthetized condition by intraperitoneal injection with sodium pentobarbital.

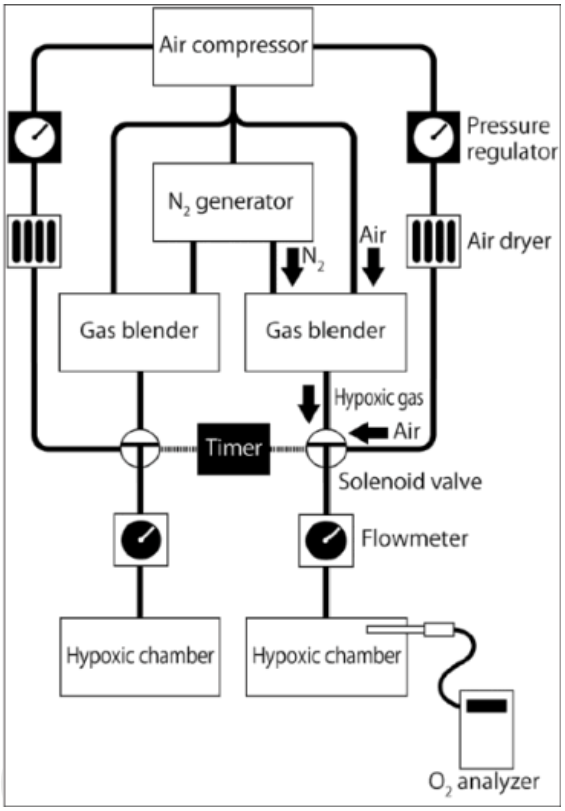

Figure 13. The schematic drawing of the IH generator.

The degree of hypoxia in the chamber was controlled by an automated system in order to induce intermittent hypoxia. Hypoxic gas and compressed air are delivered to the hypoxic chambers at the same time using a timed solenoid valve (155).

Cephalometric Analysis

To evaluate dimensional changes in the mandible, both the lateral and dorsoventral cephalometric radiographs were taken with a soft X-ray machine (SOFTEX CMB-2; SOFTEX co., Ltd., Tokyo, Japan). A pair of ear rods was used to fix each rat's head firmly into a standard head posture. The radiographs were taken with 5 mA and 30 kVp with 12-s impulses. Cephalometric landmarks are shown in Table 1 and Figures 14a-b. The landmarks consisted of sixteen linear cephalometric measurements (Table 2) as described previously (13, 156). X-rays images of the tibia were also taken for evaluation of growth in the limb region and used for the assessment of the skeletal growth index. All measurements were performed thrice by the same operator and the average values were used for analyses.

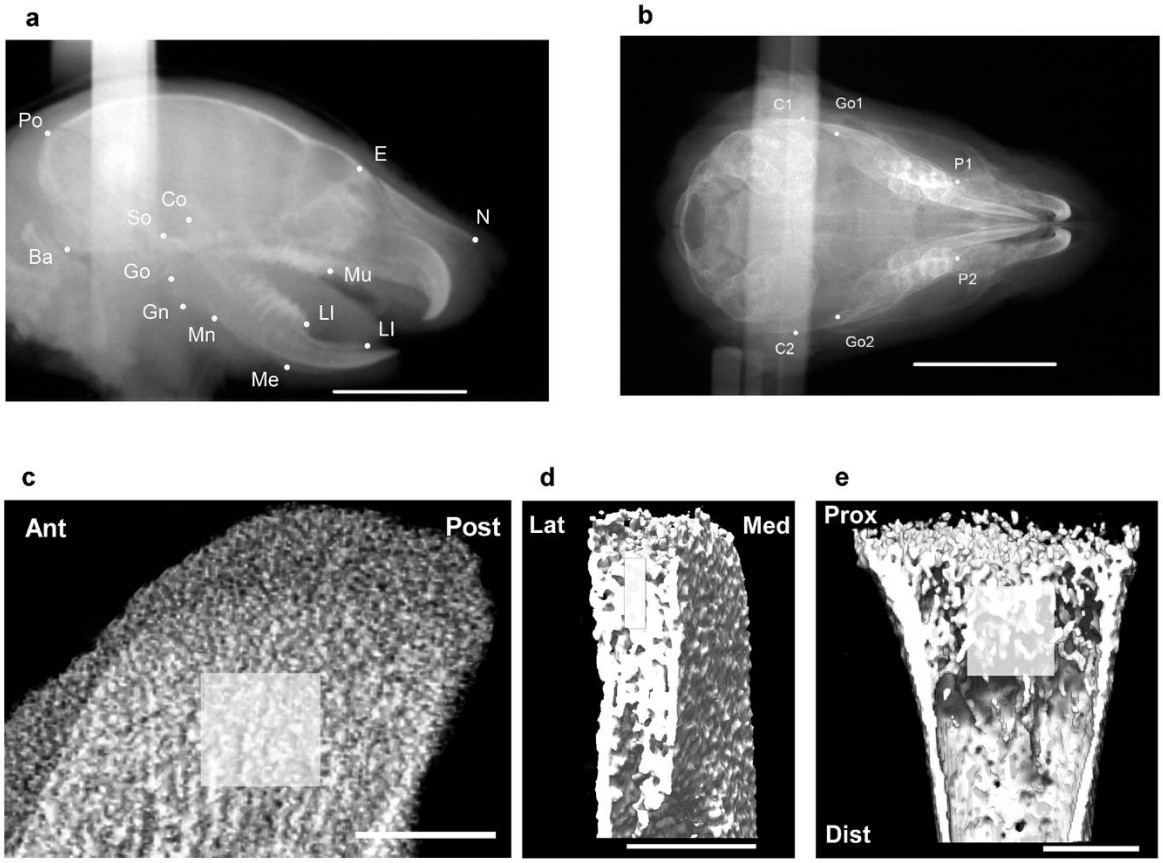

Figure 14. Cephalometric and μCT analysis landmarks.

Region of interest (ROI) in μCT images, including sagittal (c) and coronal (d) views of mandibular condyle and coronal (e) view of proximal tibial epiphysis. Boxes in c-e indicate the ROI. Scale bar = 10 mm (a-b) and 50 μm (c-e). Ant, anterior; Dist, distal; Lat, lateral; Med, medial; Post, posterior; Prox, proximal.

