Dissertation

Expression, Regulierung und Funktion von ST2/IL1RL1 in Chondrozyten der Wachstumsplatte

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Prof. Ute Schäfer/Prof. Annelie Weinberg

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Dissertation

Expression, Regulation and Function of ST2/IL1RL1 in Growth Plate Chondrocyte

submitted by

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Prof. Ute Schaefer/Prof. Annelie Weinberg

2018
Quotation

Science means constantly walking a tightrope between blind faith and curiosity; between expertise and creativity; between bias and openness; between experience and epiphany; between ambition and passion; and between arrogance and conviction - in short, between an old today and a new tomorrow.

Heinrich Rohrer

Science without religion is lame, religion without science is blind.

Albert Einstein
Declaration

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of “Good Scientific Practice”.


Graz, 23.01.2018
Ehsan Bonyadirad

Eidesstattliche Erklärung


Graz, 23.01.2018
Ehsan Bonyadirad
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1. Abbreviations

ALP: Alkaline phosphatase  
BMPs: Bone Morphogenetic Proteins  
Col I: Collagen I  
Col II: Collagen II  
Col X: Collagen X  
DAB: Diaminobenzidine  
E1a: Exon 1a  
E1b: Exon 1b  
E2: Exon 2  
ECL: Enhanced chemiluminescence  
ECM: Extracellular matrix  
ERK1/2: Extracellular signal-regulated kinases 1 and 2  
FBS: Fetal bovine serum  
FGFR: FGF receptors  
FGFs: Fibroblast growth factors  
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase  
GH: Growth Hormone  
H&E: Hematoxylin and Eosin  
HZ: Hypertrophic zone  
IGF1: Insulin-like growth factor-1  
IHC: Immunohistochemistry  
IKK: I kappa B kinase  
IL-1: Interleukin 1  
IL-1RaP: IL-1 receptor accessory protein  
IL-33: Interleukin 33  
IL1Rli: Interleukin 1 receptor-like 1  
IRAKs: IL-1 receptor associated kinases  
Ihh: Indian Hedgehog  
JNK: Jun N-terminal kinase  
MAPK: Mitogen-activated protein kinaseAP-1: Activator protein 1  
MMP-9: Matrix metalloprotease 9  
MMP-13: Matrix metalloprotease 13  
MSCs: Mesenchymal stem/stromal cells  
MyD88: Myeloid differentiation primary response 88  
NF-KB: Nuclear factor-kappa-B
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>OSC</td>
<td>Osteocalcin</td>
</tr>
<tr>
<td>OSN</td>
<td>Osteonectin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PHCs</td>
<td>Primary human growth plate chondrocytes</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid hormone-related peptide</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-transcriptionally modified</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>PZ</td>
<td>Proliferative zone</td>
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<tr>
<td>qPCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcription PCR</td>
</tr>
<tr>
<td>RZ</td>
<td>Resting zone</td>
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<tr>
<td>Runx2</td>
<td>Runt-related transcription factor 2</td>
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<tr>
<td>Runx3</td>
<td>Runt-related transcription factor 3</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>S.T.</td>
<td>Stable transfection</td>
</tr>
<tr>
<td>SOX9</td>
<td>SRY (sex determining region Y)-box 9</td>
</tr>
<tr>
<td>sST2</td>
<td>Soluble ST2</td>
</tr>
<tr>
<td>ST2L</td>
<td>ST2 transmembrane receptor</td>
</tr>
<tr>
<td>TB</td>
<td>Trabecular bone</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor-associated factor 6</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcription start site</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper cell type 2</td>
</tr>
<tr>
<td>VEGFA</td>
<td>Vascular endothelial growth factor A</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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4. Zusammenfassung

Ein prominenter Expressionsort des Gens ST2 (IL1RL1) ist Knochengewebe, wobei jedoch das Wissen über Expression, Regulierung und Funktion von ST2 in Chondrozyten begrenzt ist. Immunhistochemische Analysen der Wachstumsplatte in Mäusen zeigten eine auffallende ST2 Expression in hypertrophen Chondrozyten, sowohl im Femur als auch in der Tibia. Konträr dazu zeigte sich eine niedrige oder nicht vorhandene ST2 Expression in Ruhe- als auch Proliferationszonen.

