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The Role of Claudins in Bone Metabolism

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The Role of Claudins in Bone Metabolism

by

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

AA	Ascorbic acid
ALP	Alkaline phosphatase
βGP	β-glycerophosphate
BMD	Bone mineral density
BMMs	Bone marrow macrophages
BMPs	Bone morphogenetic proteins
BMU	Basic multicellular unit
BSA	Bovine serum albumin
BSP	Bone sialoprotein
CLC-7	Chloride channel-7
Cldn	Claudin
CS	Calf serum
Ctsk	Cathepsin K
ECL	Extracellular loop
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FHHNC	Familial hypomagnesemia with hypercalciuria and nephrocalcinosis
GWAS	Genome wide association studies
IGF-I	Insulin like growth factor-I
KO	Knockout
LRP	Lipoprotein receptor related protein

LV	lumbar vertebrae
MCSF	Macrophage colony stimulating factor
MEM	Minimum essential medium
MOI	Multiplicity of infection
NISCH	Neonatal ichthyosis and sclerosing cholangitis
Oc.S/BS	Osteoclast surface to bone surface
OPG	Osteoprotegerin
Osx	Osterix
PPIA	Peptidylpropyl isomerase A
PTH	Parathyroid hormone
RANKL	Receptor activator of nuclear factor kappa B ligand
ROI	Region of interest
SNPs	Single nucleotide polymorphisms
TGF-β	Transforming growth factor-β
TJ	Tight junction
TNF	Tumor necrosis factor
TRAP	Tartrate resistant-acid phosphatase
WT	Wild type
ZO	Zonula occludens

ABSTRACT OF THE DISSERTATION

The Role of Claudins in Bone Metabolism

by

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Doctor of Philosophy, Graduate Program in Pharmacology
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Osteoporosis, a major public health problem in the U.S., is characterized by low bone mass and structural deterioration of bone tissue. Since the imbalance between bone formation and bone resorption during bone remodeling has been documented to be a major factor in the pathogenesis of osteoporosis, it is crucial to identify novel genes and/or novel functions of known genes that regulate the formation and activity of bone forming osteoblasts and bone resorbing osteoclasts. To this end, recent evidence suggests a significant role for caudins (Cldns), which comprise a major group of tight junction proteins, in bone biology and development. This notion is based on our previous studies which demonstrated that Cldn-18 knockout (KO) mice developed osteopenia phenotype by increasing bone resorption and osteoclast differentiation. Since Cldn-18 is known to be predominately expressed in the stomach, and it was globally disrupted in these earlier studies, in this dissertation we tested the possibility that the osteopenia phenotype in these mice is due to its disruption in the stomach. We found that loss of Cldn-18 reduced gastric acidity and serum calcium in adult mice. However, correction of serum calcium deficit did not rescue the osteopenia phenotype observed in Cldn-18 KO mice, thus suggesting that increased bone resorption is likely to be due to direct effects of lack of Cldn-18 on osteoclasts, and not due to gastric pH changes caused by its loss in the

stomach. Since the Cldn family consists of 27 members, in a separate set of studies, their expression was examined during osteoblast and osteoclast differentiation. We found that several Cldns are expressed in bone, in a complex, cell type and differentiation stage-dependent fashion. Moreover, of those Cldns found to be expressed, a key member, namely Cldn-1, was found to be a positive regulator of osteoblast proliferation and differentiation, and is regulated by several osteoregulatory factors. Collectively, our studies suggest that Cldns play important non canonical functions in regulating bone cell signaling, and may lay down the foundation for the development of Cldn-based agents for diagnosis and therapeutic management of osteoporosis.

CHAPTER ONE

INTRODUCTION

Osteoporosis

Osteoporosis, a major public health problem, is characterized by low bone mass and structural deterioration of bone tissue resulting in increased bone fragility (Dempster, 2011; Raisz, 2005). According to the National Institutes Consensus Development Conference, osteoporosis is described as "a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fracture. Bone strength reflects the integration of two main features: bone density and bone quality. Bone density is expressed as grams of mineral per area or volume and in any given individual is determined by peak bone mass and amount of bone loss. Bone quality refers to architecture, turnover, damage accumulation (e.g., microfractures) and mineralization. A fracture occurs when a failure-inducing force (e.g., trauma) is applied to osteoporotic bone. Thus, osteoporosis is a significant risk factor for fracture, and a distinction between risk factors that affect bone metabolism and risk factors for fracture must be made". Osteoporosis is considered as a "silent disease" because it remains undetected until bone fractures occur (Dempster, 2011; Raisz, 2005). It is well known that osteoporosis related fractures result in both physical and psychological consequences that have a significant impact on patients' quality of life, such as decreased functionality, disability, depression, pain and anxiety (Colon-Emeric & Saag, 2006; Pasco et al., 2005). In addition, fractures are associated with increased mortality and morbidity, depending on the affected site, with hip and spine being the most common sites (Dempster, 2011). For instance, the one year survival post hip fracture is decreased by 10-20%, compared to control population (Abrahamsen, van Staa, Ariely, Olson, & Cooper, 2009; Panula et al., 2011).

In the United States, 10 million individuals have already been diagnosed with osteoporosis, and this number is expected to rise to 14 million by 2020 (http://www.nof.org/ aboutosteoporosis /bonebasics/whybonehealth, 2011). Moreover, around 34 million Americans have low bone mass and are, therefore, at increased risk of osteoporosis or an osteoporotic related fracture (http://www.nof.org/aboutosteoporosis /bonebasics/whybonehealth, 2011). In fact, according to the National Osteoporosis Foundation, it is expected that one in four men, and one out of every two women will experience an osteoporosis related fracture during their life. (http://www.nof.org/ aboutosteoporosis/bonebasics /whybonehealth, 2011). As for the economic burden of osteoporosis, it resides in direct medical cost of osteoporosis related fractures, which have been estimated in the United States at 13.7-20.3 billion dollars in 2005 (Burge et al., 2007). By 2025, the expected annual cost for treatment of osteoporosis related fractures is approximately 25.3 billion dollars (http://www.nof.org/aboutosteoporosis/bonebasics/ whybonehealth, 2011). Collectively, these aforementioned "facts" clearly demonstrate an urgent need for improving the diagnosis, prevention and treatment of osteoporosis.

Etiology and Risk Factors

It is well established that osteoporosis can result from failure to produce optimal bone mass during active growth periods and/ or the imbalance between bone formation and bone resorption during bone remodeling processes (Raisz, 2005). Osteoporosis is a multifactorial disease, with a number of risk factors implicated in its pathogenesis, including genetics, gender, age, and environmental factors such as nutrition, and physical inactivity.

As for genetic factors, they have been found to play an essential role in the regulation of bone mineral density variation, as evident from twins and family studies that suggest a high (50-85%) heritability of bone mineral density (Arden, Baker, Hogg, Baan, & Spector, 1996; Smith, Nance, Kang, Christian, & Johnston, 1973). In addition, inter-individual variance of bone formation markers was found to be attributable to genetic factors in premenopausal twins (Garnero, Arden, Griffiths, Delmas, & Spector, 1996; Harris, Nguyen, Howard, Kelly, & Eisman, 1998). These observations prompted extensive research efforts in an attempt to identify genes responsible for developing osteoporosis, by applying either a candidate gene approach or genome wide research strategy. These studies uncovered several novel single nucleotide polymorphisms (SNPs) that are associated with low bone mineral density and osteoporosis, including genes encoding nuclear receptors (e.g., vitamin D and estrogen receptors), and wnt-β-catenin signaling proteins (e.g., lipoprotein receptor related protein (LRP5) and sclerostin) (Gennari et al., 2005; Morrison et al., 1994; Richards, Zheng, & Spector, 2012; Urano & Inoue, 2014). It is noteworthy that genome wide association studies (GWAS) have identified 62 genome wide significant loci involved in the pathogenesis of osteoporosis, which indicate that it is a complex disease, regulated by multiple genes (Richards et al., 2012).

It is well established that gender plays a role in developing osteoporosis, given that it is more common in women than men (Kling, Clarke, & Sandhu, 2014). In fact, sex steroids play a significant role in regulating bone metabolism, which is evident in menopausal women as estrogen deficiency was found to be associated with increased bone turnover and loss (Raisz, 2005). To this end, the negative effect of estrogen

deficiency on the skeleton was found to be mediated by multiple mechanisms, such as enhanced osteoclast differentiation and function, decreased osteoblast differentiation, as well as increased osteoblast apoptosis (Chow, Tobias, Colston, & Chambers, 1992; Gohel, McCarthy, & Gronowicz, 1999; Syed & Khosla, 2005; Zallone, 2006). Interestingly, sex steroids also serve as important regulators of bone health in males. Estrogen and testosterone were shown to regulate bone mass, and their deficiencies resulted in bone loss in men (Chin & Ima-Nirwana, 2012; Ohlsson, Borjesson, & Vandenput, 2012).

Age is considered an important and irreversible risk factor for osteoporosis. Aside from age dependent decrease in sex hormones, increased bone turnover is considered the major cause of aging-induced bone loss (Seeman, 2003). In this connection, bone loss is known to accelerate as people age due to the reduced bone mass being subjected to the same or perhaps a higher intensity of remodeling; thus the same or larger volume of bone is removed from an already decreased bone mass (Seeman, 2003). In addition, several lines of evidence have demonstrated that bone formation is reduced with aging as a consequence of reduced osteoblasts numberans, life span, impaired differentiation and function, and enhanced adipocyte differentiation (Kassem & Marie, 2011; Khosla, 2013; Stringer et al., 2007). There are also a number of secondary causes of age related osteoporosis, including diseases such as malabsorption, medications such as corticosteroids, and physical inactivity (Khosla, 2013; Lane & Yao, 2010).

Osteoporosis is also determined by environmental factors such as nutrition, inadequate physical activity, alcohol, and smoking. Diet is very important for the development and maintenance of the skeleton. For example, calcium and vitamin D

deficiency can result in decreased bone mass and increased risk of fracture (Koo & Walyat, 2013; Murad et al., 2011). Thus, in order to prevent or reduce the risk of osteoporosis, it is important to eat a healthy balanced diet with adequate calcium and vitamin D. Additionally, some diseases and medications can contribute to vitamin D and calcium deficiency such as malabsorptive disorders (Krupa-Kozak, 2014; Targownik, Bernstein, & Leslie, 2013). As stated before, physical inactivity has a negative impact on bone; for example, prolonged bed rest may result in a significant bone loss (Bergmann et al., 2010; Inoue et al., 2000). However, it was found that loading the immobilized tibia increases periosteal bone formation, and decreases bone loss caused by immobilization (Bergmann et al., 2010; Inman, Warren, Hogan, & Bloomfield, 1999). In addition, several studies have documented that mechanical loading increases bone formation (Kesavan & Mohan, 2010; Kesavan, Mohan, Oberholtzer, Wergedal, & Baylink, 2005). Consequently, physical activity/exercise is employed as a modality to preserve bone mass and decrease risk of fractures (Tveit, Rosengren, Nilsson, & Karlsson, 2014). Taken together, these data suggest that osteoporosis is a complex disease, and is determined by interplay of a host of genetic and environmental factors.

Pharmacological Management of Osteoporosis

The goal of the therapeutic management of osteoporosis is to decrease bone loss and reduce the risk of fracture (Hosoi, 2007; Nakamura, 2008). Since osteoporosis results from an imbalance between bone formation and bone resorption, it is treated by decreasing bone resorption and/or increasing bone formation (Sweet, Sweet, Jeremiah, & Galazka, 2009). There are several Food and Drug Administration (FDA) approved anti-

resorptive drugs such as bisphosphonate, raloxifene, calcitonin, amongst others (Sweet et al., 2009). Importantly though, it is now known that the long term use of the most prescribed anti-resorptive agents is associated with an increased risk of jaw osteonecrosis and atypical fractures (Boyce, 2013). While there are many FDA approved anti-resorptive drugs, there is a shortage of anabolic drugs. Teriparatide, a recombinant form of endogenous human parathyroid hormone (PTH 1-34), is in fact the only FDA approved bone anabolic agent (Brixen, Christensen, Ejersted, & Langdahl, 2004). Therefore, in order to identify/meet the need for new anabolic agents, design safer anti-resorptive drugs, and advance our knowledge of the pathogenesis of osteoporosis, it is important to identify novel genes and/or novel function(s) of known genes as well as (better) understand the related molecular mechanisms regulating osteoblast and osteoclast functions.

The Skeleton

The skeleton makes up about 20% of our body and consists of about 213 bones (Chau, Leong, & Li, 2009; Clarke, 2008). The bones of our skeleton provide mechanical support and motility, protect internal organs, serve as a reservoir for minerals such as calcium and phosphorus, and provide an environment for hematopoiesis within the marrow spaces (Chau et al., 2009; Long, 2012). The bone is a dynamic tissue that continuously undergoes remodeling throughout life (Hadjidakis & Androulakis, 2006). As for bone remodeling, it is a process by which old bone is removed and replaced by new bone, in order to maintain the skeletal integrity, strength, and mineral homeostasis (Hadjidakis & Androulakis, 2006). The remodeling cycle consists of four sequential phases including: activation, resorption, reversal, and formation (Clarke, 2008) (Fig. 1).

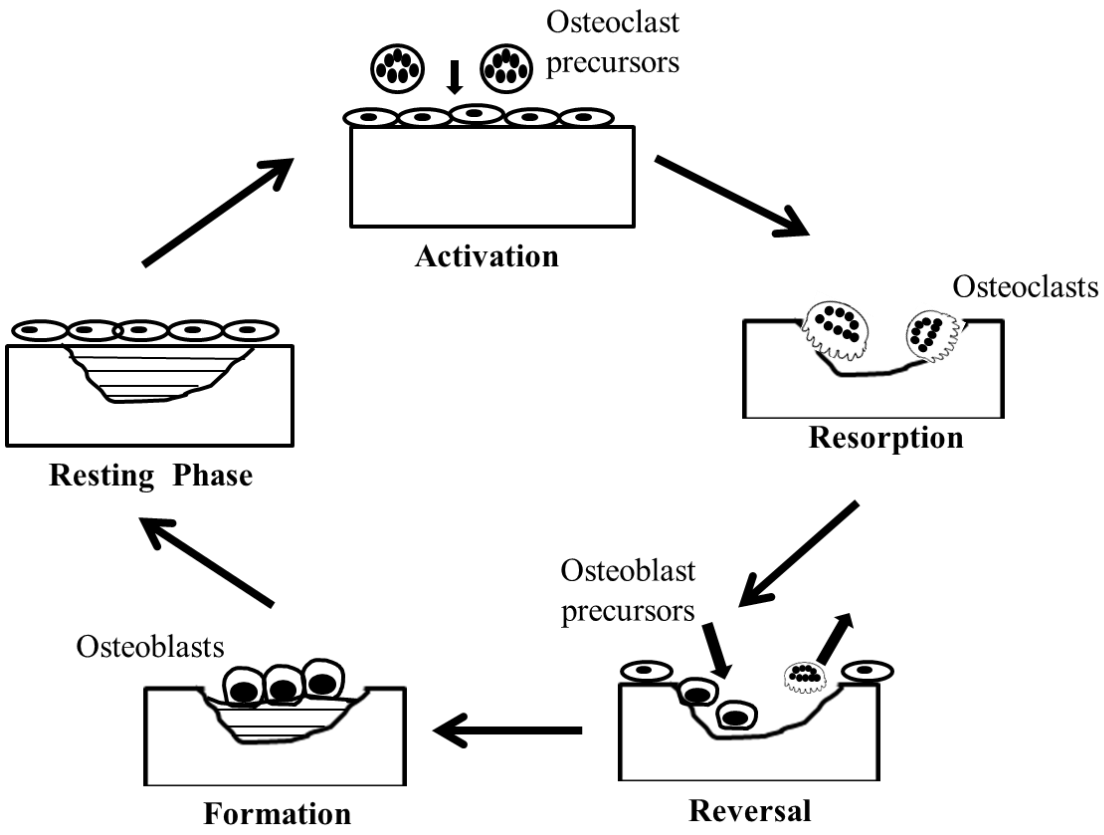

Figure 1. Bone remodeling Cycle. The remodeling cycle consists of four sequential phases including: activation, resorption, reversal, and formation. This process requires a well-coordinated activity of cells of the osteoblast and osteoclast lineages, in what is known as the basic multicellular unit (BMU); which carry out resorption of old bone and formation of new bone, in a sequential manner. Modified from (Persy & D'Haese, 2009)

Therefore, this process requires a well-coordinated activity of cells of the osteoblast and osteoclast lineages, in what is known as the basic multicellular unit (BMU); which carry out resorption of old bone and formation of new bone, in a sequential manner (Hou et al., 2009). To this end, there are three cell types in bone: 1) the bone forming osteoblasts; 2) the mechanosensory osteocytes; and 3) the bone resorbing osteoclasts. These cells are known to communicate with each other, by either direct cell-cell contact and/or indirect signaling molecules with the ultimate goal of maintaining bone homeostasis (Nakahama, 2010). Thus, it is important to understand the molecular mechanisms and signaling pathways which regulate osteoblast, osteocyte, and osteoclast functions.

Osteoblasts

As the chief bone forming cells, osteoblasts produce bone matrix proteins such as type I collagen, alkaline phosphatase (ALP), and osteocalcin (Long, 2012). Osteoblasts are mononuclear cuboidal cells with well-developed endoplasmic reticulum and a large Golgi complex supporting its secretory function (Long, 2012). Osteoblasts have also been found to express numerous genes that are not only essential but also sufficient for the formation and mineralization of bone (Long, 2012). To this end, ALP is highly expressed in osteoblasts, and hence ALP activity and staining is widely used to identify osteoblasts *in vitro* and even *in vivo (Anh, Dimai, Hall, & Farley, 1998)*. Besides their role in bone formation, osteoblasts regulate bone resorption by secreting important factors for osteoclastogenesis, such as the receptor activator of nuclear factor kappa B ligand (RANKL) (Nakahama, 2010; Proff & Romer, 2009).

Osteoblast Differentiation

Osteoblasts originate from mesenchymal stem cells, which can also differentiate into other cell types such as osteoblasts, chondrocytes, adipocytes, fibroblasts, and myoblasts, depending on the expression of lineage specific transcription factors (Chau et al., 2009). The selective expression/function of transcription factors at distinct points during osteoblast differentiation regulates the osteoblastic lineage (Fig. 2). For example, Runx-2 and osterix (Osx) are considered the key transcription factors required for osteoblast differentiation (Jensen, Gopalakrishnan, & Westendorf, 2010; Marie, 2008). Osteoblast differentiation processes are divided into several stages, including commitment to osteoprogenitors, their expansion, and maturation to functional osteoblasts; which in turn will be terminated by becoming osteocytes, bone lining cells, or dying (Fig. 2) (Chau et al., 2009; Jensen et al., 2010). As for the differentiation status of osteoblasts, it is usually evaluated by the expression level of osteogenic marker genes including ALP (early-stage marker), type I collagen, bone sialoprotein (BSP), and osteocalcin (late-stage marker). To this end, osteoblast differentiation and bone formation are regulated by hormones, growth factors, cytokines, and transcription factors.

Hormones Regulating Osteoblastogenesis

Several hormones have been shown to regulate osteoblast differentiation. For instance, estrogen exerts an anabolic effect by promoting osteoblastogenesis and inhibiting osteoblast apoptosis through modulating Runx-2 and wnt signaling (Gohel et al., 1999; Marie, 2008; Qu et al., 1998). The growth hormone also plays a fundamental role in regulating skeletal growth, and this role is primarily mediated by insulin like

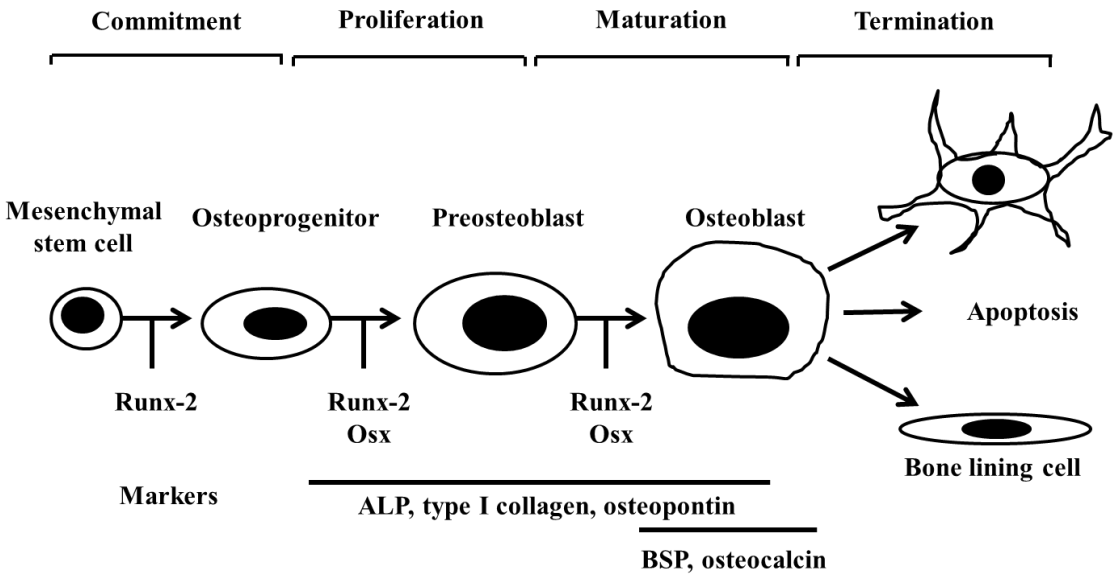

Figure 2. Regulation of osteoblast differentiation. Osteoblasts are derived from mesenchymal stem cell. Runx-2 and osterix (Osx) are considered the key transcription factors required for osteoblast differentiation and bone formation. The differentiation processes are divided into several stages, including commitment to osteoprogenitors, their expansion, and maturation to functional osteoblasts, which in turn will be terminated by becoming osteocytes, bone lining cells, or dying. The differentiation status of osteoblasts is usually evaluated by the expression level of osteogenic marker genes including ALP (early-stage marker), bone sialoprotein (BSP), and osteocalcin (late-stage marker).

growth factor-I (IGF-I) (Mohan & Kesavan, 2012). As for the parathyroid hormone (PTH), it stimulates both bone formation and resorption at physiological conditions (Aslan et al., 2012). The anabolic effects of PTH are mediated by multiple mechanisms, including increasing the expression and activity of Runx-2 and Osx, suppressing the expression of adipogenic transcription factors such as PPARγ in osteoprogenitor cells, and promoting osteoblast survival by activating wnt/β-catenin signaling (Bellido et al., 2003; Greenfield, 2012; B. L. Wang et al., 2006). Vitamin D3 also regulates bone formation by activating and/or suppressing several osteoblast genes (Ramasamy, 2006). For example, it stimulates the production of bone matrix proteins such as ALP and osteopontin, whereas a separate study has shown that it suppresses Runx-2 (Drissi et al., 2002; Prince & Butler, 1987; Ramasamy, 2006). Ascorbic acid (AA) is another key inducer of osteoblastogenesis, and its deficiency results in impaired bone formation and spontaneous fracture (Mohan et al., 2005). Several studies have shown that the addition of AA to cultured osteoblast-like cells stimulates the initial disposition of collagenous extracellular matrix proteins followed by induction of osteoblastogenic markers genes (Harada, Matsumoto, & Ogata, 1991; Mohan et al., 2005; Xing, Pourteymoor, & Mohan, 2011). Thus, it is clear that AA is essential for osteoblast formation and function.

Growth Factors Regulating Osteoblastogenesis

A number of growth factors have been demonstrated to be involved in regulating osteoblast differentiation and bone formation such, as bone morphogenetic proteins (BMPs), transforming growth factor-β (TGF-β), fibroblast growth factors (FGF), IGF-I, and wnts , amongst others.

BMPs, a subfamily of TGF-β superfamily, are one of the major transducers of osteoblast differentiation and bone formation (Chau et al., 2009). Furthermore, previous *in vitro* and *in vivo* studies have shown that the anabolic effects of BMPs on bone are mediated by regulating the expression of Runx-2 and Osx, thereby modulating several aspects of osteoblast differentiation including osteoprogenitor commitment, expansion, and activity (Chau et al., 2009). Several BMPs, such as BMP-2, -4, -5, -6, and -7, are known to have a strong osteogenic capacity (Chen, Deng, & Li, 2012). BMPs have also been found to signal through Smad-dependent and Smad-independent (e.g. MAPK) pathways, with Smad signaling being mediated by the BMP receptor (Chau et al., 2009; Chen et al., 2012). TGF-β, which is abundant in bone matrix, is another important growth factor that is known to promote osteoblast differentiation and activity (Janssens, ten Dijke, Janssens, & Van Hul, 2005). Specifically, TGF-β has been shown to play important roles in the proliferation and expansion of osteoprogenitors, and early stages of osteoblast differentiation. It has been demonstrated that TGF-β regulates Runx-2 through the canonical Smad-dependent and the non-canonical Smad-independent pathways (Chau et al., 2009; Chen et al., 2012). IGF-I, is another major growth factor that is locally produced by bone cells and acts in an autocrine/paracrine manner to regulate osteoblastogenesis (Mohan & Kesavan, 2012). The anabolic effect of IGF-I is mediated, in part, by promoting the survival, proliferation, differentiation, and function of osteoblasts (Tahimic, Wang, & Bikle, 2013). Moreover, it signals through the IGF-I receptor, which is a tyrosine kinase receptor that activates several intracellular signaling pathways, such MAPK and PI-$_3$K-AKT (Chau et al., 2009). The *wnt* family of glycoproteins is an important regulator of osteoblastogenesis, which acts at several stages

of osteoblast differentiation (Bodine & Komm, 2006; Yavropoulou & Yovos, 2007). There are two wnt pathways known to play important roles during the various osteoblast differentiation stages, from commitment to termination, i.e., the canonical β-catenin-dependent and the non-canonical β-catenin-independent pathway (e.g. JNK and PKC) (Kubota, Michigami, & Ozono, 2009; Monroe, McGee-Lawrence, Oursler, & Westendorf, 2012). The canonical wnt/β-catenin signals through frizzled receptor and LRP5/6 co-receptor thereby inactivating GSK-3β and stabilizing β-catenin. This, in turn, results in β-catenin nuclear translocation and binding to LEF/TCF transcription factors and the regulation of the expression of β-catenin target genes (Bodine & Komm, 2006; Monroe et al., 2012). In addition, wnt signaling promotes osteoblastogenesis by induction of Runx-2 and Osx, and suppression of adipogenic transcription factors such as PPARγ (Kubota et al., 2009). The wnt pathway was also found to regulate the expression of genes involved in modulating osteoblast proliferation and apoptosis (Chau et al., 2009).

Transcription Factors Regulating Osteoblastogenesis

Runx-2, a runt domain-containing transcription factor, is essential for controlling osteoblast commitment and differentiation (Komori, 2010). This notion is evident by the finding that targeted deletion of Runx-2 in mice results in complete absence of osteoblasts and mineralized bone (Komori et al., 1997; Otto et al., 1997). Indeed, Runx-2 haploinsufficiency is associated with a human disease known as cleidocranial dysplasia, which is characterized by defective bone formation and ossification of cranial bones (Mundlos et al., 1997). Furthermore, Runx-2 is expressed during all stages of osteoblast differentiation, is necessary for mesenchymal stem cell commitment towards osteoblast

lineages, as well as the control of the expression of major osteoblast specific genes, including type I collagen, osteopontin, BSP, and osteocalcin (Komori, 2010; Marie, 2008). Since the promoter of these aforementioned genes has a Runx-2 regulatory element, Runx-2 can positively or negatively regulate their expression by interacting with several transcriptional activators and repressors and other co-regulatory proteins (Marie, 2008). Osx, a zinc finger transcription factor, is also essential for osteoblastogenesis, given that its deletion in mice resulted in lack of osteoblasts and defective bone formation (Nakashima et al., 2002; Zhang, 2010). It is believed that Osx is downstream of Runx-2, as Osx expression was absent in Runx-2 deficient mice, whereas normal levels of Runx-2 were detected in Osx null mice (Jensen et al., 2010; Nakashima et al., 2002). Besides Runx-2 and Osx, several other transcription factors have been implicated in the regulation of osteoblast differentiation, including AP-1, ATF4, Dlx-3, Dlx-5, Dlx-6, Fra, and Msx2 (Jensen et al., 2010). It is thought that these transcription factors have a facilitating role in osteoblastogenesis, given that no severe phenotype was observed with their genetic manipulation (Long, 2012).

Taken together, these findings clearly demonstrate that the regulation of osteoblastogenesis is complex and mediated by several hormones, growth factors, and transcription factors. Although much progress has been made in this area, our understanding of the molecular pathways that regulate osteoblast differentiation and function is still limited. Thus, the identification of novel genes that participate in regulating osteoblast function(s) is imperative in advancing our understanding of the mechanisms that regulate bone formation.