Table 1 Definitions of landmarks in radiographic images

Lateral view landmarks	N	The most anterior point on the nasal bone
	E	The intersection of the frontal bone and floor of anterior cranial fossa
	Po	The most posterior and superior points on the skull
	Co	The most posterior and superior points on the mandibular condyle
	So	The intersection of the most anterior tympanic bulla and the superior border of the sphenoid corpus
	Ba	The most posterior and inferior points on the occipital condyle
	Go	The most posterior point on the mandibular ramus
	Gn	The most inferior point on the ramus that lies on a perpendicular bisector of the line Go-Mn
	Mn	The most concave portion of the concavity on the inferior border of the mandibular corpus
	Me	The most inferior and anterior points of the lower border of the mandible
	Mu	The junction of the alveolar bone and the mesial surface of the first maxillary molar
	Mi	The junction of the alveolar bone and the mesial surface of the first mandibular molar
	Ll	The most anterior and superior points on the alveolar bone of the mandibular incisor
Dorsoventral view landmarks	Go1, Go2	The points on the angle of the mandible that produce the widest width; Go1 is the point on the left and Go2 is the point on the right
	C1, C2	The points on the cranium that produce the widest cranial width; C1 is the point on the left and C2 is the point on the right
	P1, P2	The most anterior and medial points within the temporal fossae that produce the narrowest palatal width; P1 is the point on the left and P2 is the point on the right

Table 2. Cephalometric measurements.

Neurocranium	Po-N	Total skull length
	Po-E	Cranial vault length
	Ba-E	Total cranial base length
	So-E	Anterior cranial base length
	Ba-So	Posterior cranial base length
	Po-Ba	Posterior neurocranium height
Viscerocranium	E-N	Nasal length
	E-Mu	Viscerocranium height
Mandible	Co-Ll	Total mandibular length
	Co-Me	Length from condylar head to Me
	Co-Gn	Ramus height
	Go-Mn	Posterior corpus length
	Mi-Ll	Anterior corpus length
Transverse measurements	Go1-Go2	Bigonial width
	C1-C2	Maximum cranial width

Three-dimensional microcomputed tomography

Changes in mineralized cartilage and subchondral bone microstructure were investigated by using microcomputed tomography (micro-CT) with a desktop X-ray micro-CT system (Inspexio, Shimadzu, Japan). The output of 75 kV and 140 mA was used (157). The sample was scanned with a 20 μm scanning resolution (13, 158). An area of 1.0x1.0x0.2 mm located in the cancellous bone, distant by a 1.0 mm from the condylar epiphyseal cartilage, was chosen as the region of interest (ROI) (Figures 14c-d). An area with the same size located in the diaphysis, 50 μm inferior to the growth plate of the tibial cartilage was used for bone density analysis (159). (Figure 14e) The following four values were calculated: bone volume/trabeculae volume (BV/TV, %), bone mineral density (BMD, mg/cm^2), bone mineral content (BMC, mg) and bone mineral content/trabeculae volume (BMC/TV, mg/cm^3). The results were analyzed by using a software package (TRI/3D-BON, Ratoc, Tokyo, Japan).

Histomorphometric analysis

Fixation process

The TMJ and tibia with the surrounding tissues were dissected from the right side of the animal and fixed in 4% paraformaldehyde for 24 hour, decalcified with 4.13% EDTA (pH 7.4) for 6 weeks, at 4°C. The solution were changed every 3 days. Following fixation, the specimens were placed in suitable labelled cassettes for the dehydration process.

Dehydration and clearing process.

Since melted paraffin wax is hydrophobic, the removal of water in a specimen is necessary before it can be infiltrated with wax. The specimens were immersed in a series of increasing concentration ethanol, 70%, 90% and 100% in the following sequences;

1. 70% ethanol 60 min
2. 90% ethanol 45 min
3. 100% ethanol 45 min
4. 100% ethanol 45 min
5. 100% ethanol 60 min

After dehydration, specimens were immersed in xylene for 60 min, 3 cycles, to remove alcohol before infiltrated with paraffin wax.

Wax infiltration

After xylene infiltration, the specimen cassettes were transferred to an embedding station. The orientation of the specimens in the mold should be done carefully, as this will determine the plane of section when they are cut by the microtome. When the settings was ready, the molds were infiltrated with molted paraffin wax, all of this process had to be done on the hotplates. When the specimens were fully covered with the paraffin wax, the whole molds were transferred to the cold plate to finally set.

Sectioning

The tissue blocks containing the mandibular condyle were cut at a 5-μm thickness: parallel to the sagittal plane of the mandibular condyle. The tibiae were cut in a longitudinal direction (5 μm in thickness). Sections were stained with toluidine blue for cartilage thickness measurements.

Deparaffinized and rehydrated sections

The sections with 5-μm thickness were put in the oven at 50 ℃ for 10 minutes. After that they were deparaffinized and rehydrated with xylene and ethanol in the following sequences;

1. Xylene 5 min
2. Xylene 5 min
3. 100% Ethanol 3 min
4. 100% Ethanol 3 min
5. 90% Ethanol 3 min
6. 80% Ethanol 3 min
7. Milli-Q water

Toluidine blue staining

Prepare the Toluidine blue working solutions

1. Acetate buffer: 0.2 M

2. 0.04% Toluidine blue stain

Mix the solutions together until the pH of Toluidine blue working solution was around 3.7.

Staining procedure

1. Put the sections in 0.04% Toluidine blue solution 5-10 min
2. Rinse gently in tap water 1 min
3. Warm air dry 10 min
4. Mount with mounting media and coverslip

Image analysis and quantification

The cartilage layer of the mandibular condyle was divided into 4 layers: fibrous layer, proliferative cell layer, maturative cell layer and hypertrophic cell layer, based on cell shape, size and the intensity of staining (160). (Figure 15a) The thickness measurement of the condylar cartilage was performed at three equal parts on the central sagittal sections of the mandibular condylar head: anterior, middle and posterior parts (Figure 15b). Three other lines were drawn through the middle of each part, starting from the superior border of the fibrous layer to the inferior border of the hypertrophic cells layer (161). (Figure 15b) Then, the thickness of each layer and the total cartilage thickness were measured along the middle line of the three portions, by one observer. The average value of six sections per animal was used as a representative value for each animal.

For measurements of the tibial growth plate cartilage, 12 parallel lines, apart by 225-μm from each other, were drawn through the cartilage (162). (Figure 15c). Twelve thickness measurements obtained from these lines were averaged as representing a value for each section. Six sections from each tibia were measured, and the averaged value was used as a representative value. The following parameters were measured: total height of the growth plate (Gpc.Th), resting zone thickness (RZ.Th), proliferative zone thickness (PZ.Th) and hypertrophic zone thickness (HpZ.Th), by methods described in a previous study (163). (Figure 15d) The measurement and analysis process were done using the software NIS-Elements Analysis D 3.2 (Nikon, Tokyo, Japan).