Unterstützt wird dieser Befund durch die signifikante Expressionssteigerung des Oberflächenrezeptors (ST2L) als auch der löslichen Isoformen (sST2) während der hypertrophen Differenzierung von ATDC5 Zellen, sowie durch die Expression von hypertrophen Markern wie Collagen 10 (Col X), Runx-related transcription factor 2 (Runx2) und Matrix-Metalloproteinase 13 (MMP-13). Runx2 vermittelt als Schlüsseltranskriptionsfactor die hypertrophe Differenzierung von Chondrozyten durch Expressionsinduktion von Markern wie Col X und MMP-13 die. Funktionsmodifizierung durch „gain and loss of function“ Analysen von Runx2 unter Verwendung stabiler cDNA Transfektion sowie siRNA Knockdown offenbarte ST2 als neues Runx2-Target in Chondrozyten. Die Runx2-vermittelte Hochregulierung von ST2 wurde weiters in primären humanen Chondrozyten der Wachstumsplatte (PHCs) bestätigt. Die Entstehung der ST2 Isoformen wird von proximalen und distalen Promotern zelltypspezifisch gesteuert. Wir wiesen den proximalen Promoter als dominant für die Expression von ST2 Isoformen in ATDC5 und PHCs nach, wenngleiche auch der distale Promoter zu einer sST2 Expression in ATDC5 Chondrozyten beitragen kann.

dem vaskulären endothelialen Wachttumsfaktor A (VEGFA) und einer erhöhten Expression der beiden Proliferationsmarker Collagen II (Col II) und Sox9 in ATDC5 Zellen verbunden. Folglich legen unsere Untersuchungen nahe, dass ST2 ein neuer Regulator von früher und später Chondrozyten-Differenzierung in ATDC5 Chondrozyten ist.

Weiters wurde die Runx2-vermittelte Modulation von Runx3, einem weiteren Mitglied der runt-bezogenen Transkriptionsfaktoren-Familie, in ATDC5 und PHCs belegt. Die Auswirkung eines Runx3 Knockdowns auf Differenzierungsmarker in ATDC5 war ähnlich zu jener, die durch ST2 Silencing beobachtet wurde. Dies zeigt, dass ST2 und Runx3 proliferierend wirken und die hypertrophe Differenzierung fördern. Der supprimierende Einfluss von ST2 und Runx3 auf die Runx2-vermittelte Hochregulation der hypertrophen Marker Col X, OSC, VEGFA und MMP-13 deutet auf eine kooperative Regulation der Chondrozyten-Hypertrophie durch Runx2-assoziierte Aktivierung der Downstream-Ziele ST2 und Runx3 in ATDC5-Chondrozyten hin.

Zusammen genommen zeigt diese Studie zum ersten Mal die Expression, Regulierung und Funktion von ST2 in Chondrozyten, vor allem in postnatalen Chondrozyten der Wachstumsplatte, wo das Knochenwachstum von komplexen molekularen und biochemischen Mechanismen kontrolliert wird, die noch nicht vollständig bekannt sind. Unsere Untersuchungen liefern wichtige neue Einblicke in die Rolle von ST2 in der Funktion von Chondrozyten der Wachstumsplatte, wodurch weiteres Verständnis des hoch orchestrierten Prozesses des Knochenwachstums in Längsrichtung generiert wird.
5. Abstract

