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Understanding the Chondrocyte-Specific Roles of Gamma-Glutamyl Carboxylase in Skeletal Development

Jane Hendrickson-Rebizant, BSc, DMD

Faculty of Dentistry

McGill University

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noli illegitimi carborundum

Dedication

I dedicate this thesis to my parents, Susan Hendrickson and Terry Rebizant, and my siblings, Thor and Hanna.

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Contribution of Authors

This thesis contains data from the following manuscript which will be submitted for publication:

1. **Jane Hendrickson-Rebizant**, Omar Al Rifai, Juliana Marulanda, Geneviève Chiasson, Mathieu Ferron, and Monzur Murshed. **Understanding the Chondrocyte-Specific Roles of γ-Glutamyl Carboxylase in Skeletal Development.**

Individual contributions to the manuscript are as follows:

JHR: Characterized the *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice; maintained and genotyped the mouse colony; collected the skeletons; performed micro-CT scans of heads, reconstruction, and cephalometric measurements; performed radiographs of long bones and measurements; prepared heads and long bones for embedding, followed by sectioning, staining, and histological analyses; performed statistical analyses; prepared the manuscript and revised the final version of the manuscript with MM.

OA: Generated the initial cohorts of *Ggcx*$^{flox/flox}$ and *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice; performed Western blots and densitometric analyses to determine the levels of GGCX protein and activity in the tissues.

JM: Characterized the craniofacial phenotypes of *Mgp-/-* mice; taught JHR micro-CT cephalometric measurements and reconstruction; performed preliminary micro-CT analyses on *Ggcx*$^{flox/flox}$ and *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice.

GC: Co-supervised the study; helped to prepare manuscript.

MF: Provided the *Ggcx*$^{flox/flox}$ mice.

MM: Conceptualized, designed, and supervised the study; aided in interpretation of the data; revised the manuscript.

Abbreviations

2D: two-dimensional

3D: three-dimensional

AB: Alcian blue

ALPL: alkaline phosphatase (tissue non-specific alkaline phosphatase)

Ank: progressive ankylosis

ARSE: arylsulfatase E

bps: base pairs

BMP: bone morphogenetic protein

Col2a1: collagen type II, alpha 1

CNCC: cranial neural crest cell

CPAP: continuous positive airway pressure

CPP: calcium pyrophosphate

CPPD: calcium pyrophosphate dihydrate

DNA: deoxyribonucleic acid

ECM: extracellular matrix

Enpp1: ectonucleotide pyrophosphatase/phosphodiesterase-1

FGFR: fibroblast growth factor receptor

GAG: glycosaminoglycan

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GGCX: γ-glutamyl carboxylase

Gla: γ-carboxyglutamic acid or γ-carboxyglutamate

Glu: glutamic acid or glutamate

kDa: kilodalton

MGP: matrix Gla protein

Micro-CT: micro-computed tomography

MMA: methyl methacrylate

MV: matrix vesicle

NCC: neural crest cell

NPP: nucleotide pyrophosphatase/phosphodiesterase

OSA: obstructive sleep apnea

PCR: polymerase chain reaction

PHEX: phosphate regulating endopeptidase homolog X-linked

P_i: inorganic phosphate

PP_i: inorganic pyrophosphate

RANKL: receptor activator of nuclear factor kappa-B ligand

SMPD3: sphingomyelin phosphodiesterase 3

SNA: sella-nasion angle

SOS: spheno-occipital synchondrosis

VEGF: vascular endothelial growth factor

VKCFD: vitamin K-dependent clotting factor deficiency

VKOR: vitamin K epoxide reductase

VKVG: von Kossa van Gieson

XLH: X-linked hypophosphatemia

Abstract

Congenital abnormalities of the skeleton may lead to chondrodysplasia and formation of deformed bones with poor load-bearing capacities. A large number of genes have been found to be associated with these skeletal abnormalities. Although individually rare, the overall incidence of these diseases is estimated to be 1/5000 live births. In the current study, we investigated the congenital skeletal anomalies that are caused by loss-of-function mutations in γ-glutamyl carboxylase (GGCX). In a vitamin K-dependent manner, GGCX carboxylates specific glutamic acid (Glu) residues in proteins. These residues include blood coagulation factors and skeletal Gla proteins. Patients with inactivating *GGCX* mutations show massive bleeding and dysplasia of craniofacial and axial bones. The skeletal anomalies of these patients have been described as mimicking the traits of human Keutel syndrome, which is caused by mutations in matrix Gla protein (MGP). MGP's 5 Gla residues are γ-carboxylated by GGCX. The loss of MGP in mice results in abnormal calcification of all cartilaginous tissues, including growth plates and the nasal septum. These mice have midface hypoplasia with class III malocclusion and shortening of the endochondral bones. At present, the chondrocyte-specific role of MGP's carboxylated Gla residues and the γ-carboxylation of proteins is relatively unknown. To determine the function of these residues and proteins, we generated a novel mouse model, *Ggcx$^{flox/flox}$;Col2a1-Cre* mice, lacking GGCX only in chondrocytes. Our analyses of these mice show that unlike *Mgp-/-* mice, the *Ggcx$^{flox/flox}$;Col2a1-Cre* mice did not display nasal septum calcification or midface hypoplasia. However, all growth plates, including the spheno-occipital synchondrosis (SOS), in these mice were calcified and the long bones showed significant shortening. Our data suggest that the SOS may not play a major role in midface development in mice. Collectively, our study demonstrates the critical local role of GGCX in chondrocytes for normal endochondral bone development.

Résumé

Les anomalies congénitales du squelette peuvent entraîner la chondrodysplasie et la formation d'os déformés ayant une faible capacité de support de charge. Un grand nombre de gènes ont été associés avec ces anomalies du squelette. Bien qu'il s'agisse de cas rares, globalement à la naissance, l'incidence de ces maladies est estimée à 1 naissance vivante sur 5000. Dans la présente étude, nous avons investigué les anomalies congénitales du squelette qui sont causées par des mutations perte de fonction dans γ-glutamyl carboxylase (GGCX). De manière dépendante à la vitamine K, GGCX est responsable de la carboxylation de spécifiques résidus d'acide glutamique dans certaines protéines, y compris les facteurs de coagulation et les protéines Gla du tissu osseux. Des saignements abondants et une dysplasie des os du crâne, du visage ainsi que du squelette axial sont observés chez les patients ayant une mutation inactivatrice de GGCX. Les anomalies du squelette présentées par ces patients ont été décrites comme reproduisant les traits observés chez les humains atteints du syndrome de Keutel, lequel est causé par des mutations dans la protéine Gla de la matrice (MGP). En effet, les cinq résidus d'acide glutamique trouvés dans MGP sont γ-carboxylés par GGCX. Chez les souris, la perte de MGP se traduit par une calcification anormale de tous les tissus cartilagineux, y compris les plaques de croissance et la cloison nasale. Ces souris ont une hypoplasie de l'étage moyen de la face accompagnée d'une malocclusion de classe III et d'un raccourcissement des os endochondraux. Actuellement, le rôle spécifiquement au niveau des chondrocytes des résidus d'acide glutamique carboxylés dans MGP et, plus généralement, des protéines γ-carboxylés est inconnu. Pour y remédier, dans la présente étude, nous avons généré un nouveau modèle de souris, soit la souris *Ggcx$^{flox/flox}$;Col2a1-Cre*, dans laquelle GGCX est absente dans les chondrocytes. Nos analyses de ces souris montrent que, contrairement aux souris *Mgp-/-*, les souris *Ggcx$^{flox/flox}$;Col2a1-Cre* n'ont pas de calcification de la cloison nasale ou encore

d’hypoplasie de l’étage moyen de la face. Par contre, toutes les plaques de croissance, y compris la synchondrose sphéno-occipitale (SSO), sont calcifiées et les os longs sont significativement plus courts. Nos données suggèrent que chez la souris, la SSO ne joue pas un rôle majeur dans le développement de l’étage moyen de la face. Dans l’ensemble, notre étude démontre le rôle critique de GGCX dans les chondrocytes afin d’assurer le développement normal des os endochondraux.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Introduction and Literature Review

Cartilaginous extracellular matrices (ECMs) in the skeleton form critical anlagen for the developing endochondral bones. In addition, these tissues allow flexibility to the skeleton, act as shock absorbing cushions for the weight-bearing bones, and provide much-needed lubricated surfaces to prevent friction at the bone joints [1]. These functions are severely affected when abnormal deposition of minerals (calcification) occurs in the cartilage ECM. Ectopic calcification events in the cartilaginous tissues may lead to congenital anomalies such as chondrodysplasia, craniofacial deformities, and shortening of the long bones [2, 3]. This introductory section of the thesis will discuss the process of skeletal development, cartilaginous tissues, midface development, syndromes that manifest cartilage calcification and midface hypoplasia, the major proteins and their suggested roles in the prevention of abnormal cartilage calcification in the chondrocranium, and current treatment strategies for midface hypoplasia.

1.1. Skeletal Development: Two Modes of Ossification

Neural crest cells (NCCs) originate at the ectodermal border of the neural plate. From the ectodermal border, the neural crest cells migrate into the mesodermal mesenchyme while undergoing epithelial-to-mesenchymal cell transition. Once in the mesenchyme, the NCCs become known as ectomesenchymal cells [4]. NCCs can be categorized into four groups: cranial (called CNCCs), cardiac, vagal, and trunk [5]. NCCs migrate out of the dorsal neural tube and later, into the pharyngeal arches. CNCCs are responsible for forming the majority of the craniofacial bones (including the mandible and maxilla) and the associated cartilage, nerves, and connective tissues.

Developmentally, bones are formed by two distinct processes: intramembranous and endochondral ossification:

Intramembranous ossification is characterized by the differentiation of mesenchymal stem cells into osteoid-secreting osteoblasts. The resulting osteoid matrix contains type I collagen fibrils which enable the matrix to bind with calcium salts. The matrix continues to undergo mineralization, forming rudimentary bone containing mature osteocytes in the centre and active osteoblasts at the osteogenic front [5]. Intramembranous ossification is responsible for formation of the flat bones of the skull, the maxilla, the majority of the mandible, and the clavicles. Intramembranous ossification has been described as "especially sensitive" to biomechanical stresses and to the tensions and pressure created by bone deposition and resorption [6].

Endochondral ossification takes place in all other bones in the body, including the most distal parts of the mandible. Chondrocytes form the cartilaginous framework for bone formation during chondrogenesis and endochondral ossification. Chondrogenesis and endochondral ossification are subdivided into successive but also overlapping stages [7]. Mesenchymal cells first migrate to sites of future skeletal elements. At these sites the cells condense, resulting in increased cell density, increased direct interactions between cells, and an increased expression of molecules involved in intercellular adhesion (such as N-cadherin) [8]. A thin layer of elongated cells, called the perichondrium, then separates these cell aggregates from the surrounding tissues. The mesenchymal cells within these condensations differentiate into chondrocytes, establishing the hyaline cartilage model. Chondrocytes at this stage are marked by the expression of SOX5, 6, and 9 – these markers regulate the expression of genes encoding ECM molecules, type II collagen, and aggrecan (a cartilage-specific proteoglycan) [9-11]. This collagen anlagen provides tensile strength; aggrecan maintains hydraulic permeability and high-water content [12]. Some

chondrocytes differentiate and organize into longitudinal columns. These chondrocytes become embedded in an extensive ECM to make up the proliferative zone of cartilage in the ***growth plate***. In the core of the diaphysis, the chondrocytes stop dividing then hypertrophy and differentiate into prehypertrophic and, eventually, hypertrophic chondrocytes. Hypertrophic chondrocytes express type II and type X collagen, bone morphogenetic proteins (BMPs), and other growth factors (such as VEGF and RANKL) [3]. Type X collagen-rich ECM produced by the hypertrophic chondrocytes becomes mineralized by the deposition of hydroxyapatite [$Ca_5(PO_4)_3(OH)$] minerals. At this stage, long bones show distinct *growth plates* composed of chondrocytes at various stages of differentiation: *i) resting chondrocytes, ii) proliferating chondrocytes, iii) prehypertrophic chondrocytes, and iv) hypertrophic chondrocytes* (**Fig. A**).

VEGF produced by the hypertrophic chondrocytes stimulates angiogenesis in the mid-diaphysis, allowing some of the osteoblast precursors in the perichondrium to migrate into the ossification front [13]. The precursor cells differentiate into osteoblasts in both the outer perichondrium layer (called the periosteum/bone collar at this stage) and the inner primary ossification centre. Additionally, angiogenesis introduces a new population of undifferentiated mesenchymal cells which ultimately differentiate into osteoblasts [14]. More recent studies show that during endochondral bone development, a subset of hypertrophic chondrocytes transdifferentiate into bone-forming osteoblasts. Together with the migrating osteoblasts from the bone collar, these cells secrete type I collagen to form the primary spongiosa [15] – a mixed tissue of mineralized cartilage and bone ECM. Subsequent rounds of resorption of this mineralized matrix by osteoclasts and new bone formation by osteoblasts result in secondary spongiosa.

In the growth plates of developing long bones, multiple rounds of proliferation and differentiation of resting chondrocytes into hypertrophic chondrocytes (and their subsequent

apoptosis) continue until the end of puberty [16]. These cellular activities result in the sequential formation of newly mineralized cartilage ECM and, later, its replacement by primary and secondary spongiosa. The gradual accumulation of new bone tissue in the central region of the long bones, together with the movement of the grow plates in either direction, lead to elongation of future bones [16]. Secondary ossification centres are formed at the epiphyses via the same process at birth.

The cartilaginous growth plates between the primary and secondary ossification centres control bone growth [7]. The most abundant type of cartilage in the body is ***hyaline cartilage*** [17]. Hyaline cartilage forms most of the fetal skeleton and, later, articular cartilages of joints, costal cartilages, the cartilaginous nasal septum, cartilages of the cranial base synchondroses, the larynx, trachea, and bronchi. Hyaline cartilage provides a thin, smooth, and durable lining to the surfaces of the synovial joints which facilitate smooth movements without pain [18]. Hyaline cartilage is usually present as plates covered by a perichondrium – a vascular fibrous membrane made up of fibroblasts, collagen fibres, and vasculature, which is not able to penetrate the matrix [19]. Destructive of hyaline cartilage is associated with degenerative joint diseases.

1.2. Cartilage Extracellular Matrix (ECM)

The ECM in cartilage is secreted by chondrocytes and consists of collagen, proteoglycans, and non-collagenous glycoproteins. The ECM organizes itself into specific macromolecular assemblies which provide mechanical and structural support, compressive strength, hydraulic permeability, and an environment for cell-matrix interactions and specific signalling pathways [7]. The ECM also plays an integral role in cartilage development and homeostasis and formation of the skeletal framework during endochondral ossification. Skeletal disorders that affect growth

plates or articular cartilage are usually due to changes in chondrocyte morphology and metabolic activity, as well as ECM structure and composition [7]. Cell surface receptors, such as integrins, bind to sites on ECM molecules which results in transduction of chemical and mechanical signals to the cell interior. These signalling events significantly impact tissue-specific cell behaviours and fates.

The inner part of the perichondrium has a chondrogenic layer of osteoblasts which are responsible for secreting the intercellular matrix. While these chondroblasts bury deep in the matrix and mature into chondrocytes, daughter cells remain in lacunae to form isogenous groups [19]. The chondrocytes produce type II collagen, proteoglycans, and chondronectin, and receive nutrients via fluid exchange facilitated by blood vessels in the perichondrium. The intercellular matrix is formed from mucoproteins, chondroitin-sulphate, and a dense network of fine collagen fibres [19]. This perichondrium layer is not present in articular joints.

Collagen, together with other ECM proteins, forms a network which provides physical integrity to the tissues and organs. Via specific cell surface receptors, the collagen network interacts with cells, influencing their migration, differentiation, and biosynthetic abilities, which affects overall morphogenesis and development of these cells [20]. Aggregates of collagen peptides form supramolecular structures of triple-helical conformations which are assembled into fibrils and fibers. In total, twenty unique polypeptide chains are responsible for at least sixteen different collagen types; the majority of these collagen types in the body are I, II, and III [20]. Cartilaginous ECMs carry several different types of collagen – type II being most abundant. Type II collagen is composed of three peptides encoded by a single gene and can be found in articular, nasal, and sternal cartilages, and chondrosarcomas [20]. Although type II collagen is present in different types of cartilaginous tissues, aggrecan and type X collagen are more specifically present

in the prehypertrophic and hypertrophic zones of the growth plate, respectively. Type X collagen is a network-forming collagen found in the hypertrophic zone (making it a marker for terminally differentiated chondrocytes [21]), as well as in the calcified zone of articular cartilage. Type X constitutes approximately 1% of the total collagen in cartilage of the body [22]. However, type X collagen may constitute up to 18% of the cartilage in the hypertrophic region of epiphyseal plates because of its significant role in endochondral bone growth and development [21]. Mutations in type X collagen have been associated with chondrodysplasias in humans and mice [21]. Proteoglycans, one of the most abundant ECM molecules in cartilage, are composed of a central core protein covalently bonded to glycosaminoglycan (GAG) side chains [18]. GAGs contain negatively charged groups which attract positively charged ions such as sodium (Na^+) and, subsequently, result in water being drawn into the cartilage. Aggrecan, the major proteoglycan in articular cartilage, provides cartilage with load-bearing properties through its interactions with hyaluronic acid to form a hydrated gel surface [18].

1.3. Mineralization of the ECM

Deposition of calcium phosphate salts within the organic ECM is a physiologic process in bones, teeth, and some cartilaginous tissues. However, when this process occurs in the soft tissues, the deposition is known as ectopic mineralization/calcification. While both physiologic and pathologic ECM mineralization have many common determinants, some unique mechanisms can initiate pathologic calcification of the soft tissues [2].