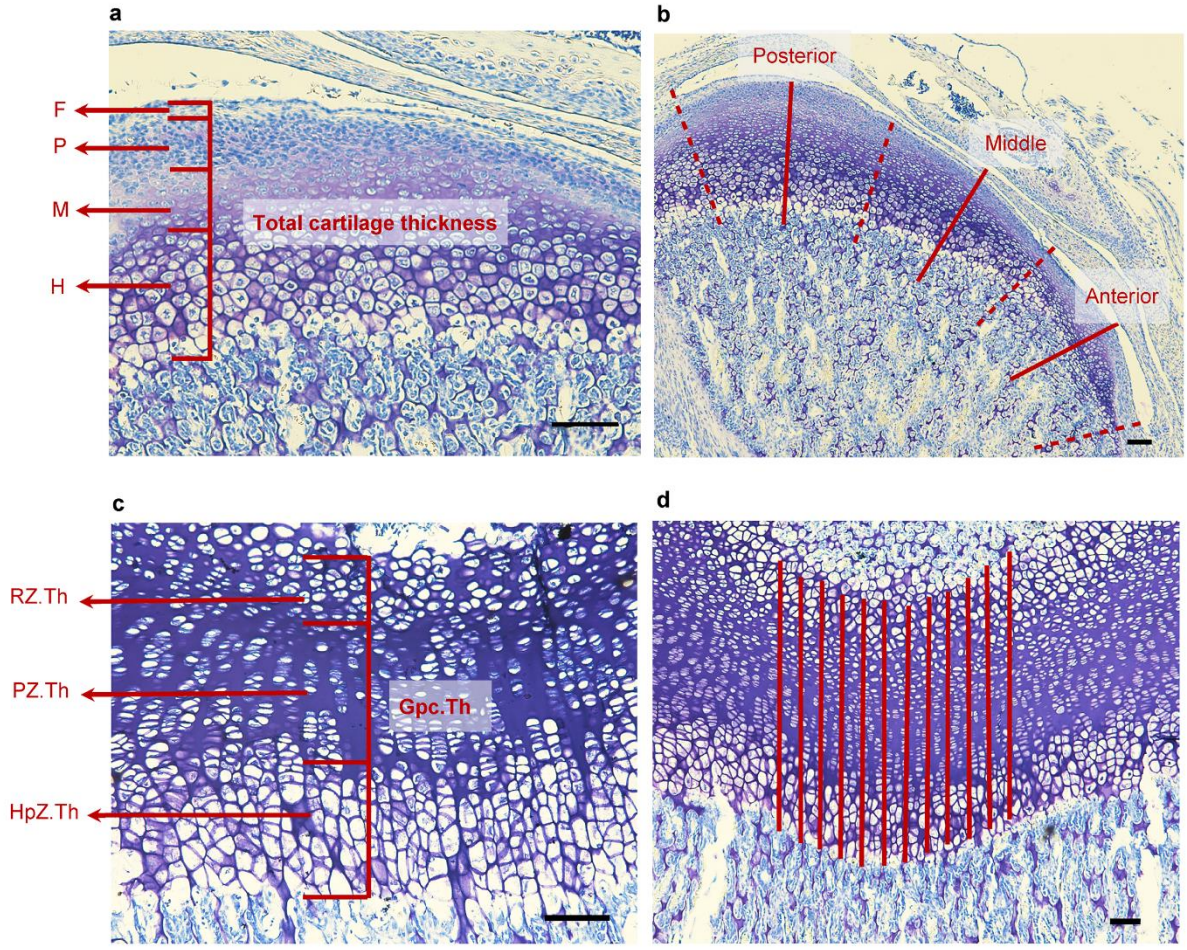

Figure 15. Histological images of mandibular condylar cartilage and tibial growth plate. The mandibular condylar cartilage and tibial growth plate from a 2-week-old rat following toluidine blue staining. (a) Sagittal section of mandibular condylar cartilage. Black dashed lines show the divided region of condyle, including the fibrous layer (F), proliferating layer (P), maturative layer (M), and hypertrophic layer (H). Red lines in (b) show the center of each part used to measure thickness of mandibular cartilage. (c) Longitudinal section of tibial cartilage, including the resting zone (RZ.Th), proliferative zone (PZ.Th), hypertrophic zone (HpZ.Th), and height of growth plate (Gpc.Th). (d) Twelve parallel lines drawn through tibial cartilage to permit measurements. Scale bar = 100 μm.

qRT-PCR

Expression of the genes related to chondrogenesis was analyzed by assessing mRNA expression of a number of genes (see below). Isolation of total RNA from the mandibular condyle and tibia tissues from the left side of the animal was done by using the PureLink™ FFPE Total RNA Isolation Kit (Invitrogen, CA, USA) according to the manufacturer's protocol.

First-stranded complementary DNA was synthesized using PrimeScript™ RT reagent Kit (Takara Bio, Shiga, Japan) according to the product's instructions. Quantitative real time PCR assays were performed in triplicate for each sample, using Premix Ex Taq™ (Takara Bio, Shiga, Japan) in StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Real-time PCR analyses were conducted with TaqMan Gene Expression Assays (Applied Biosystems). All TaqMan probe/primer sets used for real-time PCR were commercially obtained from Applied Biosystems and they were specific for rat TGF-β mRNA (Tgfb1; Rn00572010_m1), Sox9 mRNA (Sox9; Rn01751070_m1), rat type II collagen mRNA (Col2a1; Rn01637087_m1), rat type X collagen mRNA (Col10a1; Rn01408030_m1), rat COMP mRNA (Comp; Rn00563255_m1), rat Runx2 mRNA (Runx2; Rn01512298_m1), and rat hypoxanthine phosphoribosyltransferase 1 (Hprt-1) mRNA (Hprt1; Rn01527840_m1). Hprt1 expression was used as an internal control. The thermocycling conditions used were 95°C for 20 s, followed by 40 cycles of 95°C for 1 s and 60°C for 20 s. Gene

expression levels were calculated according to the ΔΔCT method of relative quantification.

Statistical analyses

All data were depicted as the mean ± standard error of the mean (SEM). Statistical calculations were performed by using IBM SPSS Statistics Version 24 (Chicago, IL, USA). We first examined the normality and the variance of the data using F-tests. Then, the comparison of animals' weight, tibia length, craniofacial dimension, bone microstructure measurements, cartilage thickness and real-time PCR results between the IH and N groups were evaluated by the independent samples t-tests. Statistical significance was accepted at a p-level less than 0.05.

Chapter IV

Results

Part I: Systemic growth of neonatal rats subjected to IH

The body weight and tibia length were recorded, as indicators for systemic growth. One week after IH exposure, the body weight of the IH group was significantly lower than the N group (Table 3), but the tibial length was not different between two groups (Table 4). These findings are consistent with the findings in the previous reports (11, 12).