Bone tissue is a well-known site of expression of ST2 (IL1RL1). However, knowledge about expression, regulation, and function of ST2 in chondrocytes is limited. Immunohistochemical analysis indicated prominent ST2 expression in hypertrophic chondrocytes of murine growth plates in both femur and tibia whilst this expression was low or absent in resting and proliferative zones. This result was verified by significant up-regulation of both surface receptor (ST2L) and soluble (sST2) isoforms during hypertrophic differentiation of ATDC5 cells concomitant with the expression of hypertrophic markers Collagen 10 (Col X), Runt-related transcription factor 2 (Runx2) and matrix metalloprotease 13 (MMP-13). Key transcription factor Runx2 promotes hypertrophic differentiation of chondrocytes through induction of markers like Col X and MMP-13. Gain and loss of Runx2 function using stable cDNA transfection and siRNA knockdown revealed ST2 to be a novel Runx2 target in chondrocytes. Runx2 mediated up-regulation of ST2 was further corroborated in primary human growth plate chondrocytes (PHCs). The ST2 isoforms arise from proximal and distal promoters in a cell-type specific manner. We demonstrate the dominant promoter driving expression of ST2 isoforms in ATDC5 and PHCs to be the proximal promoter even though distal promoter is also capable of sST2 expression in ATDC5 chondrocytes.

Consistent with pronounced ST2 expression in hypertrophic chondrocytes, we observed a perceptible hypertrophic size reduction alongside significantly diminished expression of Col X and osteocalcin (OSC) in growth plates of femur and tibia from ST2 knockout mice in comparison with wild type (WT) control. These observations were substantiated by the effect of siRNA-mediated ST2 knockdown in ATDC5 cells. The ST2 silencing was associated with a slight decrease of Col X, significant suppression of OSC and vascular endothelial growth factor A (VEGFA) and enhanced expression of proliferative stage markers Collagen II (Col II) and Sox9 in ATDC5 cells. Thus, our investigation suggests that ST2 is a new regulator of early and late chondrocyte differentiation in ATDC5 chondrocytes.

Furthermore, Runx2 mediated modulation of Runx3 another member of runt-related transcription factor family was documented in ATDC5 and PHCs. The impact of Runx3 knockdown on differentiation markers in ATDC5 was similar to
that observed by ST2 silencing indicating that ST2 and Runx3 appose proliferating and act towards promotion of hypertrophic differentiation. Eventually, the suppressive influence of ST2 and Runx3 knockdown on the Runx2 mediated upregulation of hypertrophic markers Col X, OSC, VEGFA and MMP-13 propose a cooperative regulation of chondrocyte hypertrophy through Runx2 associated activation of downstream targets ST2 and Runx3 in ATDC5 chondrocytes.

Taken together, this study points for the 1st time to the expression, regulation, and function of ST2 in chondrocyte, in particular, post-natal growth plate chondrocyte where bone growth is controlled by complex molecular and biochemical mechanisms which are not fully known. Our investigation provides an important new insight into the role of ST2 in growth plate chondrocyte function, thereby allowing further comprehension of the highly orchestrated process of longitudinal bone growth.
Introduction
6. Introduction