Osteocytes

Osteocytes are the most abundant cells in the skeleton, and comprise up to 90-95% of bone cells (Knothe Tate, Adamson, Tami, & Bauer, 2004). They are derived from osteoblast lineage cells, in which the terminally differentiated osteoblasts become embedded in the bone matrix (Bonewald, 2011). They express low levels of ALP and high levels of osteocalcin and casein kinase II, which are considered as molecular maker genes (Bonewald, 2011). In terms of morphology, osteocytes have distinct dendritic processes that radiate from their cell body, and connect to each other and different cell types via gap junctions (Schaffler & Kennedy, 2012). Originally, while they were thought to be inert cells embedded in the matrix (Bonewald, 2011; Schaffler & Kennedy, 2012), it has been demonstrated that osteocytes have multiple essential functions. These include: 1) acting as mechanosensory cells; 2) regulating bone formation mainly by secreting sclerostin, an important inhibitor of wnt signaling; 3) modulating bone resorption by secreting osteoclastogenic factors ; 4) regulating bone matrix mineralization by inducing the expression of proteins such as DMP1, PHEX, and MEPE; and 5) participating in regulation of phosphate and calcium metabolism (Schaffler & Kennedy, 2012). Collectively, osteocytes seem to be a local key orchestrator of a host of bone functions.

Osteoclasts

Osteoclasts are giant multinucleated cells that are responsible for the removal of mineralized bone during bone development, homeostasis, and repair (S. K. Lee & Lorenzo, 2006). They have unique and efficient machinery for dissolving crystalline hydroxyapatite and degrading organic bone matrix (Vaananen, Zhao, Mulari, & Halleen,

2000). Resorbing osteoclasts come into contact with bone surface and form Howship`s lacunae by undergoing cytoskeletal reorganization and cellular polarization (Bruzzaniti & Baron, 2006). Consequently, these processes result in the formation of three distinct parts of the plasma membrane: sealing zone, apical membrane (ruffled border), and basolateral membrane (Vaananen et al., 2000). As for the sealing zone, it anchors the resorbing osteoclast to bone matrix and seals the resorption lacunae from its surroundings via integrins (Bruzzaniti & Baron, 2006). A district feature of osteoclasts is their ruffled border, which consists of multiple finger like projection of plasma membrane facing the bone matrix (Vaananen et al., 2000). This border serves as the resorbing organelle by delivering hydrochloric acid to dissolve mineralized components of the bone, and vesicles containing lysosomal enzymes (e.g. cathepsin K and MMP-9) in order to degrade organic bone matrix (Vaananen & Laitala-Leinonen, 2008; Vaananen et al., 2000). As for the acid secretion machinery, it is composed of an electrogenic proton pump (H^+-ATPase), and a chloride channel in the ruffled border, as well as carbonic anhydrase in the cytoplasm,which together, maintain the acidic pH (4-5) of the resorption lacunae (Supanchart & Kornak, 2008). The degraded products are then endocytosed and transported into the basolateral membrane (Mulari, Vaaraniemi, & Vaananen, 2003). It is well known that the basolateral membrane is involved in transcytosis and exocytosis of degraded bone matrix through its functional secretory domain (Mulari et al., 2003; Salo, Lehenkari, Mulari, Metsikko, & Vaananen, 1997). Moreover, it is involved in receiving signals from different cytokines and hormones, and in cell-cell interactions with other bone cells (Vaananen & Laitala-Leinonen, 2008). Of note, tartrate resistant-acid phosphatase (TRAP) is highly expressed in osteoclasts, and therefore TRAP expression

and staining is widely used to identify osteoclasts *in vitro* and *in vivo* (Takeshita, Kaji, & Kudo, 2000*).*

Osteoclast Differentiation

Osteoclast precursors are hematopoietic cells derived from the monocyte/macrophage lineage (Boyle, Simonet, & Lacey, 2003; Bruzzaniti & Baron, 2006). The process of osteoclast formation is divided into several stages, including osteoclastic lineage commitment, proliferation, differentiation, survival and fusion (Fig. 3). Each stage is regulated by one or more cytokines and transcription factors. It is well known that macrophage colony stimulating factor (MCSF) and RANKL are sufficient for inducing osteoclast differentiation and function (Boyce, 2013; S. K. Lee & Lorenzo, 2006). The differentiation of osteoclasts is usually evaluated by measuring the expression level of osteoclastogenic marker genes including early-stage markers (e.g., RANK) and late-stage markers (e.g., TRAP, calcitonin receptor, and cathepsin K) (Boyce, 2013; Boyle et al., 2003). To this end, research efforts have thus far documented that osteoclastogenesis is regulated by several hormones, cytokines, transcription factors, amongst others (Asagiri & Takayanagi, 2007; Boyce, 2013; Yoshida et al., 1990).

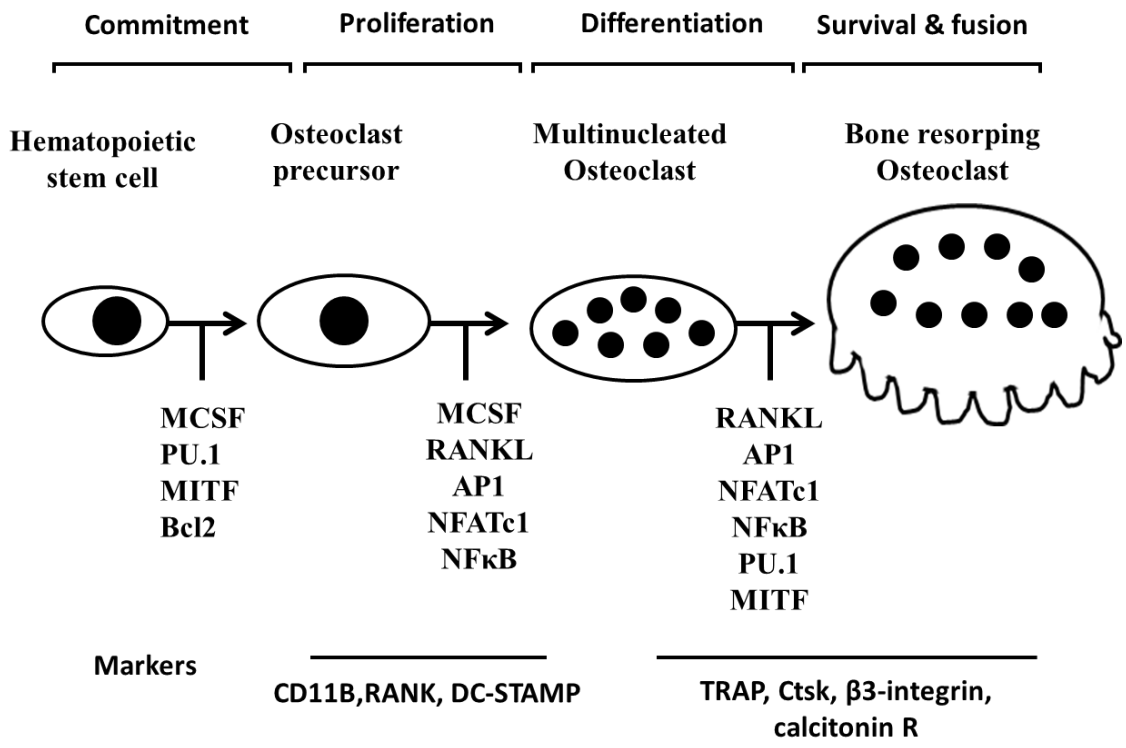

Figure 3. Regulation of osteoclast differentiation. Osteoclasts derive from monocyte/macrophage lineage hematopoietic cells, and their formation is divided into several stages, including osteoclastic lineage commitment, proliferation, differentiation, survival and fusion. Each of these stages is regulated by one or several cytokines and transcription factor. MCSF and RANKL are sufficient for inducing osteoclast differentiation and function. The differentiation of osteoclasts is evaluated by measuring the expression level of marker genes including early-stage markers (e.g., RANK) and late-stage markers (e.g., tartrate resistant acid phosphatase (TRAP) and cathepsin K (Ctsk).

Hormones Regulating Osteoclastogenesis

It is well documented that estrogen is a negative regulator of bone resorption (Zallone, 2006). The negative effects of estrogen on osteoclastogenesis are complex and is mediated by several mechanisms, including indirectly modulating the production of RANKL and osteoprotegerin (OPG; a decoy receptor for RANKL) by osteoblast lineage cells, directly modulating osteoclast activity and apoptosis, as well as reducing the production of osteoclastogenic cytokines such as TNF-α and IL-1 (Kameda et al., 1997; Lorenzo et al., 1998; Zallone, 2006). Vitamin D3 is also considered an inducer of bone resorption at supraphysiological doses (Ramasamy, 2006; Takahashi, Udagawa, & Suda, 2014). It has been demonstrated that the positive effect of vitamin D3 on osteoclastogenesis is indirect and mediated by upregulation of RANKL in osteoblast lineage cells (Ramasamy, 2006). In addition, vitamin D3 has been shown to downregulate the expression of OPG (Ramasamy, 2006). In contrast, several studies have shown that vitamin D3 inhibits bone resorption, and this suppressive effect could be mediated by its direct action on osteoclasts (Sakai et al., 2009; Takahashi et al., 2014; Takasu et al., 2006). Thus, the effect of vitamin D3 on osteoclastogenesis appears to be complex and dose dependent. PTH is another calcitropic hormone that enhances osteoclast formation and function, when it is administered continuously (Ramasamy, 2006). It positively regulates osteoclastogensis by increasing the RANKL/OPG ratio produced by osteoblasts (Ramasamy, 2006). On the other hand, calcitonin was found to be a potent inhibitor of osteoclastic bone resorption, whose function is to maintain normal calcium homeostasis (Naot & Cornish, 2008).

Cytokines Regulating Osteoclastogenesis

It is well known that MCSF and RANKL are sufficient for inducing osteoclast differentiation and function (Boyce, 2013; S. K. Lee & Lorenzo, 2006). MCSF is an essential osteoclastogenic cytokine secreted by osteoblasts (S. K. Lee & Lorenzo, 2006). It was found that MCSF was deficient in osteopetrotic mice that are lacking osteoclasts, and this osteopetrotic phenotype was rescued by MCSF injection or its selective expression in osteoblasts (Abboud, Woodruff, Liu, Shen, & Ghosh-Choudhury, 2002; Yoshida et al., 1990). Upon binding to its receptor (c-FMS) that is expressed on osteoclast precursors, MCSF upregulates the expression of RANK, and stimulates the proliferation and survival of these cells (Ross & Teitelbaum, 2005). As for RANKL, it is a member of the tumor necrosis factor (TNF) superfamily, and is a key cytokine for osteoclast differentiation and activity (S. K. Lee & Lorenzo, 2006). Targeted deletion of RANKL in mice results in osteopetrotic phenotype (characterized by high bone mass density) due to lack of osteoclasts (Kong et al., 1999). Moreover, RANKL is produced by marrow stromal and osteoblastic cells, and binds to its cognate receptor (RANK) that is expressed on osteoclasts, which in turn activates downstream signaling that is involved in osteoclastoegenesis. In contrast, OPG is a decoy receptor for RANKL, and is secreted by marrow and osteoblastic cells to negatively regulate osteoclast differentiation (Boyle et al., 2003; Lacey et al., 1998). The physiological importance of OPG was demonstrated by severe osteoporosis developed in OPG deletion mice due to increased osteoclast differentiation (Bucay et al., 1998). Therefore, the ratio of RANKL/OPG produced by marrow and osteoblastic cells has either a positive or negative impact on osteoclast differentiation and bone resorption. In addition to the MCSF/RANKL/OPG axis, several

other cytokines have been implicated in regulating osteoclast differentiation, such as TNF-α, IL-1, and IL-6 (Azuma, Kaji, Katogi, Takeshita, & Kudo, 2000; Jimi et al., 1999; S. K. Lee & Lorenzo, 2006; T. Tamura et al., 1993). These cytokines stimulate osteoclastogenesis by either exerting direct effects on osteoclasts, or indirect effects by modulating the MCSF/RANKL/OPG axis (S. K. Lee & Lorenzo, 2006).

Transcription Factors Regulating Osteoclastogenesis

As can been seen in Fig. 3, there are multiple transcription factors involved in the regulation of different stages of osteoclast formation. The commitment, proliferation, and survival of osteoclast precursors are mainly regulated by the PU.1 and MITF transcription factors. Their physiological significance was confirmed by genetic studies in which osteopetrosis was observed in mice lacking PU.1 or MITF genes (Tondravi et al., 1997; Weilbaecher et al., 2001). Additionally, PU.1 and MITF has been shown to be linked to MCSF signaling (Mellis, Itzstein, Helfrich, & Crockett, 2011). Thus, PU.1 upregulates the expression of the MCSF receptor (c-FMS), whereas MITF is activated by MCSF signaling and promotes macrophage survival by inducing the expression of an anti-apoptotic protein (Bcl-2) (DeKoter, Walsh, & Singh, 1998; McGill et al., 2002; Mellis et al., 2011). Moreover, RANK expression was found to be upregulated by the coordinated activity of PU.1 and MITF, during osteoclast differentiation (Ishii et al., 2008; Mellis et al., 2011). Activation of RANKL signaling pathways result in induction of important osteoclastogenic transcription factors, such as NFAT-c1, NFκB, and c-Fos. NFAT-c1 is the most strongly induced transcription factor by RANKL signaling, and is in fact considered as a master regulator of osteoclast differentiation (Takayanagi et al.,

2002). NFATc1-deficient embryonic stem cells fail to differentiate into osteoclasts in response to RANKL stimulation, and conditional deletion of NFAT-c1 in osteoclasts results in osteopetrosis (Aliprantis et al., 2008; Takayanagi et al., 2002). Interestingly, once NFAT-c1 is expressed in osteoclast precursors, it promotes osteoclastogenesis, even in the absence of RANKL (Takayanagi et al., 2002). Also, NFAT-c1 regulates the expression of osteoclastogenic genes such as TRAP, integrin, calcitonin receptor, and cathepsin-k (Crotti et al., 2006; Takayanagi et al., 2002). NFκB and AP-1 are transcription factors activated by RANKL signaling, are important for osteoclastogenesis, and regulate common target genes critical for osteoclastogenesis (Asagiri & Takayanagi, 2007). In this connection, targeted deletion of the c-Fos component of AP-1 in mice resulted in osteopetrosis due to defective osteoclast differentiation (Z. Q. Wang et al., 1992). Likewise, the osteopetrosis phenotype was observed in the p50/p52 double knockout (KO) mice as a consequence of osteoclast absence. However, this phenotype can be rescued by c-Fos or NFAT-c1, suggesting that they are downstream of NFκB (Iotsova et al., 1997; Yamashita et al., 2007). In summary, the regulation of osteoclast differentiation is complex and is mediated by sequential expression of several transcription factors.

Collectively, these findings clearly demonstrate that an combination of hormones, cytokines, and transcription factors act (in concert) directly and/or indirectly to regulate osteoclastogenesis. Despite this progress, the detailed molecular pathways that regulate osteoclast differentiation and function are still poorly understood. Thus, the identification of novel genes that participate in regulating osteoclast function(s) is imperative in advancing our understanding of the mechanisms that regulate bone resorption.

Tight Junctions

Cell-to-cell interaction is important in the development and maintenance of various biological tissues (Gunzel & Yu, 2013). The cell junctional complex consists of four types of proteins including gap junctions (e.g. connexins), hemidesmosomes (e.g. integrins), adherens (e.g. cadherins), and tight junctions (Elkouby-Naor & Ben-Yosef, 2010). In the skeleton, it has been shown that bone cells form a variety of intracellular junctions (Cheng, Shin, Towler, & Civitelli, 2000; Lecanda et al., 2000), and there is substantial evidence demonstrating the importance of gap junctions in skeletal development; for example, connexin 43 deficient mice exhibited reduced bone mineral density (BMD) and skeletal abnormalities (Lecanda et al., 2000). Regarding tight junctions, several investigators have documented the ability of bone cells (osteoblasts and osteocytes) to form tight junctional structures (Prele, Horton, Caterina, & Stenbeck, 2003; Soares, Arana-Chavez, Reid, & Katchburian, 1992; Weinger & Holtrop, 1974). Tight junctional strands are composed of several types of transmembrane proteins including occludin, junctional adhesion molecule, tricellulin, and caudins (Cldns) (Elkouby-Naor & Ben-Yosef, 2010). It was previously reported that occludin is expressed in osteoblasts and its targeted disruption in mice resulted in cortical bone thinning and postnatal growth retardation (Saitou et al., 2000; Wongdee et al., 2008). Of the various tight junction proteins, extensive evidence suggests that Cldns are the primary proteins responsible for the formation of tight junctional strands, and there is also evidence showing that they participate in intracellular signaling that controls cell proliferation and differentiation in a variety of cell types (Elkouby-Naor & Ben-Yosef, 2010; Gunzel & Yu, 2013; Matter, Aijaz, Tsapara, & Balda, 2005; Saitou et al., 1998). Thus, the focus of these dissertation studies is going to be on Cldns.

Claudin Structure

The Cldn family of protein molecules consists of 27 members that have been identified in mouse and human cells, with a molecular weight ranging from 20-34 kDa (Angelow, Ahlstrom, & Yu, 2008). In terms of structure, Cldns are composed of four transmembrane domains, two extracellular loops, a short intracellular loop, and cytosolic amino and carboxy termini (Chiba, Osanai, Murata, Kojima, & Sawada, 2008) (Fig. 4). While the amino acid sequence of the first and fourth transmembrane domains is highly conserved among the different Cldns, that of the second and third domains is not (Morita, Furuse, Fujimoto, & Tsukita, 1999). Structure-function studies revealed that Cldns' first extracellular loop contains several charged amino acids, which have been found to play an important role in determining their paracellular barrier charge selectivity (Colegio, Van Itallie, McCrea, Rahner, & Anderson, 2002; Elkouby-Naor & Ben-Yosef, 2010). In addition, the first extracellular loop has two signature sequences including a G-L-W motif and two highly conserved cysteine, which are thought to confer an important function in stabilizing the protein structure (Angelow et al., 2008; Gunzel & Yu, 2013; Gupta & Ryan, 2010). The second extracellular loop is folded in a helix-turn-helix motif that is involved in Cldn-Cldn interactions, and has been found- in some Cldns- to serve as a receptor for the colistridium perfringens enterotoxin (Findley & Koval, 2009; K. Fujita et al., 2000; Morin, 2005; Piontek et al., 2008). Regarding the carboxy terminus, it has been shown to possess the highest structural diversity among the Cldn family of proteins (Krause et al., 2008). It also has a PDZ-binding motif that that enables Cldns to interact with PDZ domain containing proteins such as zonula occludens (ZO)-1/2/3, MUPP1, PATJ, , amongst others (Angelow et al., 2008; Krause et al., 2008). The interactions

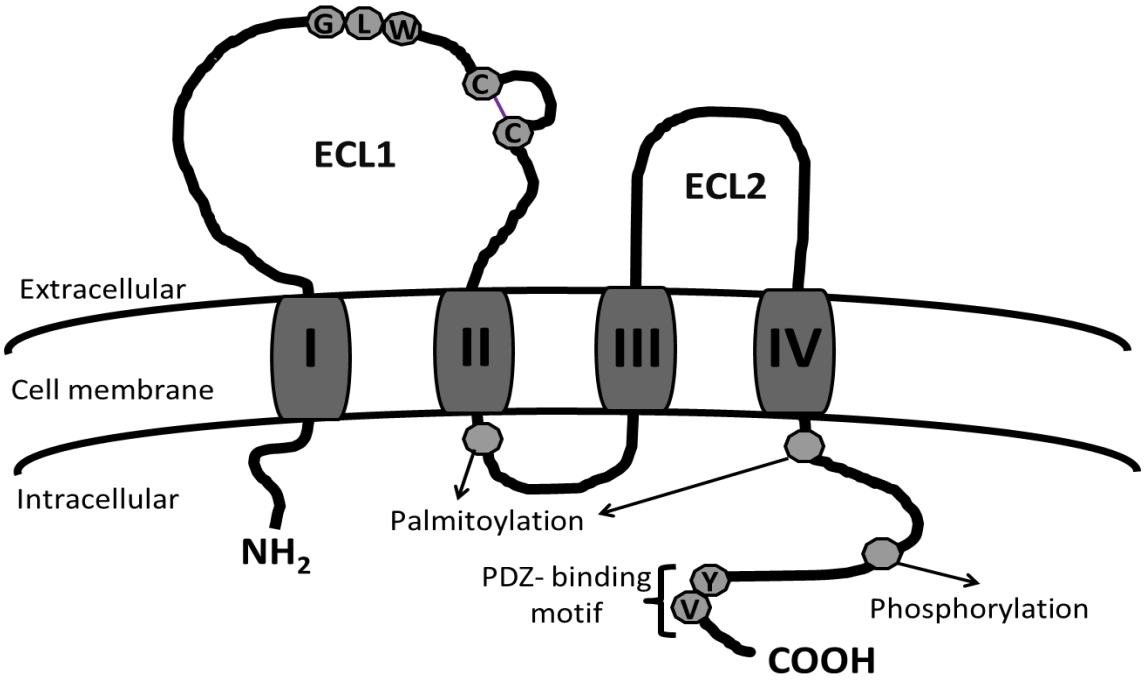

Figure 4. Schematic representation of Cldns structure. Cldns are composed of four transmembrane domains (I, II, III, and IV), two extracellular loops (ECL1 and ECL2), a short intracellular loop, and cytosolic amino and carboxy termini.

between Cldns and cytoplasmic scaffolding proteins such as ZO are predicted to be essential for linking the Cldn family of proteins to the cytoskeleton and/or for their participation in intracellular signaling (Balda & Matter, 2009; Gunzel & Yu, 2013). Additionally, the carboxy terminus has phosphorylation sites for Ser/Thr, and Tyr Kinases, which serve regulatory roles by modulating the Cldn's localization and function (Angelow et al., 2008).

Recently, several phylogenetic trees and classifications are suggested based on the structure in various species, which is comprehensively described by Gunzel et al., (Gunzel & Fromm, 2012). Based on sequence analysis and functional properties of the mouse variants, Cldns are classified into two major groups: 1. The "classic" Cldns, which encompass Cldn 1-10, 14, 15, 17, and 19, based on their close similarities; and 2. The "non-classic" Cldns, which encompass the remaining Cldns including Cldn-12, -13, -16, -18, -20, -22, and -23, as they seem to be less similar/related (Krause et al., 2008).

Expression and Distribution Profile

Cldns exhibit diverse tissue/cell type-specific patterns of expression (Table 1). Some tissues/cells such as the epidermis express several Cldns (Brandner et al., 2002), whereas others, such as Sertoli cells, express one or two Cldns (C. M. Van Itallie & Anderson, 2006). Interestingly, Cldn expression also varies within the same tissue. For example, in mouse kidney, at least 15 Cldns are expressed with distinct expression patterns in every segment of the nephron (Elkouby-Naor & Ben-Yosef, 2010). Thus, while the proximal tubules express Cldn-1, -2, -10, -11, -12, and -14, the distal tubules express Cldn-3, -7, -8, -10, and -11 (Elkouby-Naor & Ben-Yosef, 2010).

Table 1. Cldns tissue distribution and functions.

Cldn	Tissue expression	Canonical function	Non-canonical function
1	**Ubiquitous: Most epithelial tissues and vascular endothelial** (Gunzel & Yu, 2013)	**Barrier forming- cation selective** (Inai, Kobayashi, & Shibata, 1999)	**Cell proliferation** (H. Fujita et al., 2011; Pope et al., 2013) **Cell differentiation** (Hoshino et al., 2008) **Cell motility and migration (during development and in cancer cells)** (Fishwick, Neiderer, Jhingory, Bronner, & Taneyhill, 2012; Fortier, Asselin, & Cadrin, 2013; Yoon et al., 2010) **Cell signaling and regulating gene expression** (Singh et al., 2011; Suh et al., 2013) **Anti-apoptosis** (J. W. Lee et al., 2010; Y. Liu et al., 2012) **Hepatitis C virus and dengue virus entry cofactors** (Che, Tang, & Li, 2013; Meertens et al., 2008)
2	**Typical for epithelial tissues** (Gunzel & Yu, 2013; Krause et al., 2008)	**Pore forming - cation selective** (Amasheh et al., 2002; Yu et al., 2009)	**Cell proliferation** (Dhawan et al., 2011; Wada, Tamura, Takahashi, & Tsukita, 2012) **Cell signaling** (Nishida, Yoshida, Nishiumi, Furuse, & Azuma, 2013)
3	**Epithelial tissues and vascular endothelium** (Gunzel & Yu, 2013)	**Barrier forming- cation** selective (Milatz et al., 2010)	**Cell proliferation** (Okugawa et al., 2012) **Cell motility and migration (cancer cells)** (Agarwal, D'Souza, & Morin, 2005) **Gene expression** (Shang, Lin, Manorek, & Howell, 2013) **Apoptosis** (Sun et al., 2011) **Angiogenesis** (Sun et al., 2011) **Receptor for Clostridium perfringens enterotoxin** (Veshnyakova et al., 2012)
4	**Epithelial tissues** (Gunzel & Yu, 2013)	**Predominantly Barrier forming- cation selective** (C. Van Itallie, Rahner, & Anderson, 2001) **Also act as Cl- pore** (Hou, Renigunta, Yang, & Waldegger, 2010)	**Cell motility and migration (normal and cancer cells)** (Agarwal et al., 2005; Webb, Spillman, & Baumgartner, 2013) **Gene expression** (Shang et al., 2013) **Cell signaling** (Kawai et al., 2011) **Angiogenesis** (Li et al., 2009) **Receptor for Clostridium perfringens enterotoxin** (Veshnyakova et al., 2012)
5	**Typical for vascular endothelium, such as blood brain barrier** (Nitta et al., 2003) **Some epithelial tissues** (Rahner, Mitic, & Anderson, 2001)	**Barrier forming- cation selective** (Fontijn, Rohlena, van Marle, Pannekoek, & Horrevoets, 2006; Wen, Watry, Marcondes, & Fox, 2004)	**Cell motility and migration (cancer cells)** (Escudero-Esparza, Jiang, & Martin, 2012a, 2012b)

6	**Embryonic epithelium** (Turksen & Troy, 2001) **Kidney** (neonates) (Abuazza et al., 2006) Liver (A. Zheng et al., 2007)	**Barrier forming- cation selective** (Sas, Hu, Moe, & Baum, 2008)	**Cell proliferation** (Y. F. Liu et al., 2010; Zavala-Zendejas et al., 2011) **Cell differentiation** (Arabzadeh, Troy, & Turksen, 2006; Hong et al., 2005) **Cell motility and migration (Cancer cells)** (Y. F. Liu et al., 2010; Zavala-Zendejas et al., 2011) **Apoptosis** (Guo et al., 2012) **Co-receptor for hepatitis C virus** (Meertens et al., 2008; A. Zheng et al., 2007)
7	**Epithelial tissues** (Gunzel & Yu, 2013)	**Barrier and pore forming** (Alexandre, Jeansonne, Renegar, Tatum, & Chen, 2007; Alexandre, Lu, & Chen, 2005; Hou, Gomes, Paul, & Goodenough, 2006)	**Cell proliferation** (Thuma & Zoller, 2013; Zavala-Zendejas et al., 2011) **Cell motility and migration (cancer cells)** (Thuma & Zoller, 2013; Zavala-Zendejas et al., 2011) **Gene expression** (J. Y. Zheng et al., 2003) **Apoptosis** (Hoggard et al., 2013; Thuma & Zoller, 2013)
8	**Epithelial tissues** (Gunzel & Yu, 2013)	**Predominantly Barrier forming- cation selective** (Angelow, Schneeberger, & Yu, 2007; Yu, Enck, Lencer, & Schneeberger, 2003)	**Receptor for Clostridium perfringens enterotoxin** (Shrestha & McClane, 2013)
9	**Kidney (neonates)** (Abuazza et al., 2006) **Inner ear** (Kitajiri et al., 2004) **Liver** (A. Zheng et al., 2007)	**Barrier forming- cation** selective (Sas et al., 2008)	**Cell proliferation** (Zavala-Zendejas et al., 2011) **Cell motility (cancer cells)** (Zavala-Zendejas et al., 2011) **Co-receptor for hepatitis C virus** (Meertens et al., 2008)
10 a	**Epithelial tissues, mainly kidney** (Gunzel & Yu, 2013)	**Pore forming- anion selective** (C. M. Van Itallie et al., 2006)	**Cell motility and migration (cancer cells)** (Ip, Cheung, Lee, Ho, & Fan, 2007)
10 b	**Epithelial tissues, mainly kidney** (Gunzel & Yu, 2013)	**Pore forming- cation selective** (C. M. Van Itallie et al., 2006)	**Cell motility and migration (cancer cells)** (Ip et al., 2007)
11	**Sertoli cells** (Morita, Sasaki, Fujimoto, Furuse, & Tsukita, 1999) **Oligodendrocyte and myelin sheath** (Morita, Sasaki, et al., 1999) **Inner ear** (Kitajiri et al., 2004) **Choroid plexus epithelium** (Wolburg, Wolburg-Buchholz, Liebner, & Engelhardt, 2001)	**Predominantly Barrier forming- cation selective** (C. M. Van Itallie, Fanning, & Anderson, 2003)	**Cell proliferation** (Tiwari-Woodruff et al., 2001) **Cell differentiation** (Mazaud-Guittot et al., 2010) **Cell motility and migration (cancer cells)** (Agarwal et al., 2009) **Apoptosis** (Mazaud-Guittot et al., 2010)
12	**Stomach, intestine, salivary gland, epidermis, urinary bladder , and vascular endothelium** (H. Fujita et al., 2006; Gunzel & Yu, 2013)	**Not well characterized : Pore forming- cation selective** (H. Fujita et al., 2008)	**Not known**