Bone mineralization starts to occur in a human embryo during the third trimester [23]. In intramembranous bones, ECM synthesized by osteoblasts mineralizes; in endochondral bones, mineralization occurs simultaneously at two sites: the core region of cartilage and the surrounding

bone collar [24]. In the cartilage, type X collagen provides a scaffold for mineral deposition, whereas type I collagen serves this function in all bone types [24]. Systemic levels of calcium (Ca^{2+}) and inorganic phosphate (P_i) ions are important regulators of mineralization in bone. Experiments have shown that mutations in the 1,25-dihydroxyvitamin D_3 pathway lead to impaired absorption of Ca^{2+} and P_i in the gut, thereby reducing their extracellular levels [25]. In the developing bone, the α1 and α2 chains of type I collagen assemble to form a triple helix – these helices organize to first form collagen fibrils, then bundles of collagen fibres [26]. The deposited unmineralized collagen is known as osteoid. Osteoid is the site of mineral deposition within both the intra- and inter-fibrillar gaps of the collagen scaffold. Mineralization of the osteoid occurs continuously as new osteoid is being synthesized and mineralized by osteoblasts [24]. The exact mechanism of how collagen promotes mineralization is still not well understood. However, overwhelming evidence suggests that this process is driven by the absence or removal of mineralization inhibitors.

Mineralization inhibitors can be inorganic molecules such as inorganic pyrophosphate (PP_i) or polyphosphates [27]. Various metabolic pathways generate PP_i inside the cells, which can be transported to the extracellular spaces by a transmembrane protein called ANK. In addition, PP_i can be generated extracellularly by various ectonucleotide pyrophosphatases (e.g. NPPs). PP_i present in the ECM prevents its mineralization; however, in bone, PP_i can be cleaved by alkaline phosphatase (ALPL) to generate P_i. These dual actions of ALPL – cleavage of the inhibitor PP_i and generation of P_i – facilitate bone ECM mineralization [28].

In soft tissues, impaired PP_i synthesis and loss-of-function mutations in proteins like matrix Gla protein (MGP) may result in ECM mineralization [2]. Additionally, the nature of the ECM scaffold can be an important determinant for soft tissue calcification. For example, elastin- and

collagen-rich scaffolds in soft tissues like blood vessels and skin are known to promote mineral nucleation. The reduction of inhibitor levels in these ECMs and/or their altered remodeling (e.g. enzymatic degradation) may lead to ectopic calcification [27]. While soft tissue mineralization can sometimes occur due to ectopic bone formation, all soft tissue mineralization processes may not always require osteogenic or chondrogenic differentiation of the resident cells.

1.4. Role of Cartilaginous Tissues in Midface Development

The maxilla, mandible, and dentition are derived from the first pharyngeal arch. The mandible and mandible are formed from CNCCs, which are known for their multipotency and extensive migration through the embryo [29]. During early embryonic development, CNCCs migrate out of the hindbrain and travel along the dorsal-ventral axis to populate the pharyngeal arches. After first pharyngeal arch patterning is completed, a group of mesenchymal cells condense and develop into a pair of rod-shaped cartilages, the Meckel's cartilage. The Meckel's cartilage in each half of the mandible lengthens ventromedially and dorsolaterally until it fuses at the distal tip of the mandibular arch, forming the mandibular symphysis [30]. While most mammalian cartilages ossify to become bone, the tracheal, nasal, and articular cartilages retain their cartilaginous features. Bone formation begins lateral to Meckel's cartilage in the mandible, slightly preceding ossification in the maxilla. Under the control of several osteogenic transcriptional regulators, condensed mesenchymal cells differentiate into osteoblasts [31]. As mandibular ossification continues, the newly formed bony tissue approaches the rod-shaped Meckel's cartilage, becomes hypertrophic, and eventually degenerates. Multinuclear phagocytic chondroclasts resorb the calcified cartilaginous matrix; the intermediate portion of Meckel's cartilage becomes fibrous tissue. Endochondral ossification forms the most distal and proximal

regions of the symphysis, the condyle, and the mandibular angle. Development of the mandible and maxilla continues postnatally until approximately 20 years old [5].

The midface, which includes the nose, upper lip, maxillary, palatal, and zygomatic bones (**Fig. B**), is derived from the growth and fusion of seven different processes during embryogenesis. These seven processes are the frontonasal process, paired lateral and medial nasal processes, and paired maxillary processes [32]. The midface is largely populated by CNCCs. The interaction between these cells and associated epithelial cells determines midface development. An impairment of these cellular interactions can result in facial deformities, including cleft lip and palate, hypertelorism, and midface hypoplasia [33]. The overall net direction of growth of the craniofacial complex is downward and forward with lateral expansion [34]. Enlow and Hans described the growth of the maxilla and mandible as an "expanding pyramid" – the remodelling (differential apposition and resorption of bone) results in bony changes in three dimensions and, therefore, enlargement of the maxillary-mandibular complex [34]. Changes in the direction and amount of growth of this pyramid ultimately affect an individual's growth pattern, and aberrations in these patterns may result in abnormalities in the skeletal morphology and the associated occlusion [34]. Abnormal facial growth may be associated with congenital disorders and syndromes. Some of these disorders and syndromes are a consequence of embryonic abnormalities in neural crest cells, while other congenital abnormalities affecting jaw growth are due to craniosynostosis [34].

Specific structures of the craniofacial complex, such as the cranial base synchondroses and nasal septum, appear to have their own intrinsic growth potential [34]. The nasal septum is divided into two parts: bony and cartilaginous [35]. The bony septum is a singular, midline structure that comprises the perpendicular plate of the ethmoid superiorly and the vomer inferiorly [35]. The

septal cartilage is a quadrangular structure that lies in the midline of the nasal septum between the nasal bones; the ethmoid lies superiorly, and the vomer and the palate inferiorly. The cartilaginous nasal septum is a major support mechanism of the nose as it projects anteriorly to form part of the dorsal profile [35]. Even though the mandible is considered by many researchers as a factor affecting facial growth, the spheno-occipital synchondrosis (SOS) has a significant role in the development of the upper half of the craniofacial skeleton (including the height and depth of the upper face and spatial position of the maxillary teeth) and the cranial base [36, 37]. In healthy patients, the intra-occipital synchondroses fuse in childhood while the SOS remains open until adulthood [37]. Premature fusion of the SOS is associated with several disorders of midface hypoplasia such as Crouzon syndrome and obstructive sleep apnea (OSA), as well as midface hypoplasia in animal models [38]. A study by Driessen et al. found that the sella-nasion angle (SNA) measured in patients is not affected by premature closure of the SOS. This finding suggests that the SOS is not a significant contributor to midface hypoplasia and OSA. Additionally, thirty of the study patients underwent active rapid maxillary expansion treatment which resulted in an increased posterior cranial base length with no change in anterior-posterior cephalometric measurements [37].

1.5. Role of the Nasal Septum in Midface Development: Theories and Controversies

In 1953, Irish anatomist James Scott proposed the Nasal Septum Theory, which states that the nasal septum is an important growth centre that leads to overall facial growth. Furthermore, according to the Nasal Septum Theory, sutural growth is compensatory [6]. Scott's theory explains that the physical force generated by pressure-accommodating expansion of the cartilaginous portion of the nasal septum is responsible for displacing the maxilla anteriorly and inferiorly [6].

The displacement of the maxilla causes tension in the maxillary sutures which results in the enlargement of the bones at their sutures while moving in relation to each other. Many clinicians, especially those in the craniofacial field, have accepted Scott's Nasal Septum Theory and believe that the cartilaginous centres (chondrocranium, synchondroses, nasal septum) are the true centres of skull and facial growth [6].

In contrast to Scott's Nasal Septum Theory, Melvin Moss proposed the Functional Matrix Theory in 1962. This theory suggests that bone growth and displacement are in response to functional relationships established by the soft tissues associated with those bones [20] – the bone itself does not have intrinsic growth properties and, instead, relies on the functional soft tissue matrix. Moss explains that the cartilaginous nasal septum is actually a secondary and compensatory response to the primary growth of other orofacial matrices. Moss stipulates that midfacial skeletal growth is not dependent on any prior or primary growth force of the nasal septal cartilages [6]. Overall, the Functional Matrix Theory proposes that growth and displacement of all skeletal units are secondary to primary changes in their functional matrices and independent of the development of other facial structures (including the nasal septum).

1.6. Pathologies Associated with Abnormal Bone and Cartilage Mineralization

Disorders characterized by abnormal cartilage mineralization can be categorized into two subtypes: hypomineralization- and hypermineralization-associated skeletal disorders. Vitamin D deficiency, X-linked hypophosphatemia (XLH), and sphingomyelin phosphodiesterase 3 (SMPD3) deficiency all result in abnormal hypomineralization. Vitamin D deficiency rickets is a condition that affects the skeletal tissues and is caused by insufficient vitamin D in the body from a lack of sun exposure or poor dietary intake. This type of rickets usually presents initially during

infancy or childhood [39]. Since vitamin D is required for intestinal absorption of calcium and phosphorus, bone mineralization is directly affected by deficient vitamin D levels. The affected bone and cartilage mineralization manifests itself as skeletal dysplasias most commonly found at the distal forearm, knee, and costochondral junctions [40]. Patients may present with frontal bossing, costochondral junction enlargement of the ribs, widening of the wrist, and bowing of the radius, ulna, femur, and tibia [40].

XLH is an X-linked dominant disorder caused by mutations in the PHEX gene. XLH is characterized by excessive excretion of phosphate in the urine ("phosphate wasting"). Excessive excretion of phosphate results in low levels of phosphate in the blood and eventually leads to widening of the cartilaginous growth plates due to impaired apoptosis of the hypertrophic chondrocytes and soft, weak bones, usually diagnosed in childhood [41]. Most patients with XLH present with bone-related symptoms such as bone, muscle, and joint pain and weakness; long bone phenotypes due to abnormalities in the growth plates and metaphyses; abnormal gait; and abnormal tooth development [41].

In developing growth plates of the skeleton, hypertrophic chondrocytes release specialized matrix vesicles (MV) which serve as nucleation sites for hydroxyapatite crystals [42]. SMPD3, an enzyme present in the membranes of the endoplasmic reticulum and the inner leaflet of the cell membrane in bone and cartilage [43], is thought to have a role in MV-mediated mineralization of cartilaginous growth plates. Previous studies have shown that growth plate mineralization is significantly delayed in mice lacking functional SMPD3 [44], suggesting that SMPD3 is a key regulator of skeletal development. Although until now mutations in SMPD3 have not been identified in humans, mutations in enzymes acting upstream of SMPD3 have been shown to cause bone anomalies [45].

Cartilage calcification disorders which cause hypermineralization include ankylosis and chondrocalcinosis. Ankylosis is caused by a reduction in PP_i, a potent anti-mineralization molecule, in the bone joints and in soft tissues. PP_i prevents the abnormal and widespread calcification of soft tissues in vertebrates by inhibiting the incorporation of Ca^{2+} and P_i into nascent apatitic crystals; thereby, inhibiting crystal growth and calcification [24]. Mutations in *Ank* and *Enpp1* (proteins responsible for maintenance of PP_i levels in the ECM) are responsible for the ectopic mineral deposition observed in patients with ankylosis [24]. This deposition results in abnormal stiffening and rigidity of the bones. Chondrocalcinosis is a metabolic disorder precipitated by gain-of-function mutations in the *ANKH* (human ortholog of *Ank*) gene. This disorder is associated with abnormal calcium pyrophosphate (CPP) deposition. The *ANKH* gene regulates expression of human ANK protein which is involved in cellular transports of PP_i. Mutations in the *ANKH* gene lead to abnormal accumulation of PP_i in cartilage ECM which, in turn, complexes with calcium to form CPP crystals. Abnormal deposition of calcium pyrophosphate dihydrate (CPPD) crystals occurs in joint cartilage causing recurrent pain; swelling; warmth and redness of the area and, eventually, joint damage [46]. Both of these hypermineralization disorders can result in osteoarthritis which is the most common chronic condition of the joints that leads to degeneration of joint tissues [46].

Developmental disorders of cartilage calcification include Crouzon syndrome, Apert syndrome, and chondrodysplasia punctata. Crouzon syndrome is a disorder that affects signalling events involving fibroblast growth factors FGFR2 and FGFR3. Crouzon syndrome is inherited in an autosomal dominant manner. Because Crouzon syndrome affects the branchial and pharyngeal arches which give rise to the maxilla and mandible, this syndrome causes impaired maxillary growth. The most common feature of Crouzon syndrome is craniosynostosis [2]. Apert syndrome

is characterized by midface hypoplasia, craniosynostosis, and hand deformities caused by syndactyly. The syndrome may be caused by autosomal dominant mutations in FGFR2, however, most of the causal mutations are spontaneous [2]. Patients afflicted with Pfeiffer syndrome present with underdeveloped maxillas, beak-shaped noses, and deformities of the hands and feet. Mutations in FGFR1 and FGFR2 have been implicated in the syndrome, however, other contributing molecules/proteins may be present [2]. Chondrodysplasia punctata is an X-linked recessive disorder caused by mutations in arylsulfatase E (ARSE). This disorder occurs almost exclusively in males. Patients with chondrodysplasia are generally short in stature, have short finger tips and toes, and have midface hypoplasia [2]. The one facial feature common to all of the aforementioned disorders is midface hypoplasia. Considering that the focus of this thesis is the relationship between γ-glutamyl carboxylase (GGCX) and MGP, and midface hypoplasia, a more detailed discussion of this cranial phenotype follows.

1.7. Midface Hypoplasia

Midface hypoplasia is a consequence of impaired or delayed growth of the maxillary, zygomatic, and nasal bones (relative to the rest of the craniofacial complex) during embryonic development. These growth abnormalities give rise to facial features including deficient nasal length and projection, ocular proptosis, excessive scleral show, frontal bossing, acute nasolabial angle, central face concavity, and malocclusion [47]. Further complications associated with these abnormalities may include more severe systemic issues for a patient, such as sleep apnea, ophthalmic sequelae (including chronic corneal exposure), and eating difficulties.

Midface hypoplasia is often associated with congenital disorders and syndromes prompted by impaired signalling events affecting the epithelial-mesenchymal cell interactions during

development. Other syndromes and disorders with midface hypoplasia are due to prenatal or perinatal ECM abnormalities, such as premature fusion of the cranial sutures (craniosynostosis) and/or ectopic ECM calcification [2].

Craniosynostosis, first described by Hippocrates in 100 BC, is the premature closure of one or more cranial sutures, with or without ossification of the synchondroses [48]. Unusual cranial shape and overlapping sutures in a newborn with a normal head size can usually be attributed to molding of the skull during birth. This presentation generally self-corrects within the first two months after birth and does not require further work-up or treatment. However, a child with craniosynostosis will present with classic patterns of deformity, caused by the skull's inability to expand in the direction perpendicular to the stenosed suture [49]. The majority of craniosynostosis cases present with a relatively normal head circumference and growth curve. Microcephaly secondary to inadequate growth of the brain (the most common cause) and pan-synostosis with multiple suture fusions are both causes for abnormal cranial growth [49]. Pan-synostosis can present either as an isolated finding or in association with several different anomalies – the latter condition is known as syndromic craniosynostosis. Syndromic craniosynostosis has a propensity for midface hypoplasia [48].

In addition to craniosynostosis, specific structures of the craniofacial complex may have a direct impact on midface development. For instance, the cartilaginous tissues present in the cranial base synchondroses and the nasal septum appear to have their own intrinsic growth potential [34]. We have recently reported midface hypoplasia in a mouse model of Keutel syndrome. Our findings reveal abnormal calcification occurs in both the nasal septum and the SOS [50].

1.8. Midface Hypoplasia Associated with Abnormal Cartilage Calcification

Abnormal cartilage calcification is seen in several related syndromes which include vitamin K–dependent clotting factor deficiency (VKCFD), warfarin embryopathy, and Keutel syndrome. VKCFD is caused by mutations in GGCX, an enzyme essential for post-translational γ-carboxylation of glutamic acid (Glu) residues of many proteins, including blood coagulation factors and skeletal Gla proteins [51]. Vitamin K epoxide reductases (VKORs) are enzymes which generate the reduced form of vitamin K (a cofactor for the GGCX-catalyzed reaction). Inactivating mutations in VKORs may also lead to VKCFD. Pregnant mothers who are treated with warfarin, an oral vitamin K antagonist, can have newborn babies with VKCFD, including severe cartilage calcification [51]. Cartilage calcification traits caused by mutations in GGCX, VKORs, and warfarin embryopathy are thought to be products of impaired γ-carboxylation in an ECM protein called MGP.

Chondrodysplasia punctata is a skeletal dysplasia characterized by premature foci of calcification within cartilage [52]. Skeletal anomalies of this disease include abnormal stippling of the epiphyses of the long bones, vertebral column, trachea, and rib ends; distal phalangeal hypoplasia; and midface hypoplasia [52]. Fetal loss and malformations as a result of warfarin intake and other similar anticoagulant use during pregnancy have been reported since 1970 [53]. The resulting array of fetal phenotypes are referred collectively to as warfarin embryopathy. These phenotypes include nasal hypoplasia, chondrodysplasia punctata, cleft lip and/or palate, choanal stenosis, defects of the fingers and toes, ocular defects (ocular atrophy, cataracts, micropthalmia), brain malformations, stillbirth, and spontaneous abortion [53]. In humans, loss-of-function mutations in MGP cause Keutel syndrome, a rare autosomal disorder that was first described in 1972 [54]. The hallmark feature of Keutel syndrome is diffuse abnormal cartilage calcification

[55] in association with brachytelephalangism, stippled epiphyses, hearing loss, pulmonary stenosis, short stature, midface hypoplasia, and mental deficiency [56]. In addition, vascular calcification has been reported in some patients afflicted with Keutel syndrome [57-59]. Vascular smooth muscle cells and chondrocytes are two cell types that express *Mgp* at high levels. In agreement with this expression pattern, vascular and cartilaginous tissues are most severely affected in Keutel syndrome patients and MGP-deficient (*Mgp-/-*) mice [50]. Considering that MGP and GGCX are important in the prevention of ectopic cartilage calcification and their linked anti-mineralization functions via the same pathway, a more detailed discussion of these proteins is presented below.