6.1. ST2 isoforms

The ST2 known also as IL1RL1, DER4, T1, FIT1 and IL-33R was initially identified in the growth-stimulated but not resting murine fibroblast BALB/c-3T3 cells with noticeable sequence resemblance to the extracellular motif of the murine interleukin 1 receptor (Tominaga, 1989). This gene was also identified by another group in murine NIH 3T3 fibroblast cells to be induced by serum as well as Ha-ras and v-mos oncoproteins (Klemenz et al., 1989). The ST2 is regarded as an early or delayed early serum response gene (Yanagisawa et al., 1992, Kalousek et al., 1994). The ST2 is Toll-like/IL-1 receptor superfamily member and so far 4 ST2 isoforms produced by alternative splicing mechanism have been identified. The soluble ST2 (sST2) was originally identified and described as a 2.7 kb mRNA encoding an approximately 37 kD secreted the protein (Werenskiold, 1992, Takagi et al., 1993, Kakkar and Lee, 2008). The longer ST2 transcript (567 amino acids) with putative transmembrane domain and a cytoplasmic domain was subsequently identified in 1993 and designated as an ST2 transmembrane receptor (ST2L) (Yanagisawa et al., 1993). Except for a unique nine amino-acid at C-terminus, the sST2 structure is identical to the extracellular domain of ST2L and therefore sST2 lacks transmembrane and cytoplasmic domains. In addition to the two major ST2 isoforms, two other products of alternative splicing mechanism namely ST2V and ST2LV were identified in human leukemic cell line UT-7/GM and chicken embryos respectively (Tominaga et al., 1999, Iwahana et al., 2004). Experiments of human tissues indicated that ST2V is predominately expressed in the organs including spleen, stomach, intestine, colon and, testis while is absent in the other tissues such as brain, heart, liver and skeletal muscles (Tago et al., 2001). The initial in vivo assessment of the ST2L and sST2 expression pattern, however, indicated that soluble and transmembrane receptors were differentially expressed in various tissues. Whereas ST2L was abundantly found in the hematopoietic embryonic organs, the sST2 expression was mainly restricted to the nonhematopoietic embryonic tissues like bone, skin and, retina (Rossler et al., 1995). The ST2L was found to be constitutively expressed on the surface of T helper cell type 2 (Th2) as well as mast cells but not by T helper cell type 1 (Th1) or the other immune cells.
thus suggested a Th2 specific cell surface marker (Yanagisawa et al., 1997, Xu et al., 1998, Lohning et al., 1998).

6.2. ST2 gene structure and regulation

A dual promoter system namely distal and proximal was shown to be involved in the regulation of soluble and transmembrane receptor expressions. In the mouse and human ST2 gene, the distal and proximal promoters are situated with relatively 10.5 and 8 kb pairs distance from each other respectively (Iwahana et al., 1999, Gachter et al., 1998). The transcription of soluble or transmembrane receptors starting from either concomitant non-coding Exon 1a (E1a) or Exon 1b (E1b) is a cell type-specific process (Iwahana et al., 1999, Gachter et al., 1998, Hayakawa et al., 2005, Bergers et al., 1994). The ST2 gene initiates translation from the common Exon2 (E2) where the translation start codon is located disregarding whether ST2 isoforms are transcribed from the distal or proximal promoter. Figure 1 represents 5’ region of the ST2 gene structure up to E2.

For instance, murine and human fibroblasts use proximal promoter to initiate the transcription of both soluble and transmembrane ST2 isoforms. On the other hand, the distal promoter is the dominant promoter transcribing both ST2 splice variants.
in murine mast cells and thymoma cell line EL-4 (Iwahana et al., 1999, Gachter et al., 1998, Hayakawa et al., 2005). In human leukemic cell line UT-7, even though both promoters are utilized, the distal promoter is mainly used for the transcription of ST2 isoforms (Iwahana et al., 1999). In the rat homologue of ST2 gene (FIT-1) however, distal promoter expresses transmembrane receptor while the soluble form is mainly transcribed from the proximal promoter indicating that transcription of ST2 isoforms is a promoter-specific process (Bergers et al., 1994).