Table 3. Changes in body weight (g)

Day	N Group (n = 9)		IH Group (n = 9)		*P*-value
	Mean	SD	Mean	SD	
0	14.09	0.63	13.33	1.45	0.201
7	30.02	4.86	20.5	2.82	*

Table 4. Comparison of tibial length (mm) between the N and IH groups at day 7

N Group (n = 9)		IH Group (n = 9)		*P*-value
Mean	SD	Mean	SD	
8.86	0.44	8.48	0.7	0.175

Part II: Differential growth of craniofacial bones under IH condition

Of the 16 linear measurements obtained from the cephalometric images, all but posterior corpus length (Go-Mn) and palatal width (P1-P2), were significantly lower in the IH group (Figure 16a-d). Given that tibial length was not affected by IH, the ratio of each parameter to tibial length was evaluated. The ratios of the mandibular parameters, including two parameters of mandibular length (Co-Ll and Co-Me), ramus height (Co-Gn), and anterior corpus length (Mi-Ll), to the tibial length were significantly decreased with IH, while the ratios of the neurocranium and viscerocranium parameters to the tibia length were not affected by IH (Figure 16e). Data suggest that IH markedly reduces the mandibular size in the neonatal animals (Figure 16f).

Part III: Effect of IH on bone mineralization in the mandibular condyle and tibial cartilage in neonatal rats

Bone mineralization was evaluated using three-dimensional micro-computed tomography (3D μCT) analysis. The 3D micro-CT scans show, in contrast to the N group, in the mandibular condyle of the IH group a highly dense cancellous bone (Figure 17b). This effect proved to be statistically significant. Quantification of bone microstructural parameters demonstrated a higher BMD and BMC/TV of mandibular condyle cancellous bone of the IH group. No such effect was noted in tibial bone. Micro-CT analysis of the tibial cancellous bone region did not show any difference

between the N and IH groups. These results confirmed that IH significantly increased cancellous bone density in the mandible, and increased both areal bone mineral density (BMD) and bone mineral content/volumetric bone mineral density (BMC/TV) in the cancellous bone of the mandibular condyle, but not in the tibia (Figure 17 c, d).

Part IV: Histological changes in the cartilaginous layers of the mandibular condyle and tibial epiphysis

Mandibular condyle

The thicknesses of the total cartilaginous layers in the anterior, middle, and posterior areas of the condylar cartilage dramatically decreased during IH (Fig. 18a). After 1 week, the thickness of the maturative layer was largest in the middle of the N group of rats. In contrast, IH significantly reduced the maturative layer in all areas (Fig. 18b-d). IH substantially reduced the hypertrophic layer in the anterior area. However, the hypertrophic layers in the middle and posterior areas were comparable in the N and IH groups (Fig. 18b-d).

The relative thickness of each layer was then calculated to determine whether IH exposure influenced chondrocyte maturation in each layer. After 1 week of IH exposure, the relative thickness of the maturative layer was significantly reduced in the middle and posterior areas, but increased in the anterior area (Fig.

18e-g). In contrast, IH markedly decreased the hypertrophic layer in the anterior area but significantly increased the maturative layer relative to the total cartilage (Fig. 18e). IH significantly reduced the maturative layer in the middle and posterior areas, but increased the hypertrophic layer relative to the total cartilage (Fig. 18f-g).

Tibial epiphysis

IH reduced the thicknesses of all cartilage layers, including the growth plate in the proximal tibial epiphysis (Gpc.Th) (Fig. 20a,b). However, the relative thickness of each layer did not significantly differ from that of the total cartilage in the tibia (Fig. 20c).

Part V: Differential gene expression is associated with chondrocyte metabolism in the mandibular condyle and tibia during IH

IH modified the chondrocyte phenotype in the mandibular condyle. The genes related to chondrogenesis were examined in the mandibular condylar cartilage and tibial epiphyseal plate. In the former, IH significantly reduced the mRNA levels of the chondrogenesis markers TGF-β (*Tgfb1*) and SOX9 (*Sox9*) (Fig. 21a). It also increased the mRNA level of collagen X (*Col10a1*), a marker of hypertrophic chondrocytes, but not that of collagen II (*Col2a1*) mRNA (a marker of early chondrocyte differentiation in the mandibular condyle). RUNX2 (*Runx2*) mRNA expression levels were comparable between the IH and N rats. In contrast, IH did not affect the expression levels of these genes in the tibial cartilage (Fig. 21b). In neonatal mandibular