1.9. MGP, its Post-Translational Modification by GGCX, and its Role in Midface Hypoplasia

MGP is a small (14 kDa), mineral-binding protein with a potent anti-mineralization effect (**Fig. C**). MGP is expressed by vascular smooth muscle cells and chondrocytes. In agreement with this expression pattern, vascular and cartilaginous tissues are most severely affected in Keutel syndrome patients and in MGP-deficient (*Mgp-/-*) mice [50].

We have recently reported the effects of MGP deficiency on craniofacial development in mice [50]. Five-week old *Mgp-/-* skulls were examined visually and by conventional x-ray and micro-computed tomography (micro-CT). These findings included severe blunting of the snout, a wider and more rounded face, severe anterior crossbite of the incisors, abnormal calcification of the cartilaginous nasal septum, and premature closure of the SOS [50]. Additionally, detailed cephalometric analyses revealed significantly shorter antero-posterior measurements, specifically, the cranial, maxillary, and palatine lengths. The length of the nasal septum was significantly

reduced in *Mgp-/-* mice when compared to control mice at 5-weeks old. Altogether, these findings by Marulanda et al. suggest a novel form of midface hypoplasia associated with both nasal septum and SOS abnormalities. However, the relative contributions of each of these anomalies to the overall phenotype is still not well understood.

MGP undergoes two different post-translational modifications: serine residue phosphorylation and γ-carboxylation of specific Glu residues which converts them into Gla residues, the latter being vitamin K-dependent. This carboxylation reaction has been suggested as a factor in enhancing the binding of MGP to calcium ions in hydroxyapatite [55]. Uncarboxylated MGP has been associated with the presence of atherosclerosis and arterial calcification. Patients with high circulating levels of the uncarboxylated protein have been found to be at a high risk of developing prevalent cardiovascular calcification [55].

Gamma-carboxylation of MGP is accomplished by GGCX (**Fig. D**), an integral membrane enzyme located in the rough endoplasmic reticulum [60]. GGCX mutations and impairment of vitamin K metabolism affect the functions of several proteins. VKCFD is a bleeding disorder characterized by reduction of coagulation factors II, VII, IX, and X, and anticoagulant proteins C and S, following abnormalities in the vitamin K-dependent γ-carboxylation pathway [61]. VKCFD is usually diagnosed during infancy and has two subtypes: VKCFD1 (due to mutations in GGCX) and VKCFD2 (due to a deficiency in VKOR). The enzyme VKOR is responsible for the reduction of the oxidized form of vitamin K into vitamin K hydroquinone (**Fig. E**), resulting in the activation of important coagulation factors in healthy patients. The standard treatment for VKCFD is administration of high-dose phylloquinone (vitamin K1) [61]. In a case study by Tie et al., a 4-month old girl presented to the hospital with pallor, failure to thrive, skeletal traits described in Keutel syndrome, CT-confirmed intracranial hemorrhage, and bloodwork suggestive of VKCFD.

Genetic analysis of the patient and her family revealed three different missense mutations in *Ggcx*; genetic variation in *Mgp* was not a finding among these individuals. The patient's symptoms, which included both bleeding and non-bleeding effects, were found to be a result of a single GGCX mutation (D153G) that gives rise to both phenotypes described [61].

In the current study, we followed a genetic approach to ablate *Ggcx* only in chondrocytes of *Ggcx$^{flox/flox}$;Col2a1-Cre* mice. This model was analyzed to investigate the role of GGCX in the prevention of abnormal calcification of the cartilaginous tissues. Micro-CT- and histology-based techniques were used to study the craniofacial and long bone phenotypes of these mice. Unlike *Mgp-/-* mice, *Ggcx$^{flox/flox}$;Col2a1-Cre* mice do not show any calcification of the cartilaginous nasal septum. Although SOS calcification was present, its progression was slightly delayed in comparison to *Mgp-/-* mice. Cephalometric analyses confirmed the absence of midface hypoplasia in these mice. Further analyses demonstrated that long bones were shorter in *Ggcx$^{flox/flox}$;Col2a1-Cre* mice, which was associated with abnormal calcification of the growth plates. Our data suggest that local GGCX activity in the growth plate chondrocytes is essential for normal growth of the long bones and SOS calcification may not have a significant impact on midface development in mice.

1.10. Treatment of Midface Hypoplasia

Currently, the primary treatment for midface hypoplasia is surgery via a Le Fort 1 osteotomy to advance the maxilla in order to correct Class III dental malocclusions and associated soft tissue abnormalities. In addition to the initial surgery, bone grafting might be needed to improve bone healing and post-operative stability [34]. In cases that require a significant amount of movement and large quantities of grafting material, bone grafts might have to be harvested from

the iliac crest. In vertical maxillary deficiency (as seen in midface hypoplasia), elongation of the face can be achieved by bone grafting the maxilla into a more inferior position during the Le Fort 1 osteotomy. In severe cases of midface defects with infraorbital rim and malar eminence deficiency, a Le Fort III or modified Le Fort III osteotomy might be required to advance the maxillary, malar, and nasal bones [34].

Patients with severe midface hypoplasia might not be candidates for advancement surgery. In these patients, the overlying soft tissues might not be able to adapt to the acute changes and stretching that would result from a significant advancement as described above. The indicated treatment for these patients might be distraction osteogenesis [34]. Distraction osteogenesis involves an osteotomy followed by the attachment of an external appliance to facilitate gradual separation of bones and new tissue formation. The gradual tension placed on the distracting bone interface results in continuous bone formation. This tension also promotes adaptive changes in the overlying soft tissues. Distraction osteogenesis is a tedious process that has several phases: osteotomy/surgical phase, latency phase, distraction phase, consolidation phase, appliance removal phase, and remodelling phase [34]. While distraction osteogenesis can reduce the risk of post-operative complications (such as relapse and scarring), the procedure is not without its liabilities. These disadvantages include the need for two procedures (placement and removal of the distractors), increased cost, longer treatment time, and more frequent appointments with the surgeon and other specialists [34].

Obstructive sleep apnea (OSA), a severe consequence of midface hypoplasia, is a condition of apneic events during sleep. During these events patients have cessation of airflow for more than ten seconds [34]. OSA can have a multitude of side effects, ranging from sleep disruption and daytime somnolence to severe hypoxia, cardiac abnormalities, and death. Decreased muscle

tone in the palate, tongue, or pharyngeal musculature can lead to a collapse in the airway, which is the precursor of OSA [34]. OSA is also associated with mandibular and midface deficiencies from a lack of forward suspension of the tongue and associated musculature. As a consequence, OSA is exaggerated when laying down. Non-surgical treatment options for OSA include weight loss, positional changes during sleep, or a continuous positive airway pressure (CPAP) facial or nasal mask. However, an OSA patient with severe midface hypoplasia might require surgical intervention. Surgical options for treatment include uvulopalatoplasty or uvulopharyngopalatoplasty (portions of the soft palate, uvula, tonsils, and pharyngeal walls are removed), and maxillary and/or mandibular advancement [34].

Even though the surgical approaches to correcting midface hypoplasia present with a number of possible postoperative complications, such as surgical relapse, scarring, infection, excessive loading of the temporomandibular joint, and neurosensory loss [34], alternative treatment is not currently available. Our study on *Mgp-/-* and *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice has revealed possible mechanisms of cartilage-associated skeletal anomalies in some syndromic patients. A better understanding of the impaired genetic pathways in these patients and their sites of action may lead to a targeted approach of innovative treatments, such as gene or enzyme replacement therapies. These novel therapeutic approaches may circumvent many of the shortcomings associated with these complex surgical procedures.

1.11. Figures

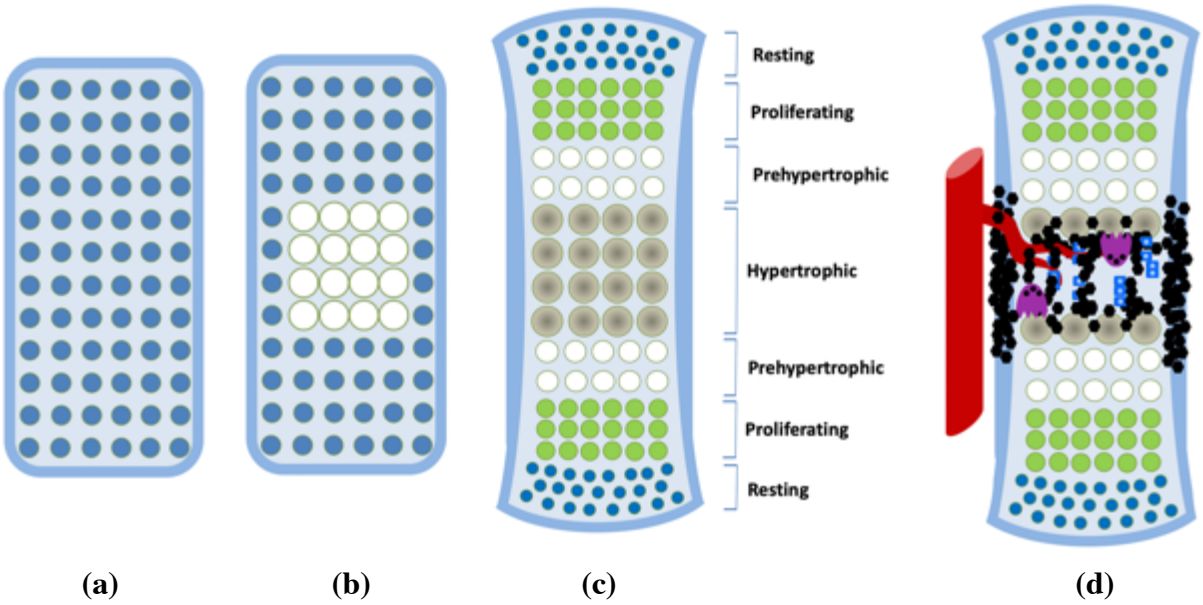

FIGURE A. Endochondral ossification. (a) The core of the cartilage precast. (b) Enlargement of the cells in the cartilage precast. (c) Four distinct zones of chondrocytes: resting, proliferating, prehypertrophic, and hypertrophic. The most advanced cells are called hypertrophic chondrocytes and they reside in the core area. (d) While hypertrophic chondrocytes release a type of collagen that is mineralized (black hexagons), angiogenesis in the core introduces osteoclasts (large purple cells) to the area. Osteoblasts (blue cells) migrate from the surrounding bone collar into the core area, synthesizing collagen that is more compact and becomes heavily mineralized. (Images taken with permission from Dr. Murshed's lecture notes.)

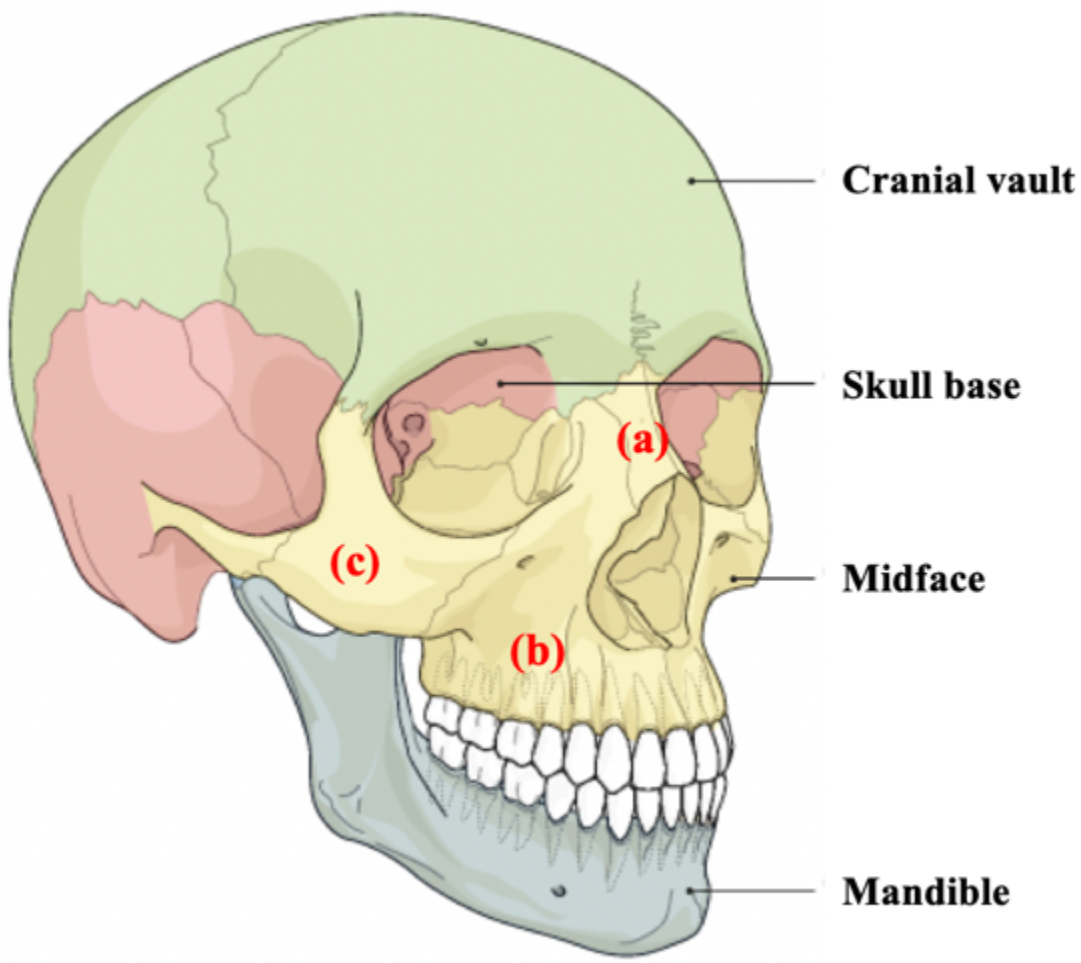

FIGURE B. The craniofacial complex. The midface is comprised of the nasal (a), maxillary (b), and zygomatic (c) bones, and the overlying soft tissues. Postnatally, the net displacement is downward and forward, and this is due to continued growth of the brain and the cranial base and is augmented by sutural expansion between the cranial base and the maxilla. (Image from www2.aofoundation.org)

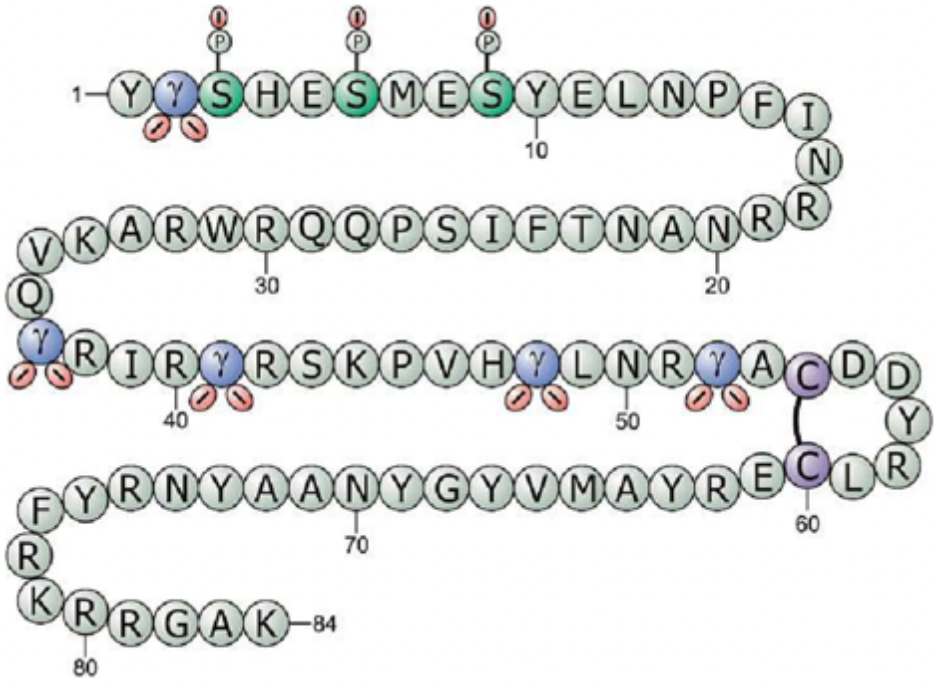

FIGURE C. Structure of MGP. MGP is a small (14 kDa) protein containing four conserved Glu residues, which undergo post-translational γ-carboxylation to form Gla (represented by the blue molecules). Additionally, MGP undergoes post-translational phosphorylation of three conserved N-terminal serine residues (green molecules). (Image from Schurgers et al., Thromb Haeomost 2008)

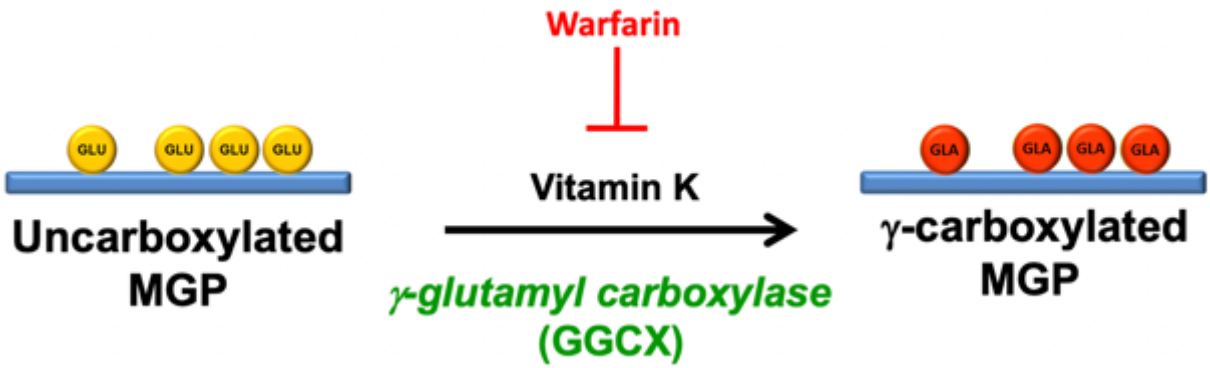

FIGURE D. Gamma-carboxylation of MGP by GGCX. Four conserved Glu residues in MGP undergo post-translational γ-carboxylation by GGCX to form Gla residues (γ-carboxylated MGP, the normal and functioning protein in the body). This reaction is vitamin K-dependent and can be inhibited by warfarin. (Image taken with permission from Dr. Murshed's lecture notes.)