6.3. The IL-33/ST2 Signaling Pathway

An important step in understanding the function of ST2 was the identification of its ligand Interleukin 33 (IL-33) in 2005 (Schmitz et al., 2005). IL-33 was attributed to the IL-1 cytokine family due to its characteristic β-sheet trefoil fold structure. It can function as both traditional cytokine and intracellular nuclear factor with transcriptional regulatory properties (Carriere et al., 2007). The IL-33 protein is able to bind and activate the ST2 receptor. For signal transduction, ST2L and IL-33 form a complex with IL-1R accessory protein (IL-1RacP), which is essential for IL-33 signaling through ST2L (Palmer et al., 2008, Ali et al., 2007, Chackerian et al., 2007). Interaction of IL-33 with its receptor ST2L induces recruitment of the adaptor protein myeloid differentiation primary response 88 (MyD88) to the TIR domain in the cytoplasmic region of ST2L (Schmitz et al., 2005). The adaptor protein MyD88 and the associated protein IL-1R-associated kinases (IRAKs) induce downstream signaling through TNF receptor-associated factor 6 (TRAF6). The TRAF6 activates mitogen-activated protein kinase (MAPK) - kinases, the extracellular signal-regulated kinases (ERK1/2), Jun N-terminal kinase (JNK) and p38 (Schmitz et al., 2005). Furthermore, TRAF6 leads to the liberation of active nuclear factor-kappa-B (NF-KB) from the inhibitor of NF-KB complex, I kappa B kinase (IKK) and to the production of the Th2-associated cytokines Interleukin 4 (IL-4), IL-5 and IL-13 (Schmitz et al., 2005). The sST2 however, can inhibit the biological activity of IL-33 through binding it and thereby acting as decoy receptor (Hayakawa et al., 2007, Kakkar and Lee, 2008)(Figure 2).
Figure 2. Scheme of the canonical IL-33/ST2 signaling pathway. Interaction of IL-33 with its receptor complex ST2L/IL-1RacP induces recruitment of MyD88 and IRAK-1/4 and activation of TRAF6. TRAF6 mediated liberation of the transcription factor NF-κB from the IKK complex, and activation of MAPK pathway, which is mediated by the activation of ERK1/2, p38, and JNK, ultimately lead to the downstream effects. The soluble ST2 isoform acts as decoy receptor binding IL-33 and thereby inhibiting its biological activity.
6.4. Skeletal development

The process of skeletal development in the vertebrate is mediated by Mesenchymal cell (MSC) condensations that eventually differentiate through either intramembranous or endochondral ossification processes under a defined expression of genes during fetal development (Kronenberg, 2003). Whereas intramembranous ossification results in the formation of flat bones of skulls and calvariae, long bones including limbs are formed via the process of the endochondral ossification (Franz-Odendaal, 2011). During intramembranous ossification, the MSCs directly differentiate into the bone forming osteoblasts whilst endochondral ossification leads to the formation of cartilaginous cells i.e. chondrocytes that are utilized as a template to be subsequently replaced by the bone (Mackie et al., 2011, Zhang et al., 2011a). Before birth, bone replaces most of the cartilage, however, some cartilage (growth plate) will be replaced by the bone during childhood and some remains during adulthood period (OpenStax, 2016).