13	**Hematopoietic tissues** (Thompson et al., 2010) **Colon and urinary bladder** (H. Fujita et al., 2006) **Kidney (neonates)** (Abuazza et al., 2006)	**Not known**	**Cell proliferation and differentiation** (Thompson et al., 2010)
14	**Kidney and liver**. (Wilcox et al., 2001) **Inner ear** (Kitajiri et al., 2004)	**Barrier forming- cation selective** (Ben-Yosef et al., 2003)	**Cell proliferation** (Baker et al., 2013) **Angiogenesis** (Baker et al., 2013) **Receptor for Clostridium perfringens enterotoxin** (Shrestha & McClane, 2013)
15	**Intestine** (H. Fujita et al., 2006) **Respiratory tract** (F. Wang et al., 2003) **Mammary epithelium** (Markov, Kruglova, Fomina, Fromm, & Amasheh, 2012)	**Predominantly pore forming - cation selective** (C. M. Van Itallie et al., 2003) **Also acts as Cl- barrier** (Colegio et al., 2002)	**Cell proliferation** (A. Tamura et al., 2008)
16	**Kidne**y (Simon et al., 1999) **Mammary epithelium** (Markov et al., 2012)	**Pore forming- cation selective** (Hou et al., 2007; Kausalya et al., 2006)	**Cell motility and migration (cancer cells)** (Martin, Harrison, Watkins, & Jiang, 2008)
17	**Kidney** (Krug et al., 2012)	**Pore forming-anion selective** (Krug et al., 2012)	**Not Known**
18-1	**Predominantly in lung** (Tureci et al., 2011) **Kidney** (Tureci et al., 2011) **Inner ear** (Kitajiri et al., 2004)	**Not known**	**Not Known**
18-2	**Predominantly in stomach** (Tureci et al., 2011) **Osteoclasts** (Linares et al., 2012a) **Osteoblasts** (Kim, Alarcon, Pourteymour, Wergedal, & Mohan, 2013) **Inner ear** (Kitajiri et al., 2004) **Esophagus** (Jovov et al., 2007)	**Barrier forming- cation selective** (Jovov et al., 2007)	**Cell differentiation** (Linares et al., 2012a)
19	**Kidney** (Konrad et al., 2006; Luk et al., 2004) **Retinal pigment epithelium** (Konrad et al., 2006) **Myelin sheath** (Miyamoto et al., 2005)	**Predominantly barrier forming- anion selective** (Hou et al., 2008) **Also act as Na barrier** (Angelow, El-Husseini, Kanzawa, & Yu, 2007)	**Not known**
20	**Intestine** (A. Tamura et al., 2008) **Brain capillary endothelial cells** (Ohtsuki, Yamaguchi, Katsukura, Asashima, & Terasaki, 2008)	**Not known**	**Not known**

21	**Intestine, stomach, liver, and kidney** (Gunzel & Yu, 2013)	**Not known**	**Not known**
22	**Brain capillary endothelial cells** (Ohtsuki et al., 2008)	**Not known**	**Not known**
23	**Intestine** (A. Tamura et al., 2008) **Brain capillary endothelial cells** (Ohtsuki et al., 2008) **skin, placenta, stomach , and germinal center B-cells** (Gunzel & Yu, 2013)	**Not known**	**Not known**
24	**Intestine, stomach kidney, and heart** (Gunzel & Yu, 2013)	**Not known**	**Not known**
25	**Intestine, stomach, liver, kidney, heart, and brain** (Gunzel & Yu, 2013)	**Not known**	**Not known**
26	**Intestine and brain** (Gunzel & Yu, 2013)	**Not known**	**Not known**
27	**Intestine and liver** (Gunzel & Yu, 2013)	**Not known**	**Not known**

There is increasing evidence that the expression of Cldns is also developmental stage specific (Gunzel & Yu, 2013). In mouse jejunum, the expression of several Cldns is increased or decreased during neonatal development (Holmes, Van Itallie, Rasmussen, & Anderson, 2006). For example, Cldn-19 expression was detected at birth, peaked at day 14, and then was undetectable by day 28 (Holmes et al., 2006). The expression of several Cldns during embryonic development has also been investigated. Thus, during mouse development (between E.7 and E.17), the expression of Cldn-21 and -24 was found to increase progressively, whereas that of Cldn-26 and -27 decreased (Gunzel & Yu, 2013; Mineta et al., 2011). There is also increasing evidence that the expression of Cldns changes in response to pathological conditions such as cancer and inflammation (Kwon, 2013). Several studies have reported up- or down-regulation of Cldns in association with various cancers (Kwon, 2013). Thus, the known complexity in the expression of patterns of Cldns in different tissues is consistent with both common and Cldn-specific functions among various Cldns.

As for Cldn distribution in bone, in older studies several investigators have documented the ability of bone cells (osteoblasts and osteocytes) to form tight junctional structures (Prele et al., 2003; Soares et al., 1992; Weinger & Holtrop, 1974). However, the evidence of the presence of tight junction associated proteins has been recently confirmed (Wongdee et al., 2008). Specifically, several Cldns including Cldn-1 to-12, -14 to -20, -22, and -23, were reported to be expressed at the mRNA level in rat osteoblasts (Prele et al., 2003; Wongdee et al., 2008). In fact, immunohistochemical analysis of decalcified tibial sections revealed that the expression of selected Cldns (Cldn-5, -11, -14, -15, and -16) was localized at the bone lining cells (inactive osteoblasts) (Wongdee et al.,

2008; Wongdee, Riengrojpitak, Krishnamra, & Charoenphandhu, 2010). Furthermore, a cell border localization of Cldn-1 was shown in an osteoblast-like cell line (Hatakeyama et al., 2008). In another study, Arana-Chavez et al., observed the presence of tight junctional structures between osteoblasts in early osteogenesis by freeze fracture and ultrathin section electron microscopy (Arana-Chavez, Soares, & Katchburian, 1995; Soares et al., 1992). Moreover, Cldn-1, -2, and -6 mRNA expression was found to be higher in osteoblast like MC3T3-E1 cells compared to osteocyte-like MLO-Y4 cells (Hatakeyama et al., 2008). By contrast, the expression of Cldn-1 and -2 in rat osteoblasts was found to be higher in the mineralization stage compared to the proliferation stage, thus suggesting that the expression levels of Cldn-1 and Cldn-2 in rat osteoblasts may be differentiation stage dependent (Prele et al., 2003). The observed changes in the expression profile of Cldns are consistent with the idea that Cldns play an important role during skeletal development.

While it is well documented that osteoblast lineage cells express Cldns, virtually little is known about their expression profiles in bone resorbing osteoclasts. Nonetheless, we recently provided the first evidence that Cldns, specifically Cldn-18 is expressed in osteoclasts (Linares et al., 2012a). Of the two tissue specific Cldn-18 isoforms, Cldn-18-1.1 (lung isoform) and Cldn-18-2.1 (stomach isoform), it was found that bone cells predominantly express the stomach isoform (Linares et al., 2012a). However, it remains to be determined if other Cldns are expressed in osteoclast line cells and if the Cldns are important for the formation and activity of multinucleated osteoclasts.

Regulation of Cldn Expression

Studies have shown that several growth factors, hormones, and cytokines play important roles in regulating Cldns expression and function. It has been demonstrated that TGF-β regulates Cldns expression via Smad dependent and Smad independent pathways in several physiological and pathological states (Gunzel & Yu, 2013). Furthermore, several inflammatory cytokines (e.g. IL-β, TNF-α, and IFN-γ) were implicated in modulating the expression of Cldns (Gunzel & Yu, 2013). For instance, it has been found that TNF-α downregulates barrier-forming Cldns (Cldn-1, -3,-4, -7, and -8) and upregulates pore-forming Cldns (e.g. Cldn-2) in the intestinal epithelium; thereby increasing epithelial cell permeability (Gunzel & Yu, 2013). In addition, the expression of intestinal Cldn-2 and Cldn-12 was found to be upregulated by vitamin-D treatment, and has been implicated to play a role in the paracellular transport of calcium in the intestinal epithelium (H. Fujita et al., 2008). Several transcription factors have been reported to regulate Cldn expression, with Snail and Slug being the most extensively studied. These transcription factors were shown to suppress the expression of various Cldns by directly binding to their gene promoters (Ikenouchi, Matsuda, Furuse, & Tsukita, 2003; Ohkubo & Ozawa, 2004; C. M. Van Itallie & Anderson, 2006). For example, overexpressing Snail was found to downregulate the expression of Cldn-1, -2, -3, -4 and -7 (Gunzel & Yu, 2013; Ohkubo & Ozawa, 2004). Other transcription factors such as GATA-4, CDX-1, Grhl2, and SP1 have also been reported to regulate Cldn expression (Gunzel & Yu, 2013). The issue of whether these transcription factors are involved in mediating the transcriptional effects of growth factors and cytokines on Cldn expression in target cells remains to be determined.

In terms of skeletal tissues, little is known about the physiological regulators of Cldn expression. A study by Hatakeyama et al., reported that Cldn-1, -2, and -6 expression in MC3T3-E1 mouse osteoblasts was upregulated by IGF-I, a key bone formation regulator (Hatakeyama et al., 2008). Despite the increased mRNA expression level of the three aforementioned Cldns, upregulation at the protein level was only observed for Cldn-1 (Hatakeyama et al., 2008). This study also demonstrated that the mechanism by which IGF-I upregulates Cldn-1 expression is mediated by the MAP-kinase pathway, as inhibition of this pathway diminished the increase in Cldn-1 expression (Hatakeyama et al., 2008). Recent work by our laboratory on bone cells revealed that Cldn-18 expression is regulated by estrogen (Kim et al., 2013). In fact, the mRNA levels of Cldn-18 were found to be reduced by 93% in bones of ovariectomized (estrogen deficient) mice compared with sham operated animals, whereas estrogen treatment increased Cldn-18 mRNA levels in bone cells, *in vitro* (Kim et al., 2013). Furthermore, Cldn-18 is thought to be a novel estrogen target in the skeleton, as its deletion protects from estrogen deficiency induced bone loss in mice (Kim et al., 2013).

Known Functions of Cldns

The role of individual Cldns has been investigated by employing loss or gain of function models in cells and whole animals (Steed, Balda, & Matter, 2010). These studies showed that KO of Cldns resulted in diverse phenotypes in different tissues as listed in Table 2. For instance, targeted disruption of Cldn-1, -5, and -7 in mice resulted in postnatal lethality by affecting the skin epithelial barrier, blood brain barrier, and kidney functions, respectively (Furuse et al., 2002; Nitta et al., 2003; Tatum et al., 2010).

Table 2. Phenotypic changes caused by Cldn gene deletions and transgenics in mice

Cldn	Phenotype
Cldn-1	KO: Skin barrier defect resulting in dehydration and neonatal lethality
Cldn-2	KO: Defect in leaky and cation selective barriers in kidney proximal tubule
Cldn-5	KO: Blood brain barrier defect and neonatal lethality
Cldn-6	Overexpressing transgenic mice: Skin barrier defect and neonatal lethality
Cldn-7	KO: Growth retardation renal salt wasting, chronic dehydration, and neonatal lethality
Cldn-9	Mutant: Deafness
Cldn-11	KO: CNS myelin defect, male sterility, deafness
Cldn-14	KO: Deafness
Cldn-15	KO: mega-intestine
Cldn-16	KO: Renal divalent ion wasting resemble FHHNC in humans but without nephrocalcinosis Knock down: Renal calcium and magnesium wasting resemble FHHNC in humans
Cldn-18.2	KO: Atrophic gastritis KO: Bone loss due to direct (non-canonical) effect on osteoclast differentiation.
Cldn-19	KO: Peripheral nervous system deficit resulted in behavioral changes and neuropathy Knock down: Renal calcium and magnesium wasting resemble FHHNC in humans
Cldn-2/-15	Double KO: Malnutrition due to decrease nutrient absorption resulting in neonatal lethality
Cldn-11/-14	Double KO: CNS myelin defect, male sterility, deafness

By contrast, Cldn-16 overexpression in transgenic mice resulted in postnatal lethality, as a consequence of a defect in the skin epithelial barrier (Turksen & Troy, 2002). Several renal function defects were observed in Cldn-2, -7, -16, and -19 KO mice. Also, the lack of Cldn-9, -11,and -14 resulted in deafness (Ben-Yosef et al., 2003; Gow et al., 2004; Nakano et al., 2009). In addition, loss of Cldn-11 caused CNS nerve conduction abnormalities and male sterility (Gow et al., 2004; Gow et al., 1999).

To understand the interaction between Cldns, a double KO mouse approach has been employed. Specifically, the double Cldn-11/-14 KO phenotype was a combination of those observed in each of the single deletion animals, including male sterility, neurological deficits, and deafness. These findings suggest a lack of cooperation between these two Cldns (Elkouby-Naor, Abassi, Lagziel, Gow, & Ben-Yosef, 2008). On the other hand, Cldn-2/-15 double deletion in mice resulted in infant death as a consequence of malnutrition (in a manner more dominant than in each of the single KOs), which highlights a cooperative interaction between these two Cldns in maintaining nutrient absorption in the intestine (Wada, Tamura, Takahashi, & Tsukita, 2013).

In agreement with data from mouse models, the importance of Cldns in modulating various biological systems have been confirmed by linking mutations of Cldn genes to human diseases. Mutations in human Cldn-1 and Cldn-14 have been found in neonatal ichthyosis and sclerosing cholangitis (NISCH) and nonsyndromic hearing loss, respectively (Hadj-Rabia et al., 2004; K. Lee et al., 2012; Wilcox et al., 2001). Several mutations in human Cldn-16 and -19 are associated with a hereditary renal disease known as familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) (Konrad et al., 2006; Simon et al., 1999).

With regards to the skeleton, our previous studies have shown that mice with targeted disruption of Cldn-18 (stomach isoform) exhibited a 20-25% decrease in areal BMD of the total body, vertebrae, and long bones (Linares et al., 2012a). Although bone size was found not to be affected by Cldn-18 deficiency, μCT analysis revealed a 20% reduction in cortical thickness at the femoral mid-diaphysis (Linares et al., 2012a). Additionally, Cldn-18 KO mice exhibited a dramatic decrease in trabecular bone volume, trabecular thickness, and trabecular number, whereas trabecular separation at both the lumber vertebra 5 and distal femur metaphysis was increased (Linares et al., 2012a). Histomorphometric analysis and *in vitro* assays of bone formation revealed that bone formation parameters were not affected by Cldn-18 deficiency (Linares et al., 2012a). By contrast, Cldn-18 KO mice exhibited an 87% increase in TRAP labeled osteoclast surface in the trabecular bone, as well as an increase in osteoclast number (Linares et al., 2012a). The increased bone resorption observed in Cldn-18 KO was also confirmed by measuring the serum and mRNA levels of osteoclastogenic marker genes (Linares et al., 2012a). In addition, the number of TRAP positive multinucleated cells was greater in bone marrow macrophage cultures derived from Cldn-18 KO mice that were induced to differentiate in the presence of RANKL and MCSF, *in vitro* (Linares et al., 2012a). These data clearly demonstrate that the reduced bone mineral density reported in Cldn-18 mice was due to increased osteoclast formation and bone resorption which highlights Cldn-18 as a novel negative regulator of bone resorption. Interestingly, humans with a Cldn-14 variant demonstrated a lower bone mineral density in the spine and the hip than their normal counterparts, which provides further evidence that Cldns play an important function in bone (Thorleifsson et al., 2009). Taken together, it is clear that while much is known

regarding the role of Cldn-18, the skeletal phenotype of other Cldns and their contribution to the genesis of osteoporosis is poorly defined.

Molecular Mechanisms of Cldn Action

Cldns are multifunctional proteins: they regulate paracellular transport of ions, solutes, and water; and serve as a fence that divides apical and basolateral domains of plasma membranes, which is known as the "canonical function". Aside from functioning as tight junctions, interestingly, Cldns have also been shown to participate in intracellular signaling that controls cell proliferation and differentiation, which is known as the "non-canonical function".

Cldns are the major determinant of paracellular permeability in epithelial and endothelial cells, acting (canonically) as barriers or pores to decrease or increase permeability, respectively. Several *in vitro* studies have demonstrated that both barrier and pore forming Cldns are size and charge selective (Table 1) (Gunzel & Yu, 2013). In MDCK and LLC-PK1 cell lines, while Cldn-2, -10b, -15, and -16, selectively increased cation permeability in tight junctions, Cldn-1, -4, -5, -6, -8, -11, and -14 decrease it. Furthermore, Cldn-7, -10a, and -17 were found to serve as anion pores, but in contrast, Cldn-7 and -19 act as anion barriers (Gunzel & Yu, 2013; Krause et al., 2008). As mentioned before, the Cldn carboxy-terminus possesses several phosphorylation sites for various kinases including PKC, PKA, WNK, MLCK, MAPK, RhoK, and c-Src, which have been implicated in regulating tight junction assembly and function (Banan et al., 2005). In fact, several studies have reported that the barrier/pore function of Cldns can be negatively or positively regulated by phosphorylation (Findley & Koval, 2009). It has

been demonstrated that phosphorylation of Cldn-1 and Cldn-4 by PKC promoted their assembly into tight junctions and increased the barrier function (Banan et al., 2004; D'Souza, Agarwal, & Morin, 2005; French et al., 2009). Conversely, dephosphorylation of Cldn-1 by PP2A resulted in their disassembly and decreased barrier function (Nunbhakdi-Craig et al., 2002). As for negative regulation, Tanaka et al., reported that phosphorylation of Cldn-4 by EphA4 decreased its integration into tight junctions and increased paracellular permeability (Tanaka, Kamata, & Sakai, 2005). It is to be noted though that the consequence of phosphorylation on the barrier function is rather complex and controversial. In fact, there are kinases that can phosphorylate distinct residues on the same Cldn with the outcome (positive or negative) depending on the external stimuli, and the physiological, and pathological conditions (Gonzalez-Mariscal, Tapia, & Chamorro, 2008). Besides phosphorylation, it remains to be determined whether other post-translational modifications regulate canonical functions of Cldns.

The canonical function of Cldns in regulation of the paracellular transport of ions in bone has been suggested by several studies (Bushinsky, Chabala, & Levi-Setti, 1989; Hatakeyama et al., 2008; Marenzana, Shipley, Squitiero, Kunkel, & Rubinacci, 2005; Rubinacci, Benelli, Borgo, & Villa, 2000; Wongdee et al., 2010). There is substantial evidence that osteoblast and bone lining cells form an epithelial like bone membrane to control the paracellular ion exchange and maintain differential ion compositions between the plasma and bone extracellular fluid (Bushinsky et al., 1989; Hatakeyama et al., 2008; Marenzana et al., 2005; Rubinacci et al., 2000; Wongdee et al., 2008; Wongdee et al., 2010). The expression and localization of certain Cldns in the bone lining cells support their function as barriers, a notion that was confirmed by measuring the transepithelial

resistance of an osteoblast monolayer (Wongdee et al., 2008). As for regulation of the Cldn barrier function, more work must be done to address this issue. Nonetheless, in osteoblast like MC3T3-E1 cells, a reduction of paracellular permeability was observed as a consequence of MAPK activation by IGF-I (Hatakeyama et al., 2008). To this end, our previous study demonstrated that Cldn-18 disruption/overexpression did not influence paracellular transport of calcium ions in osteoclasts, thereby supporting a potential non-canonical function of Cldns in bone cells (Linares et al., 2012a). Thus, some but not other Cldns may exert their canonical functions in bone cells.

Besides their canonical function as a barrier, gate, and fence, Cldns have recently started to emerge as mediators of cell signaling, e.g., proliferation and differentiation (Table 1) (Matter et al., 2005). The non-canonical Cldn functions have been shown to involve interaction with adaptor proteins that shuttle between the plasma membrane and the nucleus, thereby regulating gene expression, cell proliferation, and differentiation (Balda & Matter, 2009). As mentioned earlier, Cldns have the capacity to interact with other PDZ domain containing cytoplasmic scaffolding proteins such as ZO-1/2/3, via their carboxy- terminus PDZ-binding motif (Angelow et al., 2008; Krause et al., 2008). Although much remains to be investigated regarding the physiological significance of this interaction, research to date indicates that the interaction between Cldns and PDZ domain containing proteins is of importance in certain biological processes (Chung, Shikano, Hanyu, & Li, 2002; Guillemot, Paschoud, Pulimeno, Foglia, & Citi, 2008; Matter & Balda, 2003; Sierralta & Mendoza, 2004). Of the several adaptor proteins, ZO-1, the first tight junction protein identified, has been extensively studied (Stevenson, Siliciano, Mooseker, & Goodenough, 1986). ZO-1 has three PDZ and SH3 domains that

have been implicated in the regulation of cell proliferation (Laing, Chou, & Steinberg, 2005). The negative effect of ZO-1 on cell proliferation is thought to be mediated by its SH3 domain interaction with the Y-BOX transcription factor ZONAB (Balda, Garrett, & Matter, 2003). The resultant cytoplasmic sequestration of ZONAB was found to reduce its interaction with important cell cycle regulators and cell cycle target genes (e.g., CK4, and cyclin D) (Balda et al., 2003; Balda & Matter, 2000; Matter & Balda, 2007; Sourisseau et al., 2006). Cldns have been also implicated in regulating cell differentiation (S. K. Lee et al., 2005). For example, overexpression of Cldn-1 in intestinal epithelium was found to activate Notch-signaling, and in turn inhibit goblet cell differentiation (Pope et al., 2013).

In the recent studies, we found that Cldn-18 regulates RANKL-induced osteoclast differentiation via stimulating non-canonical signaling (Linares et al., 2012a). We found that overexpression of Cldn-18 resulted in a dramatic inhibition of RANKL induced osteoclast differentiation *in vitro,* while bone marrow-derived macrophage precursors-derived from Cldn-18 KO mice formed fewer osteoclasts in the presence of MCSF and RANKL, thus suggesting that Cldn-18 is a negative regulator of osteoclast differentiation (Linares et al., 2012a). In terms of mechanisms for Cldn-18 action in osteoclasts, we found that overexpression of full-length Cldn-18 but not a mutant form of Cldn-18 with a deleted C-terminal PDZ binding motif had an effect on RANKL induced osteoclastogenesis, thus suggesting that the biological effects of Cldn-18 on osteoclasts is mediated by the PDZ binding motif (Linares et al., 2012a). Of the several PDZ domain-binding proteins reported to directly bind to the C-terminal YV sequence of several Cldns, ZO-2 was found to be highly expressed and significantly upregulated by RANKL

in osteoclasts (Linares et al., 2012a). Consistent with this data, the Cldn-18 and ZO-2 interaction was confirmed by immunoprecipitation (Linares et al., 2012a). Importantly, overexpression of Cldn-18 reduced ZO-2 nuclear translocation induced by RANKL, and this effect was abrogated with the deletion of the Cldn-18 C-terminal PDZ binding motif (Linares et al., 2012a). Thus, we concluded that the negative effect of Cldn-18 on osteoclasts is mediated via sequestering ZO-2 in the membrane complex. Furthermore, we found that shRNA knockdown of ZO-2 inhibited RANKL-induced osteoclast differentiation, *in vitro*. Consistent with this prediction, there is a significant body of evidence that ZO-2 has nuclear localization signals and that nuclear translocation is associated with increased gene expression (Betanzos et al., 2004; Islas, Vega, Ponce, & Gonzalez-Mariscal, 2002; Traweger et al., 2003; Traweger et al., 2008). In this regard, ZO-2 knockdown in an osteoclast like cell line resulted in reduction of RANKL induced TRAP and cathepsin k expression, and inhibition of NF-κB and NFAT transcriptional activity (Linares et al., 2012a). Collectively, we believe that the non-canonical effect of the loss of Cldn-18 in the regulation of RANKL-induced osteoclast differentiation is mediated by disruption of the interaction with ZO2, resulting in increased nuclear translocation of ZO2. In turn, this translocation increases the expression of important transcription factors involved in RANKL-induced osteoclast differentiation, which ultimately leads to increased bone resorption.

While our studies have laid down a foundation for the non-canonical function of Cldn-18 in regulating bone homeostasis, whether other Cldn family members are also expressed and exhibit a function during osteoblastogenesis and osteoclastogenesis remains to be investigated. Based on the role of Cldn-18 in regulating bone metabolism

and the established importance of Cldn family members in the development of various tissues, **we hypothesize that Cldns are important regulators of bone cell function. Our long term goal is to characterize the role of specific Cldns in skeletal growth and maintenance.** The following specific aims are designed to address the above hypothesis:

1- **Investigate whether the osteopenia phenotype in Cldn-18 KO mice is due to disruption of Cldn-18 in tissues other than bone, i.e., stomach.**
2- **Investigate the expression profile of Cldn family members during osteoblast and osteoclast differentiation.**
3- **Examine the role of Cldn-1 in the regulation of osteoblast proliferation and differentiation, *in vitro*.**

Rationale and Hypothesis

AIM 1: Investigate Whether The 0steopenia Phenotype in Cldn-18 KO Mice is Due to Disruption of Cldn-18 in Tissues Other Than Bone, i.e., Stomach

Our earlier studies utilized Cldn-18 that was globally disrupted, and this Cldn is known to be expressed in tissues separate from bone (Linares et al., 2012a; Tureci et al., 2011). Thus, the purpose of this aim is to investigate the possibility that the osteopenia phenotype of Cldn-18 KO mice is mediated in part by its disruption in other tissues, i.e., the stomach (Krause et al., 2008; Tureci et al., 2011). In this connection, it has previously been found that Cldn-18 is the predominant form of Cldns in the stomach, and plays a critical function in paracellular transport of small molecules including H^+ (Hayashi, Tamura, Tanaka, Yamazaki, Watanabe, Suzuki, Sentani, et al., 2012; A. Tamura et al., 2012). Moreover, deletion of Cldn-18 in mice resulted in abnormalities in gastric mucosa

and atrophic gastritis via decreasing paracellular barrier against H^+ in the stomach epithelium (Hayashi, Tamura, Tanaka, Yamazaki, Watanabe, Suzuki, Sentani, et al., 2012). It is noteworthy that the role of gastric acidity in calcium absorption/metabolism has received more attention recently (Boyce, 2009; Wright, Proctor, Insogna, & Kerstetter, 2008). Also, calcium solubilization is thought to be prerequisite for its absorption in the small intestine, with the stomach's acidic environment inducing the dissolution of calcium salts and the release of ionized calcium (Bo-Linn et al., 1984; Sipponen & Harkonen, 2010). Interestingly, several clinical studies have shown that gastric acidity is important for calcium absorption in the small intestine (Wright et al., 2008). In this regard, we observed that levels of serum PTH (whose reduced levels are an indicator of calcium deficiency) were elevated in Cldn-18 KO mice fed a normal calcium diet compared to control mice fed a normal calcium diet (Linares et al., 2012a). Thus, this data suggests that Cldn-18 KO mice may be calcium deficient. Based on these findings, we hypothesize that the loss of Cldn-18 decreases gastric acidity, and the osteopenia phenotype observed in Cldn-18 KO mice is partly a consequence of decreased calcium absorption due to the low gastric acidity.

The findings pertaining to this Aim are summarized in Chapter 2.

AIM 2: Investigate the Expression Profile of Cldn Family Members During Osteoblast and Osteoclast Differentiation

Cldns exhibit complex patterns of expression that is tissue/cell type and developmental stage specific (Alshbool & Mohan, 2014; Gunzel & Yu, 2013). While much is known regarding the expression patterns and function(s) of many Cldn family

members in several tissues, little is known about their expression and role in bone. There is evidence that a number of Cldns are expressed in osteoblasts; however their differential expression during osteoblast differentiation is still not well defined. In addition, aside from Cldn 18, nothing is known about the expression of other Cldns in osteoclasts. Based on the above consideration, we hypothesize that the expression of Cldns is differentiation stage and cell type specific in bone.

The findings pertaining to this Aim are summarized in Chapter 3.

AIM 3: Examine The Role of Cldn-1 in The Regulation of Osteoblast Proliferation and Differentiation, in Vitro

Cldn-1 plays important roles in the development of various biological systems. In fact, targeted disruption of Cldn-1 in mice resulted in postnatal lethality immediately after birth (Furuse et al., 2002). Furthermore, there is substantial evidence that Cldn-1 participates in intracellular signaling that controls cell proliferation and differentiation (H. Fujita et al., 2011; Hoshino et al., 2008; Pope et al., 2013). It has also been found that Cldn-1 expression in MC3TE-E1 mouse osteoblasts is regulated by IGF-I, a key bone formation regulator (Hatakeyama et al., 2008). While Cldn-1 is known to be expressed in osteoblasts, little is known about its regulation and role during osteoblastogenesis (Wongdee et al., 2008). In this regard, our data demonstrated an interesting differential expression pattern of Cldn-1 during osteoblastogenesis (i.e., upregulation of Cldn-1 during early stages of osteoblast differentiation, as opposed to downregulation during late stages). On this basis, we hypothesize that Cldn-1 modulates osteoblast proliferation and differentiation.