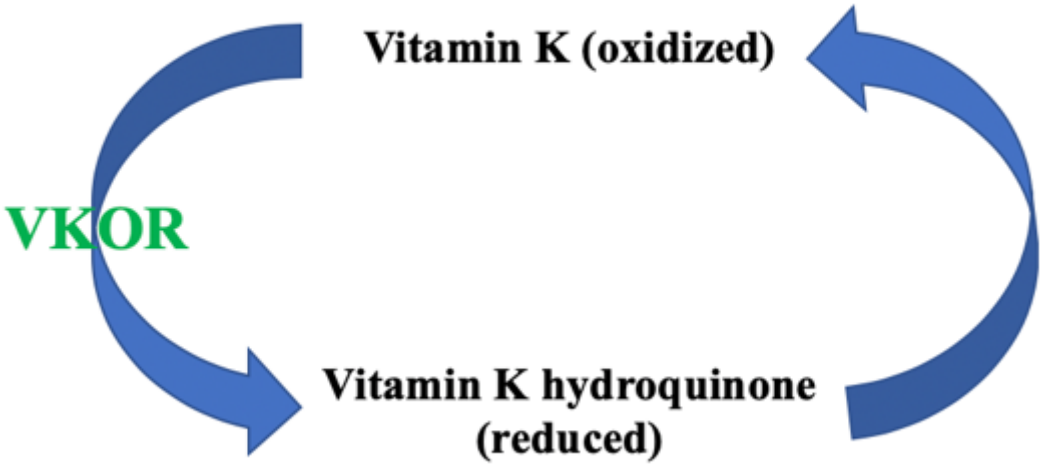

FIGURE E. VKOR. The enzyme VKOR is required for the conversion of oxidized vitamin K to reduced vitamin K, which is needed for the synthesis and activation of coagulation factors. Abnormalities in this pathway may lead to bleeding disorders such as VKCFD.

CHAPTER 2: OBJECTIVES

Objectives

Overarching goal: To study the chondrocyte-specific roles of GGCX in skeletal development.

MGP is a potent inhibitor of mineralization in the body. Loss-of-function mutations in MGP have been associated with diffuse ectopic calcification of cartilaginous tissues resulting in both craniofacial and skeletal abnormalities. Although MGP mutations have been implicated in the development of Keutel syndrome in humans, the individual contribution of its upstream enzyme, GGCX, is not well-known. GGCX is responsible for the post-translational γ-carboxylation of MGP's Glu residues, converting these residues into Gla. This conversion results in the formation of γ-carboxylated MGP, which is the normal and functioning form of the protein in the body. While patient data suggest a role for GGCX in the prevention of cartilage calcification, the local role of this enzyme in chondrocytes has yet to be confirmed.

Specific aims:

i) To generate and validate a mouse model (*Ggcx$^{flox/flox}$;Col2a1-Cre)* with chondrocyte-specific ablation of *Ggcx*.

Rationale: In addition to γ-carboxylation of MGP, GGCX is also required for the carboxylation of the coagulation factors and other proteins in the body. Until now, an animal model for the whole-body knockout of *Ggcx* was not available to study, given that these mice die prematurely due to massive bleeding from vessels. In order to successfully study the roles of GGCX in cartilage during skeletal development, a mouse model with chondrocyte-specific ablation of *Ggcx* was generated. Once these *Ggcx$^{flox/flox}$;Col2a1-Cre* mice were obtained, they were validated by genotyping PCR and Western blot analyses performed on nasal septum and sternal tissues.

ii) To compare the craniofacial and long bone phenotypes of *Ggcx$^{flox/flox}$;Col2a1-Cre* mice to *Mgp-/-* mice.

Rationale: Previous studies have shown that MGP-deficient mice faithfully recapitulate the features of Keutel syndrome including midface hypoplasia. Craniofacial anomalies have also been reported in humans with GGCX mutations. In order to study the effects of GGCX on craniofacial development, the heads of *Ggcx$^{flox/flox}$;Col2a1-Cre* mice were scanned by micro-CT, analyzed, and measured for cephalometric data. Because some of the human traits have been reported in Keutel syndrome, the long bones of the *Ggcx$^{flox/flox}$;Col2a1-Cre* mice were then analyzed. The tibiae and femurs were collected, radiographed and measured, then embedded in plastic, sectioned, and stained with VKVG for microscopic analysis of the growth plates.

CHAPTER 3: MANUSCRIPT FOR PUBLICATION

Understanding the Chondrocyte-Specific Roles of γ-Glutamyl Carboxylase in Skeletal Development

Jane Hendrickson-Rebizant[1], Omar Al Rifai[2], Juliana Marulanda[1], Geneviève Chiasson[1], Mathieu Ferron[2], and Monzur Murshed[1,3,4*]

[1]Faculty of Dentistry, McGill University, Montréal, Québec, Canada

[2]Institut de recherches cliniques de Montréal, Montréal, Québec, Canada

[3]Department of Medicine, McGill University, Montréal, Québec, Canada

[4]Shriners Hospital for Children, Montréal, Québec, Canada

***Corresponding author**:

Monzur Murshed, PhD

Address: 1003 Boulevard Décarie, Room 05-21

Montréal, Québec H4A 0A9, Canada

E-mail: monzur.murshed@mcgill.ca

Phone: 514 282 8255

Fax: 514 842 5581

Abstract

Congenital abnormalities of the skeleton may lead to chondrodysplasia and formation of deformed bones with poor load-bearing capacities. A large number of genes have been found to be associated with these skeletal abnormalities. Although individually rare, the overall incidence of these diseases is estimated to be 1/5000 live births. In the current study, we investigated the congenital skeletal anomalies that are caused by loss-of-function mutations in γ-glutamyl carboxylase (GGCX). In a vitamin K-dependent manner, GGCX carboxylates specific glutamic acid (Glu) residues in proteins, including blood coagulation factors and skeletal Gla proteins. Patients with inactivating *GGCX* mutations show massive bleeding from vessels and dysplasia of craniofacial and axial bones. The skeletal anomalies of these patients have been described to mimic the traits of human Keutel syndrome, which is caused by mutations in matrix Gla protein (MGP). MGP's 5 Gla residues are γ-carboxylated by GGCX. The loss of MGP in mice results in abnormal calcification of all cartilaginous tissues, including growth plates and the nasal septum. These mice have midface hypoplasia with class III malocclusion and shortening of the endochondral bones. At present, the chondrocyte-specific role of MGP's carboxylated Gla residues, and γ-carboxylation of proteins is relatively unknown. To determine the chondrocyte-specific role of these residues and proteins, in the current study, we generated a novel mouse model (*Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice) lacking GGCX only in chondrocytes. Our analyses of these mice demonstrate that, unlike *Mgp-/-* mice, neither nasal septum calcification nor midface hypoplasia were apparent in the *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice. However, all of the growth plates, including the spheno-occipital synchondrosis (SOS), were calcified along with a significant shortening of the long bones. Our data suggest that the SOS may not play a major role in midface development in mice. Collectively,

our study demonstrates the critical local role of GGCX in chondrocytes for normal endochondral bone development.

Introduction

Cartilaginous extracellular matrices (ECMs) in the skeleton form critical anlagen for the developing endochondral bones. These tissues also allow flexibility to the skeleton, act as shock absorbing cushions for weight-bearing bones, and provide much-needed lubricated surfaces to prevent friction at bone joints [1]. These functions are severely affected when abnormal deposition of minerals (calcification) occurs in the cartilage ECM. Such ectopic calcification events in the cartilaginous tissues may lead to congenital anomalies such as chondrodysplasia, craniofacial deformities, and shortening of the long bones [2, 3].

Abnormal cartilage calcification is evident in several related syndromes including vitamin K–dependent clotting factor deficiency (VKCFD), warfarin embryopathy, and Keutel syndrome. VKCFD is caused by mutations in γ-glutamyl carboxylase (GGCX), an enzyme essential for post-translational γ-carboxylation of glutamic acid (Glu) residues of many proteins, including blood coagulation factors and skeletal Gla proteins [50]. Vitamin K epoxide reductases (VKORs) are enzymes which generate reduced vitamin K (a cofactor for the GGCX-catalyzed reaction). Inactivating mutations in VKORs may also lead to VKCFD. Pregnant mothers who are treated with warfarin, an oral vitamin K antagonist, can have newborn babies with VKCFD, including severe cartilage calcification [51]. Cartilage calcification traits caused by mutations in GGCX, VKORs, and warfarin embryopathy are thought to be products of impaired γ-carboxylation in an ECM protein called MGP.

MGP is a small (14 kDa), mineral-binding protein with a potent anti-mineralization effect largely attributed to its Gla residues. MGP carries four conserved Glu residues, which are post-translationally γ-carboxylated by GGCX to form Gla residues. This post-translational carboxylation reaction has been suggested to enhance binding of MGP to calcium ions in

hydroxyapatite minerals [55]. Uncarboxylated MGP has been associated with the presence of atherosclerosis and arterial calcification [55]. In addition to γ-carboxylation, MGP undergoes another post-translational modification: three of its conserved N-terminal serine residues are phosphorylated by a yet unknown kinase [62].

In humans, loss-of-function mutations in MGP cause Keutel syndrome, a rare autosomal disorder that was first described in 1972 [54]. The hallmark feature of Keutel syndrome is diffuse abnormal cartilage calcification [55] in association with brachytelephalangism, stippled epiphyses, hearing loss, pulmonary stenosis, short stature, midface hypoplasia, and mental deficiency [56]. In addition, vascular calcification has been reported in some patients [57-59]. Vascular smooth muscle cells and chondrocytes are two cell types that express *Mgp* at high levels. In agreement with this expression pattern, vascular and cartilaginous tissues are most severely affected in Keutel syndrome patients and in MGP-deficient (*Mgp-/-*) mice [50].

Our group has extensively characterized the ectopic calcification traits in *Mgp-/-* mice. We previously reported that among two related skeletal Gla proteins, MGP and osteocalcin, only MGP possesses anti-mineralization functions [63]. When *Mgp* was overexpressed in bone forming osteoblasts, a cell type that expresses *Mgp* at lower levels, we observed a marked increase of unmineralized bone ECM. This increase was completely abolished when MGP's conserved Glu residues were mutated to aspartic acid [63]. However, until now, the functional roles of these Gla residues in MGP's anti-mineralization functions in vascular and cartilaginous tissues have not been tested in vivo. The overall objective of our current study is to investigate the effects of protein γ-carboxylation in the cartilaginous tissues.

As shown earlier, *Mgp-/-* mice show severe growth plate cartilage calcification, leading to the abnormal loss of the growth plates and shortening of the long bones [64]. More recently, we

reported the effects of MGP deficiency on midface development in mice. These mice show blunting of the snout, a wider and more rounded face, and severe anterior crossbite of the incisors. Abnormal calcification of the cartilaginous nasal septum and premature closure of the spheno-occipital synchondrosis (SOS) were also observed in these mice [50]. Additionally, detailed cephalometric analyses revealed severe midface hypoplasia with significantly shorter antero-posterior measurements, specifically, the cranial, maxillary, and palatine lengths. Altogether, these findings suggest a novel form of midface hypoplasia associated with both nasal septum and SOS abnormalities. However, the relative contributions of each of these anomalies to the overall phenotype are still not well understood [50].

Midface hypoplasia is a result of impaired or delayed growth of the maxilla, zygomatic bones, and nasal bones, relative to the rest of the craniofacial complex, during embryonic development. These growth abnormalities give rise to facial features including deficient nasal length and projection, ocular proptosis, excessive scleral show, frontal bossing, acute nasolabial angle, central face concavity, and malocclusion [47]. Further complications associated with these abnormalities may include more severe systemic issues for a patient, such as sleep apnea, ophthalmic sequelae (including chronic corneal exposure), and eating difficulties.

Premature fusion of the cranial sutures (craniosynostosis) has long been associated with midface hypoplasia. Although rare, pan-synostosis with multiple suture fusions can be present either as an isolated finding or in association with several different anomalies – the latter condition is known as syndromic craniosynostosis. Syndromic craniosynostosis is highly associated with midface hypoplasia [48, 49]. A child with craniosynostosis will present with classic patterns of deformity, due to the skull's inability to expand in the direction perpendicular to the stenosed suture [49]. In addition to craniosynostosis, specific structures of the craniofacial complex, such

as the cranial base synchondroses and nasal septum, may affect midface development. Cartilaginous tissues of these structures appear to have their own intrinsic growth potential [34], which can be affected by the cell and/or ECM abnormalities at these sites.

In the current study, we followed a genetic approach to ablate *Ggcx* only in chondrocytes of *Ggcx$^{flox/flox}$;Col2a1-Cre* mice. This model was analyzed to investigate the local role of GGCX in preventing abnormal calcification of the cartilaginous tissues. We used micro-CT- and histology-based techniques to study the craniofacial and long bone phenotypes of these mice. Unlike *Mgp-/-* mice, *Ggcx$^{flox/flox}$;Col2a1-Cre* mice do not show any calcification of the cartilaginous nasal septum. Although SOS calcification was present, its progression was slightly delayed in comparison to *Mgp-/-* mice. Cephalometric analyses confirmed the absence of midface hypoplasia in these mice. Further analyses demonstrated that long bones were shorter in *Ggcx$^{flox/flox}$;Col2a1-Cre* mice, which was associated with abnormal calcification of the growth plates. Our data suggest that local GGCX activity in the growth plate chondrocytes is essential for the normal growth of the long bones and SOS calcification may not have a significant impact on midface development in mice.

Materials and Methods

Mice

The generation and genotyping of *Ggcx*$^{flox/flox}$ and *Col2a1-Cre* mice were previously described [65, 66]. Genotypes of the offspring obtained by the described breeding scheme (see Results section) were determined by polymerase chain reaction (PCR) on genomic DNA isolated from tail biopsies. DNA was isolated using the phenol-chloroform method. The presence of the 'floxed' allele in the gene-targeted mice was confirmed using the primer pair 5'-TCCAAGTGCGTCTTTAACTCC-3' (P3) and 5'-TCATTGAGTCCTTCCCGAAC-3' (P1) (amplicon sizes, 180 bps ['floxed' allele] and 140 bps [wild-type allele]). The *Cre* transgene was detected in a separate PCR using the primer pair 5'-GCCTGCATTACCGGTCGATGCAACGA-3' and 5'-GTGGCAGATGGCGCGGCAACACCCATT-3' (amplicon size, ~700 bps).

Western blotting

The nasal septum and sternum from *Ggcx*$^{flox/flox}$ and *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice were homogenized in protein lysis buffer containing 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 1 mM PMSF, and 1× protease inhibitor cocktail. Western blot analyses were performed following standard procedure as described previously [67]. Proteins were resolved on 7.5% tris-glycine SDS-PAGE gel and were detected using a rabbit anti-GGCX antibody (Proteintech, 16209-1-AP), anti-Gla antibody previously reported [68], and rabbit anti-GAPDH (Cell Signaling Technologies, 5174). Densitometric analysis was then performed using the Image Lab software.

Conventional radiography and micro-computed tomography (micro-CT)

Radiographic analyses of the long bones were performed at the Centre for Bone and Periodontal Research Core Facility at McGill University with an XPERT x-ray imaging system (Kubtec). Measurements of the tibiae and femurs in these radiographs were done using ImageJ software. Micro-CT scanning of mouse heads was performed at the Shriners Hospital for Children in Montréal with a SkyScan model 1072 instrument set at a resolution of 8.0 μm and 0.5-mm Al filter. Micro-CT image processing and analyses were performed with version 2.2f of the SkyScan software. Cephalometric measurements and analyses of the sutures and cranial base were done using Data Viewer software (SkyScan). Measurements on micro-CT scans of whole heads were done on 5- and 10-week old mice following a previously reported method [69]. The 3D reconstructions of head scans were performed with CtAn and CtVol software (SkyScan).