6.5. Post-natal growth plate (epiphyseal plate) and endochondral ossification

By the time the fetus skeleton formation is completed, the cartilage remains on the surface of the joints named articular cartilage as well as in between of diaphysis and epiphysis of the long bones termed growth/epiphyseal plate (OpenStax, 2016). This cartilaginous organ located at the end of the long bones orchestrates longitudinal bone growth postnatally. The growth plate contains chondrocytes at 3 principal stages of differentiation organized into resting, proliferative/columnar and hypertrophic zones (Figure 3). During endochondral ossification within the growth plate, avascular cartilage gradually converts into a highly vascularized bony tissue thus leading to longitudinal bone growth, a process that ends at late adolescence (Gerber and Ferrara, 2000, Olsen et al., 2000). In resting zone (RZ) and closest to the epiphysis, low proliferative irregularly scattered small spherical cells are present in a bed of extracellular cartilage matrix (Abad et al., 2002). In vivo study has proven that RZ regenerates complete growth plate 1 week after removal of proliferative (PZ) and hypertrophic zones (HZ) demonstrating RZ to have stem-like
cells with renewal ability of the PZ (Abad et al., 2002). In the PZ, the flattened cells resembling “stack of coins” are aligned in columns and highly proliferate in parallel towards the long axis of the bones (Hunziker and Schenk, 1989, Nilsson and Baron, 2004)(Figure 3). During hypertrophic differentiation, however, chondrocytes cease proliferation and enlarge in size of approximately 10-folds by an active process of increase in organelles like endoplasmic reticulum (ER) and mitochondria, which lead to the intracellular volume enhancement (Hunziker et al., 1987, Hunziker, 1994). The chondrocyte hypertrophy plays a key role in longitudinal bone growth. In addition to volume increase, the chondrocyte hypertrophy and terminal maturation phase is accompanied with expression of hypertrophic and terminally differentiated markers such as Indian Hedgehog (Ihh), Runx2, Col X, OSC, MMP-9, MMP-13, alkaline phosphatase (ALP), VEGF and extracellular matrix remodeling (Studer et al., 2012, Kwon et al., 2011, Zhang et al., 2004, Kozhemyakina et al., 2015). The Hypertrophic chondrocytes subsequently mineralize the matrix through apatitic crystal deposition (calcification) which serve as an appropriate scaffold for the invasion of chondroclast/osteoclasts, bone progenitor/forming cells and blood vessels (Yin et al., 2002). Vascular invasion occurs simultaneously with the hypertrophic chondrocytes apoptosis even though according the recent proofs, terminally differentiated hypertrophic chondrocytes are also capable to trans-differentiate to osteoblasts (Kronenberg, 2003, Cheung et al., 2003, Shapiro et al., 2005, Gibson et al., 1997, Yang et al., 2014, Zhou et al., 2014, Enishi et al., 2014). The mechanisms dictating hypertrophic chondrocytes trans-differentiation to osteoblasts are not, however, understood as yet. The vascular invasion is mediated by hypertrophic chondrocytes secretion of the VEGFA (Colnot and Helms, 2001). The bone is subsequently deposited by the osteoblasts on the remnants of the growth plate (Mackie et al., 2008) thus remodeling the matrix. By the end of the endochondral bone ossification process, the mineralized cartilage is replaced by the endochondral bone tissue thus the process leads to longitudinal bone growth. It worth emphasizing that the skeletal bone growth would continue as long as the growth plates generate chondrocytes (Gilbert, 2000) and this process would cease by the end of the pubertal stage at the late adolescence (Shim, 2015).
Figure 3. Scheme representing structure of the growth plate: chondrocytes at different stages of differentiation are organized into resting, proliferative/columnar and hypertrophic zones. The process of endochondral ossification within the growth plate converts the avascular cartilaginous tissue into vascular bony tissue thus leading to longitudinal bone growth. RZ: resting zone, PZ: proliferative zone, HZ: hypertrophic zone.
6.6. Local and systemic factors controlling growth plate metabolism

A complex network of systemic and local factors precisely modulates the chondrocytes behavior towards the complete process of endochondral ossification even though the detailed mechanisms of these regulations are not fully known. Below, some of the most important factors are briefly described.

6.6.1. The systemic factors (hormones)

The effects of some of the important hormones (such as growth hormone, thyroid hormone, and estrogen) on the regulation of the growth plate metabolism are briefly discussed in this section.

6.6.1.1. Growth hormone (GH)

As known for many years, the GH is the key regulator of the post-natal bone growth. The GH effect is highly dependent on the induction of the downstream factor insulin-like growth factor-1 (IGF1) in the liver as well as peripheral tissues (Sjogren et al., 1999). Decreased level of circulating liver-derived IGF1 for instance, results in a reduced longitudinal growth in mice (Yakar et al., 2002). These results were corroborated by the IGF1 effect following administration in the mice and human with inactivating mutations in GH receptor (Guevara-Aguirre et al., 1997, Mackie et al., 2008). Reviewed in (Mackie et al., 2008), the paracrine mode of the IGF-1 action has been also shown indicating that the local IGF-1 expression would play a major role in longitudinal growth. IGF-1 affects longitudinal growth through regulation of the various stages of the chondrocytes differentiation (Hunziker et al., 1994).

6.6.1.2. Thyroid hormone

Another known hormone influencing growth plate and skeletal development is thyroid hormone (Shao et al., 2006). In human, the hypothyroidism results in severe hypertrophic differentiation impairment decreased longitudinal bone growth
and thus height deficit (Mackie et al., 2008, Rivkees et al., 1988). An investigation by O’Shea PJ et. Al (O’Shea et al., 2005), indicated the skeletal hypothyroidism, retarded intramembranous and endochondral ossification, postnatal growth delay and reduced mineralization in trabecular bone in mice harboring thyroid hormone receptor alfa-1 mutation as reviewed in (Karimian et al., 2011). Furthermore, in both in vitro and in vivo studies, the transition between proliferation to hypertrophy has been shown to be importantly regulated by the thyroid hormone as reviewed in (Shao et al., 2006).