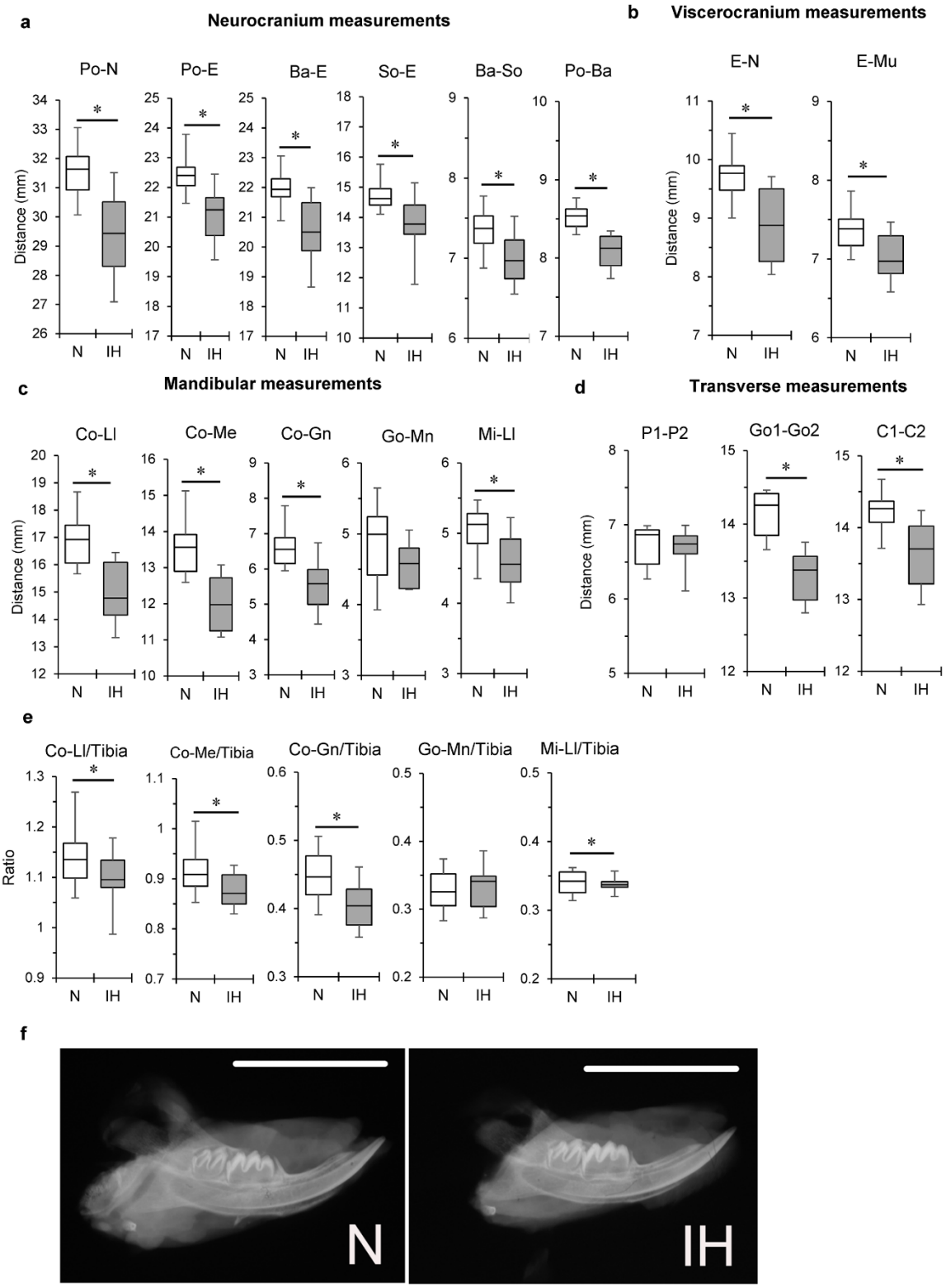

Figure 16. Differential craniofacial growth in neonatal rats subjected to IH.

*Linear craniofacial measurements obtained from lateral and transverse cephalometric images of 2-week-old rats. Neurocranial (a), viscerocranial (b), mandibular (c), and transverse (d) parameters of normoxic (N) and IH group cephalometry. (e) Normalization of mandibular dimensions to tibial length in N and IH rats. Ratios of mandibular length (Co-Ll, Co-Me), ramus height (Co-Gn), and anterior corpus length (Mi-Ll) to tibial length were significantly smaller in the IH group than those in the N group. (f) Representative lateral radiographs of hemi-mandible from neonatal rat after one week of IH exposure. The abbreviations are explained in Fig. 1A and Tables 1A and 1B. Scale bar = 10.0 mm. Data are mean ± SEM for each group. *P < 0.05.*

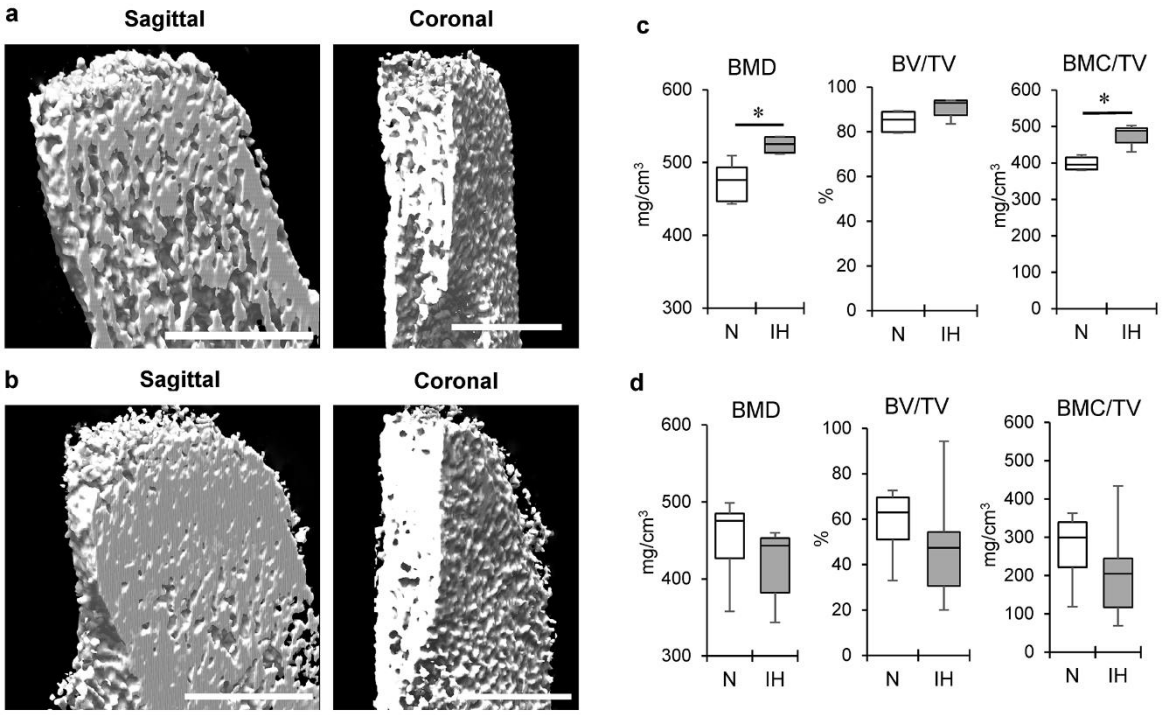

*Figure 17. IH-induced high bone mineral density in mandibular condyle and tibia. IH-induced high bone mineral density in mandibular condylar head in contrast to tibial epiphysis. Representative sagittal and coronal sections of mandibular condyle in the N (a) and IH (b) groups on 3D μCT imaging. Panel (c) shows comparative quantification of bone microstructural parameters in mandibular condyle cancelous bone between N and IH groups. Panel (d) shows comparisons of cancelous bone of proximal tibial epiphysis in both groups. BMD, bone mineral density; BV/TV, bone volume/trabecular volume; BMC/TV, bone mineral content/trabecular volume. Scale bar = 50 μm. Data are mean ± SEM for each group. *P < 0.05.*