Histology and tissue imaging

For bone histomorphometry analysis, the tibia and femur were dissected out together, fixed in 10% formalin, dehydrated in graded ethanol series, and embedded in methyl methacrylate (MMA) resin. Von Kossa and van Gieson (VKVG) staining was performed using 7 μm sections to determine the calcified tissue volume over total growth plate volume. To determine the ECM types in the SOS, VKVG and Alcian blue staining were performed on undecalcified and decalcified plastic sections. Histomorphometric analyses were performed using the OsteoMeasure Analysis System (OsteoMetrics). All histological images were captured using a digital camera (DP72, Olympus Canada Inc.), acquired with DPR-BSW software (XV3.0, Olympus Canada Inc.), and processed using Adobe PhotoShop software.

Statistical analysis

Results are reported as mean ± standard deviation (SD). Statistical analyses were performed using Student's *t* test using Microsoft Excel. P-values were tested against a significant level of 0.05. All experiments were repeated at least three times or performed on at least three independent animals.

Study approval

All animal experiments were performed according to Animal Use Protocol 7132, approved by the Animal Care Committee of McGill University.

Results

Generation of the Ggcx$^{flox/flox}$;Col2a1-Cre mice

In order to study the cartilage-specific role of GGCX, particularly in craniofacial development, we ablated *Ggcx* in chondrocytes in mice. To achieve this ablation, we used *Ggcx$^{flox/flox}$* mice previously reported by us. In these mice, the *Ggcx* locus was 'floxed' by introducing two loxP sites – one before Exon 1 and one after Exon 2 (**Fig. 1A**). These *Ggcx$^{flox/flox}$* mice were then mated with *Col2a1-Cre* mice to eventually generate *Ggcx$^{flox/flox}$;Col2a1-Cre* mice.

Genotypes of the mice that we obtained from the above breeding were determined by PCR. Two DNA primers, P1 (5' reverse) and P3 (5' forward), were designed to anneal at the genomic sequences flanking the upstream loxP site. PCR performed using this primer pair results in a 140 bps band for the wild-type allele and 180 bps for the targeted allele. An additional PCR was performed to detect the presence or absence of a 700 bps band representing the *Col2a1-Cre* transgene in the samples (**Fig. 1B**). A deletion PCR was performed to detect the deletion of the 'floxed' region in the *Ggcx$^{flox/flox}$;Col2a1-Cre* mouse model (**Fig. 1B**). A total of six genotypes were obtained: wild-type, *Col2a1-Cre,Ggcx$^{+/flox}$, Ggcx$^{flox/flox}$, Ggcx$^{+/flox}$;Col2a1-Cre, and Ggcx$^{flox/flox}$;Col2a1-Cre* (**Fig. 1B**). This breeding scheme was sufficient to generate viable *Ggcx$^{flox/flox}$;Col2a1-Cre* mice for our study. Unlike the whole-body *Ggcx* ablation model, early lethality was not observed in *Ggcx$^{flox/flox}$;Col2a1-Cre* mice (**Fig. 1C**).

GGCX protein levels and activity are reduced in the cartilaginous tissues in Ggcx$^{flox/flox}$;Col2a1-Cre mice

Western blot analyses were performed on the total proteins extracted from the nasal septum and sternum of *Ggcx$^{flox/flox}$* and *Ggcx$^{flox/flox}$;Col2a1-Cre* mice. We first detected the presence of

GGCX in the blots using an anti-GGCX antibody. Densitometric analyses showed a marked reduction of the protein when normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in both the nasal septum and sternum. However, we observed a partial reduction of the protein content (**Fig. 1D)**. Next, blots were examined for levels of γ-carboxylated (Gla) proteins using anti-Gla antibody. As expected, we observed a marked reduction of different Gla proteins in the nasal septum and sternum extracts on the blots, some of which were confirmed by densitometric analyses (**Fig.1E)**.

GGCX deficiency does not affect the cranial sutures in Ggcx$^{flox/flox}$;Col2a1-Cre mice

The frontonasal, coronal, and lamboidal sutures were examined in 2D micro-CT lateral views of 10-week old control *Ggcx$^{flox/flox}$* and *Ggcx$^{flox/flox}$;Col2a1-Cre* heads. These images did not show abnormal calcification or closure of these cranial sutures (craniosynostosis) (**Fig. 2A**).

GGCX deficiency does not cause nasal septum calcification in Ggcx$^{flox/flox}$;Col2a1-Cre mice

In order to investigate the effects of GGCX deficiency on the nasal septum in our mouse model, 2D micro-CT lateral images of 10-week old *Ggcx$^{flox/flox}$* control and *Ggcx$^{flox/flox}$;Col2a1-Cre* heads were compared to those of *Mgp-/-* mice. The scanned images were examined for areas of ectopic calcification within the cartilaginous nasal septum. *Ggcx$^{flox/flox}$;Col2a1-Cre* nasal septa were virtually identical to that of the controls, as ectopic calcification was not present. Note that the cartilaginous tissues of *Mgp-/-* nasal septa are already heavily calcified in 5-week old *Mgp-/-* mice. *Ggcx$^{flox/flox}$;Col2a1-Cre* nasal septa did not present with abnormal calcification (**Fig. 2B**).

GGCX deficiency causes abnormal calcification of the SOS in Ggcx$^{flox/flox}$;Col2a1-Cre mice

We next examined the effect of GGCX deficiency on the development and anatomy of the SOS, a cartilaginous tissue that is prematurely calcified/ossified in *Mgp-/-* mice [50]. Reconstructed 3D images of the head scans of 5-week old *Ggcx$^{flox/flox}$;Col2a1-Cre* mice showed abnormal deposition of minerals within the SOS, while the control *Ggcx$^{flox/flox}$* SOS did not show this abnormal deposition of minerals. The severity of this phenotype was comparable to that of the *Mgp-/-* SOS at 5-weeks old (**Fig. 2C**). The amount of deposited minerals appeared to increase over time since 10-week old mice showed a more occluded SOS (**Fig. 2C**). Examination of the VKVG undecalcified SOS sections showed an increase in the amount of mineralized tissue in the *Ggcx$^{flox/flox}$;Col2a1-Cre* mice when compared to the control *Ggcx$^{flox/flox}$* SOS (**Fig. 3A**). Comparison of the VKVG and Alcian blue staining of the decalcified SOS sections showed that mineral deposition occurred on a cartilaginous ECM, rather than a type I collagen-rich bone ECM (**Fig. 3B, C**).

No midface hypoplasia in Ggcx$^{flox/flox}$;Col2a1-Cre mice

Two-dimensional lateral, axial, and frontal micro-CT images of heads from 5- and 10-week old mice were first examined visually to assess the effects of *Ggcx* deficiency on the development of the craniofacial skeleton. The axial and lateral views failed to show obvious blunting of the snout or Class III malocclusion, which were observed in the *Mgp-/-* mice [50]. The control mice and the *Ggcx$^{flox/flox}$;Col2a1-Cre* mice showed grossly normal snouts and Class I occlusion.

To confirm our visual findings as described above, detailed cephalometric analyses were performed on the skulls. The antero-posterior cranial measurements – specifically, the maxillary, palatine, and cranial lengths – were comparable in both *Ggcx$^{flox/flox}$* and *Ggcx$^{flox/flox}$;Col2a1-Cre*

mice at both 5- and 10-weeks (**Fig. 4**). These measurements were found to be significantly shorter in the *Mgp-/-* mice [50].

Abnormal shortening of the long bones in Ggcx$^{flox/flox}$;Col2a1-Cre mice

Considering that some endochondral bones in patients lacking GGCX or MGP have been reported to be shortened, the tibiae and femurs of our 10-week old mice were radiographed and measured in order to verify any shortening of these bones. These long bones of the *Ggcx$^{flox/flox}$;Col2a1-Cre* mice at 10-weeks of age measured significantly shorter than those of the control *Ggcx$^{flox/flox}$* mice (**Fig. 5A**). In order to investigate the potential causes for shortening of the long bones that we observe in *Ggcx$^{flox/flox}$;Col2a1-Cre* mice, 7-μm plastic sections of the distal femur and proximal tibia were stained with VKVG. Microscopic analysis of these sections revealed that the presence of ectopic calcifications had disrupted the continuity of the growth plates in both the long bones of 10-week old *Ggcx$^{flox/flox}$;Col2a1-Cre* mice. These abnormal findings were not seen in the control *Ggcx$^{flox/flox}$* mice (**Fig. 5B**). We then measured the calcified tissue area and found an approximately 30-fold increase in the amount of calcified tissue in these growth plates (**Fig. 5C**).

Discussion

GGCX deficiency has been associated with Keutel syndrome-like phenotypes which include midface hypoplasia [55, 61]. While nasal septum calcification has been reported in Keutel syndrome patients [70], this trait is currently unreported in patients with GGCX deficiency. As well, information regarding the premature fusion of the SOS in patients with GGCX is not available. While our newly generated *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice do not fully recapitulate all of the reported human traits, the abnormal cartilage calcification of these mice and *Mgp-/-* mice begs a thorough analyses of all cartilaginous tissues in these patients. The presence of elongated snouts raises some concerns about the use of rodent models to study facial anomalies of humans. However, our work and other published data suggest that analyses of these models may provide valuable information on the pathophysiology of these diseases.

Recently, a patient with a GGCX mutation has been reported with Keutel syndrome-like skeletal anomalies [61]. These anomalies are also common in chondrodysplasia punctata and warfarin embryopathy. Although studies have found that the inactivation of arylsulfatase E (ARSE) leads to chondrodysplasia, the actual substrate and function of this enzyme remains unknown [71]. Previous studies have suggested that mutations in ARSE lead to impaired vitamin K metabolism which subsequently inhibits the carboxylation of Gla proteins including MGP. While warfarin, a commonly used anti-coagulant, may inhibit ARSE in vitro [71], the primary pharmacological target of warfarin in vivo is vitamin K epoxide reductase (VKOR). This effect of warfarin on VKOR would explain why Keutel syndrome-like craniofacial abnormalities are prevalent in babies born to pregnant mothers treated with warfarin (or similar blood thinning agents) and in patients with GGCX mutations [71-74]. While the mutations in Keutel syndrome

and these related disorders are now well-established, the mechanisms of action of these proteins and their linked pathways in the developing skeleton still need to be clarified.

As previously mentioned, γ-carboxylation of MGP is accomplished by GGCX in a vitamin K-dependent manner. Several other proteins, including coagulation factors II, VII, IX, and X, also undergo carboxylation by GGCX [60]. A whole-body knockout of GGCX in mice leads to perinatal lethality due to massive bleeding of vessels [66]. To reduce the potential lethality in whole-body knockout mice, we developed a mutant model for conditional ablation of *Ggcx* in order to study the tissue-specific functions of this enzyme. In the current study, we used *Ggcx*$^{flox/flox}$ mice already reported by us [65]. Chondrocyte-specific ablation of *Ggcx* was achieved by mating these mice with *Col2a1-Cre* mice – a strategy which has been successfully used in many studies [65, 66]. As expected, early lethality that was observed in the whole-body knockouts was not seen in *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice. This new mouse model allowed us to study the phenotypes of various cartilaginous tissues lacking GGCX at different time points.

In *Mgp-/-* mice, the cartilaginous portion of the nasal septum starts calcifying by the end of the first week after birth [50]. Interestingly, we did not observe any calcification of the septal cartilage at any time point in *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice. This absence of calcification is most likely due to partial ablation of *Ggcx,* as confirmed by our Western blot analyses. GGCX levels were reduced by nearly 80% in the nasal septum. Accordingly, in our Western blots performed using an anti-Gla antibody, we observed partial reduction of γ-carboxylation of various Gla proteins present in the septal cartilage. While partial γ-carboxylation of MGP (or other yet unknown inhibitors of calcification) might be sufficient to prevent septal cartilage calcification, Gla residues might not play a significant role in the prevention of calcification in this tissue. Our observation that the replacement of MGP's conserved Glu residues to alanine did not cause

vascular calcification suggests that the Gla residues are dispensable for MGP's anti-mineralization function in the vascular tissues (unpublished data). *Ggcx* ablation in the sternum, a representative cartilaginous tissue, occurred at lower levels than in the nasal septum which can be explained by the presence of multiple cell types and contamination by other tissues.

Although the nasal septum was not calcified, the SOS was abnormally calcified in *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice. However, calcification of the SOS in these mice appears to be less severe in comparison to *Mgp-/-* mice at 5 weeks of age. The complete absence of nasal septum calcification at all time points, as well as milder SOS calcification during the early phases of life, possibly prevents the development of midface hypoplasia in *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice. However, by 10 weeks of age, the SOS is completely fused by abnormal mineral deposits; contrarily, our cephalometric analyses showed an absence of abnormalities involving the maxillary, palatine, and cranial bones in these mice. Note that the SOS of the control *Ggcx*$^{flox/flox}$ mice is still unfused at this age. (*Mgp-/-* mice were not analyzed at 10 weeks, since they do not survive past 8 weeks [75].) Collectively, these findings suggest that SOS calcification has minimal or no significant contribution to midface development. With regard to the multiple mixed cell types and smaller size of the SOS, we did not analyze the ablation levels of GGCX or examine its activity in this tissue. However, the presence of calcific deposits in the SOS and its premature fusion suggest that GGCX activity is essential for the normal development of this synchondrosis. The absence of calcification in the nasal septum, but its presence in the SOS of *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice, provided us a unique opportunity to determine the relative contributions of these two sites in midface development.

In the existing literature, premature closure of synchondroses has been defined as ossification [76, 77]. However, we did not detect any bone-like staining of the ECM (typically

stained red), in the decalcified SOS of 10-week old *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice. Instead, the Alcian blue staining of the ECM found in our specimens signified that the cartilaginous ECM directly undergoes calcification without changing to bone-like ECM. This finding is in agreement with recent reports suggesting that ectopic calcification may not always require ossification [50, 78].

Currently, the primary treatment for midface hypoplasia is surgical osteotomy, which is a surgical procedure involving the advancement of the maxillary, malar, and nasal bones. These procedures involve multiple invasive surgeries before any facial or dental anomalies can be reasonably corrected [34]. Distraction osteogenesis may be indicated for more severe cases. Distraction osteogenesis involves an osteotomy followed by the attachment of an external appliance in order to facilitate gradual separation of bones and new tissue formation [34]. Although these surgeries can be very traumatic for the patient and patient's family and present with a variety of possible postoperative complications, alternative treatment is currently not available. Our study on *Mgp-/-* and *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice has revealed possible mechanisms of cartilage-associated skeletal anomalies in some syndromic patients. A better understanding of the impaired genetic pathways in these patients and their sites of action may lead to a targeted approach of innovative treatments, such as gene or enzyme replacement therapies. These novel therapeutic approaches may circumvent many of the shortcomings associated with these complex surgical procedures.

Our analyses of *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice provide conclusive data on the role of GGCX in the prevention of premature calcification of the SOS and other growth plates – the latter being associated with severe shortening of the long bones. The isolated premature SOS calcification in *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice, without any presence of craniosynostosis or nasal septum

calcification, provided us a unique opportunity to study the role of the SOS in midface development. At present, research has not determined if GGCX activity is required for the prevention of ectopic calcification of the nasal septum. Future work involving selective mutations of the conserved Gla residues in MGP, which are targeted by GGCX, may establish the role of protein γ-carboxylation in the nasal septum. In agreement with available clinical data, our study demonstrates the critical local role of GGCX in chondrocytes for normal development of endochondral bones.

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Figure Legends

Fig. 1. Generation of the *Ggcx$^{flox/flox}$;Col2a1-Cre* mice. (A) Gene ablation strategy. Primers used for genotyping are indicated. (B) Genotyping PCR demonstrating the generation of *Ggcx$^{flox/flox}$;Col2a1-Cre* mice. Genotypes obtained are: i) wild-type, ii) *Col2a1-Cre, iii) Ggcx$^{+/flox}$, iv) Ggcx$^{flox/flox}$, v) Ggcx$^{+/flox}$;Col2a1-Cre, and vi) Ggcx$^{flox/flox}$;Col2a1-Cre*. Gene ablation was confirmed by PCR using the P3-P2 primer pairs. (C) Breeding scheme to generate *Ggcx$^{flox/flox}$;Col2a1-Cre* mice and table depicting the survival of control *Ggcx$^{flox/flox}$* and *Ggcx$^{flox/flox}$;Col2a1-Cre* mice. (D) Western blot analyses showing reduction of GGCX protein levels in the nasal septum of *Ggcx$^{flox/flox}$;Col2a1-Cre* mice, however not an appreciable decrease in the sternum. (E) Western blot analyses showing significant reduction of γ-carboxylation in both the nasal septum and sternum of *Ggcx$^{flox/flox}$;Col2a1-Cre* mice.

Fig. 2. Ectopic calcification of the SOS in GGCX-deficient mice. (A) 2D images of micro-CT scans of *Mgp-/-, Ggcx$^{flox/flox}$* (control), *and Ggcx$^{flox/flox}$;Col2a1-Cre* mice, showing the absence of craniosynostosis in the GGCX-deficient mice (demonstrated by normal frontonasal, coronal, and lamboidal sutures). (B) 3D images of micro-CT scans of *Mgp-/-, Ggcx$^{flox/flox}$* (control), and *Ggcx$^{flox/flox}$;Col2a1-Cre* mice, showing the absence of calcification in the cartilaginous nasal septum of *Ggcx$^{flox/flox}$;Col2a1-Cre* mice, compared to the heavy calcification present in the nasal septum in *Mgp-/-* mice. (C) 3D images of the SOS in *Mgp-/-, Ggcx$^{flox/flox}$* (control), and *Ggcx$^{flox/flox}$;Col2a1-Cre* mice. Ectopic calcification and premature closure of the SOS present in the *Ggcx$^{flox/flox}$;Col2a1-Cre* mice, similar to *Mgp-/-* mice (top panel: 5-weeks old; bottom panel: 10-weeks old).