6.6.1.3. Estrogen

Investigation in humans has suggested that the low estrogen level accompanied by the high GH level is a significant contributing factor in growth lunge whilst the elevated level of estrogen leads to the growth arrest revealing estrogen as a potent inhibitor of the longitudinal growth if expressed beyond a certain concentration (van der Eerd en et al., 2003). The estrogen hormone has been discovered to be involved in the fusion of the growth plate after puberty. The patients diagnosed with either of mutations in the aromatase gene which results in estrogen deficiency or mutations in the estrogen receptor-α that leads to the estrogen resistance failed to normally fuse the growth plate (Morishima et al., 1995, Smith et al., 1994). Although not fully known, the fusion of epiphyseal plate is apparently mediated by the estrogen-dependent acceleration of the programmed senescence in the growth plate chondrocytes through the promotion of the proliferative potential exhaustion (Weise et al., 2001).

6.6.2. The local factors

6.6.2.1. Indian hedgehog (Ihh)

The morphogen Indian hedgehog (Ihh) is a family member of hedgehog signaling proteins (Gritli-Linde et al., 2001) playing several crucial roles in bone and cartilage development. The Ihh has been initially found to be expressed by the pre-hypertrophic chondrocytes in the vicinity to the proliferative zone in the developing cartilage of mice embryos (Bitgood and McMahon, 1995, Vortkamp et
The function of the Ihh during skeletal development has been assessed by the ablation of this protein in mice. The Ihh-deficient mice (Ihh -/-) displayed a reduced proliferative zone and abnormalities in the formation of hypertrophic chondrocytes. Furthermore, mature osteoblasts were absent in endochondral bones indicating the implication of Ihh in regulating chondrocyte differentiation and bone formation (Bitgood and McMahon, 1995). These reports thus show that Ihh plays several key roles in the process of endochondral bone development.

6.6.2.2. Parathyroid hormone-related peptide (PTHrP)

The PTHrP seemingly belongs to the parathyroid hormone family (Martin et al., 1991). The PTHrP and its receptor mRNA have been detected in skeletal tissue in developing rat embryos (Lee et al., 1995). The PTHrP null mice (PTHrP (-/-)) develops extensive abnormalities in the growth plate development including the dramatic decrease in growth plate size, premature mineralization and a faster bone formation suggesting a vital influence of the PTHrP gene in the process of endochondral bone development in mice (Karaplis et al., 1994). The further PTHrP study in PTHrP (-/-) mice has verified the paracrine importance of this protein mainly in the proliferative stage of chondrocyte differentiation. In a report by Lee, K. et. al (Lee et al., 1996), the PTHrP (-/-) mice has shown a substantial reduction in the proliferating zone and accelerated process of chondrocyte terminal differentiation i.e. hypertrophic, apoptosis and mineralization substantiating the significant PTHrP effect on retaining the chondrocyte proliferation and delaying the terminal differentiation. The Ihh promotes the PTHrP expression (Vortkamp et al., 1996). In this view, the initiation of the chondrocyte hypertrophy is inhibited/delayed by the Ihh mediated induction of PTHrP signaling in periarticular chondrocytes at the ends of long bones representing a feedback loop mechanism that regulates chondrocyte differentiation (Schipani et al., 1997, Hirai et al., 2011, Kronenberg, 2003).
6.6.2.3. Bone Morphogenetic Proteins (BMPs)