The findings pertaining to this Aim are summarized in Chapter 3.

CHAPTER TWO

A HIGH CALCIUM DIET FAILED TO RESCUE AN OSTEOPENIA PHENOTYPE IN CLAUDIN-18 KNOCKOUT MICE

The work presented in this chapter has been published.

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Abstract

We have recently demonstrated that mice with disruption of claudin-18 (Cldn-18) gene exhibited osteopenia due to increased bone resorption (BR). In this study, we found that gastric pH was significantly higher in Cldn-18 knockout (KO) mice compared to heterozygous control mice at 10 wks of age. To test the possibility that the increased BR in the Cldn-18 KO mice fed a normal Ca diet is a consequence of decreased Ca absorption caused by increased stomach pH, we subjected KO and control mice to a normal Ca and high Ca diet at birth. Serum Ca levels were significantly lower in Cldn-18 KO mice compared to control mice at a normal Ca diet but not at high Ca diet. DEXA revealed that a high Ca diet significantly increased lumbar BMD but had no effect on femur/tibia BMD in both Cldn-18 KO and control mice compared to a normal Ca diet. While a high Ca diet did not affect volumetric BMD, trabecular, and cortical parameters of the lumbar vertebrae (LV) as measured by uCT, the size of the LV did increase, in both genotypes due to reduced BR. Comparison of the skeletal phenotype of high Ca Cldn-18 KO and control mice revealed that an osteopenia phenotype seen at a normal Ca diet was still maintained at different skeletal sites in the KO mice till 10 weeks of age. In conclusion, our findings suggest that increased BR is likely caused by direct effects of a lack of Cldn-18 on osteoclasts rather than gastric pH changes.

Introduction

Osteoporosis is a major clinical problem in which loss of bone strength leads to skeletal fractures (13). Osteoporosis mediated bone fragility can result from failure to produce optimal bone mass and/or the imbalance between bone formation and bone resorption during bone remodeling (25). It is well known that bone resorption is dependent on the number and activity of osteoclasts, which is in turn regulated by a number of local and systemic factors including macrophage colony stimulating factor (MCSF) and receptor activator of nuclear factor kappa B ligand (RANKL). While the MCSF and RANKL are sufficient for inducing osteoclast activity and function, the role of other regulatory molecules and their signaling pathways has not been determined (6, 18).

Tight junctions (TJs) play important roles in different biological systems (10). Claudins (Cldns) comprise a major group of TJ proteins and consist of 27 members in mice and humans (20, 23, 24). Cldns serve as a multifunctional complex: they regulate paracellular transport of ions, solutes, and water; and serve as a fence that divides apical and basolateral domains of plasma membranes. Moreover, Cldns act non-canonically by regulating a variety of signaling molecules that control cell differentiation, proliferation, and polarity (10, 20, 23, 30). Given that Cldns have major functions in different biological systems, the role of individual Cldns has been investigated by employing loss of function models in vivo, and by transgenic studies in cells in vitro (14). However, the role of Cldns in bone homeostasis is still poorly defined. Nonetheless, we have recently demonstrated that one of the Cldns, namely, Cldn-18 is expressed in bone and that targeted disruption of Cldn-18 in mice resulted in markedly decreased total body bone mineral density, trabecular volume, and cortical thickness (22).

In our previous studies, we provided the first evidence that Cldn-18 is expressed in osteoclasts and that the underlying cause of decreased bone mass observed in Cldn-18 KO mice was increased bone resorption, but not impaired bone formation (22). We investigated the mechanism by which loss of Cldn-18 increased bone resorption and found that osteoclast differentiation was increased by regulating RANKL signaling (22). The non-canonical effect of the loss of Cldn-18 on RANKL actions was shown to be mediated by disruption of the interaction with a scaffold protein called zonula occludens (ZO)-2 that resulted in increased nuclear translocation of ZO2 (22). In turn, this translocation increased the expression of important transcription factors involved in RANKL-induced osteoclast differentiation (22). Since Cldn-18 was globally disrupted and Cldn-18 is expressed in other tissues, the purpose of this study was to investigate the possibility that the osteopenia phenotype of Cldn-18 KO mice is mediated in part by disruption of Cldn-18 in other tissues, i.e., the stomach (20, 32).

It has been found that Cldn-18 is the predominant form of Cldns in the stomach, and has been shown to play a major role in the physiology and pathology of the stomach epithelial barrier (16, 31). The expression level of stomach Cldn-18 is significantly down regulated in atrophic gastritis and gastric cancer in humans (26). Moreover, targeted disruption of Cldn-18 in mice resulted in abnormalities in gastric mucosa and atrophic gastritis via decreasing paracellular barrier against H+ in stomach epithelium (16). Interestingly, several clinical studies have shown that gastric acidity is important for calcium absorption in the small intestine (35). In this regard, we observed that serum parathyroid hormone (PTH) levels were elevated in Cldn-18 KO mice fed a normal calcium diet compared to control mice, thus suggesting that Cldn-18 KO mice may be

calcium deficient. Based on these findings, we undertook studies to characterize the impact of a lack of cldn-18 on the gastric pH and serum calcium levels, and whether a dietary manipulation of calcium homeostasis can rescue the osteopenia phenotype in the Cldn-18 KO mice. Collectively, our findings revealed that a high calcium diet failed to rescue an osteopenia phenotype in Cldn-18 KO mice.

Materials and Methods

Animals

The generation of Cldn-18 deficient mice by homologous recombination and genotyping was previously described (22). Since we did not observe any apparent differences in the skeletal phenotype between heterozygous and wild type animals, we opted to use the heterozygous littermates as control mice. Homozygous Cldn-18 KO and heterozygous littermate controls were generated by breeding heterozygous with homozygous Cldn-18 KO. Mice were housed at Jerry L. Pettis Memorial VA Medical Center Medical Unit (Loma Linda, CA, USA) under standard approved laboratory conditions. All animal experiments were performed in compliance with and approved by the Institutional Animal Care and Use Committee.

Experimental Design

Cldn-18 KO and heterozygous control mice were subjected to either normal calcium diet or high calcium diet by feeding their mothers the indicated diet at birth. After weaning, Cldn-18 KO and heterozygous control mice were kept on their respective diets until 10 weeks of age. The normal calcium diet (TD.04200, containing 0.6% calcium carbonate and 0.4% phosphate by weight) and the high calcium diet (TD.96348,

containing 2% calcium carbonate and 1.25% phosphate by weight) were purchased from Harlan Teklad (Madison, WI, USA).

Measurement of Gastric pH

Gastric pH was assessed in 10 week old mice fed a normal calcium diet using a modified method of Waisberg et al., (34). After the mice were euthanized, the stomach was clamped at the esophageocardial and pylorodoudenal junctions, before being removed, and 0.5 ml sterile water injected into the gastric lumen. The stomach fluid contents were then collected, and gastric pH was measured using a PHR-146 microcombination pH electrode.

Total Calcium Serum Measurement

The level of total serum calcium was determined in 10 week old mice fed either a normal calcium or a high calcium diet using Stanbio Total Calcium LiquiColor© (Arsenazo III) kit as per manufacturer`s instructions (STANBIO, lab, Boerne, TX, USA).

Bone Densitometry

Total and multiple skeletal site bone mineral density (BMD) was measured by dual energy X-ray absorptiometry with a PIXImus instrument (Lunar-Corp, Madison, WI, USA) at 3, 6, and 10 weeks of age as previously described (38). To obtain bone mass parameters of the total body, a region of interest (ROI) rectangle was moved and re-sized to cover the whole body, and the animal's head was set as an exclusion zone. Femur,

tibia, and lumbar vertebrae 5 BMD were obtained by moving the ROI rectangle to the specific region and resized to cover the assigned area. The precision for the BMD was ± 1% for repeat measurements of the same bone several times.

Micro-CT Analysis

Micro-CT analysis of lumbar vertebrae 5 was carried out using a "vivaCT 40" microCT system (Scanco Medical, Bassers-drof, Switzerland). Transverse slices were acquired for the entire vertebra body, as previously described (15). Trabecular bone was evaluated in a region 0.3 mm below the cranial and above the caudal growth region. The cortical parameters and cross sectional areas were evaluated for 100 slices by delineating a region of interest around the entire vertebral body. The thresholds were set at 220 for trabecular bone, and 260 for cortical bone. The length of lumbar vertebrae 5 was recorded at the time of scanning.

Dynamic Calcein Labeling and Histomorphometry

Cldn-18 KO and heterozygous control mice were injected intraperitoneally with calcein 8 days (20 mg/kg of body weight) and 2 days prior to euthanization in order to label mineralized bone surfaces. Lumbar vertebrae 5 were fixed in 10% formalin overnight, the bones were washed, dehydrated, and embedded in methyl methacrylate for sectioning. The sections were stained for tartrate resistant acid phosphatase (TRAP), and the cortical bone and trabecular bone that adjacent to the growth plate were excluded. The trabecular surface and TRAP labeled surface were measured using the OsteoMeasure software (Osteometric, Inc. Decatur, GA) (36)

Cell Culture

Primary osteoclast precursors or bone marrow macrophages (BMMs) were isolated from femurs and tibias of Cldn-18 KO and heterozygous control mice, as described previously (7), and maintained in α-MEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 ug/ml), and macrophage colony stimulating factor (MCSF; 25 ng/ml). Twenty four hours later, non-adherent cells (BMMs) were collected and treated with MCSF (50 ng/ml) and receptor activator of nuclear factor kappa B ligand (RANKL; 100 ng/ml) for 6 days; with fresh differentiation media added every 3 days. MCSF and RANKL were obtained from R&D Systems (Minneapolis, MN, USA).

RNA Extraction and Gene Expression Analysis

RNA was extracted using Trizol and chloroform, and isolation was completed using E.Z.N.A.® RNA Isolation Kits (Omega Bio -Tek). The expression was determined by real-time RT-PCR. The housekeeping gene peptidylpropyl isomerase A (PPIA) was used as internal control in the PCR reaction and the ΔΔCT method was used for relative quantification of gene expression. Values are presented as fold change of control heterozygous mice.

Statistical Analysis

Data were expressed as the mean ± SEM and were analyzed using Student`s t-test or ANOVA (Statistica software).

Results

Cldn-18 Deficiency Affects Gastric pH

In order to determine the effect of Cldn-18 on gastric acidity, the stomach pH was measured in both 10 week old Cldn-18 KO and heterozygous control mice. We found that the lack of Cldn-18 dramatically increased the gastric pH (pH= 6.1) compared to heterozygous control mice (pH=2.6) (Table. 3).

The Effect of Dietary Calcium on Serum Calcium Levels in 10 Week Old Cldn-18 KO and Heterozygous Control Mice

To determine the effect of Cldn-18 KO on serum calcium levels and verify the effectiveness of giving high calcium in the diet to correct calcium deficit in Cldn-18 KO mice, we measured total serum calcium levels in Cldn-18 KO and heterozygous control animals. Expectedly, the high calcium diet significantly increased serum calcium levels in both Cldn-18 KO and heterozygous control mice (Table. 3). Interestingly, serum calcium levels were found to be lower in Cldn-18 mice fed a normal calcium diet compared to heterozygous control mice fed the same diet (Table. 3). However, serum calcium levels were not different between Cldn-18 KO and heterozygous control mice on a high calcium diet (Table. 3).

Table 3. The effect of dietary calcium on gastric pH, serum calcium, and lumbar vertebrae 5 trabecular and cortical morphology in 10 week old Cldn-18 KO and heterozygous control mice.

	Normal calcium diet		High calcium diet		P value			
	Control	Cldn-18 KO	Control	Cldn-18 KO	Control High vs. Control Normal	KO High vs. KO Normal	KO Normal vs. Control Normal	KO High vs. Control High
Gastric pH	2.56± 0.08	6.11± 0.05	N.D.	N.D.	N.D.	N.D.	***	N.D.
Serum Ca (mg/dl)	9.61± 0.13	9.26± 0.06	10.01± 0.07	9.98± 0.1	*	***	*	N.S.
Trabecular Bone								
BV/TV	0.34±0. 01	0.29±0. 006	0.33±0. 01	0.28± 0.007	N.S.	N.S.	***	***
Connectivity density (mm-3)	215.54± 12.15	187.38± 5.57	223.79± 8.51	204.98± 8	N.S.	N.S.	*	*
Tb.N (1/mm)	6.14± 0.19	5.46± 0.11	6.16± 0.18	5.45± 0.13	N.S.	N.S.	**	**
Tb.Th(mm)	0.057± 0.0008	0.054± 0.0008	0.056± 0.001	0.052± 0.009	N.S.	N.S.	**	**
Tb.Sp (mm)	0.171± 0.005	0.188± 0.004	0.169± 0.005	0.187± 0.005	N.S.	N.S.	*	*
Cortical Bone								
Total vBMD (mg/cm3)	347.91± 6.09	323.55± 3.69	333.74± 6.95	311.605 ±4.66	N.S.	N.S.	**	*
Cortical vBMD (mg/cm3)	960.91± 1.81	941.19± 1.66	954.19± 3.92	935.27± 3.02	N.S.	N.S.	***	**
Cort. Th (mm)	0.073± 0.0001	0.066± 0.001	0.071± 0.001	0.064± 0.001	N.S.	N.S.	***	***

Data represents the mean ±SEM. n= 15-17/group

BV/TV: bone volume/total volume; Tb.N: trabecular number; Tb.Th: trabecular thickness; Tb.Sp: trabecular separation; vBMD: volumetric BMD; Cort. Th: cortical thickness; N.S.: not significant; N.D.: not determined. *: $p<0.05$; **: $p<0.01$; and ***: $p<0.001$.

The Effect of Dietary Calcium on Bone Mineral Density (BMD) of The Cldn-18 KO and Heterozygous Control Mice at Different Skeletal Sites

We have previously reported a decrease in bone mass and an increase in bone resorption in Cldn-18 KO mice (22). To evaluate whether these observations are a consequence of decreased calcium absorption due to low gastric acidity, Cldn-18 KO and heterozygous control mice were subjected to either a high calcium (2%) or normal calcium (0.6%) diet at birth, that was continued until 10 weeks of age. First, and as expected, the body weight and animal length increased with age in both genotypes (Fig. 5 A, B). However, neither body weight nor body length was significantly different between Cldn-18 KO and heterozygous control mice fed either a normal or high calcium diet (Fig. 5 A, B). The high calcium diet increased total areal BMD in heterozygous control mice compared to a normal calcium diet at 3 and 6 weeks of age, by 8% and 5%, respectively (Fig. 6 A). A similar trend was observed in the Cldn-18 KO mice, as a high calcium diet increased whole body areal BMD compared to a normal calcium diet at all ages (Fig. 6 A). Expectedly, the total areal BMD was decreased significantly in Cldn-18 KO mice fed a normal diet in comparison to heterozygous control mice fed a normal calcium diet (Fig. 6 A). Moreover, total body areal BMD was significantly lower in the high calcium Cldn-18 KO group compared to the high calcium heterozygous control group at 6 and 10 weeks. Therefore, this data suggests that the high calcium diet did not rescue the reduced whole body BMD phenotype in the KO mice (Fig. 6 A). Phenotypic differences due gender-genotype-diet interaction were not observed and, therefore, data from both genders were pooled for analyses. Evaluation of BMD at different skeletal sites revealed

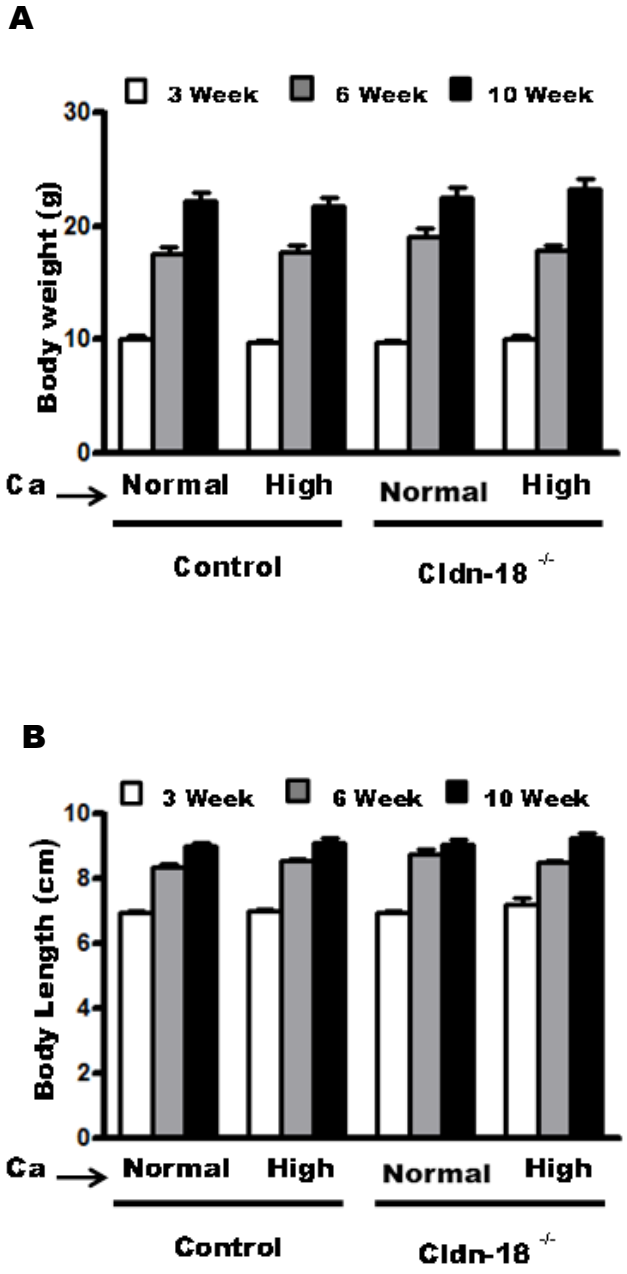

Figure 5. The effect of dietary calcium on body weight and length of Cldn-18 KO and heterozygous control mice. (A) Body weight of 3, 6, and 10 week old Cldn-18 KO and heterozygous control mice (n=13-19/group). (B) Body length of 3, 6, and 10 week old Cldn-18 KO and heterozygous control mice (n=13-19/group, mixed gender).

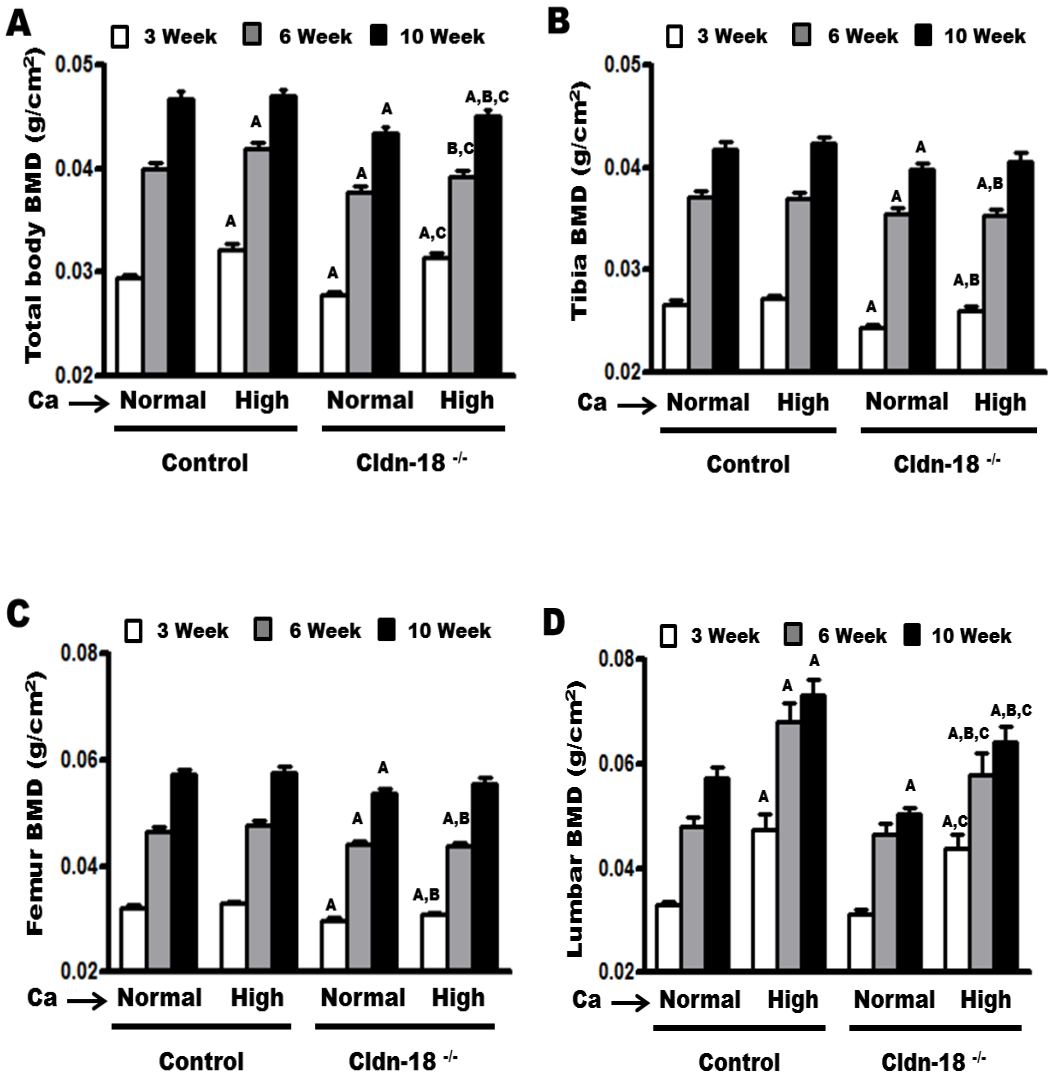

Figure 6. The effect of high calcium diet on areal BMD of Cldn-18 KO and heterozygous control mice at different ages. (A) Total body BMD as determined by DXA at 3, 6, and 10 weeks of age. (B) Tibia BMD. (C) Femur BMD. (D) Lumbar vertebrae BMD (n=13-19/group, mixed gender). A=p< 0.05 versus heterozygous control mice (normal calcium diet), B=p<0.05 versus heterozygous control mice (high calcium diet), C=p<0.05 versus KO mice (normal calcium diet).

that the high calcium diet had no significant effect on femur and tibia BMD in both Cldn-18 KO and heterozygous control mice compared to mice on a normal calcium diet, at any age (Fig. 6 B, C). However and as previously documented, Cldn-18 KO mice exhibited a significant decrease in tibia and femur BMD compared to heterozygous control mice on a normal calcium diet (Fig. 6 B, C). Moreover, Cldn-18 KO mice treated with a high calcium diet exhibited a significantly lower femur and tibia BMD compared to heterozygous control mice treated with a high calcium diet at 3 and 6 weeks of age (Fig. 6 B, C). By contrast, the lumbar BMD increased significantly in the high calcium heterozygous control group by 44%, 42%, and 27% at 3, 6, and 10 week of age, respectively, compared to the normal calcium heterozygous control group (Fig. 6 D). The same trend was observed in Cldn18 KO mice fed a high calcium diet compared to a normal calcium diet (Fig. 6 D). Even though lumbar BMD was lower in normal calcium fed Cldn-18 KO mice compared to heterozygous control mice, the difference was not significant until the mice reached 10 week of age (Fig. 6 D). However, lumbar BMD was significantly lower in the high calcium Cldn-18 KO group compared to the high calcium heterozygous control group at 6 and 10 weeks (Fig. 6 D). Analysis of variance indicated that the genotype alone had a significant effect on areal BMD and the age alone had a significant effect on areal BMD (Table. 4). Furthermore, areal BMD was significantly affected by diet alone (Table. 4). However, there was no significant genotype-diet-age interaction for total areal BMD and skeleton-specific BMD (Table. 4).

Table 4. The effects of genotype, diet, and age on areal BMD using ANOVA analysis.

Grouping Variable	Total areal BMD	Tibia BMD	Femur BMD	Lumbar BMD
Genotype	<0.001	<0.001	<0.001	<0.001
Diet	<0.001	<0.001	<0.001	<0.001
Age	<0.001	<0.001	<0.001	<0.001
Genotype+ diet	N.S.	N.S.	N.S.	N.S.
Genotype +age	N.S.	N.S.	N.S.	N.S.
Diet + age	<0.05	<0.05	N.S.	N.S.
Genotype + diet +age	N.S.	N.S.	N.S.	N.S.

Data represent p values of the interaction of y- axis variables with x –axis parameters calculated using ANOVA.

Areal BMD was measured in 3, 6, and 10 week old Cldn-18 KO and heterozygous control mice fed either a normal or a high calcium diet using Piximus. N.S.: not significant**.**

The Effect of Dietary Calcium on Lumbar Vertebrae 5 Bone Mass Parameters of 10 Week Old Cldn-18 KO and Heterozygous Control Mice

To further characterize the effect of the calcium diet on the lumbar vertebrae, trabecular and cortical bone parameters were evaluated at lumbar vertebrae 5 from 10 week old Cldn-18 KO and heterozygous control mice using uCT. The high calcium diet had no significant effect on trabecular bone parameters compared to the normal calcium diet in heterozygous control mice (Table. 3, Fig. 7). A similar finding was observed in the Cldn-18 KO mice, as a high calcium diet had no effect on trabecular parameters compared to a normal calcium diet (Table. 3, Fig. 7). However and as expected, the trabecular bone parameters were found to have significantly deteriorated in Cldn-18 KO compared to heterozygous control mice fed a normal calcium diet (Table. 3, Fig. 7). Moreover, Cldn-18 KO mice treated with a high calcium diet exhibited a significantly deteriorated trabecular architecture compared to heterozygous control mice under similar dietary conditions. The same trend was observed for cortical bone parameters (Table. 3). Thus, these data provide further evidence that a high calcium diet failed to rescue the osteopenia phenotype at lumbar vertebrae 5 (Table. 3).

The Effect of Dietary Calcium on Lumbar Vertebrae 5 Bone Size Parameters of 10 Week Old Cldn-18 KO and Heterozygous Control Mice

Since areal BMD is influenced by size parameters, the length of lumbar vertebrae 5 was measured to see if the change in lumbar BMD observed with piximus data is due to a size difference. The length of lumbar vertebrae 5 was increased by 5% ($P<0.05$) in the high

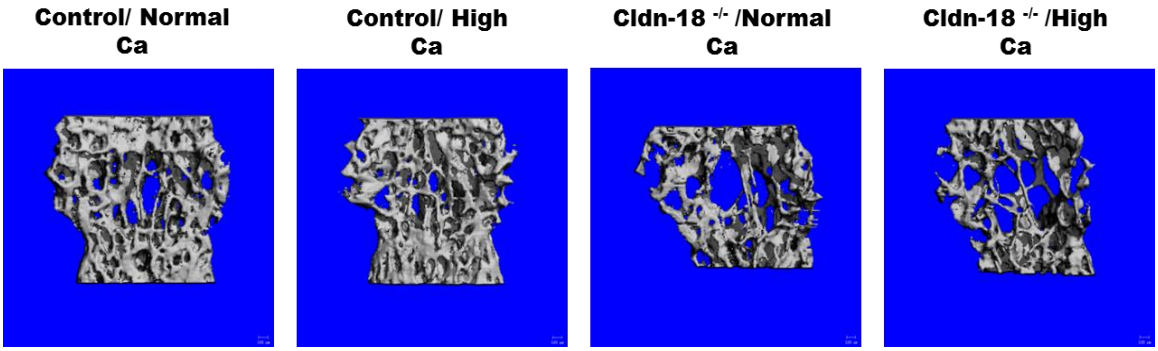

Figure 7. The effect of dietary calcium on lumbar vertebrae 5 trabecular morphology. Three-dimensional μ-CT images of lumbar trabecular bone from 10 week old Cldn-18 KO and heterozygous control mice

calcium heterozygous control group compared to the normal calcium heterozygous control group. A similar increase was also seen in the Cldn-18 KO mice (Fig. 8 A).Furthermore, the cross sectional area of lumbar vertebrae 5 was also increased significantly in both Cldn-18 KO and heterozygous control mice fed with high dietary calcium compared to normal dietary calcium (Fig. 8 B).