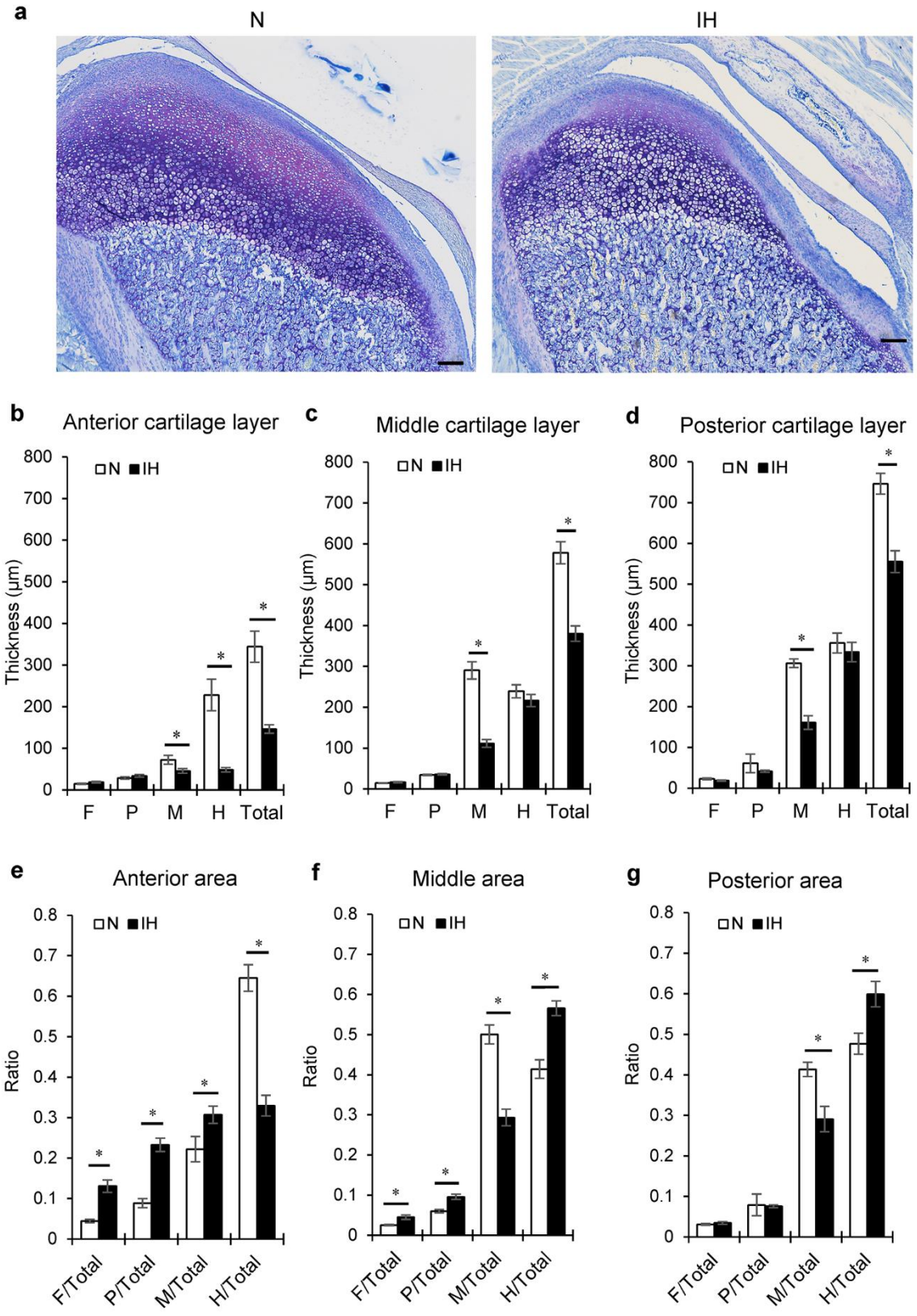

Figure 18. Histological changes in mandibular condylar cartilage of IH rats.

(a) Representative images of toluidine blue-stained sagittal sections of mandibular condyle from the N and IH groups. Thickness of each cartilage layer of anterior (b), middle (c), and posterior (d) parts in mandibular condyle. Ratios of each cartilage layer to total cartilage thickness shown in panels (e)–(g). F, fibrous layer; P, proliferating layer; M, maturative layer; H, hypertrophic layer. Scale bar = 100 μm. Data are mean ± SEM for each group. *P < 0.05.

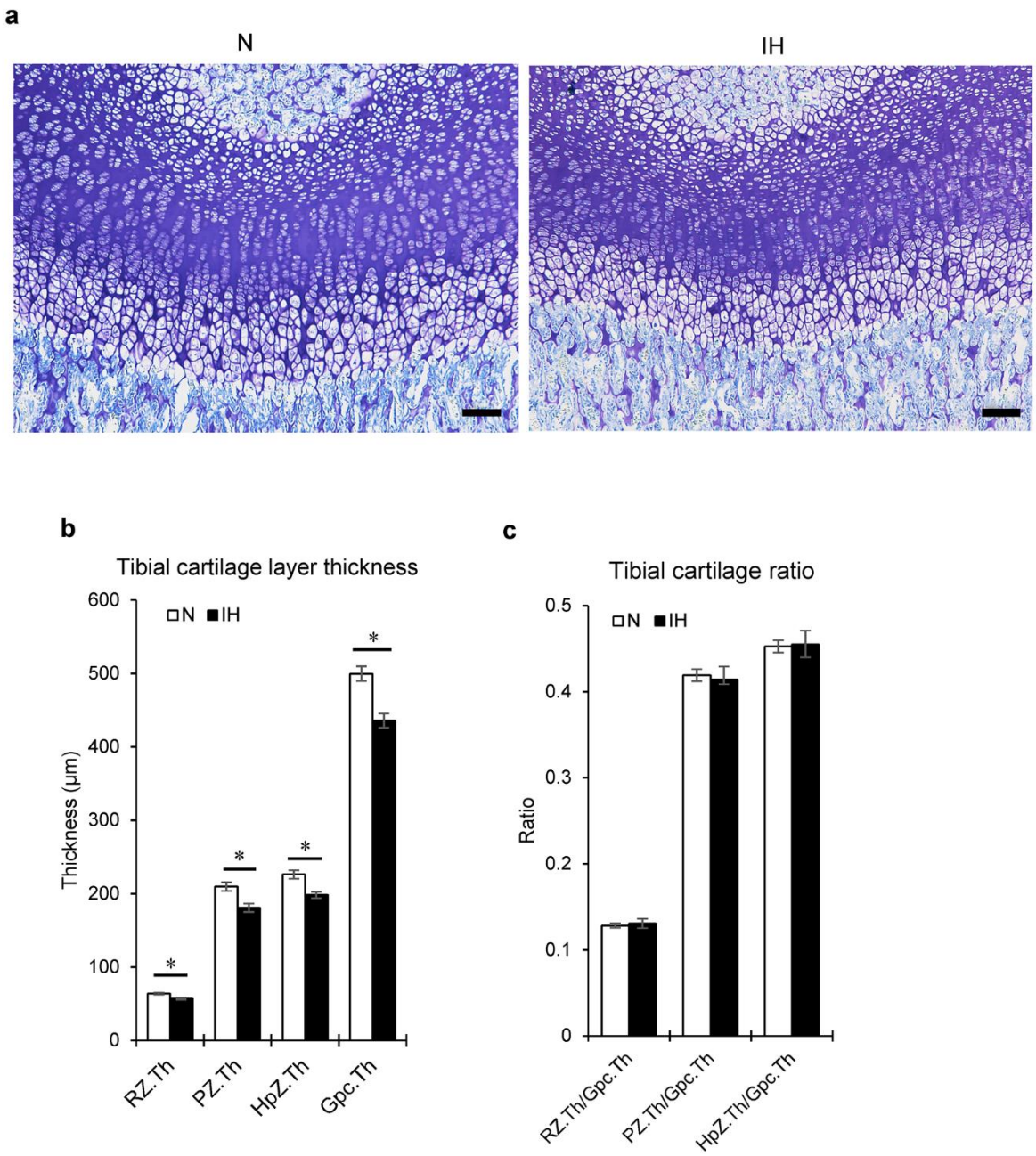

Figure 19. Histological changes in tibial cartilage of IH rats.
*(a) Representative images of toluidine blue-stained longitudinal sections of tibial cartilages from the N and IH groups. (b) Comparison of thicknesses of cartilage layers and growth plates between the N and IH groups. (c) Ratios of cartilage layer thickness to growth plate thickness. RZ.Th, tibial cartilage layers; RZ.Th, resting zone; PZ.Th, proliferative zone; HpZ.Th, hypertrophic zone; Gpc.Th, height of growth plate. Scale bar = 100 μm. Data are mean ± SEM for each group. *P < 0.05.*