Fig. 3. Histological analysis of the SOS in *Ggcx$^{flox/flox}$;Col2a1-Cre* mice. (A) Histological analyses of undecalcified SOS sections stained with von Kossa and van Gieson (VKVG; stains minerals in black and unmineralized bone ECM in bright red) show the presence of calcified tissues in the growth plates of the SOS in *Ggcx$^{flox/flox}$;Col2a1-Cre* mice (arrow in A_2), but not in control *Ggcx$^{flox/flox}$* mice. For each of the panels, the magnified views of the areas marked by the dashed lines (A_1-C_1 and A_2-C_2) have been presented on the right. Consecutive SOS sections from both genotypes were decalcified and stained with (B) VKVG (stains decalcified bone ECM in bright red) and (C) Alcian blue (AB; stains cartilage ECM in blue). Light microscopy image shows that the calcified area does not stain red, indicating the absence of bone ECM (arrow in B_2). However, the presence of blue stain in the same area suggests that the calcified area of the SOS growth plate in *Ggcx$^{flox/flox}$;Col2a1-Cre* mice is cartilaginous in nature. These observations demonstrate that cartilage ECM was not converted to bone ECM prior to mineral deposition. Scale bars = 100 μm and 50 μm (magnified).

Fig. 4. The effect of GGCX-deficiency on craniofacial measurements. Results from detailed cephalometric analysis show that the cranial, maxillary, and palatine lengths in *Ggcx$^{flox/flox}$;Col2a1-Cre* mice measure normally at (A) 5-weeks and (B) 10-weeks old, compared to the significantly shorter measurements reported in *Mgp*-/- mice.

Fig. 5. Analysis of the long bones in *Ggcx$^{flox/flox}$;Col2a1-Cre* mice. (A) Radiographs of the tibia and femur in *Ggcx$^{flox/flox}$;Col2a1-Cre* mice at 10-weeks old show that both long bones are significantly shorter, confirmed by quantification analysis (B). (C) VKVG staining showing areas of ectopic calcification throughout the growth plate of the tibia in a 10-week old

Ggcx$^{flox/flox}$*;Col2a1-Cre* mouse. This observation is confirmed by quantification analysis, which illustrates a statistically significant increase in the amount of calcified tissue in the GGCX-deficient growth plate (D). Scale bars = 100 μm.

Supplemental

A. Cephalometric analyses in *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice at two time points. Cranial, maxillary, and mandibular measurements in *Ggcx*$^{flox/flox}$ (control) and *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice at 5-weeks and 10-weeks of age. Subsequent statistical analysis demonstrates no statistically significant difference between the two genotypes.

Figures

Fig. 1

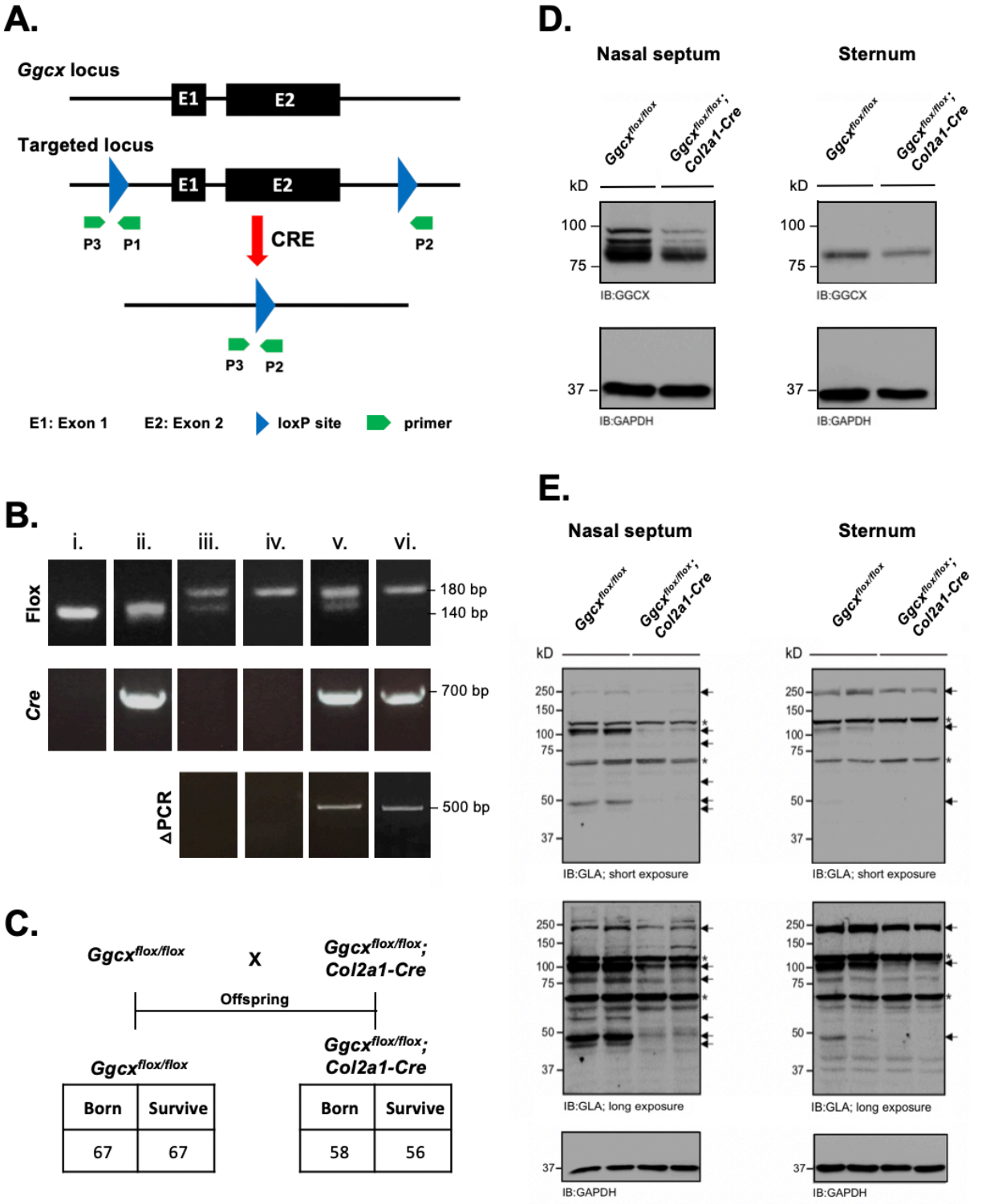


Fig. 2

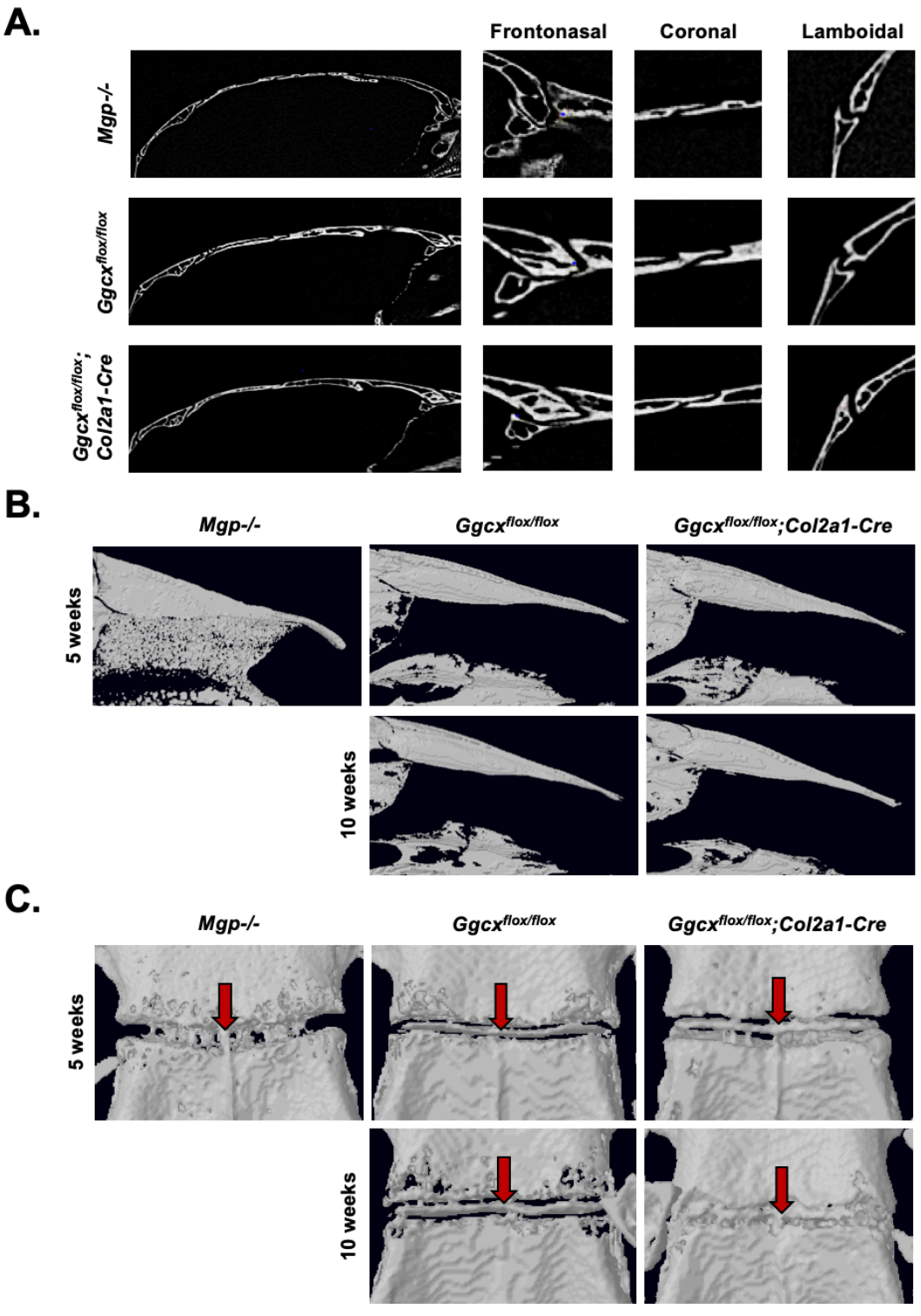

A.
Frontonasal
Coronal
Lamboidal
Mgp-/-
Ggcx^flox/flox
Ggcx^flox/flox; Col2a1-Cre
B.
Mgp-/-
Ggcx^flox/flox
Ggcx^flox/flox;Col2a1-Cre
5 weeks
10 weeks
C.
Mgp-/-
Ggcx^flox/flox
Ggcx^flox/flox;Col2a1-Cre
5 weeks
10 weeks

Fig. 3

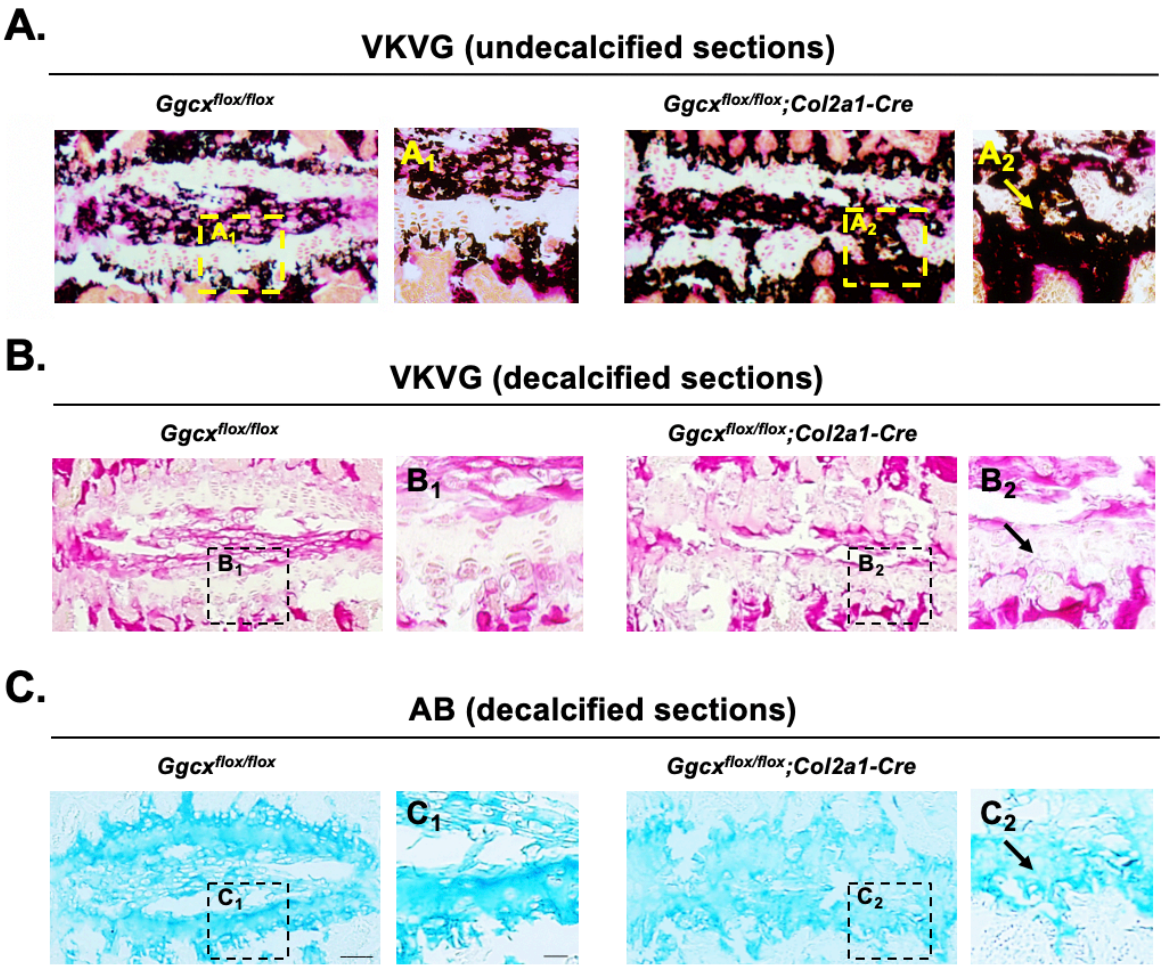

A.
VKVG (undecalcified sections)
Ggcx flox/flox
Ggcx flox/flox;Col2a1-Cre
A1
A2
B.
VKVG (decalcified sections)
Ggcx flox/flox
Ggcx flox/flox;Col2a1-Cre
B1
B2
C.
AB (decalcified sections)
Ggcx flox/flox
Ggcx flox/flox;Col2a1-Cre
C1
C2