The Effect of Dietary Calcium on Histomorphometric Parameters of Lumbar Vertebrae 5 of 10 Week Old Cldn-18 KO and Heterozygous Control Mice

Since the lumber BMD was dramatically increased in both Cldn-18 KO and heterozygous control mice fed a high Ca diet and the lack of Cldn-18 was previously reported to affect bone resorption, we performed histomorphometric analysis at lumbar vertebrae 5 and measured TRAP stained surfaces of the trabecular bone. Our analysis showed that the osteoclast surface to bone surface (Oc.S/BS) was decreased significantly in the high calcium Cldn-18 KO group compared to the normal calcium Cldn-18 KO group (Fig. 9). A similar trend was observed in the heterozygous control mice, as a high calcium diet decreased Oc.S/BS compared to a normal calcium diet; however, this reduction in Oc.S/BS was not statistically significant which could be due to either the low baseline resorption of control animals, or the small sample size examined (Fig. 9). Expectedly, Oc.S/BS was increased significantly in Cldn-18 KO mice fed a normal diet in comparison to heterozygous control mice fed a normal calcium diet (Fig. 9). Moreover, Oc.S/BS was significantly higher in the high calcium Cldn-18 KO group compared to the high calcium heterozygous control group. Therefore, these findings suggest that the high calcium diet did not rescue the increased bone resorption observed in the KO mice.

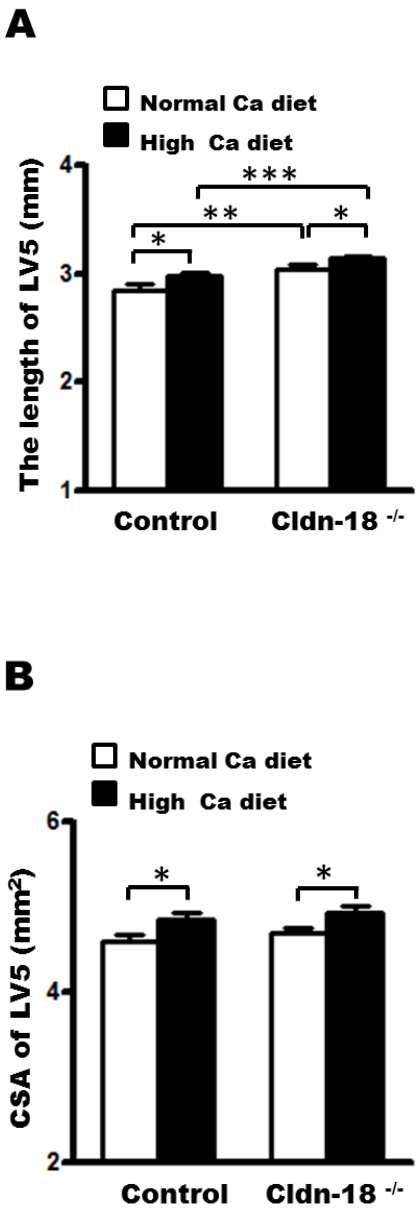

Figure 8. The effect of dietary calcium on bone size parameters of lumbar vertebrae 5 (LV5) in 10 week old Cldn-18 KO and heterozygous control mice. (A) The length of lumbar vertebrae 5. (B) The cross sectional area (CSA) calculated in the middle region of lumbar vertebrae 5 using the uCT (n=12-17/group, mixed gender). *: $p<0.05$; **: $p<0.01$; and ***: $p<0.001$.

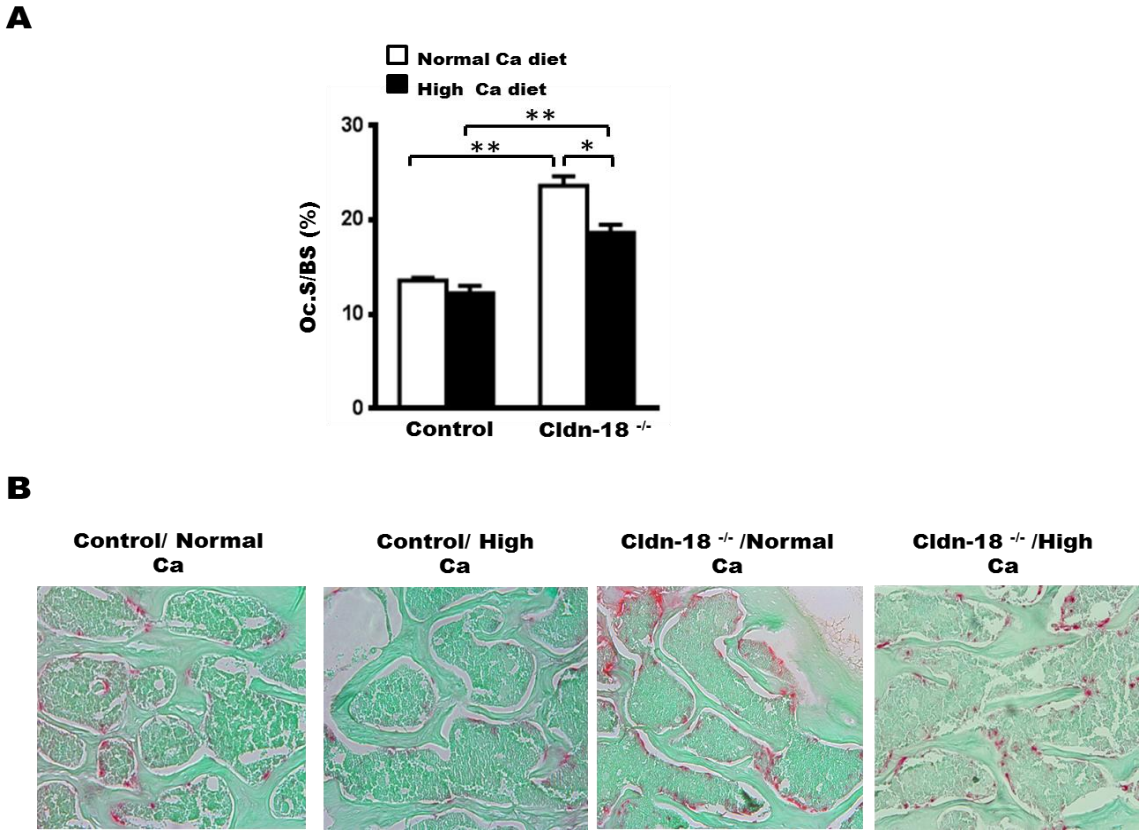

Figure 9. The effect of dietary calcium on lumbar vertebrae 5 TRAP labeled trabecular surface of Cldn-18 KO and heterozygous control mice at 10 weeks of age. (A) Osteoclast surface to bone surface (Oc.S/BS) was measured at trabecular bone of lumbar vertebrae 5 (n= 5-6/group, mixed gender). (B) Representative micrograph of lumbar vertebrae 5 sections stained with TRAP (10X). *: $p<0.01$; and **: $p<0.001$.

The Effect of Cldn-18 Deficiency on Acid Secretion by Osteoclasts

To test the possibility that increased bone resorption observed in Cldn-18 KO mice is due to increased acid secretion from mature osteoclasts, we measured mRNA levels of carbonic anhydrase II, chloride channel-7 (CLC-7), and H+ pump in primary osteoclasts derived from Cldn-18 KO and heterozygous control mice. The expression levels of carbonic anhydrase II, CLC-7, and H+ pump was up regulated in Cldn-18 KO compared to heterozygous control mice (Fig. 10). Because the number of TRAP positive multinucleated cells was greater in RANKL/MCSF treated BMM cultures-derived from Cldn-18 KO mice (21), we normalized mRNA levels in carbonic anhydrase II, CLC-7, and H+ pump to that of TRAPC5b mRNA, a well-established marker of osteoclast number. Upon normalization to expression levels of TRAPC5b, no significant change was observed in these "normalized" values between Cldn-18 KO and heterozygous control mice (Fig. 10). Additionally, the expression of calcitonin receptor was also not significantly different between the two genotypes after normalization to TRAPC5b mRNA levels (Fig. 10).

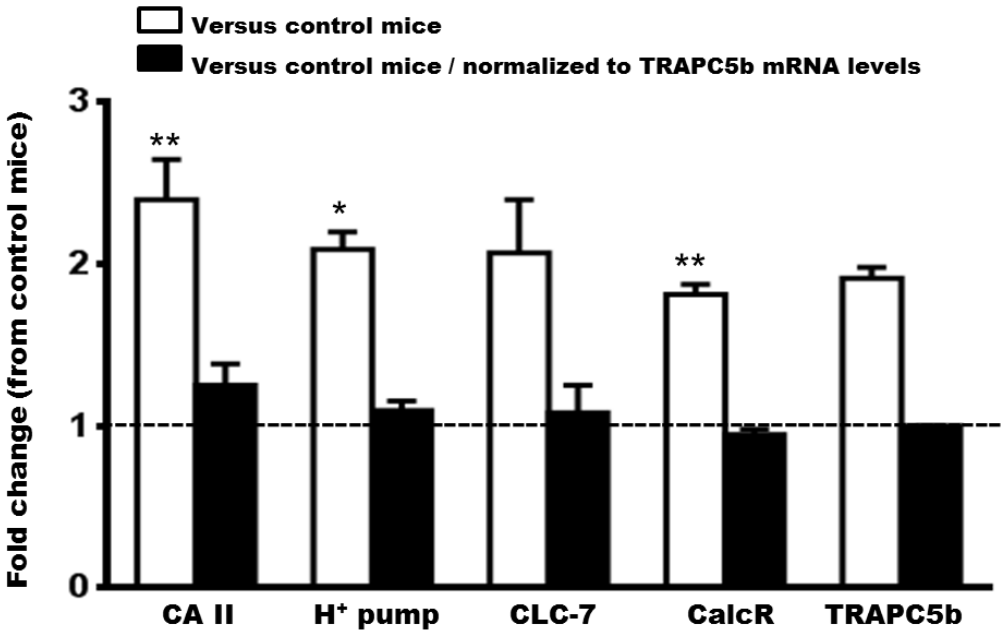

Figure 10. The effect of Cldn-18 deficiency on the expression of osteoclastic acid secreting machinery. The expression levels of carbonic anhydrase II (CA II), CLC-7, H^+ pump, calcitonin receptor (CalcR), and TRAPC5b were determined by real -time RT-PCR of mature osteoclasts derived from Cldn-18 KO and heterozygous control mice. Values are presented as fold change of control heterozygous mice (n=4-5/group). *: $p<0.05$; and **: $p<0.01$ versus heterozygous control mice.

Discussion

In this study, we found that the loss of Cldn-18 negatively affected gastric acidity in adult mice. Consistent with this observation, Sanada et al., have reported that Cldn-18 was down regulated in gastric cancer and atrophic gastritis (26). Furthermore, Hayashi et al., have recently demonstrated that Cldn-18 deficient mice developed atrophic gastritis and the gastric pH was significantly higher in Cldn-18 KO mice compared to WT mice at day 14 postnatally (16). Additionally, these authors found that the H+ leakage into the submucosal layer of gastric tissues was higher in Cldn-18 KO mice compared to WT mice, and was associated with the up-regulation of pro- inflammatory cytokines which in turn induced gastritis (16, 31). Furthermore, the expression of H+-K+ ATPase was found to be down regulated in Cldn-18 KO mice, which is caused by a decrease in the total number of well differentiated parietal and chief cells (16). Because Cldn-18 is a novel negative regulator of bone resorption, we tested the possibility that Cldn-18 deficiency affects acid secretion machinery in osteoclasts. We did not find significant differences in the expression levels of any of the three markers of acid secretion between Cldn-18 KO and heterozygous control mice after adjustment for differences in osteoclast number using TRAPC5b mRNA levels. Based on these and published data, it appears that the increased gastric pH (due to less acid secretion) observed in Cldn-18 KO mice is due to a defect in the H+ resistant paracellular barrier between gastric epithelial cells and a decrease in the total number of parietal cell induced by inflammation while the increased bone resorption in Cldn-18 KO mice is due to MCSF/RANKL signaling-induced increase in osteoclast number and not due to increased acid secretion per se per osteoclast. Further studies are, however, needed to verify these conclusions.

It is noteworthy that the role of gastric acidity in calcium absorption/metabolism has received more attention recently (5, 35). Calcium solubilization is thought to be prerequisite for calcium absorption in the small intestine, with the stomach's acidic environment inducing the dissolution of calcium salts and the release of ionized calcium (3, 29). To this end, several clinical studies reported a positive association between the long term use of acid suppressing agents and bone fractures (17, 33, 37). Moreover, short and long term use of acid suppressing agents decreased bone mineral density (1, 19). Although there is limited experimental evidence regarding the mechanism of acid suppressing agent-induced bone loss, it seems to be more related to the inhibitory effect of acid on calcium absorption (35). Recently, Schinke et al., have reported that mice deficient in cholecystokinin B-gastrin receptor that affects acid secretion by parietal cells, exhibited an osteoporotic phenotype, mediated by an alteration in calcium homeostasis (27). These authors also found that a high calcium diet fully rescued hypo-chlorhydria induced bone loss in these mice. Similarly, a high calcium diet is routinely used in the literature to correct calcium deficit induced by gene mutations (2, 11, 27). The efficacy of the dietary approach we employed in this study was in fact reported and validated in several animals model studies in which calcium absorption and homeostasis were perturbed. For example, the bone phenotype of vitamin D receptor deficient mice was completely rescued by feeding them a high calcium diet (2, 11). Additionally, several studies have demonstrated that feeding pregnant mothers with a high calcium diet significantly increases calcium content in the milk, thereby providing an effective means to correct calcium deficiency in the pups (8, 28). Furthermore, our data that areal BMD of vertebra is increased in high calcium diet groups of both genotypes and that serum

calcium levels were significantly higher in high calcium diet groups compared to normal calcium diet groups are in support of the notion that the approach that we used was effective in correcting calcium deficit in the Cldn-18 KO mice.

In agreement with our previous studies in which the serum PTH levels were found to be elevated in Cldn-18 KO mice fed a normal calcium diet compared to control mice, we found the serum calcium levels were lower in Cldn-18 KO mice compared to heterozygous control mice fed a normal calcium diet. In addition, the serum calcium levels were significantly higher in Cldn-18 KO mice fed a high calcium diet compared to a normal calcium diet. Moreover, bone resorption (Oc.S/BS) was lower in high calcium diet fed groups compared to normal calcium diet fed groups. Together these results suggest that Cldn-18 KO mice may be calcium deficient and are in support of the possibility that the high systemic calcium achieved by high calcium diet was effective in correcting the calcium deficit in Cldn-18 KO mice. Importantly, the finding that the high dietary calcium did not rescue the osteopenia phenotype in Cldn-18 KO mice supports the idea that low gastric acidity may not be responsible for the Cldn-18 deficiency mediated bone loss. In this regard, we have recently obtained evidence suggesting that the low BMD observed in Cldn-18 KO mice may derive from the lack of Cldn-18 in bone cells. Specifically, we have shown that Cldn-18 is expressed in osteoclasts and that lack of Cldn-18 increased osteoclast differentiation and bone resorption (22). Taken together, future studies will elucidate the direct role of Cldn-18 on bone cells using mice with a conditional disruption of Cldn-18 in osteoclasts.

We also found that various skeletal sites respond differently to increased dietary calcium. Thus, a high calcium diet increased lumbar BMD in both Cldn-18 KO and

heterozygous control mice compared to their corresponding genotypes on a normal calcium diet, whereas tibia and femur BMD were unaffected by dietary calcium in both genotypes. Consistent with our finding, Datta et al., showed an increase in lumbar vertebrae 5 BMD in both WT and phosphorylation deficient PTH1R knock-in mice fed a high calcium diet starting at 4 week of age that was stopped at 8 week of age compared to the genotype matched mice fed a normal calcium diet (12). On the other hand, these authors observed that femur BMD was unaffected by a high calcium diet in both of these genotypes (12). To our knowledge, our study is the first to document a positive association between spine BMD and a high calcium diet that is started at birth, in mice. In contrast to our results, it has been previously reported that tibia BMD increased in 3 week old WT and parathyroid hormone deficient mice fed by dams who received a high calcium diet compared to those on a normal calcium diet (8, 28). Skeletal site selectivity in response to calcium supplementation is well documented in human studies during childhood and adolescence, but the mechanism of this site specific effect remains to be elucidated (4, 9).

Although lumbar BMD increased significantly in response to a high calcium diet in both Cldn-18 KO and heterozygous control mice, we did not find a significant change in either the trabecular or cortical bone parameters at lumbar vertebrae 5, as measured by uCT. One explanation is the size difference in the lumbar vertebrae 5 between the high calcium diet group and the normal calcium diet. We also found that the loss of Cldn-18 in mice fed a normal calcium diet decreased the total body BMD, trabecular, and cortical bone parameters which was consistent with our previous report on the Cldn-18 deficient mice bone phenotype (22). However, increased dietary calcium intake in Cldn-18

deficient mice did not rescue this phenotype at different skeletal sites. Furthermore, the lumbar bone resorption (Oc.S/BS) was still significantly higher in Cldn-18 KO mice fed a high calcium diet compared to heterozygous control mice fed a high calcium diet. Collectively, correction of serum calcium deficit did not correct decreased BMD and increased bone resorption observed in Cldn-18 KO mice, thus ruling out the possibility that gastric abnormalities contributed to the osteopenia phenotype in these mice. While these data suggest a direct role of Cldn-18 in osteoclasts, more direct evidence from the use of osteoclast-specific Cldn-18 KO mice are warranted to convincingly demonstrate a non-canonical role of Cldn-18 in osteoclasts.

The limitations of this study are as follows. First, we did not measure the impact of changes in gastric pH in Cldn-18 KO mice on calcium absorption by directly measuring dietary calcium uptake using calcium isotopic tracers. Second, we used heterozygous mice lacking one functional allele of Cldn-18 based on the earlier finding that skeletal phenotype was not different between wild type and heterozygous mice (20) and based on the homozygous KO X heterozygous breeding strategy which yielded 50% KO and 50% heterozygous mice that were used as control. Third, we have not provided direct evidence using mice with conditional KO of Cldn-18 in osteoclasts to rule out any role for Cldn-18 expressed in the stomach for the osteopenia phenotype in Cldn-18 KO mice. Fourth, we did not measure serum PTH and calcitonin levels to check whether the enhanced lumber BMD and decreased bone resorption observed in both genotypes in response to high dietary calcium were due to either suppression of PTH and/or due to secretion of calcitonin. In this regard, the calcitonin receptor expression was not different after adjustment for differences in TRAP positive osteoclasts between the two genotypes.

In conclusion, we demonstrated that Cldn-18 deficiency negatively affects gastric acidity. In addition, we found that a high calcium diet increased lumbar BMD and decreased bone resorption in both Cldn-18 KO and heterozygous control mice, whereas correcting the deficit in serum calcium in Cldn-18 KO by feeding a high calcium diet did not correct the osteopenia phenotype and the increase in bone resorption. Therefore, these data suggest that the osteopenia phenotype observed in Cldn-18 KO mice is related to the changes in bone resorption rather than a deficit in calcium absorption.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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CHAPTER THREE

DIFFERENTIAL EXPRESSION OF CLAUDIN FAMILY MEMBERS DURING OSTEOBLAST AND OSTEOCLAST DIFFERENTIATION: CLDN-1 IS A NOVEL POSITIVE REGULATOR OF OSTEOBLASTOGENESIS

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Keywords: Claudins, bone, osteoblast, osteoclast, differentiation, proliferation, non-canonical.

Abstract

Claudins (Cldns), a family of 27 transmembrane proteins, represent major components of tight junctions. Aside from functioning as tight junctions, Cldns have emerging roles as regulators of cell proliferation and differentiation. While Cldns are known to be expressed and have important functions in various tissues, their expression and function in bone cells is ill-defined. In this study, the expression of Cldns was examined during osteoblast and osteoclast differentiation. The expression of Cldn-1, -7, -11, and -15 was downregulated during early stages of osteoclast differentiation, whereas Cldn-6 was upregulated. Moreover, the expression of several Cldns increased 3-7 fold in fully differentiated osteoclasts. As for osteoblasts, the expression of several Cldns was found to increase more than 10-fold during differentiation, with some peaking at early, and others at late stages. By contrast, only expression of Cldn-12, and -15 decreased during osteoblast differentiation. In subsequent studies, we focused on the role of Cldn-1 in osteoblasts as its expression was increased by more than 10 fold during osteoblast differentiation and was found to be regulated by multiple osteoregulatory agents including IGF-1 and Wnt3a. We evaluated the consequence of lentiviral shRNA-mediated knockdown of Cldn-1 on osteoblast proliferation and differentiation using MC3T3-E1 mouse osteoblasts. Cldn-1 knockdown caused a significant reduction in MC3T3-E1 cell proliferation and ALP activity. Accordingly, expression levels of cyclinD1 and ALP mRNA levels were reduced in Cldn-1 shRNA knockdown cells. We next determined if Cldn-1 regulates the expression of Runx-2 and osterix, master transcription factors of osteoblast differentiation, and found that their levels were reduced significantly as a consequence of Cldn-1 knockdown. Moreover, knocking down Cldn-1 reduced β-catenin level. In conclusion, the expression of Cldn family members during

bone cell differentiation is complex and involves cell type and differentiation stage-dependent regulation. In addition, Cldn-1 is a positive regulator of osteoblast proliferation and differentiation.

Introduction

Osteoporosis is a major public health problem in the U.S. that is characterized by low bone mass and structural deterioration of bone tissue, resulting in increased bone fragility [1,2]. It can result from failure to produce optimal bone mass during active growth periods and/or the imbalance between bone formation and bone resorption during bone remodeling processes [2]. It is well known that bone formation is mediated by osteoblasts, which are derived from mesenchymal stem cells [3]. On the other hand, bone resorption is mediated by osteoclasts, which are generated from hematopoietic stem cells derived from a macrophage/monocyte lineage [4,5]. In terms of osteoblast and osteoclast differentiation, such processes are divided into multiple stages and regulated by temporal and sequential expression of several genes [6-8]. Although a number of systemic hormones and local regulatory factors have been shown to regulate bone formation and resorption, our understanding of the molecular pathways that modulate the formation and function of osteoblasts and osteoclasts is, to date, still limited. Thus, the identification of novel genes that participate in regulating osteoblast and osteoclast differentiation and functions is imperative for advancing our understanding of the pathogenesis of osteoporosis.

Tight junctions, which are composed of several types of transmembrane proteins including Claudins (Cldns), junctional adhesion molecule, occludin, and tricellulin [9], are important in the development and maintenance of various tissues [10]. It is noteworthy that there is significant evidence suggesting that Cldns are the principal proteins responsible for the formation of tight junction strands [9-11]. The Cldn family of proteins is comprised of 27 members in mouse and human cells with a molecular weight

ranging from 20-34 kDa [10,12], and are divided into two major groups, i.e., "classic" and "non-classic" according to sequence analysis and functional properties of the mouse variants [13]. Moreover, Cldns exhibit complex patterns of expression that is tissue/cell type and developmental stage specific [10,14], where certain tissues/cells express several Cldns, and others express only one or two [10,15]. Interestingly, Cldns expression varies within the same tissue in a developmental/ differentiation stage dependent fashion [16]. In terms of their regulation, it has been demonstrated that the expression and function of Cldns is controlled by several transcription factors, hormones, and cytokines [14]. As for their function, Cldns act canonically as barriers/pores to regulate paracellular permeability of ions and small molecules, and serve as a fence that divides apical and basolateral domains of plasma membranes [14]. Recently, a distinct role for Cldns has emerged, in which they were shown to serve as mediators of cell signaling. Thus, a "non-canonical function" for Cldns was observed, as they were found to control cell proliferation and differentiation [14,17]. Even though progress has been made, our knowledge of the expression patterns and function(s) of many Cldn family members in various tissues, their expression, regulation, and role in bone remains ill defined.

It has been shown that bone cells (osteoblasts and osteocytes) form tight junctional structures [18-20], and that rat osteoblasts express several Cldns at the mRNA level [21]. Furthermore, recent work by our laboratory have documented that osteoclasts express Cldn-18 and that its deletion, in mice, results in an osteopenia phenotype, i.e., markedly decreased total body bone mineral density, cortical thickness, and trabecular volume [22]. Interestingly, the negative effect of a lack of Cldn-18 on the skeleton was found to be independent of tight junction functions and mediated by increased bone

resorption and osteoclast differentiation [23,24]. While our studies have laid down the foundation for the non-canonical function of Cldn-18 in regulating bone homeostasis, whether other Cldn family members are expressed and have a function during osteoblastogenesis and osteoclastogenesis remains to be investigated. We, therefore, undertook studies to evaluate the expression of Cldn family members during osteoblast and osteoclast differentiation at the mRNA level, and to examine the regulation and function of selected Cldns. Our findings demonstrate that both osteoblasts and osteoclasts express several Cldns and that their expression levels vary depending on cell type and differentiation stage. We have also determined Cldn-1 to be a positive regulator of osteoblast proliferation and differentiation, and is regulated by several osteoregulatory factors. In addition, knocking down Cldn-1 reduced β-catenin level, suggesting that it may be involved in Cldn-1 regulation of osteoblastogenesis.

Materials and Methods

Reagents

Ascorbic acid (AA) and β-glycerophosphate (βGP) were purchased from Sigma Chemicals (St. Louis, MO). Minimum essential medium α (α-MEM) and AA free α-MEM were from Life Technologies (Carlsbad, CA). Calf serum (CS) was purchased from Hyclone (Logan, UT). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). Wnt3a, recombinant macrophage colony stimulating factor (M-CSF), and receptor activator of nuclear factor kappa B ligand (RANKL) were from R&D systems (Minneapolis, MN, USA). IGF-1 was a gift from Upjohn pharmacia (Stockholm, Sweden). Cldn-1 specific polyclonal antibody was from cell signaling technology (Danvers, MA). Antibodies to β-actin and β-catenin, and the MISSION

shRNA lentiviral particles against Cldn-1 and control non-target short-hairpin RNA (shRNA) lentiviral particles were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture

Primary osteoclast precursor or bone marrow macrophages (BMMs) were isolated from femurs and tibias of C57BL/6J mice, as described previously [25], and maintained in α-MEM supplemented with 10% FBS, P/S, and MCSF (25 ng/ml). Twenty four hours later, non-adherent cells (BMMs) were collected and treated with M-CSF (25 ng/ml) and RANKL (50 ng/ml) for different time periods (0, 1, and 6 days); with fresh differentiation media added every 3 days. Primary calvarial osteoblasts were isolated from the calvarias of 3 days old C57BL/6J mice, as previously described [26]. To induce differentiation, calvarial osteoblasts were incubated with AA free α-MEM supplemented with 10% CS, penicillin (P, 100 units/ml), streptomycin (S, 100 ug/ml), βGP (10 mM), and AA (100 ug/ml) for different time periods (0, 4, 6, 8, 13, 19, and 24 days); with fresh differentiation media added every 3 days. All mice were housed in VA Loma Linda Health Care System VMU (Loma Linda, CA, USA) under standard approved laboratory conditions. All animal experiments were performed in compliance with and approved by the Institutional Animal Care and Use Committee. MC3T3-E1 mouse preosteoblast cells were grown in AA free α-MEM supplemented with 10% CS, and P/S. Twenty four hours prior to treatment, cells were incubated in a serum free medium containing AA free α-MEM, 0.1% bovine serum albumin (BSA), and P/S. Cells were then treated with different osteoregulatory agents (BMP-7, IGF-1, vitamin-D3, and Wnt3a) for 72 hrs. All the treatments were made in AA free α-MEM, 0.1% (BSA), and P/S. Both control short-

hairpin RNA (shRNA) and Cldn-1 shRNA MC3T3-E1 cells were grown in AA free α-MEM supplemented with 10% CS, and P/S. Prior to treatment, cells were incubated in a serum free medium containing AA free α-MEM, 0.1% bovine serum albumin (BSA), and P/S for 24 hrs. Subsequently cells were treated with βGP (10 mM) and AA (100 ug/ml) for 24 hrs and 6 days.

RNA Extraction and Gene Expression Analysis

RNA was extracted from primary cultures and MC3T3-E1 cells using Trizol and chloroform, and isolation was completed using E.Z.N.A.® RNA Isolation Kits (Omega Bio –Tek, Norcross, GA). The purity of RNA was determined by the ratio of the absorbance at 260 and 280 nm. Reverse transcription was accomplished using SuperScript® II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and the cDNA were used for real-time RT-PCR. The housekeeping gene peptidylpropyl isomerase A (PPIA) was used as an internal control in the PCR reaction and the fold change compared to control was calculated according to the formula $2^{-\Delta\Delta Ct}$. Primers used for real-time RT-PCR are listed in Table. 5.