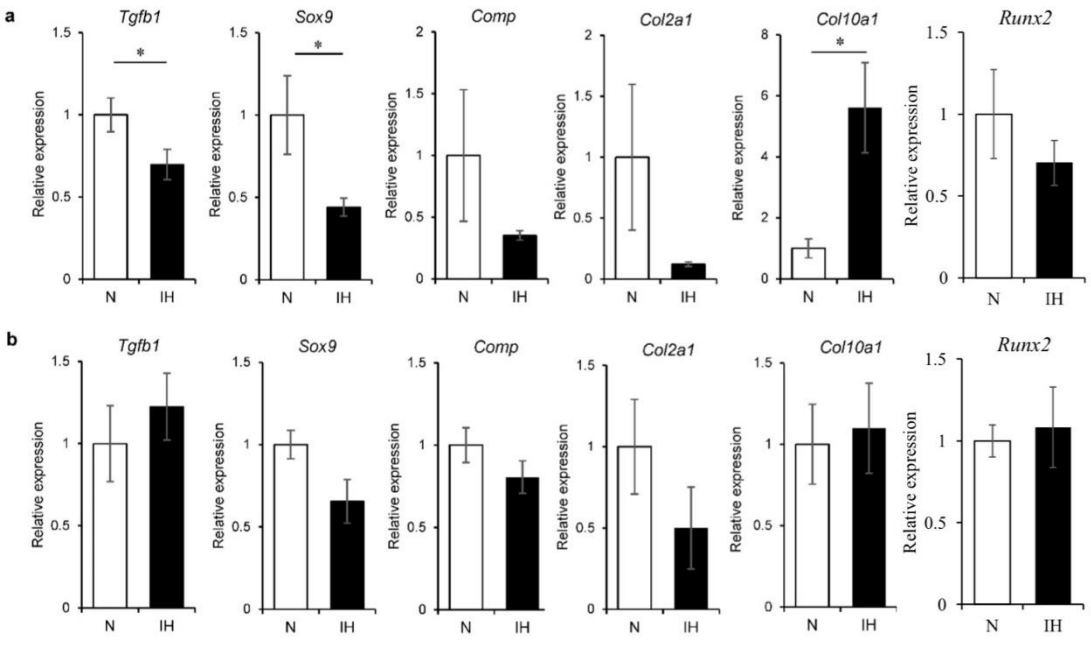

Figure 20. Differential effects of IH on expression levels of genes.

*Differential effects of IH on expression levels of genes associated with chondrocyte and bone metabolism in mandibular condyle and tibia. Relative mRNA expression levels of bone metabolic regulator TGF-β (Tgfb1) and chondrocyte markers SOX9 (Sox9), COMP (Comp), Collagen II (Col2a1), Collagen X (Col10a1), and RUNX2 (Runx2) in mandibular condylar cartilage (a) and tibial cartilage (b). Data are mean ± SEM for each group. *P < 0.05.*

Chapter VI

Discussion and Conclusion

To the best of our knowledge, this study is the first to demonstrate that IH exposure impairs mandibular fibrocartilage growth and induces chondrocyte hypertrophy and endochondral ossification in neonatal rats. In contrast, IH did not induce these effects in the tibial growth plate (hyaline) cartilage. One week of IH exposure repressed the chondrogenesis regulators TGF-β (Tgfb1) and SOX9 (Sox9) in the fibrocartilage of the rodent mandible condyle. The mRNA of collagen X (Col10a1), a hypertrophic chondrocyte marker, was upregulated in the mandibular condyle of neonatal rats. IH induced BMD and BMC/TV in the mandibular condyle, thereby promoting chondrocyte hypertrophy and cartilage calcification. In contrast, IH had no such effects on hyaline cartilage or tibial growth plate bone. Therefore, IH impedes mandible growth in neonatal rats, and mandibular condylar fibrocartilage is sensitive to IH.

Since the rat is the most commonly used model in numerous animal studies, the differences in anatomy, physiology, and development between different species must be considered. Information on the correlation between animal and human age is necessary. However, the method of translating the time points in rats into human developmental stages is not completely clear. Hence, our findings should be interpreted with caution (164).

Lateral and dorsoventral cephalometric radiographs revealed relatively reduced mandibular length (Co-Ll and Co-Me), ramus height (Co-Gn), and bigonial width (Go1-Go2) in neonatal rats exposed to IH. These findings are similar to those reported for the mandibular morphology of pubescent rats subjected to IH (13, 14). However, no reduction in the posterior corpus length (Go-Mn) was observed in neonatal rats exposed to IH. Human and rodent mandibles consist mainly of a horizontal portion (mandibular body) and two perpendicular portions (mandibular ramus). Bone growth in the mandibular body and ramus is complex (15, 16). The posterior corpus (Go-Mn) is the main site of masseter muscular attachment in the rat mandible. Masticatory muscle development strongly influences bone formation (165). Masseter muscle development begins in rats at the weaning stage (3 weeks) when the animals begin biting and chewing solid food (166). We measured the ramus portion, where the mandibular condyle controls skeletal growth (88). In rodents, bone growth is very rapid during the first 3 weeks of life (167). In our 1–2-week neonatal rat models, mandibular underdevelopment was explained by the IH-induced changes in condylar cartilaginous growth, rather than by masticatory muscle development.

The μCT data revealed dense epiphyseal bone in the mandibular condylar heads of neonatal rats exposed to IH. The IH treatment significantly upregulated BMD and BMC/TV in the cancellous bone region of the mandibular condyle. The bony microstructural changes occurring in adult patients with obstructive sleep apnea

(OSA) are controversial (168). Positive BMD responses were reported for the alveolar bone of adolescent rats (12) and the lumbar vertebrae of adult rats (169) exposed to IH. However, a neonatal rat model exposed to IH for 1 week presented with reduced BMD in the mandible (170). Relative to the control, IH exposure did not significantly modify BMD in adult mice (171). To the best of our knowledge, no reports have clarified the roles of hypoxia in cartilage and bone metabolism in IH-exposed neonates.