Fig. 4

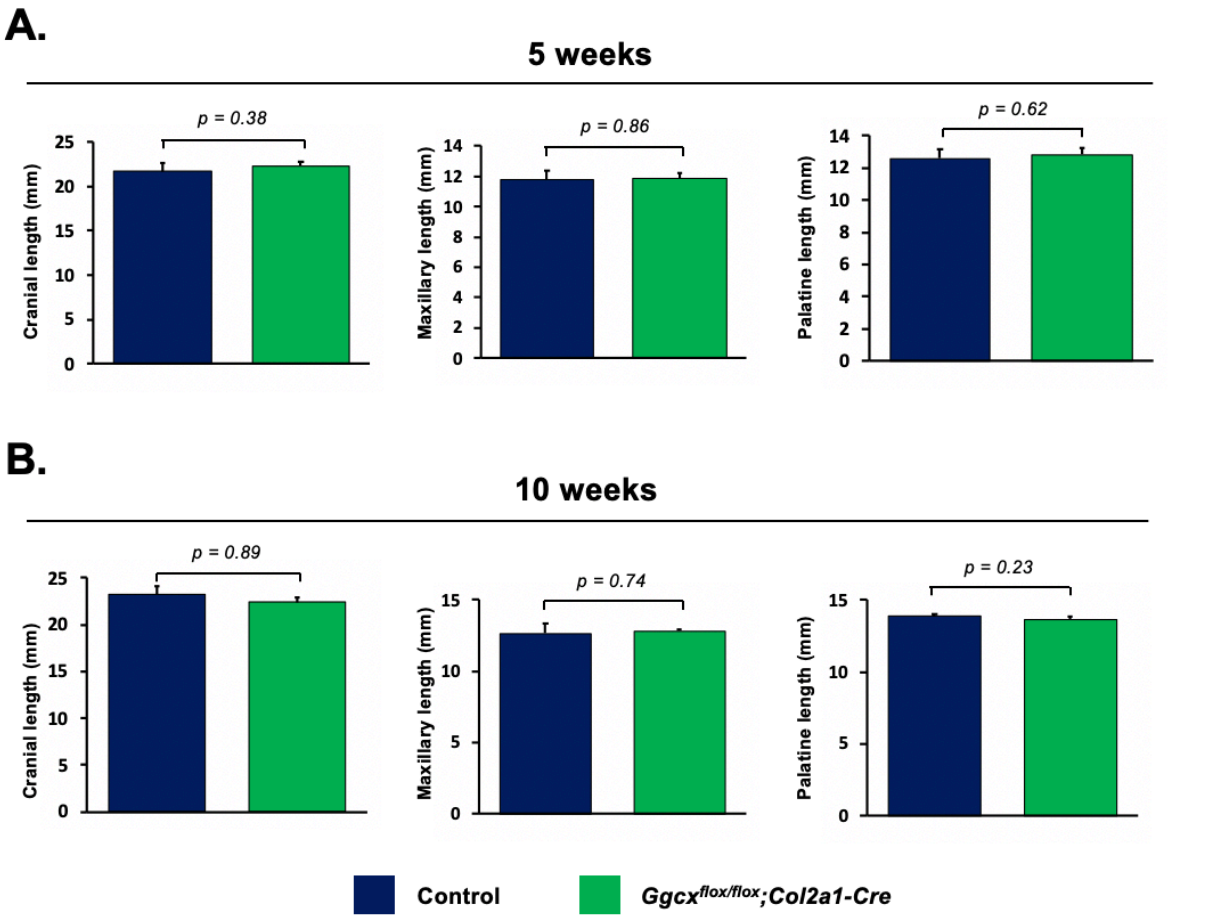
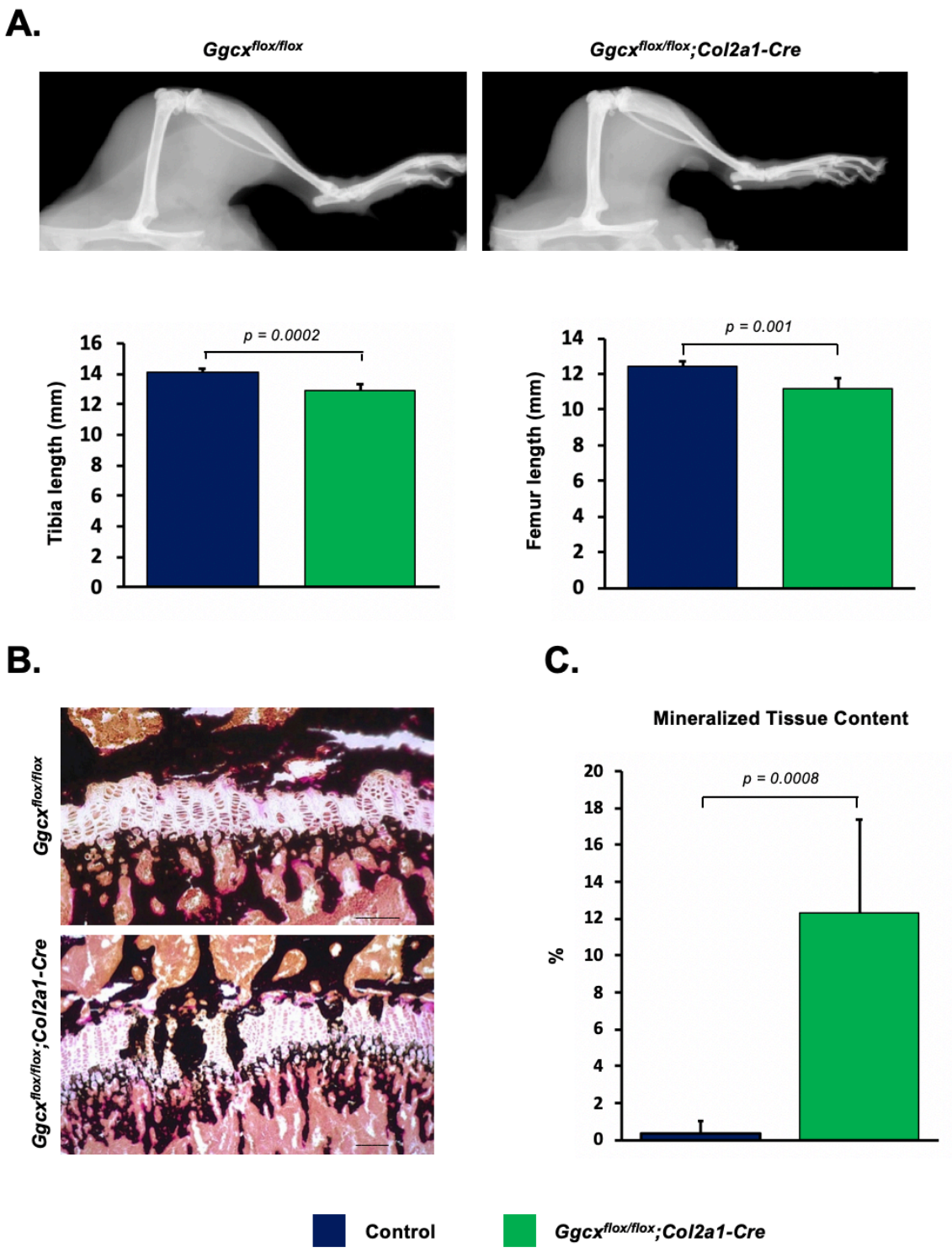

A.
5 weeks
p = 0.38
p = 0.86
p = 0.62
Cranial length (mm)
Maxillary length (mm)
Palatine length (mm)
B.
10 weeks
p = 0.89
p = 0.74
p = 0.23
Cranial length (mm)
Maxillary length (mm)
Palatine length (mm)
Control
Ggcx flox/flox;Col2a1-Cre

Fig. 5


A.
Ggcx^flox/flox
Ggcx^flox/flox;Col2a1-Cre
Tibia length (mm)
p = 0.0002
Femur length (mm)
p = 0.001
B.
Ggcx^flox/flox
Ggcx^flox/flox;Col2a1-Cre
C.
Mineralized Tissue Content
%
p = 0.0008
Control
Ggcx^flox/flox;Col2a1-Cre

A.

Cephalometric analysis – 5 weeks

Measurement (mm)	*Ggcx*$^{flox/flox}$	SD	*Ggcx*$^{flox/flox}$; *Col2a1-Cre*	SD	p
Craniofacial					
Cranial length	21.68	1.04	22.36	0.47	0.38
Skull height	9.28	0.27	9.61	0.08	0.16
Internasal distance	3.43	0.12	3.56	0.04	0.19
Interorbital length	4.12	0.19	4.18	0.15	0.68
Interzygomatic distance	11.87	0.52	12.11	0.17	0.51
Bitemporal distance	8.98	0.15	8.90	0.27	0.72
Maxillary					
Maxillary length	11.76	0.58	11.84	0.37	0.86
Palatine length	12.57	0.61	12.81	0.45	0.62
Anterior cranial height	3.47	0.26	3.50	0.19	0.88
Upper incisor height	2.97	0.13	2.94	0.10	0.75
Intermolar maxillary distance	4.31	0.16	4.42	0.08	0.39
Mandibular					
Effective mandibular length	10.83	0.52	11.05	0.22	0.55
Mandibular plane	10.34	0.49	10.49	0.27	0.68
Mandibular axis	2.97	0.10	3.07	0.07	0.22
Inferior incisor height	4.24	0.29	4.51	0.34	0.36
Ascending ramus height	5.69	0.22	0.10	0.17	0.74
Posterior mandibular distance	4.38	0.10	4.42	0.17	0.74
Intermolar mandibular distance	4.11	0.17	4.27	0.001	0.23

Cephalometric analysis – 10 weeks

Measurement (mm)	*Ggcx*$^{flox/flox}$	SD	*Ggcx*$^{flox/flox}$; *Col2a1-Cre*	SD	p
Craniofacial					
Cranial length	23.16	0.90	23.08	0.09	0.89
Skull height	9.48	0.23	9.52	0.08	0.76
Internasal distance	3.59	0.07	3.54	0.05	0.37
Interorbital length	4.39	0.29	4.26	0.26	0.55
Interzygomatic distance	12.32	0.12	12.40	0.10	0.42
Bitemporal distance	9.82	0.64	9.67	0.27	0.72
Maxillary					
Maxillary length	12.64	0.73	12.80	0.11	0.74
Palatine length	13.85	0.17	13.63	0.19	0.23
Anterior cranial height	3.64	0.44	3.41	0.23	0.50
Upper incisor height	2.82	0.20	3.13	0.18	0.12
Intermolar maxillary distance	4.37	0.09	4.40	0.07	0.66
Mandibular					
Effective mandibular length	11.52	0.27	11.70	0.41	0.57
Mandibular plane	10.96	0.12	10.78	0.14	0.16
Mandibular axis	3.12	0.48	3.05	0.33	0.85
Inferior incisor height	4.59	0.35	4.71	0.27	0.68
Ascending ramus height	5.95	0.23	6.06	0.20	0.60
Posterior mandibular height	4.58	0.14	4.51	0.25	0.73
Intermolar mandibular distance	4.31	0.10	4.21	0.10	0.31

CHAPTER 4: DISCUSSION

Discussion

Keutel syndrome is caused by mutations in MGP, which is post-translationally carboxylated by GGCX. The cartilage anomalies, including midface hypoplasia, associated with Keutel syndrome are also present in humans with GGCX mutations [55, 61]. However, while nasal septum calcification has been reported in Keutel syndrome patients [70], this trait is currently unreported in patients with GGCX deficiency. As well, information regarding the premature fusion of the SOS in patients with GGCX is not available. While our newly generated *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice do not fully recapitulate all of the reported human traits, the abnormal cartilage calcification in these mice and *Mgp-/-* mice begs a thorough analyses of all cartilaginous tissues in these patients. The presence of elongated snouts raises some concerns about the use of rodent models to study facial anomalies of humans. However, our work and other published data suggest that analyses of these models may provide valuable information on the pathophysiology of these diseases.

A patient with a GGCX mutation presenting with Keutel syndrome-like skeletal anomalies has been reported recently [61]; these anomalies are also frequently reported in chondrodysplasia punctata and warfarin embryopathy. Although studies have found that the inactivation of ARSE leads to chondrodysplasia punctata, the actual substrate and function of this enzyme remain unknown [71]. Previous studies have suggested that mutations in ARSE lead to impaired vitamin K metabolism, which subsequently inhibits the carboxylation of MGP and other Gla proteins. While it has been reported that the commonly used anticoagulant warfarin may inhibit ARSE in vitro [71], the primary pharmacological target of warfarin in vivo is VKOR. This effect of warfarin on VKOR would explain why Keutel syndrome-like craniofacial abnormalities are prevalent in babies born to women who were treated with warfarin (or other similar blood thinning agents) and

in patients with GGCX mutations [71-74]. While the mutations in Keutel syndrome and these related disorders are now well-established, the mechanisms of action of these proteins and their connecting pathways in the developing skeleton still need to be clarified.

GGCX γ-carboxylates MGP in a vitamin K-dependent manner. Several other proteins, including coagulation factors II, VII, IX, and X, also undergo carboxylation by GGCX [60]. A whole-body knockout of GGCX in mice results in perinatal lethality due to massive bleeding of vessels [66]. To reduce the potential lethality in whole-body knockout mice, we developed a mutant animal model for conditional ablation of *Ggcx* in order to study the tissue-specific functions of this enzyme. In the current study, we used *Ggcx*$^{flox/flox}$ mice already reported by us [65] and mated them with *Col2a1-Cre* mice, a breeding scheme that has been successfully used in many studies [65, 66]. We did not observe early lethality in *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice, which is present in whole-body knockouts. This new mouse model allowed us to study the phenotypes of various cartilaginous tissues lacking GGCX at different time points.

The cartilaginous portion of the nasal septum starts calcifying by the end of the first week after birth in *Mgp-/-* mice [50]. Interestingly, we did not observe any calcification of the septal cartilage at any time point in *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice. This absence of calcification is most likely due to partial ablation of *Ggcx,* as confirmed by our Western blot analyses. GGCX levels were reduced by nearly 80% in the nasal septum. Accordingly, in our Western blots performed using an anti-Gla antibody, we observed partial reduction of γ-carboxylation of various Gla proteins present in the septal cartilage. While partial γ-carboxylation of MGP (or other yet unknown inhibitors of calcification) might be sufficient to prevent septal cartilage calcification, it is still possible that Gla residues might not play a significant role in the prevention of calcification in this tissue. Our recent observation that the replacement of MGP's conserved Glu residues to

alanine did not result in vascular calcification suggests that the Gla residues are dispensable for MGP's anti-mineralization function in the vascular tissues (unpublished data). *Ggcx* ablation in the sternum, a representative cartilaginous tissue, occurred at lower levels than in the nasal septum, which can be explained by the presence of multiple cell types and contamination by other tissues.

Although we did not see nasal septum calcification in *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice, abnormal calcification of the SOS was present. However, calcification of the SOS in these mice appears to be less severe when compared to *Mgp-/-* mice at 5 weeks of age. The complete absence of nasal septum calcification at all time points, as well as milder SOS calcification during the early phases of life, possibly prevents the development of midface hypoplasia in *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice. By 10 weeks of age, we found the SOS to be completely fused by abnormal mineral deposits; however, our cephalometric analyses showed an absence of abnormalities involving the maxillary, palatine, and cranial bones in these mice. At this time point in control *Ggcx*$^{flox/flox}$ mice, the SOS remained unfused. (*Mgp-/-* mice were not analyzed at 10 weeks, since they do not survive past 8 weeks [75].) Collectively, these findings suggest that SOS calcification has minimal or no significant contribution to midface development. With regard to the multiple mixed cell types and smaller size of the SOS, we did not analyze the ablation levels of GGCX or examine its activity in this tissue. However, the presence of calcific deposits in the SOS and its premature fusion suggest that GGCX activity is essential for the normal development of this synchondrosis. The absence of calcification in the nasal septum, but its presence in the SOS of *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice, provided us a unique opportunity to determine the relative contributions of these two sites in midface development.

In the existing literature, premature closure of synchondroses has been defined as ossification [76, 77]. However, we did not detect any bone-like staining of the ECM (typically

stained red) in the decalcified SOS of 10-week old *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice. Instead, the Alcian blue staining of the ECM found in our specimens signified that the cartilaginous ECM directly undergoes calcification without changing to bone-like ECM. This finding is in agreement with recent reports suggesting that ectopic calcification may not always require ossification [50, 78].

Currently, the primary treatment for midface hypoplasia is surgical osteotomy, which is a surgical procedure involving the advancement of the maxillary, malar, and nasal bones. These procedures involve multiple invasive surgeries before any facial or dental anomalies can be reasonably corrected [34]. Distraction osteogenesis may be indicated for more severe cases. Distraction osteogenesis involves an osteotomy followed by the attachment of an external appliance in order to facilitate gradual separation of bones and new tissue formation [34]. Although these surgeries present a variety of possible postoperative complications, alternative treatment is currently not available. Our study on *Mgp-/-* and *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice has revealed possible mechanisms of cartilage-associated skeletal anomalies in some syndromic patients. A better understanding of the impaired genetic pathways in these patients and their sites of action may lead to a targeted approach of innovative treatments, such as gene or enzyme replacement therapies. These novel therapeutic approaches may circumvent many of the shortcomings associated with these complex surgical procedures.

Weaknesses:

1) Midface hypoplasia has been reported in patients with GGCX mutations. However, our model does not show any such trait. The absence of midface hypoplasia in our specimens prompts one to wonder if the mouse is a good model to study GGCX deficiency. The

absence of midface hypoplasia in our study could be due to a combined effect of the lack of nasal septum calcification and a relatively milder calcification of the SOS in our model. Further experiments (see below) are required to address this issue.

2) A weakness of the Cre-loxP system for conditional gene ablation, unlike conventional gene ablation models, is that complete ablation of a gene is usually not achieved. Because an absence of phenotype was noticed in a tissue (such as absence of nasal septum calcification), we cannot conclude whether the absence of nasal septum calcification is due to a possible non-functional role of GGCX in nasal septum or due to the presence of residual GGCX activity in this tissue after the incomplete conditional gene ablation.
3) In our study, we did not examine the level of *Ggcx* ablation in the SOS. However, we did observe a strong premature calcification of the SOS in our mice. This finding confirms a role for this enzyme in this tissue.
4) Our work did not confirm or rule out MGP as the downstream effector of GGCX to prevent soft tissue calcification. Currently, a reliable antibody is not available to detect γ-carboxylated mouse MGP. However, this issue can be addressed via alternative experimental approaches (see below).

Strengths:

1) While patient data suggest a role for GGCX in the prevention of cartilage calcification, our study is the first to confirm a local role for this enzyme in chondrocytes.
2) Comparing the traits of a patient with a GGCX mutation [61] to that of Keutel syndrome patients with loss-of-function MGP mutations, studies have concluded that these patients manifest overlapping phenotypes. Our work shows that the individual loss of these proteins

results in overlapping traits, including abnormal calcification of the SOS and growth plates and shortening of the limbs. Our comparison of traits of *Ggcx$^{flox/flox}$;Col2a1-Cre* mice to those reported in *Mgp-/-* mice reinforces the link between VKCFD and Keutel syndrome.

3) To our knowledge, *Ggcx$^{flox/flox}$;Col2a1-Cre* mice are the only model that shows an isolated abnormal calcification of the SOS. This model will be a valuable tool to dissect out the effects of this phenotype on midface development.
4) We used an animal model for a known human syndrome, which is a strength of this study.

Significance:

1) Our published analyses of *Mgp-/-* mice and the data presented in this thesis strongly suggest that the nasal septum is a key growth site in midface development. This information will help future site-specific treatment strategies to treat midface hypoplasia in VKCFD, Keutel syndrome, and other related diseases. For example, gene and/or protein replacement therapies can be targeted to the nasal septum to prevent ectopic calcification and other site-specific anomalies present in these pathologies.
2) The findings of our previous and current studies indicate that the GGCX-MGP pathway is central in preventing cartilage calcification in the SOS and other growth plates. Unknown Gla proteins may act as inhibitors of ECM mineralization in these sites. However, considering that other Gla proteins with anti-mineralization activity have not been identified, the possibility that unknown Gla proteins act as inhibitors is unlikely.
3) The absence of nasal septum calcification in *Ggcx$^{flox/flox}$;Col2a1-Cre* mice suggests that other functional features, besides the Gla residues in MGP, are required to prevent nasal septum calcification. MGP carries 3 N-terminal serine residues which have been shown to

prevent in vitro hydroxyapatite crystal growth. Additionally, our unpublished data demonstrate that the Gla residues in MGP may not be required for MGP's anti-mineralization function in the vascular tissues. Different functional residues in MGP might be responsible in preventing ectopic calcification at various sites.