Protein Extraction and Western Blotting

Primary calvarial osteoblasts were isolated from 3 days old mice and incubated with AA free α-MEM +10% CS+P/S+ βGP ± AA (100 ug/ml) for 6 days. Both control

Table 5. Primer sequences used in real-time RT-PCR

Gene	Forward (5'-3')	Reverse (5'-3')	Ref
PPIA	CCATGGCAAATGCTGGACCA	TCCTGGACCCAAAACGCTCC	[46]
ALP	ATGGTAACGGGCCTGGCTACA	AGTTCTGCTCATGGACGCCGT	[47]
Cyclin-D1	AATGTACTCTGCTTTGCTGAA	ATGAGACCACTAGAGGTCG	[24]
Osteocalcin	CTCTCTCTGCTCACTCTGCT	TTTGTAGGCGGTCTTCAAGC	[24]
Osterix	AGAGGTTCACTCGCTCTGACGA	TTGCTCAAGTGGTCGCTTCTG	[46]
Runx-2	AAAGCCAGAGTGGACCCTTCCA	ATAGCGTGCTGCCATTCGAGGT	[46]
TRAP	CACTCAGCTGTCCTGGCTCAA	CTGCAGGTTGTGGTCATGTCC	[24]
Cldn-1	GATGTGGATGGCTGTCATTG	CGTGGTGGGTAAGAGGT	[48]
Cldn-2	ATGTCCTCGCTGGCTTGTATTAT	GCCATGAAGATTCCAAGCAACTG	[49]
Cldn-3	CAGTGTACCAACTGCGTACAAGAC	ACCGGTACTAAGGTGAGCAGAG	[49]
Cldn-4	TCGTGGGTGCTCTGGGGAT	GCGGATGACGTTGTGAGCG	[49]
Cldn-5	GCTGGCGCTGGTGGCACTCTTT	GCGAACCAGCAGAGCGGCAC	[49]
Cldn-6	TGCCCACTCTATCATCCAGGACTTC	AGGCCTGAGGCTGCCCAG	[50]
Cldn-7	TTTCATTGTGGCAGGTCTTG	CCAGAAGGACCAGAGCAGAC	[51]
Cldn-8	GGCAACCTACGCTCTTCAAA	CAGGGAGTCGTAGACCTTGC	[51]
Cldn-9	AAGAGAGAACTGGGGGCTTC	AACGGGAAGGGATGGAGTAG	[51]
Cldn-10	ATCTGCGTTACCGATTCCAC	GATCTGAGCCTCCGACTTTG	[52]
Cldn-11	CTGCCGAAAAATGGACGAACTG	TGCACGTAGCCTGGAAGGATGA	[50]
Cldn-12	ACTGCTCTCCTGCTGTTCGT	TGTCGATTTCAATGGCAGAG	[51]
Cldn-13	TAGTGTTGGCCTTCTGATGC	AGCCAAGCAATGGGTTAAAG	[53]
Cldn-14	GCTCCTAGGCTTCCTGCTTA	CTGGTAGATGCCTGTGCTGT	[53]
Cldn-15	CATCTTTGAGAACCTGTGGTACAGC	GATGGCGGTGATCATGAGAGC	[54]
Cldn-16	ATTCATCACCCTGCTCCTTG	AGAGGAGCGTTCGACGTAAA	[51]
Cldn-17	TCGTTCTGATTCCAGTGTCC	TCCTCCAAGTTCTCGCTTCT	[53]
Cldn-19	CCCAGCACTCCTGTCAATG	GTGCAGCAGAGAAAGGAACC	[55]
Cldn-20	GGTACACCAAGGAGATCATAGCG	TACAGGGCTCCTCCAGGTTCATA	[54]
Cldn-22	CTTCCGAACGGCAACGCA	CCTCCCGACTTCCTCCTGG	[56]
Cldn-23	ACAGGGACACCAGCAAGCTCAA	CGGAGTCACAGGGCAGCGAA	[53]

shRNA and Cldn-1 shRNA MC3T3-E1 cells were grown in AA free α-MEM supplemented with 10% CS, and P/S. Prior to treatment, cells were incubated in a serum free medium containing AA free α-MEM, 0.1% bovine serum albumin (BSA), and P/S for 24 hrs. Subsequently, cells were treated with βGP (10 mM) and AA (100 ug/ml) for 48 hrs. Whole cell extracts were prepared as previously described [24]. Equivalent amounts of protein were resolved on 10% SDS polyacrylamide gel under denaturing conditions and were transferred to a PVDF membrane (Millipore, Billerica, MA). Membranes were blocked with 10% non-fat dry milk dissolved in Tris buffered saline with 0.1% Tween-20 overnight with rotation at 4°C. The following day, membranes were probed with Cldn-1, β-catenin, and β-actin antibodies for 1 hr at room temperature. The membranes were washed and probed with appropriate horseradish peroxidase conjugated secondary antibody (1:15,000; Sigma-Aldrich; St. Louis, MO). Chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA) was used to detect the bands.

Lentivirus Transduction

MC3T3-E1 cells were transduced with control shRNA or Cldn-1 shRNA lentiviral particles using a multiplicity of infection (MOI) of 10 in the presence of hexadimethrine bromide (8ug/ml), as previously described [27]. The following day, the lentiviral particles containing media was changed with fresh media, followed by puromycin selection (10 ug/ml) for 7 days. The knockdown efficiency was evaluated by real-time RT-PCR and western blot.

Proliferation Assay

Cells were plated at 3,000 cells/well in 96-well plates with α-MEM (AA free) + 10% CS +P/S. The following day, the medium was changed to serum free α-MEM (AA free) + 0.1% BSA for 24 hrs, before the cells were treated with βGP ± AA for 48 hrs, rinsed with PBS and frozen at -80C°. Cell proliferation was assessed using a CYQUANT© cell proliferation kit as per the manufacturer`s instructions (Life Technologies, Carlsbad, CA). Fluorescence measurements were made using a microplate reader with excitation at 485 nm and emission detection at 530 nm.

Alkaline Phosphatase (ALP) Activity Assay

Cells were plated at 5,000 cells/well in 96-well plates with α-MEM (AA free) + 10% CS +P/S. The following day, the medium was changed to serum free α-MEM (AA free) + 0.1% BSA for 24 hrs. The cells were then treated with βGP ± AA for 72 hrs, before being rinsed with PBS and permeabilized with ALP activity buffer containing 0.1% triton X-100 in 250 mM NaHCO3. Protein concentration was determined by the BCA method (Thermo Fisher Scientific; Waltham, MA) and ALP activity was detected as previously described [28].

ALP Staining

Cells were incubated with AA free α-MEM +10% CS+P/S+ βGP ± AA (100 ug/ml) for 6 days. Cells were washed with PBS, fixed with 10% formalin for 20 minutes at room temperature, and incubated at 37C° for 30 min in staining buffer containing 50 mM Tris-HCL, pH 8.6, 100 mM NaCl, 5 mM KCl, 1 mM MgCl2, 0.8 mg/ml naphthol

AS-TR phosphate, and 0.6 mg/ml fast red violet LB diazonium (Sigma Aldrich, St. Louis, MO). Quantification of ALP-stained areas was measured with ImageJ software.

Statistical Analysis

Data are expressed as the mean ± SEM (n=4-8) and were analyzed using one-way ANOVA (GraphPad Prism6) for the expression of Cldns during osteoblast and osteoclast differentiation and the Student`s t test (Microsoft Excel) for all other experiments. Newman- Keuls was used as a post hoc test.

Results

Expression Profiles of Cldn Family Members During Osteoclast Differentiation

To examine the expression of Cldns during osteoclast differentiation, we used primary osteoclast precursors isolated from bone marrow (i.e., BMMs) and treated them with MCSF and RANKL for different time periods (0, 1, and 6 days). Firstly, osteoclast differentiation was verified by measuring the bone resorption marker gene, TRAP, which is known to increase as osteoclasts differentiate (Fig. 11) [4]. Our findings indicated that some of the "Classic" Cldns including Cldns-1, -7, and -15 were downregulated during early stages (day 1) of osteoclast differentiation, whereas Cldn-6 was upregulated. As for the expression of "Classic" Cldns, several of them (i.e., -1, -2, -3, -5, -6, -15, and -17), were found to be increased 3-7 fold in fully differentiated osteoclasts compared to undifferentiated osteoclasts (day 6 vs. day 0; Fig. 12). As for the remaining "Classic"

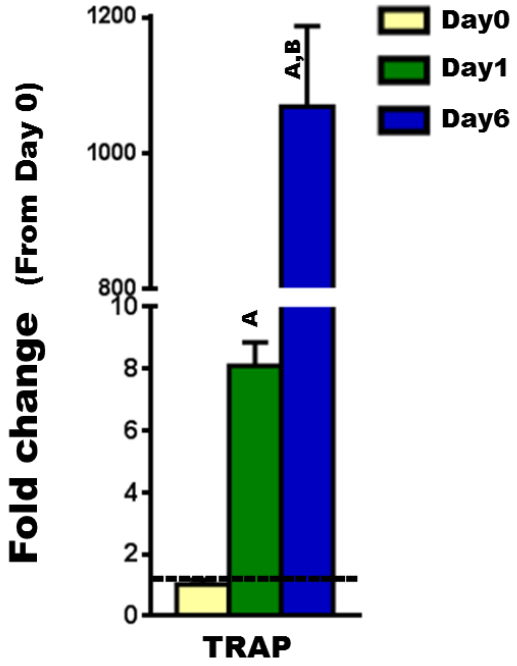


Figure 11. The expression level of tartrate resistant acid phosphatase (TRAP), an osteoclastogenic marker gene, during RANKL induced osteoclast differentiation. The expression level of TRAP during primary osteoclast differentiation was evaluated by real time RT-PCR, at different time points (0, 1, and 6 days). Values are (means ± SEM; n = 4) presented as fold change from day 0 (untreated cells); data were analyzed using one-way ANOVA (GraphPad Prism6). A = < 0.05 (from day 0), and B = < 0.05 (from day 1).

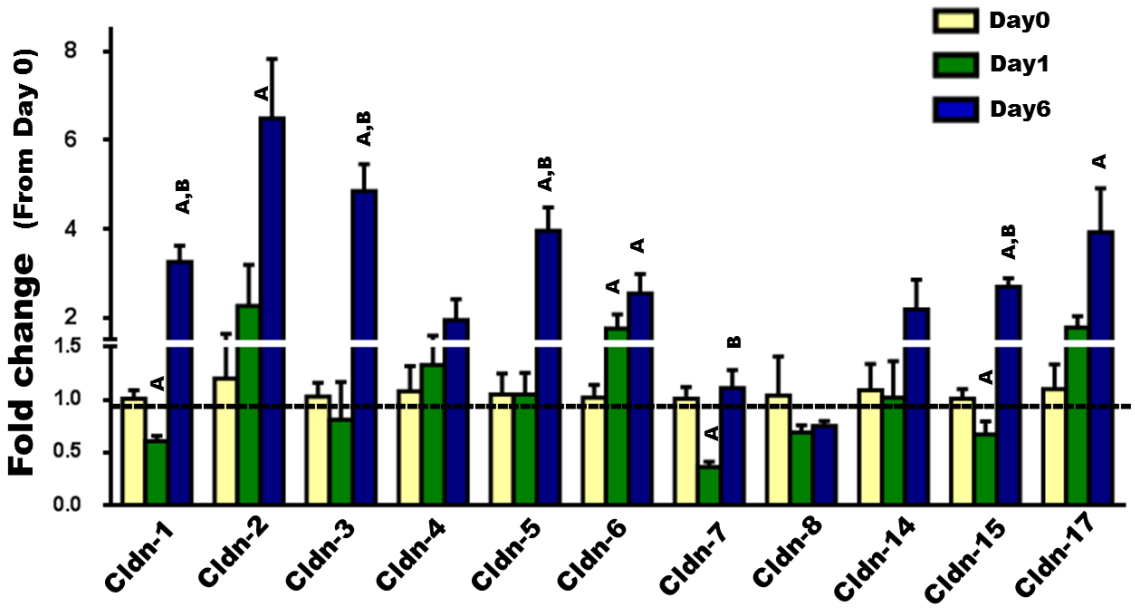


Figure 12. The expression level of "classic" Cldns during RANKL induced osteoclast differentiation. The expression level of "classic" Cldns (Cldn 1-8, -14, -15, and -17) during primary osteoclast differentiation was evaluated by real time RT-PCR, at different time points (0, 1, and 6 days). Values (means ± SEM; n = 4) are presented as fold change from day 0 (untreated cells)), data were analyzed using one-way ANOVA (GraphPad Prism6). A = < 0.05 (from day 0), and B = < 0.05 (from day 1).

Cldns (-4,-8, and -14), no significant change in their expression levels was observed (Fig. 12). Of the "non-classic" Cldns, Cldn-11 was downregulated during the early stage (day 1) of osteoclast differentiation; Cldn-12, -13, and -22 were upregulated during late stages (day 6); while no changes in Cldn-20 and -23 mRNA levels were detected (Fig. 13). Taken together, these findings suggest that Cldns are differentially expressed and highly regulated during osteoclast differentiation.

Expression Profiles of Cldn Family Members During Osteoblast Differentiation

To investigate the expression of Cldns during osteoblast differentiation, primary osteoblasts were isolated from calvarias of 3 days old mice and differentiated with AA for different time periods (0, 4, 6, 8, 13, 19, and 24 days). The expression of Cldns was then evaluated by real-time RT-PCR. We first confirmed osteoblast differentiation by measuring osteoblast marker genes such as ALP and osteocalcin, which are known to increase, in a time-dependent manner, as osteoblasts differentiate (Fig. 14) [29]. Given that Cldns are divided into "classic" and "non-classic" groups [13], for the sake of simplicity and ease of comprehension, the "classic" ones were subdivided into three clusters based on their phylogenetic tree close proximity, as follows: (1) Cluster A, which includes Cldn-3, -4, -5, -6 , -8, -9, and -17; (2) Cluster B, which includes Cldn-1, -2, -7, -14, and -19; and (3) Cluster C, which includes Cldn-10 and -15. On the other hand, the less similar/related Cldns were classified as "non-classic", and include Cldn-11, -12, -13, -16, -18, -20, -22, and -23. Our results revealed that the expression levels of all "Classic" Cldns were upregulated during osteoblast differentiation, except Cldn-15 which was found to be downregulated (Fig. 15A, B, C). Interestingly, expression levels of Cldn-1,-2,

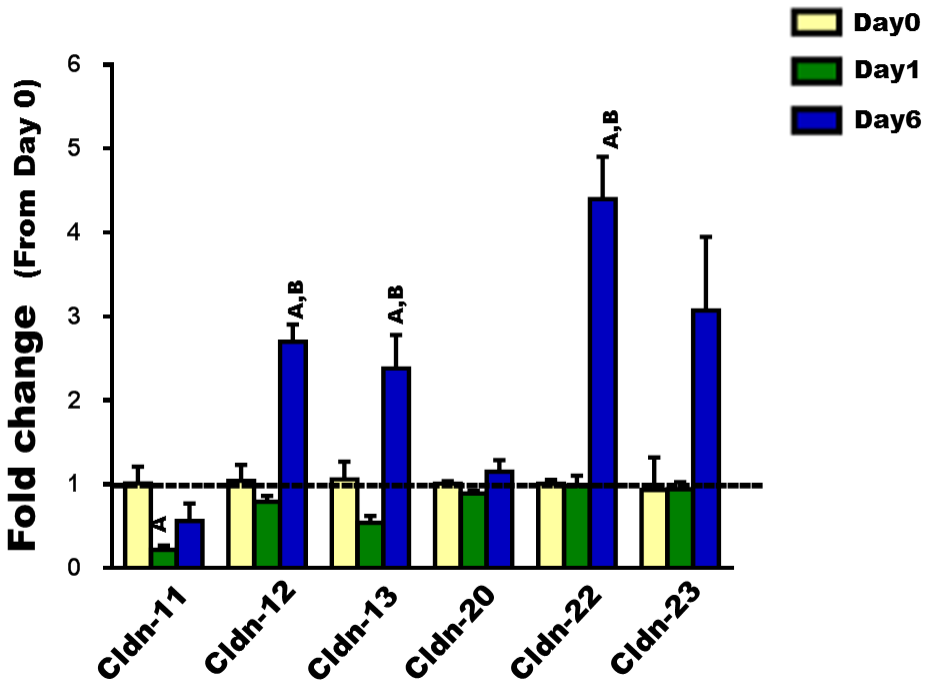


Figure 13. The expression level of "non-classic" Cldns during RANKL induced osteoclast differentiation. The expression level of "non-classic" Cldns (Cldn 11-13, -20, -22, and -23) during primary OC differentiation was evaluated by real time RT-PCR, at different time points (0, 1, and 6 days). Values (means ± SEM; n = 4) are presented as fold change from day 0 (untreated cells), data were analyzed using one-way ANOVA (GraphPad Prism6). A = < 0.05 (from day 0), and B = < 0.05 (from day 1).

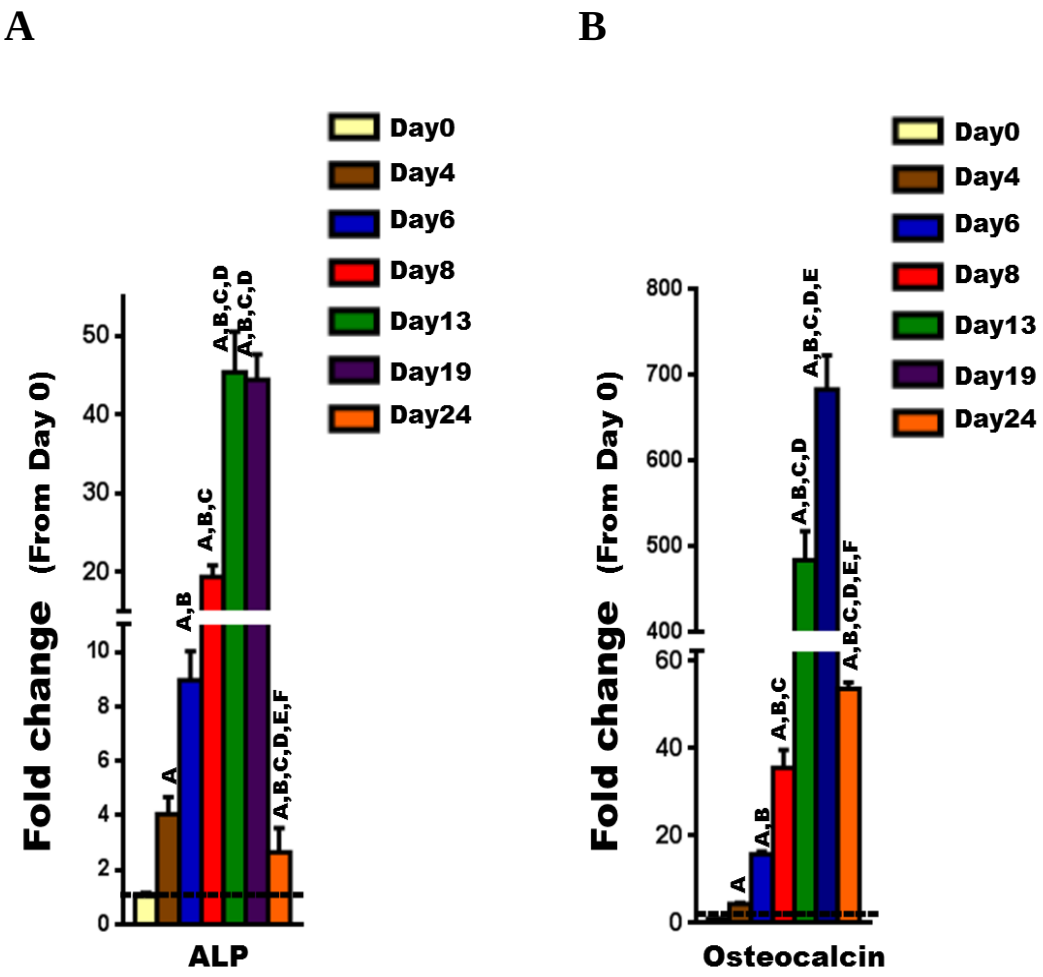
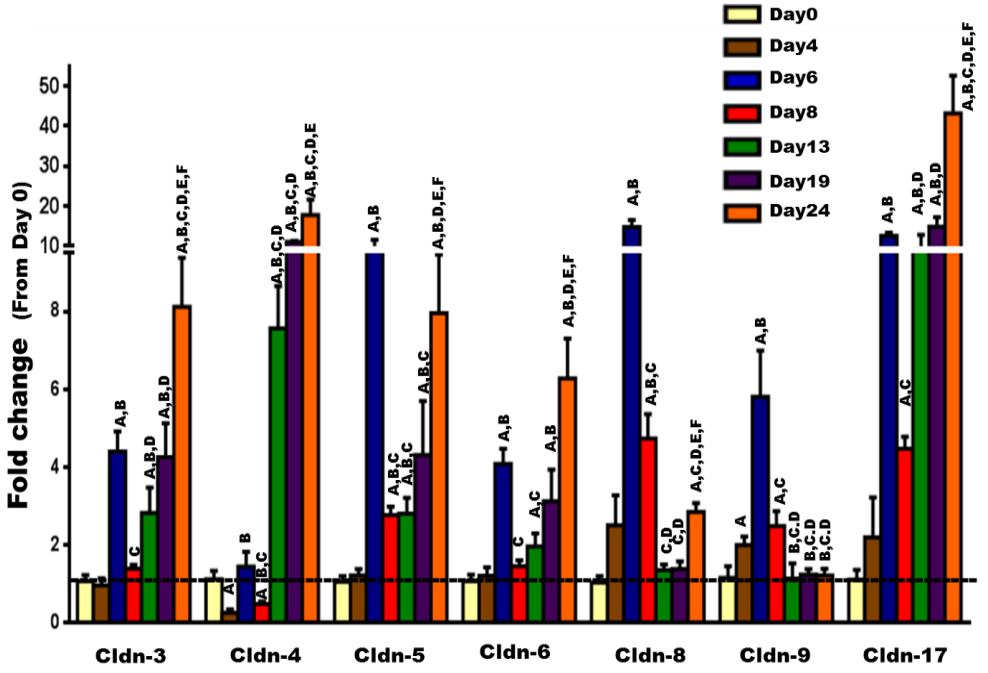


Figure 14. The expression level of osteoblastogenic marker genes during ascorbic acid induced osteoblast differentiation. A) The expression of alkaline phosphatase (ALP), an early stage maker gene of osteoblast differentiation, as determined by real time RT-PCR. B) The expression of osteoclacin, a late stage maker gene of osteoblast differentiation, as determined by real time RT-PCR. Values (means ± SEM; n = 6) are presented as fold change from day 0 (untreated cells), data were analyzed using one-way ANOVA (GraphPad Prism6). A= < 0.05 (from day 0), B = < 0.05 (from day 4), C = < 0.05 (from day 6), D = < 0.05 (from day 8), E = < 0.05 (from day 13), and F = < 0.05 (from day 19).

A


Day0
Day4
Day6
Day8
Day13
Day19
Day24
Fold change (From Day 0)
Cldn-3
Cldn-4
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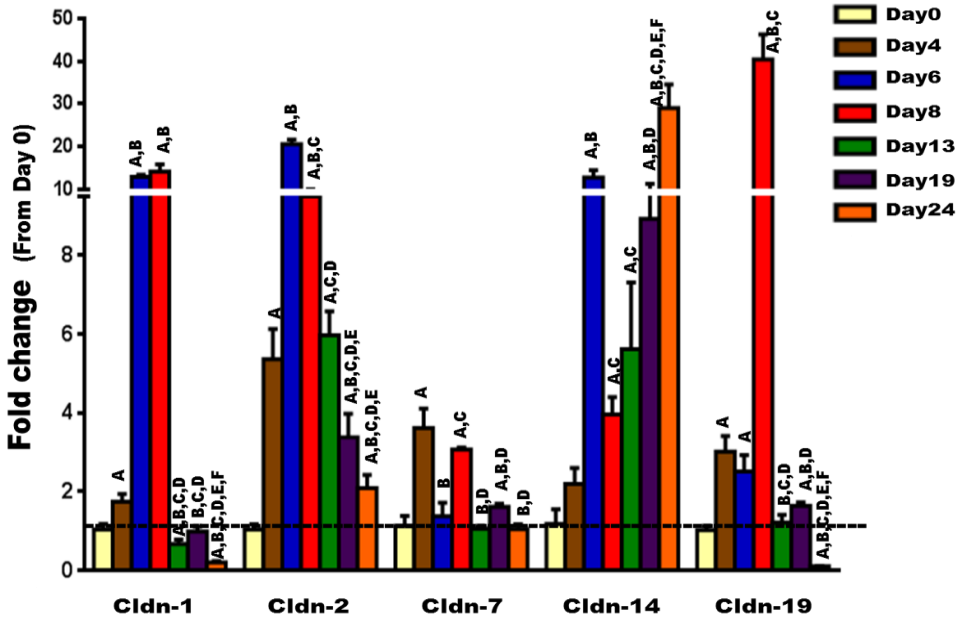
Cldn-6
Cldn-8
Cldn-9
Cldn-17

B


Day0
Day4
Day6
Day8
Day13
Day19
Day24
Fold change (From Day 0)
Cldn-1
Cldn-2
Cldn-7
Cldn-14
Cldn-19

C

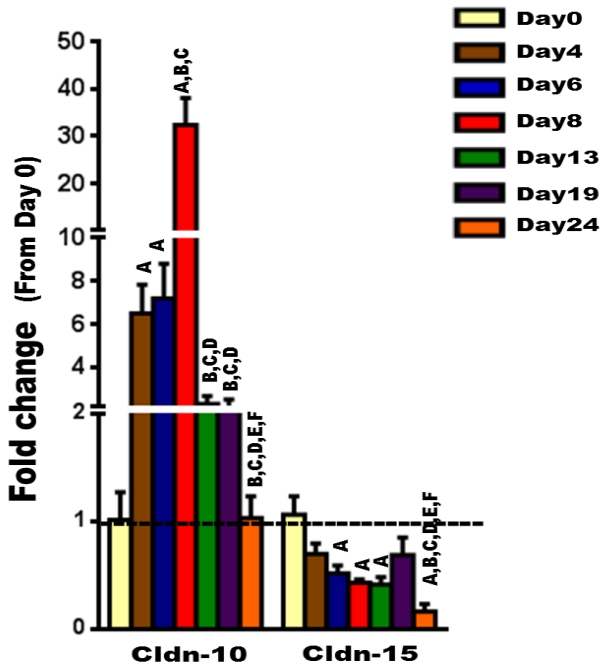


Figure 15. The expression level of "classic" Cldns during ascorbic acid induced osteoblast differentiation. The expression level of "classic" Cldns during primary osteoblast differentiation was measured by real time RT-PCR, at different time points (0, 4, 6, 8, 13, 19, 24 days). A) The expression level of cluster A of the "classic" Cldns, which includes Cldn-3, -4, -5, -6 , -8, -9, and -17. B) The expression level of cluster B of the "classic" Cldns, which includes Cldn-1, -2, -7, -14, and -19. C) The expression level of cluster C of the "classic" Cldns, which includes Cldn-10 and -15. Values (means ± SEM; n = 6) are presented as fold change from day 0 (untreated cells) , data were analyzed using one-way ANOVA (GraphPad Prism6). A = < 0.05 (from day 0), B = < 0.05 (from day 4), C = < 0.05 (from day 6), D = < 0.05 (from day 8), E = < 0.05 (from day 13), and F = <0.05 (from day 19).

-5, -7 to -10, and -19 were increased significantly during early stages of osteoblast differentiation, i.e., peaked between day 6 and 8, whereas the mRNA levels of Cldn-3, -4, -6, -14, and -17 peaked at late stages (Fig. 15A, B, C). It is noteworthy that several "classic" Cldns were found to be upregulated by more than 10 fold, at certain stages of osteoblast differentiation, including Cldn-1, -2, -4, -5, -8, -10, -14, -17, and -19 (Fig. 15A, B, C).

Regarding the "non-classic Cldns", our results indicate that they exhibit a diverse expression profile. While the expression levels of Cldn-11, -13, -22, and -23 were increased significantly during osteoblast differentiation and peaked during either early or late stages (Fig. 16), those of Cldn-12 decreased significantly (Fig. 16). Moreover, no change in the expression level of Cldn-20 was observed during osteoblast differentiation (Fig. 16). Of note, the mRNA of Cldn-16 was found to be undetectable by real-time RT-PCR, at least under our experimental conditions. Collectively, these data suggest that the regulation of expression of Cldns during osteoblast differentiation is complex and differentiation stage dependent.