Rapid calcified matrix removal and marrow cavity formation are essential for growth plate development during endochondral bone biosynthesis (167). Usui and colleagues suggested that hypertrophic chondrocytes regulate osteoclastogenesis in the growth plate and remove calcified matrix (172). This process is mediated by BMP2-induced RANKL expression in normal bone. Here, the markedly increased BMD observed in the mandibular condyles of neonatal rats exposed to IH was not accompanied by any change in the Runx2 mRNA level. The latter has been associated with bone metabolism. The mechanisms underlying the effect of IH on cartilage and bone metabolism in neonatal rats remain to be determined. Hypoxia and osteogenesis are related via activation of the hypoxia-inducible factor (HIF) pathway, which occurs when the oxygen level is low, leading to marked changes in bone density together with increased vascular endothelial growth factor-A (VEGF-A) levels (173). A previous in vitro study reported increased expression of HIF-1α and VEGF in condylar chondrocytes under chemically induced hypoxia (174). HIF-1α has

protective and anti-apoptotic effects on chondrocytes during skeletogenesis. A study on HIF-1α gene-knockout mice reported varied skeletal growth through HIF1α-regulated chondrogenesis (138). Hence, we also performed RT-PCR for HIF-1α and HIF-2α gene expression in the mandibular condylar cartilage and tibial cartilage of neonatal rats exposed to IH. No significant difference in HIF-1α expression was evident in condylar chondrocytes (Figure 21). Consistent with our results, Bianchi and colleagues reported age-related HIF-1α expression after IH exposure. The neonatal rats did not show any changes in HIF-1α expression. The authors suggested that this might be due to the adaptation ability of neonates (175). Based on these findings, bone morphogenetic protein 2-induced RANKL and HIFs, and their downstream gene expression should be evaluated in future studies. In addition to qRT-PCR, western blot analysis for protein detection is necessary to confirm these findings. Furthermore, the previous biomarker study demonstrated excessive reactive oxygen species (ROS) generation in rodents exposed to IH (8). Oxidative stress arrests chondrocyte proliferation and degrades cartilage in the growth plate (21). Accordingly, further investigations on the role of ROS in these conditions are warranted. Additionally, small interfering RNA or RNA interference studies may prove useful in revealing the direct causality between chondrocyte metabolism and related gene expression during IH exposure.

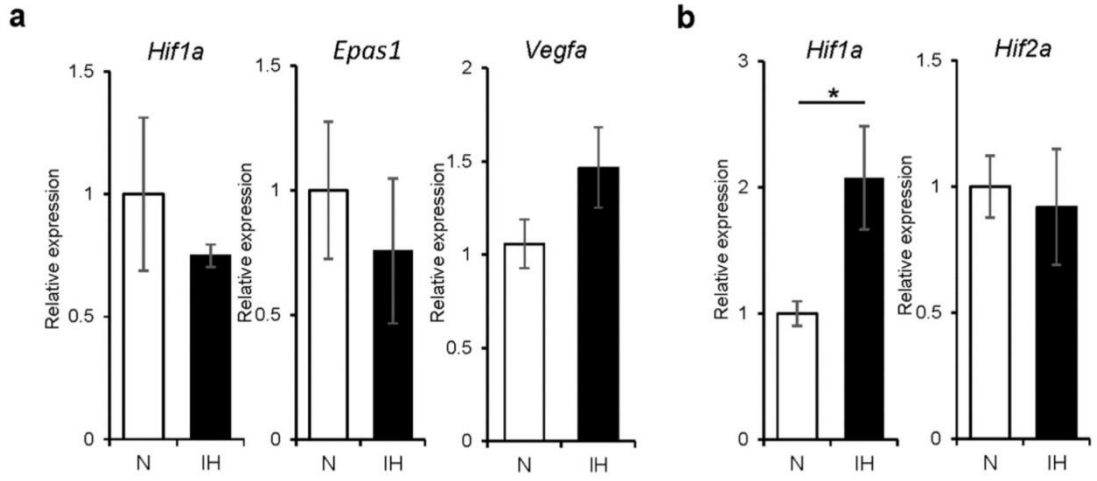

*Figure 21. Gene expression levels of HIFs and VEGF in mandibular condyle and tibia. Relative gene expression levels of HIF-1α (Hif1a), HIF-2α (Epas1) and VEGF (Vefga) were compared by TaqMan Gene Expression Assays (Hif1a, Rn01472831_m1; Epas1; Rn00576515_m1; VEGF; Rn01511602_m1; Hprt1 (internalcontrol), Rn01527840_m1). Relative mRNA expression levels of Hif1a, Epas1 and Vegfa in the mandibular condylar cartilage (a) and relative mRNA expression levels of Hif1a and Epas1 in the tibial cartilage (b). Data are presented as mean ± SEM for each group. *: p < 0.05.*

Regiospecific changes in the maturative and hypertrophic layers were observed in the mandibular epiphyseal cartilage of neonatal rats exposed to IH for 1 week. The qRT-PCR analysis revealed significantly downregulated TGF-β (Tgfb1) and SOX9 (Sox9) and upregulated collagen X (Col10a1) mRNA, which is relevant to our histomorphology appearance, in the mandibular condylar cartilage of the IH group compared to that in the N group. SOX9 inhibits chondrocyte maturation from the proliferation to prehypertrophy stages and the development of an osteoblastic phenotype (27). Sox9-mutant mice present with dwarfism and their proliferative layers are shortened (27, 176). Advanced endochondral ossification occurs in the

growth plate cartilage of the long bones because of premature chondrocyte prehypertrophy and matrix mineralization. Sox9 knockdown promotes pathological heart valve calcification via RUNX2-mediated osteogenic target gene activation (177). The regulation of SOX9 expression is complex, although it is known to be involved in various mechanisms at the gene and mRNA levels. Many pathways in chondrogenesis, including the Hedgehog, BMP-2, TGF-β, FGF-2, hypoxia signaling, and inflammatory pathways have been reported to play a role in SOX9 upregulation and downregulation (98, 99, 178). A previous in vitro study reported that Sox9 transduced articular chondrocytes under lowered oxygen conditions (5%) had a better ability to increase cell proliferation and DNA production compared to those in normal conditions (179). Hence, we speculated that the increase in the hypertrophic layer and endochondral ossification in the mandibular condylar cartilage after IH exposure may be related to a specific mechanism in the SOX9 pathway. However, to support this hypothesis, further experiments on SOX9 overexpression and condylar chondrocyte morphology under IH should be conducted in vivo. Moreover, to provide a better demonstration of the changes in condylar chondrocytes after IH exposure, performing the time-line experimental design or dynamic histomorphology to present the chondrocyte growth pattern at different time points might help clarify these findings.

Conclusions: The present study demonstrates that IH contributes to mandibular condyle growth impediments and morphometric deficits in condylar chondrocytes of neonatal rats, and also alters the expression of chondrogenic regulators. These findings provide a better understanding of the condylar cartilage tissue responses to IH.

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