4) Together with the reported findings in VKCFD patients, our study further reinforces the importance for vitamin K as an essential nutritional factor for normal skeletal growth.
5) SOS calcification is somewhat milder in 5 week-old *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice than the calcification observed in *Mgp-/-* mice. Because of this finding, we cannot rule out any potential contribution of the SOS to midface development. However, considering that the SOS is fully calcified by 10 weeks of age in *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice, but absent in control *Ggcx*$^{flox/flox}$ mice, we can conclude that the role of the SOS during midface development must be during the very early growth phase.

CHAPTER 5: CONCLUSION AND FUTURE DIRECTIONS

Conclusion and Future Directions

Our analyses of *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice provide conclusive data on the role of GGCX in the prevention of premature calcification of the SOS and other growth plates – the latter being associated with severe shortening of the long bones. The isolated premature SOS calcification in *Ggcx*$^{flox/flox}$*;Col2a1-Cre* mice, without any presence of craniosynostosis or nasal septum calcification, provided us a unique opportunity to study the role of the SOS in midface development. At present, research has not determined if GGCX activity is required for the prevention of ectopic calcification of the nasal septum. Future work involving selective mutations of the conserved Gla residues in MGP, which are targeted by GGCX, may establish the role of protein γ-carboxylation in the nasal septum. In agreement with available clinical data, our study demonstrates the critical local role of GGCX in chondrocytes for normal development of endochondral bones.

Future directions:

1) Our study examined the effect of GGCX ablation in the cartilaginous tissues of the skeleton in mice. In order to understand whether various syndromes with cartilage calcification are interconnected and are caused by the impairment of a common pathway, future work should be focused on comparative analyses of the traits of these syndromic patients.
2) To determine the definitive roles of protein γ-carboxylation by GGCX and particularly the role of this post-translational modification in MGP in the nasal septum, generation of a new genetic model will be required in the future. In this model, native MGP can be replaced by an altered MGP in which the conserved Gla residues undergoing γ-carboxylation can

be mutated. This experiment will confirm or rule out the role of MGP's Gla residues and, indirectly, the role of GGCX in the prevention of nasal septum calcification.

3) In the event that the loss of MGP's Gla residues does not cause nasal septum calcification in the above future experiment, this finding will suggest the role of other functional residues in MGP. In such a scenario, the role of the conserved serine residues can be investigated in vivo.

4) An important future experiment may involve a diet-based approach to promote P_i-induced calcification in *Ggcx$^{flox/flox}$;Col2a1-Cre* mice. We have shown earlier that reduction of P_i levels in *Mgp-/-* mice prevents nasal septum calcification. Of importance would be a study to determine whether an increased uptake of dietary phosphate in *Ggcx$^{flox/flox}$;Col2a1-Cre* mice can induce nasal septum calcification and also further intensify SOS calcification. The findings from such a study would suggest a role for GGCX activity and vitamin K in the prevention of phosphate-induced calcification.

CHAPTER 6: REFERENCES

References

1. Maldonado, M. and J. Nam, *The role of changes in extracellular matrix of cartilage in the presence of inflammation on the pathology of osteoarthritis*. Biomed Res Int, 2013. **2013**: p. 284873.
2. Marulanda, J. and M. Murshed, *Role of Matrix Gla protein in midface development: Recent advances*. Oral Dis, 2018. **24**(1-2): p. 78-83.
3. Karsenty, G. and E.F. Wagner, *Reaching a genetic and molecular understanding of skeletal development*. Dev Cell, 2002. **2**(4): p. 389-406.
4. Loring, J.F. and C.A. Erickson, *Neural crest cell migratory pathways in the trunk of the chick embryo*. Dev Biol, 1987. **121**(1): p. 220-36.
5. Yuan, Y. and Y. Chai, *Regulatory mechanisms of jaw bone and tooth development*. Curr Top Dev Biol, 2019. **133**: p. 91-118.
6. Berkowitz, S., *Cleft Lip and Palate*. 2013, Berline Heidelberg: Springer-Verlag.
7. Prein, C. and F. Beier, *ECM signaling in cartilage development and endochondral ossification*. Curr Top Dev Biol, 2019. **133**: p. 25-47.
8. Delise, A.M. and R.S. Tuan, *Analysis of N-cadherin function in limb mesenchymal chondrogenesis in vitro*. Dev Dyn, 2002. **225**(2): p. 195-204.
9. Lefebvre, V. and B. de Crombrugghe, *Toward understanding SOX9 function in chondrocyte differentiation*. Matrix Biol, 1998. **16**(9): p. 529-40.
10. Lefebvre, V., *Toward understanding the functions of the two highly related Sox5 and Sox6 genes*. J Bone Miner Metab, 2002. **20**(3): p. 121-30.
11. Lefebvre, V. and P. Smits, *Transcriptional control of chondrocyte fate and differentiation*. Birth Defects Res C Embryo Today, 2005. **75**(3): p. 200-12.
12. Han, L., A.J. Grodzinsky, and C. Ortiz, *Nanomechanics of the Cartilage Extracellular Matrix*. Annu Rev Mater Res, 2011. **41**: p. 133-168.
13. Gerber, H.P., et al., *VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation*. Nat Med, 1999. **5**(6): p. 623-8.
14. Rabie, A.B., et al., *The correlation between neovascularization and bone formation in the condyle during forward mandibular positioning*. Angle Orthod, 2002. **72**(5): p. 431-8.
15. Touaitahuata, H., et al., *The mineral dissolution function of osteoclasts is dispensable for hypertrophic cartilage degradation during long bone development and growth*. Dev Biol, 2014. **393**(1): p. 57-70.
16. Mackie, E.J., L. Tatarczuch, and M. Mirams, *The skeleton: a multi-functional complex organ: the growth plate chondrocyte and endochondral ossification*. J Endocrinol, 2011. **211**(2): p. 109-21.
17. Hossler, F., *Ultrastructure Atlas of Human Tissues*. 2014: John wiley & Sons, Inc.
18. Kiani, C., et al., *Structure and function of aggrecan*. Cell Res, 2002. **12**(1): p. 19-32.
19. Slipka, J. and Z. Tonar, *Outlines of Histology*. 2017, Charles University, Prague: Karolinum Press.
20. Miller, E.J., *Collagen Types: Structure, Distribution, and Functions*, in *Collagen*. 2017, CRC Press.
21. Shen, G., *The role of type X collagen in facilitating and regulating endochondral ossification of articular cartilage*. Orthod Craniofac Res, 2005. **8**(1): p. 11-7.

22. Eyre, D.R., *The collagens of articular cartilage*. Semin Arthritis Rheum, 1991. **21**(3 Suppl 2): p. 2-11.
23. Kovacs, C.S., *Bone development and mineral homeostasis in the fetus and neonate: roles of the calciotropic and phosphotropic hormones*. Physiol Rev, 2014. **94**(4): p. 1143-218.
24. Murshed, M., *Mechanism of Bone Mineralization*. Cold Spring Harb Perspect Med, 2018. **8**(12).
25. Dardenne, O., et al., *Targeted inactivation of the 25-hydroxyvitamin D(3)-1(alpha)-hydroxylase gene (CYP27B1) creates an animal model of pseudovitamin D-deficiency rickets*. Endocrinology, 2001. **142**(7): p. 3135-41.
26. Shoulders, M.D. and R.T. Raines, *Collagen structure and stability*. Annu Rev Biochem, 2009. **78**: p. 929-58.
27. Fleisch, H. and S. Bisaz, *Mechanism of calcification: inhibitory role of pyrophosphate*. Nature, 1962. **195**: p. 911.
28. Murshed, M., et al., *Unique coexpression in osteoblasts of broadly expressed genes accounts for the spatial restriction of ECM mineralization to bone*. Genes Dev, 2005. **19**(9): p. 1093-104.
29. Chai, Y., et al., *Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis*. Development, 2000. **127**(8): p. 1671-9.
30. Richany, S.F., T.H. Bast, and B.J. Anson, *The development of the first branchial arch in man and the fate of Meckel's cartilage*. Q Bull Northwest Univ Med Sch, 1956. **30**(4): p. 331-55.
31. Baek, W.Y., et al., *Osterix is required for cranial neural crest-derived craniofacial bone formation*. Biochem Biophys Res Commun, 2013. **432**(1): p. 188-92.
32. Cox, T.C., *Taking it to the max: the genetic and developmental mechanisms coordinating midfacial morphogenesis and dysmorphology*. Clin Genet, 2004. **65**(3): p. 163-76.
33. Suzuki, A., et al., *Molecular mechanisms of midfacial developmental defects*. 2016. **245**(3): p. 276-93.
34. Tucker, M.R., B.B. Farrell, and R.E. Bauer, *Correction of Dentofacial Deformities*, in *Peterson's Principles of Oral and Maxillofacial Surgery*.
35. Steele, N.P. and J.R. Thomas, *Surgical Anatomy of the Nose*, in *Rhinology and Facial Plastic Surgery*, F.J. Stucker, et al., Editors. 2009, Springer.
36. Coben, S.E., *The spheno-occipital synchondrosis: the missing link between the profession's concept of craniofacial growth and orthodontic treatment*. Am J Orthod Dentofacial Orthop, 1998. **114**(6): p. 709-12; discussion 713-4.
37. Driessen, C., et al., *The effect of early fusion of the spheno-occipital synchondrosis on midface hypoplasia and obstructive sleep apnea in patients with Crouzon syndrome*. J Craniomaxillofac Surg, 2017. **45**(7): p. 1069-1073.
38. Schmidt, M.J., et al., *Comparison of closure times for cranial base synchondroses in mesaticephalic, brachycephalic, and Cavalier King Charles Spaniel dogs*. Vet Radiol Ultrasound, 2013. **54**(5): p. 497-503.
39. Holick, M.F., *Resurrection of vitamin D deficiency and rickets*. J Clin Invest, 2006. **116**(8): p. 2062-72.
40. Sahay, M. and R. Sahay, *Rickets-vitamin D deficiency and dependency*. Indian J Endocrinol Metab, 2012. **16**(2): p. 164-76.
41. Alenazi, B., et al., *X-linked hypophosphatemic rickets (PHEX mutation): A case report and literature review*. Sudan J Paediatr, 2017. **17**(1): p. 61-65.

42. Wuthier, R.E. and G.F. Lipscomb, *Matrix vesicles: structure, composition, formation and function in calcification*. Front Biosci (Landmark Ed), 2011. **16**: p. 2812-902.
43. Li, J., et al., *Smpd3 Expression in both Chondrocytes and Osteoblasts Is Required for Normal Endochondral Bone Development*. Mol Cell Biol, 2016. **36**(17): p. 2282-99.
44. Khavandgar, Z., et al., *A cell-autonomous requirement for neutral sphingomyelinase 2 in bone mineralization*. J Cell Biol, 2011. **194**(2): p. 277-89.
45. Pekkinen, M., et al., *Osteoporosis and skeletal dysplasia caused by pathogenic variants in SGMS2*. JCI Insight, 2019. **4**(7).
46. Abhishek, A. and M. Doherty, *Pathophysiology of articular chondrocalcinosis--role of ANKH*. Nat Rev Rheumatol, 2011. **7**(2): p. 96-104.
47. Greig, A.V., et al., *Correcting the typical Apert face: combining bipartition with monobloc distraction*. Plast Reconstr Surg, 2013. **131**(2): p. 219e-230e.
48. Katzen, J.T. and J.G. McCarthy, *Syndromes involving craniosynostosis and midface hypoplasia*. Otolaryngol Clin North Am, 2000. **33**(6): p. 1257-84, vi.
49. Wexler, A., *Craniofacial Anatomy*, in *Craniofacial Surgery*, S.R. Thaller, J.P. Bradley, and J.I. Garri, Editors. 2007, CRC Press.
50. Marulanda, J., et al., *Matrix Gla protein deficiency impairs nasal septum growth, causing midface hypoplasia*. J Biol Chem, 2017. **292**(27): p. 11400-11412.
51. De Vilder, E.Y., J. Debacker, and O.M. Vanakker, *GGCX-Associated Phenotypes: An Overview in Search of Genotype-Phenotype Correlations*. Int J Mol Sci, 2017. **18**(2).
52. Alrukban, H. and D. Chitayat, *Fetal chondrodysplasia punctata associated with maternal autoimmune diseases: a review*. Appl Clin Genet, 2018. **11**: p. 31-44.
53. Chan, K.Y., E. Gilbert-Barness, and G. Tiller, *Warfarin embryopathy*. Pediatr Pathol Mol Med, 2003. **22**(4): p. 277-83.
54. Keutel, J., G. Jorgensen, and P. Gabriel, *[A new autosomal-recessive hereditary syndrome. Multiple peripheral pulmonary stenosis, brachytelephalangia, inner-ear deafness, ossification or calcification of cartilages]*. Dtsch Med Wochenschr, 1971. **96**(43): p. 1676-81 passim.
55. Cranenburg, E.C., et al., *Circulating matrix gamma-carboxyglutamate protein (MGP) species are refractory to vitamin K treatment in a new case of Keutel syndrome*. J Thromb Haemost, 2011. **9**(6): p. 1225-35.
56. Ziereisen, F., C. De Munter, and N. Perlmutter, *The Keutel syndrome. Report of a case and review of the literature*. Pediatr Radiol, 1993. **23**(4): p. 314-5.
57. Ayyildiz, Z.A., et al., *Echocardiographic assessment in children with Gaucher disease receiving enzyme replacement therapy*. Anadolu Kardiyol Derg, 2012. **12**(2): p. 191-2.
58. Hur, D.J., et al., *A novel MGP mutation in a consanguineous family: review of the clinical and molecular characteristics of Keutel syndrome*. Am J Med Genet A, 2005. **135**(1): p. 36-40.
59. Meier, M., et al., *Tracheobronchial stenosis in Keutel syndrome*. Eur Respir J, 2001. **17**(3): p. 566-9.
60. Oldenburg, J., et al., *The vitamin K cycle*. Vitam Horm, 2008. **78**: p. 35-62.
61. Tie, J.K., et al., *Characterization of vitamin K-dependent carboxylase mutations that cause bleeding and nonbleeding disorders*. Blood, 2016. **127**(15): p. 1847-55.
62. Price, P.A., J.S. Rice, and M.K. Williamson, *Conserved phosphorylation of serines in the Ser-X-Glu/Ser(P) sequences of the vitamin K-dependent matrix Gla protein from shark, lamb, rat, cow, and human*. Protein Sci, 1994. **3**(5): p. 822-30.

63. Murshed, M., et al., *Extracellular matrix mineralization is regulated locally; different roles of two gla-containing proteins*. J Cell Biol, 2004. **165**(5): p. 625-30.
64. Luo, G., et al., *Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein*. Nature, 1997. **386**(6620): p. 78-81.
65. Ferron, M., et al., *GGCX and VKORC1 inhibit osteocalcin endocrine functions*. J Cell Biol, 2015. **208**(6): p. 761-76.
66. Azuma, K., et al., *Liver-specific gamma-glutamyl carboxylase-deficient mice display bleeding diathesis and short life span*. PLoS One, 2014. **9**(2): p. e88643.
67. Mahmood, T. and P.C. Yang, *Western blot: technique, theory, and trouble shooting*. N Am J Med Sci, 2012. **4**(9): p. 429-34.
68. Lacombe, J., et al., *VKOR paralog VKORC1L1 supports vitamin K-dependent protein carboxylation in vivo*. JCI Insight, 2018. **3**(1).
69. Eimar, H., et al., *Craniofacial and Dental Defects in the Col1a1Jrt/+ Mouse Model of Osteogenesis Imperfecta*. J Dent Res, 2016. **95**(7): p. 761-8.
70. Munroe, P.B., et al., *Mutations in the gene encoding the human matrix Gla protein cause Keutel syndrome*. Nat Genet, 1999. **21**(1): p. 142-4.
71. Weaver, K.N., et al., *Keutel syndrome: report of two novel MGP mutations and discussion of clinical overlap with arylsulfatase E deficiency and relapsing polychondritis*. Am J Med Genet A, 2014. **164A**(4): p. 1062-8.
72. Rost, S., et al., *Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2*. Nature, 2004. **427**(6974): p. 537-41.
73. Howe, A.M., et al., *Binder's syndrome due to prenatal vitamin K deficiency: a theory of pathogenesis*. Aust Dent J, 1992. **37**(6): p. 453-60.
74. Watzka, M., et al., *Bleeding and non-bleeding phenotypes in patients with GGCX gene mutations*. Thromb Res, 2014. **134**(4): p. 856-65.
75. Borras, T., M.H. Smith, and L.K. Buie, *A Novel Mgp-Cre Knock-In Mouse Reveals an Anticalcification/Antistiffness Candidate Gene in the Trabecular Meshwork and Peripapillary Scleral Region*. Invest Ophthalmol Vis Sci, 2015. **56**(4): p. 2203-14.
76. Alhazmi, A., et al., *Timing and rate of spheno-occipital synchondrosis closure and its relationship to puberty*. PLoS One, 2017. **12**(8): p. e0183305.
77. Krishan, K. and T. Kanchan, *Evaluation of spheno-occipital synchondrosis: A review of literature and considerations from forensic anthropologic point of view*. J Forensic Dent Sci, 2013. **5**(2): p. 72-6.
78. Chan, E.D., et al., *Calcium deposition with or without bone formation in the lung*. Am J Respir Crit Care Med, 2002. **165**(12): p. 1654-69.

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