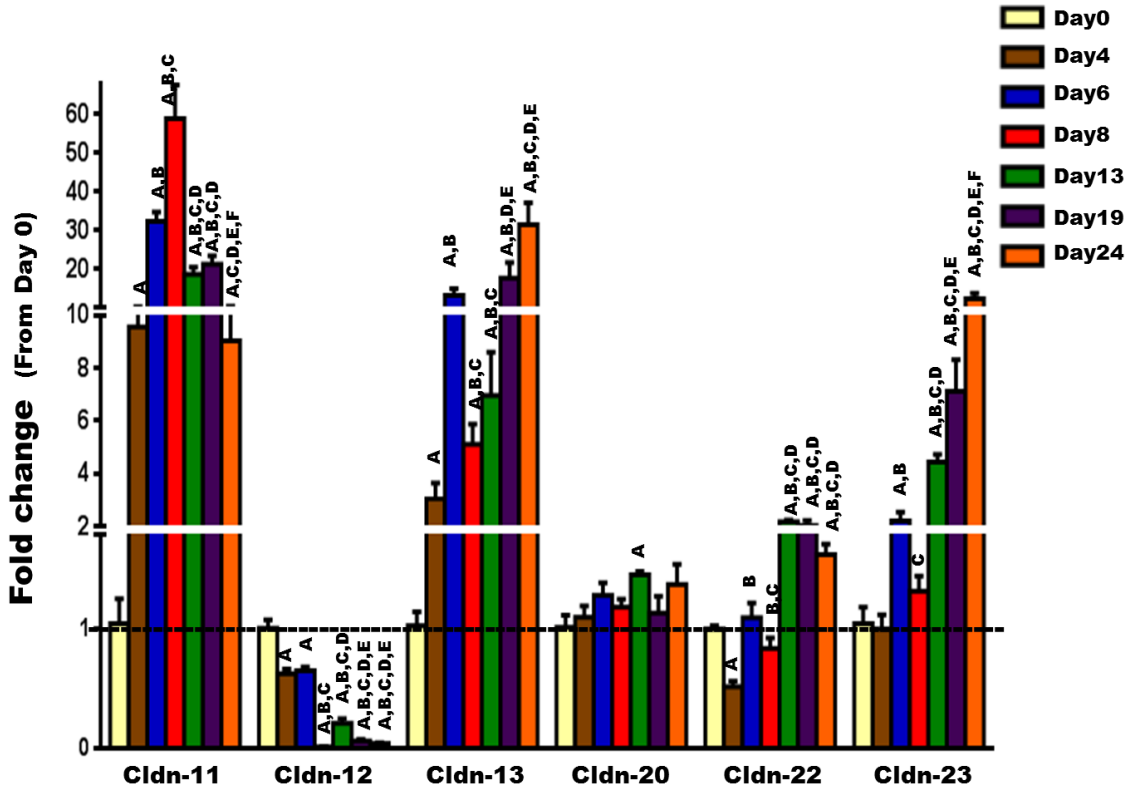


Figure 16. The expression level of "non-classic" Cldns during ascorbic acid induced osteoblast differentiation. The expression level of "non-classic" Cldns during primary osteoblast differentiation was evaluated by real time RT-PCR at different time points (0, 4, 6, 8, 13, 19, 24 days). Values (means ± SEM; n = 6) are presented as fold change from day 0 (untreated cells), data were analyzed using one-way ANOVA (GraphPad Prism6). A = < 0.05 (from day 0), B = < 0.05 (from day 4), C = < 0.05 (from day 6), D = < 0.05 (from day 8), E = < 0.05 (from day 13), and F = < 0.05 (from day 19).

Regulation of Cldn-1 Expression

Among the several Cldns expressed in osteoblasts, Cldn-1 is of particular interest because it is known to be regulated by the key bone formation regulator IGF-1 [30], and its expression, as shown by our data, increased by more than 10 fold during osteoblast differentiation (Fig. 15B). We next sought to confirm that the increase in mRNA level of Cldn-1 during early stages (between day 6 and 8) of osteoblast differentiation was commensurate with an increase at the protein level using western blot analysis. Figure 17 shows that AA treatment for 6 days caused a 2-fold increase in Cldn-1 protein levels in newborn mouse calvarial osteoblasts. Osteoblasts are known to produce a number of growth factors which act in an autocrine/paracrine manner to regulate proliferative and differentiative functions [8]. In order to determine if bone growth factors regulate Cldn-1 expression, Cldn-1 mRNA level was measured after treating MC3T3-E1 mouse preosteoblast cells with different osteoregulatory agents (BMP-7, IGF-1, vitamin-D3, and Wnt3a) for 72 hours. As expected and previously reported, IGF-1 (30 ng/ml) treatment increased the expression of Cldn-1 in MC3T3-E1cells [30], whereas treatment with Wnt3a (10 ng/ml) decreased Cldn1 expression by 80% (Fig. 18). On the other hand, Cldn-1 expression was not affected by BMP-7 (30 ng/ml) or vitamin-D3 (10 nM) treatment (Fig. 18).

The Effect of Knocking Down Cldn-1 Expression on MC3T3-E1 Cell Proliferation and Differentiation

Cldn-1 is known to regulate cell proliferation and differentiation in a variety of cell types [31-33]. Therefore, the role of Cldn-1 in regulating osteoblast proliferation and

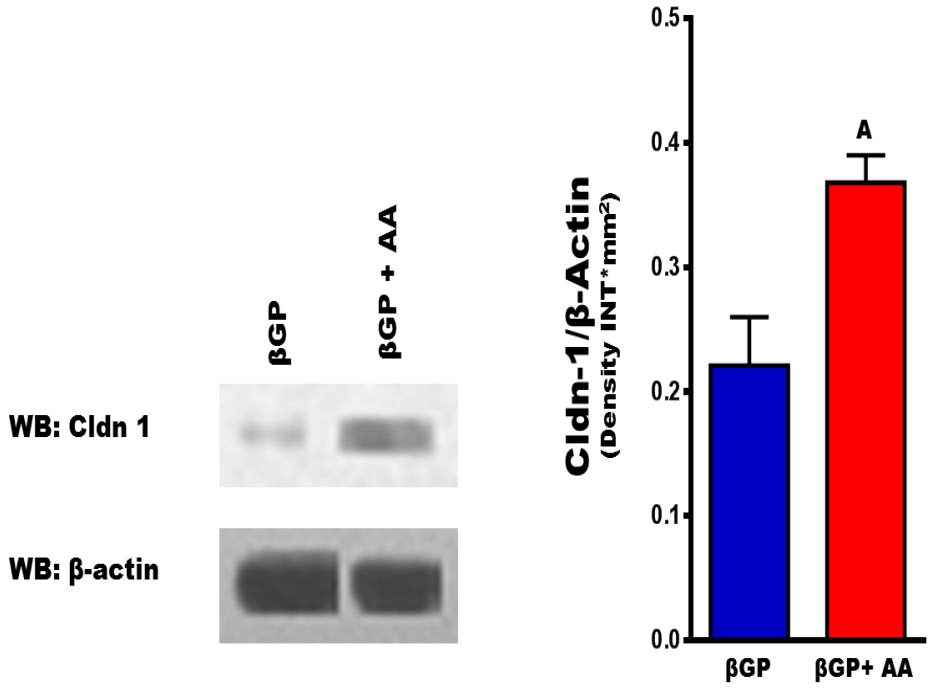


Figure 17. Cldn-1 protein levels during early stages of ascorbic acid induced osteoblast differentiation. Primary osteoblasts isolated from calvarias were treated with βGP ± AA for 6 days, before Cldn-1 expression was evaluated by western blotting. Values (means ± SEM; n = 4). A = < 0.05 vs. untreated cells (βGP alone).

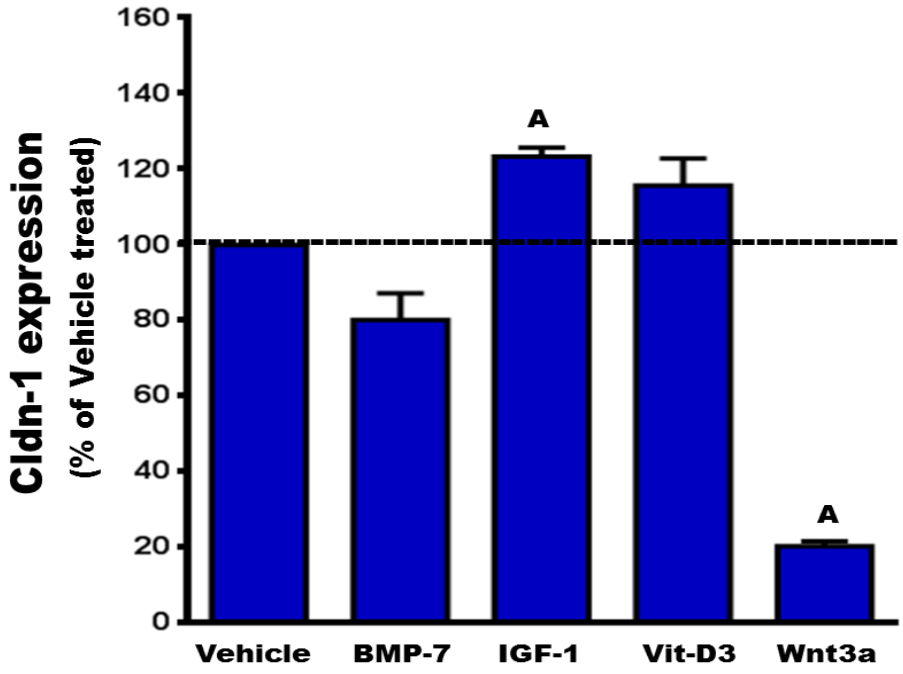


Figure 18. The regulation of Cldn-1 expression. MC3T3-E1 cells were treated with vehicle, BMP-7, IGF-1, vitamin-D3 (Vit-D3), and Wnt3a for 72 hrs, before the expression level of Cldn-1 was examined by real time RT-PCR. Values (means ± SEM; n = 4) are represented as % vehicle-treated control. A = < 0.05 vs. vehicle control.

differentiation was determined by knocking it down, using lentivirus shRNA, in MC3T3-E1 cells. First, we confirmed that MC3T3-E1 cells express Cldn-1 and that its expression was increased by 3 fold ($P<0.01$) by AA treatment at day 6 (data not shown). It was observed that Cldn-1expression was significantly decreased at both the mRNA and protein levels, in cells expressing Cldn-1 shRNA compared to control shRNA (Fig. 19A, B). It was found that cell proliferation was reduced by 18% and 13%, compared to control shRNA cells, when treated with βGP or AA, respectively (Fig. 19C). Moreover, in agreement with reduced cell proliferation in Cldn-1 shRNA cells, the expression of the osteoblast proliferation marker gene cylcin-D1 was reduced by 41% (Fig 19D).

Given that Cldn-1 expression is upregulated during early stages of osteoblast differentiation, we examined whether it plays a role in regulating expression of osteoblast differentiation markers. Knockdown of Cldn-1 expression in MC3T3-E1 cells reduced ALP activity by 61% (Fig. 20A). AA treatment caused a 4-fold increase in ALP activity in control shRNA treated cells, as expected. However, the effect of AA on ALP activity was reduced by 80% upon Cldn-1 knockdown (Fig. 20A). Consistent with these data, inhibition of Cldn-1 reduced ALP staining, in cells treated with AA (Fig. 20B).

To further characterize the positive effect of Cldn-1 on osteoblast differentiation, mRNA levels of osteoblast specific differentiation markers were determined in control and Cldn-1 shRNA cells, treated with or without AA. While the mRNA levels of ALP and bone sialoprotein were not found to be significantly decreased in Cldn-1 shRNA compared to control 24 hrs after AA treatment, their levels were decreased by 92% 6 days after AA treatment (Fig. 20C). Even though knocking down Cldn-1 appeared to reduce osteoclacin expression (a late osteoblast differentiation marker gene) at these time

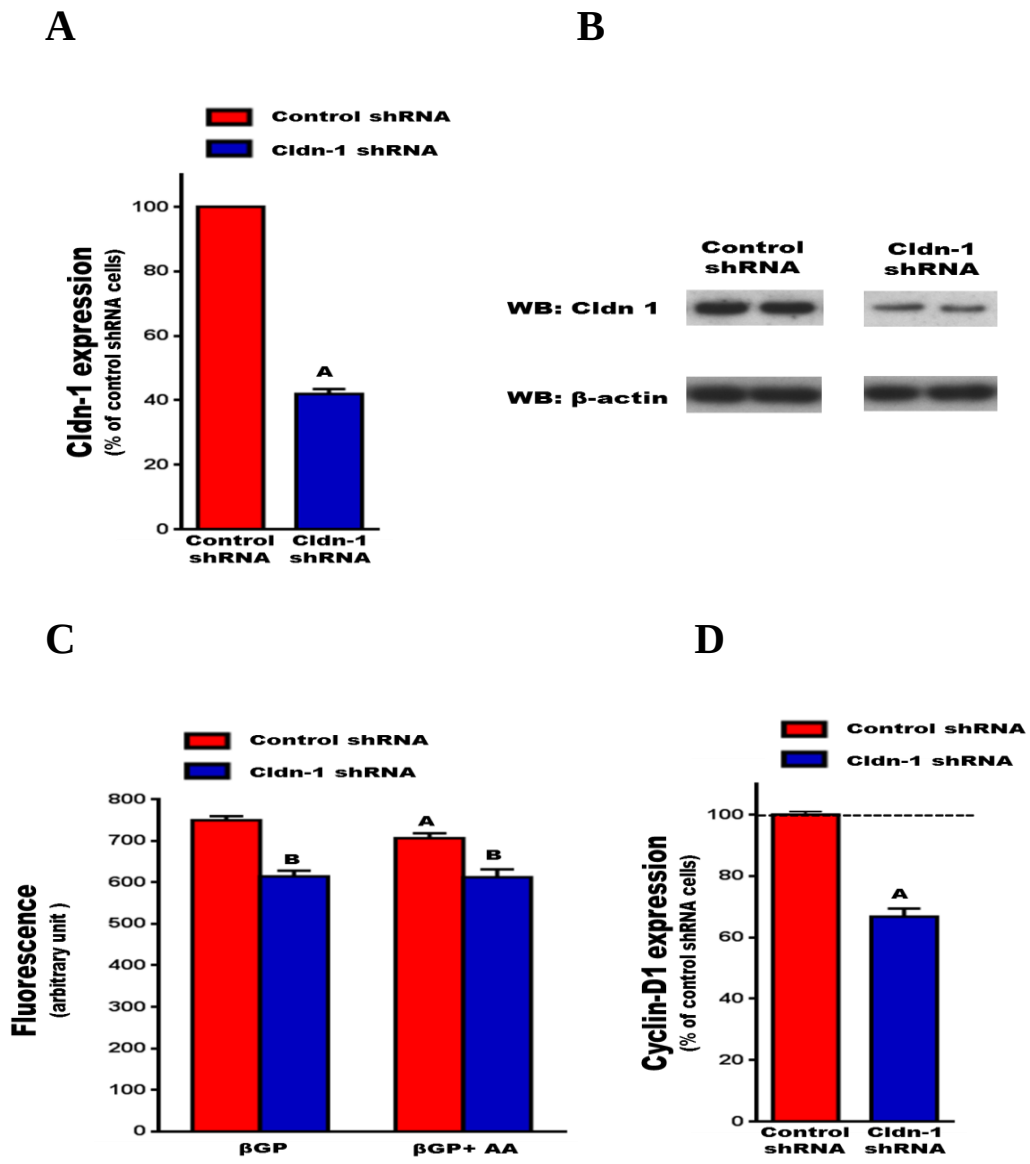


Figure 19. The effect of Cldn-1 knockdown on osteoblast proliferation in MC3T3-E1 cells: A) Cldn-1 expression in MC3T3-E1 cells transduced with control shRNA or Cldn-1 shRNA as determined by real time RT-PCR. Values (means ± SEM; n = 4). A = < 0.0001 vs. control shRNA. B) Cldn-1 protein level in MC3T3-E1 cells transduced with control shRNA or Cldn-1 shRNA as determined by western blot using whole cell lysates. C) Cell proliferation: MC3T3-E1 cells transduced with control or Cldn-1 shRNA and treated with βGP ± AA for 48 hrs, before cell proliferation was assessed using the CYQUANT© cell proliferation kit by measuring fluorescence after excitation at 485 nm and by emission detection at 530 nm. Values (means ± SEM; n = 8). A = < 0.05 vs. βGP treated, and B = <0.05 vs. control shRNA at corresponding treatment. D) The expression of a cell proliferation marker gene (cyclin-D1) in MC3T3-E1 cells transduced with control shRNA or Cldn-1 shRNA and treated with βGP ± AA for 24 hrs, as determined by real time RT-PCR. Values (means ± SEM; n = 4) are presented as % of control shRNA. A=<0.05 vs. control shRNA cells.

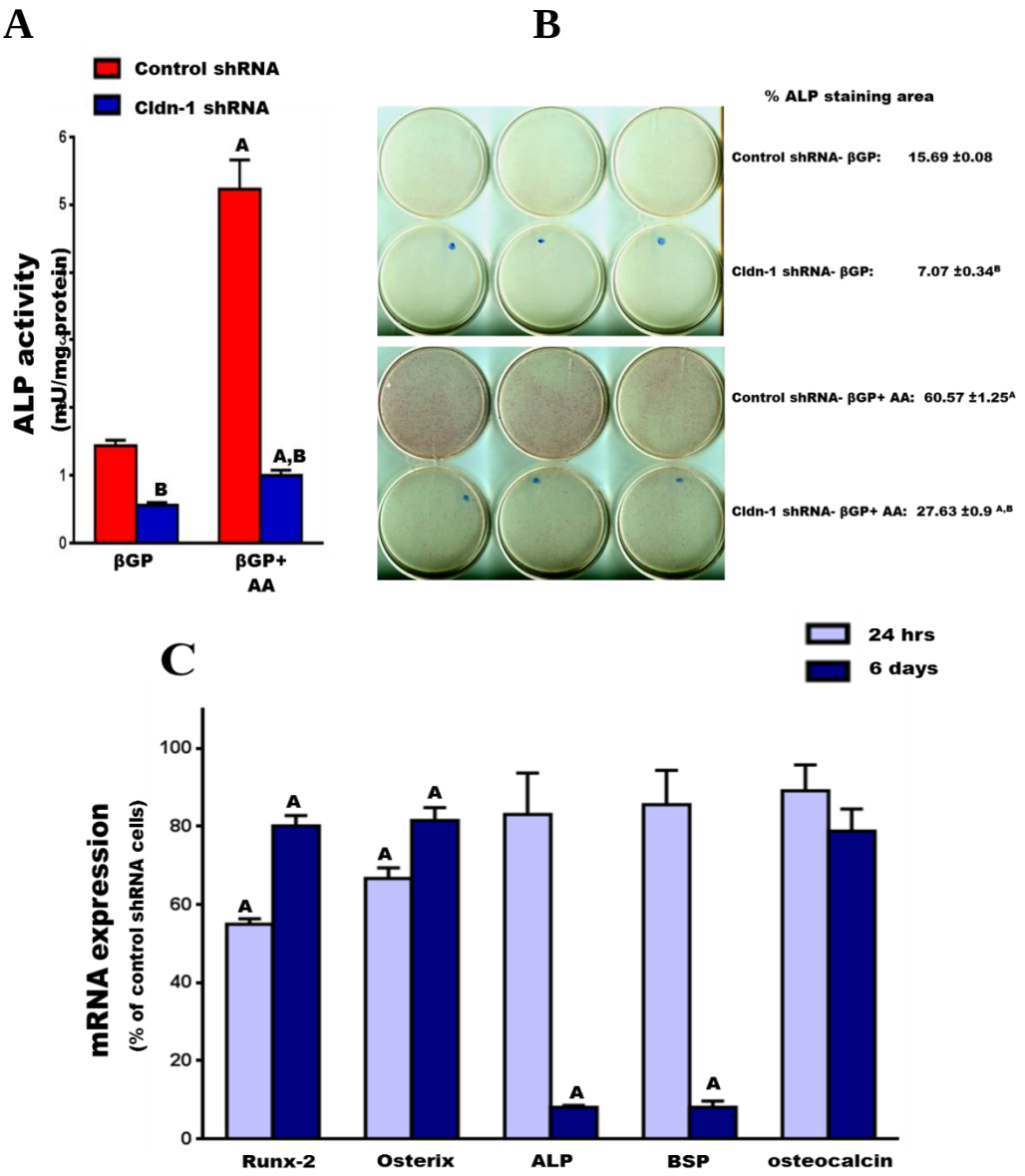


Figure 20. The effect of Cldn-1 knockdown on osteoblast differentiation in MC3T3-E1 cells: A) ALP activity was determined in MC3T3-E1 cells transduced with control or Cldn-1 shRNA and treated with βGP ± AA for 72 hrs. Values (means ± SEM; n = 8). A = < 0.05 vs. βGP treated, and B = < 0.05 vs. control shRNA at the corresponding treatment. B) ALP staining was determined on MC3T3-E1 cells transduced with control or Cldn-1 shRNA and treated with βGP ± AA for 6 days, followed by ALP activity staining. Values (means ± SEM; n = 5) are represented as % ALP stained area. A = < 0.05 vs. βGP treated, and B = < 0.05 vs. control shRNA at the corresponding treatment. C) The expression of osteogenic master transcription factor genes (osterix and Runx-2) and osteogenic marker genes (ALP, bone sialoprotein (BSP), and osteocalcin) was evaluated in control and Cldn-1 shRNA MC3T3-E1 treated β-glycerophosphate (βGP) with or without ascorbic acid (AA) for 24 hrs and 6 days using real time RT-PCR. Values (means ± SEM; n = 4) are presented as % of control shRNA. A=<0.05 vs. control shRNA cells.

points, the difference did not reach statistical significance (Fig. 20C). Finally, the expression levels of transcription factors that are required for osteoblast differentiation was evaluated in control and Cldn-1 shRNA cells. We found that down regulating Cldn-1 expression reduced the expression of both osterix and Runx-2 mRNA levels, by 33% and 45% respectively, when treated with AA for 24 hrs (Fig. 20C). This effect was still maintained in the Cldn-1 shRNA cells, even after 6 days of AA treatment, albeit to a lower extent (Fig. 20C).

The Effect of Knocking Down Cldn-1 Expression on β-catenin Levels

To gain more insight into the molecular pathway(s) underlying Cldn-1 function in osteoblasts, we tested the effect of its knockdown on β-catenin signaling. This is based on β-catenin's established importance in regulating osteoblastogenesis [34,35], as well as reports that inhibition and overexpression of Cldn-1 in a colon cancer cell line leads to a reduction and activation of β-catenin signaling, respectively [36,37]. Thus, β-catenin protein level was evaluated in Cldn-1 shRNA compared to control shRNA cells, treated with AA for 48 hrs. Indeed, we found that β-catenin protein level decreased by 28% as a consequence of Cldn-1 knockdown (Fig 21), suggesting that it may be involved in Cldn-1 regulation of osteoblastogenesis.

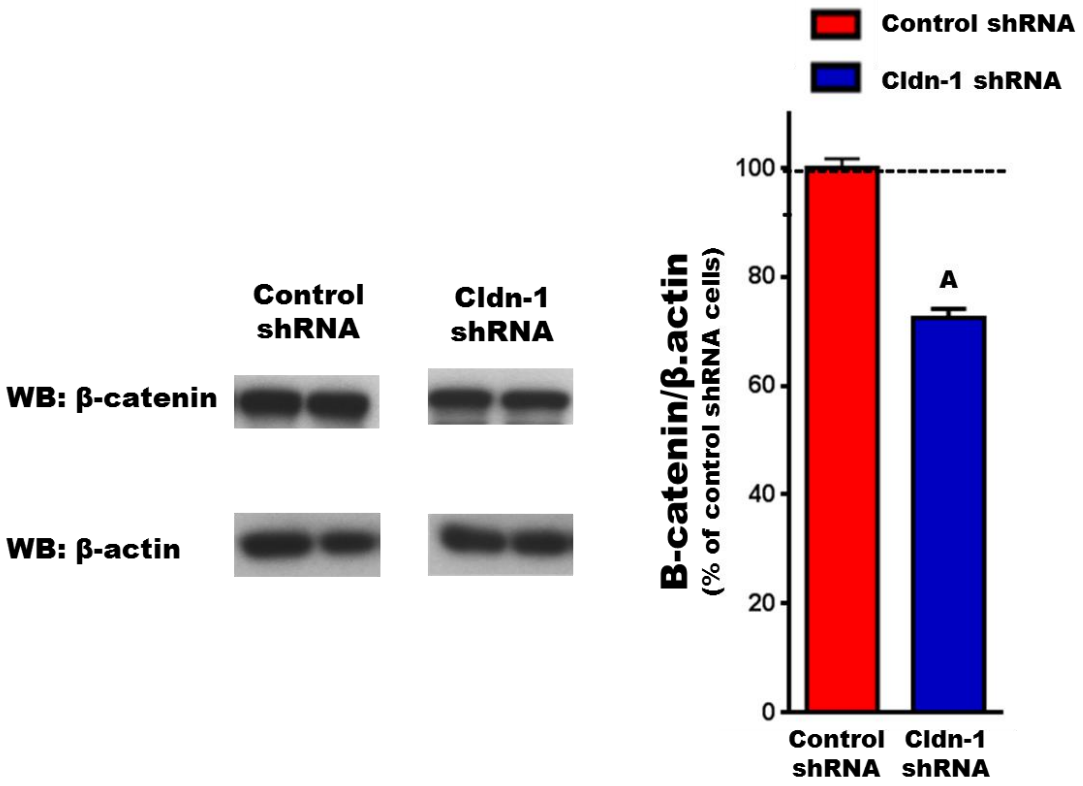


Figure 21. The effect of Cldn-1 knockdown on β-catenin. Control shRNA or Cldn-1 shRNA MC3T3-E1 cells were treated with AA for 48 hrs, then β-catenin expression was evaluated by western blotting using whole cell lysates. Values (means ± SEM; n = 4). A=<0.05 vs. control shRNA cells.

Discussion

This study provides the first comprehensive investigation of the expression of Cldn family members during bone cell differentiation, in mice. Specifically, we report for the first time that primary mouse osteoclasts differentially express several Cldns in a time dependent manner. Furthermore, Cldns were found to be differentially expressed and highly regulated during primary osteoblast differentiation In an attempt to decode the function of Cldns in regulating skeletal development and maintenance, we focused on the role of Cldn-1 during osteoblastogenesis. We determined that Cldn-1 expression is regulated by several osteoregulatory agents such as IGF-1 and Wnt3a and that Cldn-1 is a positive regulator of osteoblast proliferation and differentiation. In addition, knockdown of Cldn-1 in osteoblasts leads to a reduction in β-catenin protein levels, suggesting that this pathway is possibly involved in Cldn-1-mediated modulation of osteoblastogenesis.

In agreement with the complex expression patterns of Cldns reported in different tissues, we found that Cldns also exhibit cell type and differentiation stage specific patterns of expression, during bone cells differentiation. As for osteoclasts, we were the first to provide evidence that Cldns, specifically Cldn-18, are expressed in osteoclasts [24]. Aside from Cldn-18, the expression profile of other Cldn family members has not been previously reported in the literature. In this regard, the current study demonstrates, for the first time, that several Cldns were expressed in primary osteoclasts, in a differentiation stage dependent fashion. Furthermore, three distinct expression profiles were observed during osteoclast differentiation: 1. upregulation or downregulation during early stages, suggesting that these Cldns may have potential functions during the proliferation stage; 2. up regulation during late stages, supporting possible roles in regulating osteoclast function and activity; and 3. no apparent change in the expression of

some Cldns, which may indicate a lack of a role during osteoclastogenesis. Regarding their function, Cldn-18 is the only family member shown to play a role in osteoclastogenesis [24]. While these studies clearly document a critical non-canonical role for Cldn-18 in regulating osteoclast differentiation, the current study suggests that other Cldn family members may also have potential roles in regulating these processes. In addition, the issue of whether Cldn-18 interacts with other Cldns to regulate bone resorption remains to be elucidated.

As for osteoblasts, their differentiation processes in vitro are divided into several stages and regulated by differential and sequential expression of several genes [7,8]. While rat osteoblasts are known to express several Cldns [20,21], the consequence of cellular differentiation on Cldn expression has not been documented. Our study revealed for the first time that the expression pattern of Cldns may be osteoblast differentiation stage-dependent. Many Cldns showed an increase in their expression levels, some were found to be downregulated, and others exhibited no change, indicating possible diverse roles for Cldns during different stages of osteoblastogenesis. Consistent with our finding, it has been shown that the expression of Cldn-1 and -2 was higher in osteoblast like MC3T3-E1 cells compared to osteocyte-like MLO-Y4 cells [30]. By contrast, an earlier study showed that Cldn-1 and Cldn-2 mRNA levels were upregulated during the mineralization stage compared to the proliferation stage [20]. While expression levels of several Cldns seem to vary depending on differentiation stage of osteoblasts, we have not determined whether these changes are biologically significant. Therefore, our future experiments will evaluate the biological role of the various Cldn family members in regulating osteoblast proliferation/differentiation to determine if their differentiation

stage-dependent expression has physiological relevance. In-line with differentiation stage dependent patterns of Cldns expression, it has been shown that other cells of mesenchymal origin differentially express Cldns [38,39]. For example, Cldn-6 was found to be upregulated and plays an important role during adipocyte differentiation [38]. Moreover, our findings that all "classic" Cldns mRNA levels were increased during osteoblast differentiation suggest that these closely related Cldns may exert similar/redundant functions, and possibly regulated in a similar manner. By contrast, the diverse expression patterns observed with the "non-classic" Cldns supports the idea that they may have district functions, and may, therefore, be regulated differently. Thus, even though these data suggest potential roles of Cldn family members in regulating osteoblastogenesis, our future studies will examine their role in skeletal development and maintenance, by employing both gain and loss of function experiments in vivo and in vitro.

In terms of the regulation of Cldn expression, multiple studies in other tissues have shown that Cldn expression and function is regulated by a host of growth factors, hormones, and cytokines [14]. The observed complex expression patterns of Cldns during osteoblast differentiation appear to indicate that Cldn expression is tightly regulated. Thus, these tight regulation processes of Cldn expression during AA induced osteoblast differentiation could be explained by either direct effects of AA treatment and/or indirect effects. The latter could be mediated by growth factors and/or bone matrix proteins secreted during osteoblast differentiation, which, in turn, act in an auto/paracrine fashion. In order to better understand the regulation and function of Cldns in bone, our efforts focused on Cldn-1 based on its interesting differential expression pattern, (i.e.,

upregulation of Cldn-1 during early stages of osteoblast differentiation, whereas downregulation during late stages); and its established importance in tissue development as mice with targeted disruption of the Cldn-1 gene failed to survive after birth [40]. Consistent with an important role for Cldn-1 are our findings that its expression is regulated by IGF-1 and Wnt3a. The increased expression of Cldn-1 following IGF-1 treatment is similar to a previous report in which IGF-1 was found to upregulate Cldn-1 expression, via a MAPK dependent pathway (25). On other hand, Wnt3a, a known stimulator of bone formation, significantly decreased the expression of Cldn-1. Interestingly however, an opposite regulation pattern was observed in colon cancer cells supporting the notion that Cldns regulation by Wnt signaling may be tissue/cell type specific [41,42].

Regarding Cldn-1 function, it has been shown that Cldn-1 acts canonically as a cation restrictive barrier in epithelial as well as endothelial tissues [10,43]. Aside from this function, there is substantial evidence that Cldn-1 participates in intracellular signaling that controls cell proliferation and differentiation [31-33]. While the canonical function of Cldn-1 in bone cells has been suggested in the literature, virtually nothing is known about its non-canonical function, i.e., as a mediator of cell signaling, in such cells [30]. Consequently, knocking down Cldn-1 reduced osteoblast proliferation and the expression of the cell proliferation marker gene cyclin-D1, indicating that Cldn-1 promotes osteoblast proliferation. In addition, several studies in other cell types have demonstrated that Cldn-1 is a promoter, inhibitor, or has no effect on cell proliferation, thereby supporting the notion that the “proliferative” function of Cldn-1 is cell type-dependent [32,33,36]. Besides regulating proliferation, Cldn-1 appears to play an

important role in regulating early stage osteoblast differentiation as revealed by data from Cldn-1 knockdown experiments. In agreement with the observed upregulation of Cldn-1 during early stages of osteoblast differentiation, Cldn-1 deficiency had no significant effect on the expression of the late stage osteogenic marker gene osteocalcin. In addition, previous studies demonstrated that Cldn-1 can modulate the expression of transcription factors in various tissues [37,44,45]. Accordingly, we determined if Cldn-1 regulates the expression of Runx-2 and osterix, master transcription factors of osteoblast differentiation, and found their levels to be reduced significantly as a consequence of Cldn-1 knockdown. It seems that Cldn-1 regulates the expression of transcription factors during early time points, which seems to be consistent with their consequent regulation of the expression of osteogenic marker genes. The finding that the effect of Cldn-1 knockdown on expression of osteogenic transcription factors was maintained after 6 days, suggests that Cldn-1 deficiency inhibits osteoblast differentiation. However, the issue whether inhibition of Cldn-1 prevents or delays differentiation will be addressed in future studies. Together these findings underscore Cldn-1 as a positive regulator of (early) osteoblastogenesis, which is consistent with its reported function in early dentinogenesis [31]. On the contrary, it has been reported that Cldn-1 inhibits differentiation of other cell types [33], thereby, providing evidence that its regulation of differentiation is cell type and stage dependent.

In terms of potential mechanism(s) for Cldn-1 actions, we evaluated the effect of Cldn-1 knockdown on β-catenin, which is a major pathway known to be critical for osteoblastogenesis [34,35]. We found that knockdown Cldn-1 in osteoblasts leads to a reduction in β-catenin protein levels. In support of our findings, it has been reported that

inhibition and overexpression of Cldn-1 in a colon cancer cell line leads to reduction and activation of β-catenin signaling, respectively [36,37]. However, the detailed mechanism by which Cldn-1 modulates β-catenin signaling remains to be determined, and the cause and effect relationship between reduced β-catenin level and reduction in proliferation/differentiation caused by Cldn1 knockdown in osteoblasts will be the focus of future experiments. Nonetheless, one of the potential mechanisms may be through phosphorylation/inactivation of GSK3, which normally results in β-catenin degradation. Another possible mechanism is by direct binding, which will make β-catenin less accessible to the destruction complex [36]. On the other hand, Cldn-1 itself has been found to be a target for β-catenin signaling in colon cancer [41]. Therefore, we cannot exclude the possibility that β-catenin signaling is also upstream of Cldn-1 and/or there exists some kind of cross talk or feedback loop between them in regulating osteoblastogenesis. While our data suggests that Cldn-1 may regulate β-catenin signaling, we cannot exclude the involvement of other pathways such as TGF-β, BMP, and Notch signaling. Thus, the delineation of the molecular pathway(s) by which Cldn-1 acts should advance our understanding of the regulation of osteoblast differentiation.

In conclusion, here we show for the first time that Cldn family members are expressed and tightly regulated in both primary osteoblasts and osteoclasts, in a differentiation stage dependent manner. Furthermore, we provide compelling evidence that Cldn-1 is a novel positive regulator of osteoblast differentiation and proliferation, and its regulation is complex and mediated by several osteoregulatory factors. Collectively, the observed complexity in the expression patterns of Cldns during bone cells differentiation, and the finding that Cldn-1 regulates osteoblast differentiation

suggests that Cldns may have potential roles in regulating bone homeostasis . Finally, our future understanding of how Cldns regulate osteoblast and osteoclast function, and overall bone homeostasis could lead to the development of Cldn-based drug targets for diagnosis and therapeutic management of osteoporosis.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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CHAPTER FOUR

DISCUSSION

Osteoporosis, a major public health problem, is characterized by low bone mass and structural deterioration of bone tissue resulting in increased bone fragility (Dempster, 2011; Raisz, 2005). The imbalance between bone formation and resorption during bone remodeling has been documented to be a major factor in the pathogenesis of osteoporosis (Raisz, 2005). It is well known that bone mass is maintained by a well-coordinated activity of cells of the osteoblast and osteoclast lineages, in what is known as the basic multicellular unit (BMU), which carry out resorption of old bone and formation of new bone, in a sequential manner (Hou et al., 2009). While a number of systemic hormones and local growth factors have been implicated in regulating bone formation and resorption, our knowledge of the molecular pathways that regulate the activity of osteoblasts and osteoclasts is, to date, still limited. Thus, it is crucial to identify novel genes and/or novel functions for some of the known genes that regulate the formation and activity of these cell types, which, in turn, is expected to advance our understanding of the pathogenesis of osteoporosis, and aid in designing novel regimens for its therapeutic management.

To this end, recent evidence suggests a significant role for the tight junction proteins, Cldns, in the regulation of bone remodeling processes. In our previous studies, we have demonstrated that Cldn-18 is expressed in bone cells and its targeted disruption in mice resulted in osteopenia phenotype by increasing bone resorption (Linares et al., 2012a). In fact, Cldn-18 was found to act non-canonically by regulating RANKL mediated osteoclast differentiation (Linares et al., 2012b). While our previous data suggest a direct non-canonical function in regulating osteoclast differentiation, it is

known that Cldns act "canonically" as key determinants of paracellular permeability of ions, solutes, and water across the plasma membrane (Gunzel & Yu, 2013). Thus, it is possible that the phenotype of Cldn-18 KO mice is in part mediated by its canonical function. Since Cldn 18 is known to be predominately expressed in the stomach, and it was globally disrupted in these earlier studies (Hayashi, Tamura, Tanaka, Yamazaki, Watanabe, Suzuki, Sentani, et al., 2012; A. Tamura et al., 2012), we tested the possibility that the osteopenia phenotype in Cldn-18 KO mice is due to its disruption in the stomach. In our study, we found that the loss of Cldn-18 negatively affected gastric acidity in adult mice. Consistent with this observation, Sanada et al., have reported that Cldn-18 was down regulated in gastric cancer and atrophic gastritis (Sanada et al., 2006a). Furthermore, Hayashi et al., have recently demonstrated that Cldn-18 deficient mice developed atrophic gastritis and their gastric pH was significantly higher compared to WT mice, at day 14 postnatally (Hayashi, Tamura, Tanaka, Yamazaki, Watanabe, Suzuki, Sentani, et al., 2012). Additionally, these authors found that the H^+ leakage into the submucosal layer of gastric tissues was higher in Cldn-18 KO mice compared to WT mice (Hayashi, Tamura, Tanaka, Yamazaki, Watanabe, Suzuki, Sentani, et al., 2012; A. Tamura et al., 2012). Together, these findings provide strong evidence that Cldn-18 plays an important role in the physiology and pathology of stomach function and may provide the paracellular barrier against H^+ leakage in the stomach.

Given the documented role of gastric acidity in calcium absorption/metabolism, as calcium solubilization by stomach's acidic environment is thought to be prerequisite for calcium absorption in the small intestine (Bo-Linn et al., 1984; Boyce, 2009; Sipponen & Harkonen, 2010; Wright et al., 2008) , there is a possibility that the increased

bone resorption in the Cldn-18 KO mice is a consequence of reduced calcium absorption due to the increased stomach pH. To address this issue, Cldn-18 KO and control mice were subjected to a normal or high calcium diet at birth, for a 10-week period. Serum calcium levels were significantly lower in Cldn-18 KO mice compared to control mice on a normal calcium diet but not on high calcium diet. This finding is consistent with our previous studies in which the serum PTH levels (and indicator of calcium homeostasis) were found to be elevated in Cldn-18 KO mice fed a normal calcium diet compared to control mice (Linares et al., 2012a), suggesting that Cldn-18 KO mice may be calcium deficient. In support of the effectiveness of the dietary approach we employed in this study, the serum calcium levels were significantly higher in Cldn-18 KO mice fed a high calcium diet compared to a normal calcium diet. Moreover, bone resorption was lower in high calcium diet fed groups compared to normal calcium diet fed groups. Consistent with our previous report on the Cldn-18 deficient mice bone phenotype, we also found that these mice exhibited decreased total body BMD, trabecular, and cortical bone parameters when fed a normal calcium diet (Linares et al., 2012b). However and importantly, increased dietary calcium intake in Cldn-18 deficient mice did not rescue this phenotype at different skeletal sites. Furthermore, the lumbar bone resorption was still significantly higher in Cldn-18 KO mice fed a high calcium diet compared to control mice fed a high calcium diet. Collectively, correction of serum calcium deficit did not rescue decreased BMD and increased bone resorption observed in Cldn-18 KO mice; thus ruling out the possibility that gastric abnormalities contributed to the osteopenia phenotype in these mice. Therefore, these findings and our previous study support a

direct non-canonical role for Cldn-18 in regulating osteoclast differentiation and bone resorption (Linares et al., 2012b).

While our studies have laid down the foundation for the non-canonical function of Cldn-18 in regulating bone homeostasis, whether other Cldn family members are expressed and modulate osteoblastogenesis and osteoclastogenesis remains to be investigated. Cldns exhibit complex patterns of expression that are tissue/cell type and developmental stage specific (Alshbool & Mohan, 2014; Gunzel & Yu, 2013). Some tissues/cells such as the epidermis express several Cldns (Brandner et al., 2002), whereas others such as Sertoli cells express only one or two Cldns (C. M. Van Itallie & Anderson, 2006). Interestingly, Cldn expression also varies within the same tissue. For example, in mouse kidney, at least 15 Cldns are expressed with distinct expression patterns in every segment of the nephron (Elkouby-Naor & Ben-Yosef, 2010). Thus, while the proximal tubules express Cldn-1, -2, -10, -11, -12, and -14, the distal tubules express Cldn-3, -7, -8, -10, and -11 (Elkouby-Naor & Ben-Yosef, 2010). There is also increasing evidence that the expression of Cldns is developmental stage specific (Gunzel & Yu, 2013). In the mouse jejunum, the expression of several Cldns is increased or decreased during neonatal development (Holmes et al., 2006). For example, the expression of Cldn-19 was predominant in 2 week old mouse jejunum and was no longer detected after 4 weeks (Holmes et al., 2006). While much is known regarding the expression patterns of many Cldn family members in several tissues, little is known about their expression in bone. In the present study, we provide the first experimental evidence that several Cldns are expressed in bone, in a complex, cell type and differentiation stage-dependent fashion. Specifically, several Cldns were found to be expressed in primary osteoclasts, in a

distinct differentiation stage dependent fashion, including: upregulation or downregulation during early stages, and upregulation during late stages. On the other hand, some exhibited no apparent changes in their expression profile. The aforementioned three distinct expression profiles observed during osteoclast differentiation suggest that some Cldns have potential roles in regulating osteoclast proliferation and/or osteoclast activity and function; whereas others might have no role. As mentioned above, while Cldn-18 is the only family member with an established role in regulating osteoclastogenesis (Linares et al., 2012a), the current study suggests that other Cldn family members may also have potential roles in regulating these processes. It would be interesting to determine whether Cldn-18 interacts with other Cldns or tight junction proteins to regulate bone cell functions. One might find that Cldn-18 or other Cldns act in concert or independently in regulating osteoclast function. It is noteworthy that the double KO mouse approach has served as a powerful tool in understanding the interaction between Cldns in different tissues. Therefore, our future studies will examine the interaction between Cldn-18 and other candidate Cldns in regulating osteoclastogensis, by employing this double loss of function approach, both *in vivo* and *in vitro.*

As for osteoblasts, multiple Cldns have been shown to be expressed in rat osteoblasts (Prele et al., 2003; Wongdee et al., 2008), however the consequence of changes in Cldn expression on cellular differentiation has not yet been documented. Our study has revealed that, similar to osteoclasts, their expression pattern is osteoblast differentiation stage dependent in which many Cldns exhibited an increase in their expression levels, either during early or late stages of differentiation; and some were

found to be downregulated, whereas others did no change. The observed differentiation stage dependent patterns of expression, such as early upregulation of Cldn-1, -2, -5, -7 to -11, and -19 suggests that these Cldns modulate early stages of osteoblast differentiation. Conversely, upregulation of Cldn-3, -4, -6, -13, -14, -17, -22, and -23 during terminal (mineralization) stages, in which osteoblasts are converted into osteocytes or inactive bone lining cells, appears to indicate a function during late stages of osteoblast differentiation. Consistent with this finding, it has been shown that the expression of Cldn-1 and -2 was higher in osteoblast like MC3T3-E1 cells compared to osteocyte-like MLO-Y4 cells (Hatakeyama et al., 2008). In contrast, an earlier study showed that Cldn-1 and Cldn-2 mRNA levels are upregulated during the mineralization stage compared to the proliferation stage (Prele et al., 2003). In-line with differentiation stage dependent patterns of Cldns expression, it has been shown that other cells of mesenchymal origin differentially express Cldns (Hong et al., 2005; L. Wang et al., 2012). For example, Cldn-6 was found to be upregulated and to play an important role during adipocyte differentiation (Hong et al., 2005). Collectively, the observed changes in the expression profile of Cldns are consistent with the notion that they potentially play important roles during osteoblastogenesis. However, the issue of why multiple Cldns are expressed and whether they have redundant, overlapping, or distinct functions remains to be investigated. In epithelial tissue, it was found that a combination of Cldns determines the characteristics and functionality of the resulting tight junction (Elkouby-Naor & Ben-Yosef, 2010). In fact, some Cldns need another Cldn (e.g., Cldn-16 and -19) to translocate into tight junction and become functional, which in turn highlights a cooperative interaction between them (Gunzel & Yu, 2013; Hou et al., 2009; Hou et al.,

2008). On the other hand, a lack of interaction between Cldns was observed in other cell systems, for example; the phenotype of double Cldn-11/-14 KO was a combination of those observed in each of the single deletion animals which suggests redundant functions (Elkouby-Naor et al., 2008). To this end, our findings that mRNA levels of all "classic" Cldns were increased during osteoblast differentiation suggest that the closely related Cldns exert similar/redundant functions in regulating bone formation, and may even be regulated in a similar manner. By contrast, the diverse expression patterns observed in the "non-classic" Cldns supports the notion that they have district functions during osteoblast differentiation, and may, therefore, be regulated differently. Taken together, the observed complexity in the expression patterns of Cldns in osteoblasts suggests potential common and member-specific functions among various Cldns. This notion remains to be investigated by manipulating the expression of Cldn family members individually or in combination, *in vivo* and *in vitro.*

Based on the functions of Cldns known to date, it appears that Cldns do not follow a simple functional paradigm in various tissues including bone. Cldns act canonically as a major determinant of paracellular permeability in epithelial and endothelial cells; and serve as a fence that divides apical and basolateral domains of plasma membranes. For example, it has been found that Cldn-18 (stomach isoform) acts canonically as paracellular barriers against cations, such as sodium and hydrogen in the stomach (Hayashi, Tamura, Tanaka, Yamazaki, Watanabe, Suzuki, Suzuki, et al., 2012; Jovov et al., 2007). In addition, Cldn-18 was found to be downregulated in gastric cancer, in which it was assumed that the loss of cell polarity contributes to tumorigenesis (Sanada et al., 2006b). On the other hand, our previous study demonstrated that Cldn-18

disruption/overexpression did not influence paracellular transport of calcium ions in osteoclasts, thereby supporting the concept that Cldn-18 function is cell type specific (Linares et al., 2012a). Thus, some but not other Cldns may exert their canonical functions in bone cells. In this connection, there is evidence that osteoblast and bone lining cells form an epithelial like bone membrane to control the paracellular ion exchange and maintain differential ion compositions between the plasma and bone extracellular fluid (Bushinsky et al., 1989; Hatakeyama et al., 2008; Marenzana et al., 2005; Rubinacci et al., 2000; Wongdee et al., 2008; Wongdee et al., 2010). The expression and localization of certain Cldns in the bone lining cells suggests that they function as barriers, a notion that was confirmed by measuring the transepithelial resistance of an osteoblast monolayer (Wongdee et al., 2008). Although a canonical Cldns function in controlling paracellular transport of ions across the bone lining cells has been suggested in the literature, compelling evidence for this function is still thus far lacking. This field is still in its infancy with many intriguing questions remaining unanswered: 1) What is/are the identity of ion restrictive/permeable Cldns in bone barriers? 2) Do they resemble the same functions documented in epithelial tissues? 3) Given the large number of Cldn proteins, how do these multiplicities influence their barrier function? and 4) What is the interaction between these Cldns in regulating paracelluar permeability?

Beyond functioning as a tight junction, it is now clear that Cldns exert non-canonical functions by regulating cell signaling; which is becoming an emerging area of research. The non-canonical Cldn functions have been shown to involve interaction with adaptor proteins that shuttle between the plasma membrane and the nucleus, thereby

regulating gene expression, cell proliferation, and differentiation (Balda & Matter, 2009). As mentioned earlier, Cldns have the capacity to interact with other PDZ domain containing cytoplasmic scaffolding proteins such ZO-1/2/3, via their carboxy- terminus PDZ-binding motif (Angelow et al., 2008; Krause et al., 2008). For example, the non-canonical effect of the loss of Cldn-18 in the regulation of RANKL-induced osteoclast differentiation is mediated by disruption of the interaction with ZO2, resulting in increased nuclear translocation of ZO2 (Linares et al., 2012a). In turn, this translocation increases the expression of important transcription factors involved in RANKL-induced osteoclast differentiation, which ultimately leads to increased bone resorption. In line with the novel and emerging functions of Cldn-18, a number of studies have shown that Cldn-18 is upregulated in various types of cancers, which suggests that its role in the regulation of cancer cell behavior extends beyond just forming tight junctions (Halimi et al., 2013; Sahin et al., 2008). Moreover, it has been recently demonstrated that suppression of Cldn-18 promotes the proliferation of gastric cancer cells (Oshima et al., 2013). Since little is known about the biological role of Cldn-18 in any tissues, our novel finding that Cldn-18 has a direct non-canonical role in regulating osteoclast differentiation is not only relevant for improving our understanding of bone biology, but may provide fundamental and perhaps mechanistic information that may be applicable to other tissues. In terms of the non-canonical functions of other Cldn family members, it is noteworthy that some Cldns do not have a PDZ binding motif (e.g., Cldn-12, 19a, -12, and -24 to-27) suggesting that not all Cldns may exert non-canonical functions or their functions are mediated by another novel/unknown mechanism(s) (Gunzel & Yu, 2013). While several signaling systems have been linked to Cldns non-canonical functions, we

have just started to understand their underlying molecular mechanisms (Matter & Balda, 2003). Thus, it is crucial to understand the emerging role of Cldn family members in bone cell signaling and biology, a knowledge that can perhaps be extrapolated to other cell systems.

In order to better understand the function of Cldns in bone, our efforts focused on Cldn-1. This selection was based on its interesting differential expression pattern, (i.e., upregulation of Cldn-1 during early stages of osteoblast differentiation, whereas downregulation during late stages); and its established importance in tissue development (Furuse et al., 2002). In this study we found that Cldn-1 is a positive regulator of osteoblast differentiation and proliferation. In fact, knocking down Cldn-1 reduced osteoblast proliferation and differentiation, and decreased the expression of osteogenic marker genes. In agreement with our findings, there is substantial evidence that Cldn-1 participates in intracellular signaling that controls cell proliferation and differentiation in different tissues; which is known as a "non-canonical function" (H. Fujita et al., 2011; Hoshino et al., 2008; Pope et al., 2013). However, it appears that its "non-canonical" function is cell type-dependent. For example, it has been demonstrated that Cldn-1 is a promoter, inhibitor, or has no effect on cell proliferation and differentiation in other cell types (Dhawan et al., 2005; H. Fujita et al., 2011; Hoshino et al., 2008; Pope et al., 2013). Since Cldn-1 is also expressed in osteoclasts, there is a possibility that it may regulate osteoclastogenesis and/or be involved in a coupling mechanism between osteoblasts and osteoclasts during bone remodeling. Consequently, our future studies will evaluate the skeletal phenotype of Cldn-1 deficient mice by its conditional disruption in bone cells.

In terms of potential mechanism(s) for Cldn-1 actions, we evaluated the effect of Cldn-1of knockdown on β-catenin, which is a major pathway known to be critical for osteoblastogenesis (Bodine & Komm, 2006; Yavropoulou & Yovos, 2007). We found that knockdown Cldn-1 in osteoblasts leads to a reduction in β-catenin protein levels. In support of our findings, it has been reported that inhibition and overexpression of Cldn-1 in a colon cancer cell line leads to reduction and activation of β-catenin signaling, respectively (Dhawan et al., 2005; Singh et al., 2011). However, the detailed mechanism by which Cldn-1 modulates β-catenin signaling remains to be determined, and will be the focus of future experiments. Nonetheless, one of the potential mechanisms may be through phosphorylation/inactivation of GSK3, which normally results in β-catenin degradation. Another possible mechanism is by direct binding, which will make β-catenin less accessible to the destruction complex (Dhawan et al., 2005). On the other hand, Cldn-1 itself has been found to be a target for β-catenin signaling in colon cancer (Miwa et al., 2001). Therefore, we cannot exclude the possibility that β-catenin signaling is also upstream of Cldn-1 and/or there exists some kind of cross talk or feedback loop between them in regulating osteoblastogenesis. While our data suggests that Cldn-1 may regulate β-catenin signaling, we cannot exclude the involvement of other pathways such as TGF-β, BMP, and Notch signaling. Thus, the delineation of the molecular pathway(s) by which Cldn-1 acts should advance our understanding of the regulation of osteoblast differentiation.

Taken together, our studies on Cldn-18 and Cldn-1 clearly document a critical non-canonical function of Cldns in regulating bone homeostasis, which may also be applicable in other cell systems or tissues. Given that Cldns are classically thought to

simply function as structural elements of tight junctions, our findings clearly demonstrate that they play a much wider role in biology. Moreover, our work opens a broad range of avenues for research focused on understanding Cldns' emerging role in bone cell signaling and biology. Accordingly, Cldns are one example of proteins that have multiple roles beyond their canonical function as tight junction molecules, which thereby argues against the concept of protein "stereotyping". Collectively, we hope to provide a framework for guiding future research on understanding how Cldns modulate osteoblast and osteoclast function and overall bone homeostasis. Such studies should provide valuable insights into the pathogenesis of osteoporosis, and may define Cldns and/or identify Cldn-based agents for treating such disease states.

Summary and Conclusions

1) In the study described in chapter 2, we demonstrated that Cldn-18 deficiency negatively affects gastric acidity. In addition, we provide evidence that serum calcium levels were lower in Cldn-18 KO mice compared to heterozygous control mice fed a normal calcium diet suggesting that these mice may be calcium deficient. Moreover, we found that a high calcium diet increased lumbar BMD and decreased bone resorption in both Cldn-18 KO and heterozygous control mice, whereas correcting the deficiency in serum calcium in Cldn-18 KO by feeding a high calcium diet did not correct the osteopenia phenotype and the increase in bone resorption. In conclusion, the failure to rescue the osteopenia phenotype by high calcium diet in Cldn-18 KO mice suggests that increased bone resorption is likely to be due to direct effects of lack of Cldn-18 on osteoclasts and not due to gastric pH changes caused by its loss in the stomach.

2) In the study described in chapter 3, we provide the first comprehensive investigation of the expression of Cldn family members during bone cell differentiation, in mice. We report for the first time that primary mouse osteoclasts differentially express several Cldns in a differentiation-stage dependent manner. Furthermore, Cldns were found to be differentially expressed and highly regulated during primary osteoblast differentiation. In addition, we provide compelling evidence that Cldn-1 is a novel positive regulator of osteoblast differentiation and proliferation, and that its regulation is complex and mediated by several osteoregulatory factors such as IGF-1 and Wnt3a. In addition, knocking down Cldn-1 reduced β-catenin level, suggesting that it may be involved in Cldn-1 regulation of osteoblastogenesis. Therefore, our data suggest that Cldns have a potential role in regulating bone homeostasis.

Future Directions

1) In our study on Cldn-18, the failure to rescue the osteopenia phenotype by high calcium diet in Cldn-18 KO mice suggests that increased bone resorption is likely to be due to direct effects of lack of Cldn-18 on osteoclasts and not due to gastric pH changes caused by lack of Cldn-18 in the stomach. However, more direct evidence using mice with conditional KO of Cldn-18 in osteoclasts are warranted to convincingly demonstrate a local non-canonical function of Cldn-18.
2) While our previous and current findings are consistent with the important role of Cldn-18 in regulating bone resorption, the issue whether Cldn-18 also affects bone formation needs to be evaluated. In this regard, bone formation was found not to be affected by Cldn-18 deficiency under resting/normal conditions (Linares

et al., 2012a), but this does not exclude a potential role for Cldn-18 in regulating bone formation. In fact, Cldn-18 was found to be expressed in osteoblasts, suggesting that it may regulate bone formation under conditions when bone formation rates are severely compromised (Wongdee et al., 2008). Thus, future studies will elucidate the direct role of Cldn-18 in the skeleton, using mice with a conditional disruption of Cldn-18 under pathological conditions.

3) Besides Cldn-18, the finding that several Cldns are expressed in osteoclasts in a differentiation stage dependent manner suggests potential roles of other Cldn family members in regulating osteoclastogenesis. Thus, the issue of whether Cldn-18 interacts with other Cldns to regulate bone resorption remains to be elucidated. Our future studies will examine the interaction between Cldn-18 and other candidate Cldns in regulating osteoclastogensis, by employing double loss of function approach *in vivo* and *in vitro*. Of note, the skeletal phenotype of Cldn-18/-11 double KO mice is currently under investigation.
4) The observed complexity in the expression patterns of Cldns during osteoblast differentiation is consistent with both common and Cldn-specific functions among various Cldns. Our strategy to decode the role of candidate Cldns in skeletal development and maintenance is by employing both gain and loss of function experiments *in vivo* and *in vitro.*
5) In our studies on Cldn-1, we found that it is a positive regulator of osteoblast proliferation and differentiation. Additionally, knocking down Cldn-1 reduced β-catenin level, suggesting that it may be involved in Cldn-1 regulation of osteoblastogenesis. While our *in vitro* studies suggest a non-canonical function

for Cldn-1 in regulating osteoblastogenesis, the detailed mechanism by which Cldn-1 modulates bone cell functions remains to be determined. Therefore, our future studies will determine the direct non-canonical function and delineate signaling pathways of Cldn-1, by employing transgenic overexpression or knockdown of gene expression, *in vitro* and *in vivo*.

6) Finally, an important issue that needs to be addressed is the involvement of Cldns in the pathogenesis of bone related diseases, such as osteoporosis. As mentioned earlier, a sequence variant of human Cldn-14 was found to be associated with a lower bone mineral density in the spine and hip of affected individuals. However, whetheher sequence polymorphisms in other Cldns contribute to variation in the skeletal phenotype remains to be evaluated.

In conclusion, the results of this dissertation start to unravel novel roles of Cldns in bone biology, and open a broad range of avenues for research focused on understanding such emerging role(s) in bone cell signaling. We believe that a better understanding of the role and molecular mechanisms of Cldn function in bone could lead to the identification of novel targets for diagnosis and treatment of metabolic bone diseases including osteoporosis.

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