The BMPs belong to the transforming growth factor-β (TGFβ) superfamily and initially discovered as the potent inducers of the endochondral bone formation (Wozney et al., 1988, Tsumaki and Yoshikawa, 2005). Extensive investigations in mice have shown the several BMPs function in osteoblasts, osteoclasts, and chondrocyte differentiation as well as subsequent bone remodeling (Tsumaki and Yoshikawa, 2005). In chondrocytes, retroviral overexpression of BMP-2 and BMP-4 during limb development of chick resulted in a delay in chondrocyte hypertrophy and substantial elevation in the number of chondrocytes not due to the increased proliferation but apparently through differentiation of perichondrium or surrounding mesenchyme into the chondrogenic cells (Duprez et al., 1996). In another study, evaluation of the role of two type 1 BMP receptors, BMPR-IA and BMPR-IB showed that whereas BMPR-IA is involved in the regulation of chondrocyte hypertrophy as a downstream target of Ihh, BMPR-IB expression is essentially required for the early stages of chondrogenesis during development of the chick limb (Zou et al., 1997).

6.6.2.4. Fibroblast growth factors (FGFs)

The FGF family of secreted proteins consists of at least 22 identified proteins that bind to and activate 4 FGF receptors (FGFR) (Ornitz and Marie, 2002). The chondrocyte differentiation as indicated by several genetic studies is critically regulated by the FGF signaling (Kronenberg, 2003). The expression of many of these FGF and FGFRs has been detected during different stages of endochondral bone formation. When growth plate is formed, FGFR3 expression begins in resting and proliferating chondrocytes that is followed by the expression of FGFR1 in later stages of pre-hypertrophic and hypertrophic differentiation (Ornitz and Marie, 2002). The overexpression of FGFR 2 has been detected in resting zone and FGFR 4 overexpression during resting and proliferative zones (Lazarus et al., 2007). Whilst FGFR3 knockout mice developed extended chondrocyte columns via enhancement in the rate of chondrocyte proliferation, transgenic mice carrying FGFR3 activating mutation led to the inhibition of chondrocyte proliferation and
deceleration of differentiation (Deng et al., 1996, Colvin et al., 1996, Naski et al., 1998).

6.6.2.5. Vascular endothelial growth factors (VEGFs)

The VEGF is a mitogen for the vascular endothelial cells and it was initially identified as a growth factor for the endothelial cells from bovine pituitary follicular cells (Yang et al., 2012). Seven members of the VEGF family namely, VEGF-A (Know also as VEGF), -B, -C, -D, -E, -F and placenta growth factor have been identified (Ferrara and Davis-Smyth, 1997, Roy et al., 2006). The main mediators of angiogenesis, i.e., VEGFs, play crucial roles in a vascular invasion in the growth plate to successfully complete the process of endochondral bone formation. In addition, VEGF is suggested to be involved in the diverse bone developing processes including osteoblast and chondrocyte differentiation as well as recruitment of osteoclasts (Yang et al., 2012). The VEGFs signal through tyrosine kinase receptors VEGF receptor 1 and VEGF receptor 2 (Yang et al., 2012). During endochondral ossification process, the VEGF expressed by hypertrophic chondrocytes mediates blood vessel invasion bringing undifferentiated mesenchymal cells into hypertrophic/mineralization zone. These cells will subsequently undergo differentiation into the osteoblasts depositing bone matrix (Rabie and Hagg, 2002). The VEGF or VEGF receptor blockage inhibits blood vessel invasion, adversely influencing the growth plate structure by the expansion of hypertrophic zone, trabecular bone impairment, and reduced differentiation and/or recruitment of chondroclasts (Wedge and Ogilvie, 2000, Gerber et al., 1999). The termination of VEGF blockage reversed the effects and resulted in the normal growth plate function including re-occurrence of capillary invasion, resorption of the hypertrophic chondrocyte and restoration of bone growth (Gerber et al., 1999).

6.6.2.6. Wnt/β-catenin signaling

The Wnt proteins play several major roles during skeletal development. The Wnt signaling functions either via β-catenin, which is known as canonical Wnt pathway or independent of β-catenin, called as non-canonical pathway (Usami et al., 2016).
During mice and chick cartilage development, several Wnt proteins including Wnt4, 5a, 5b, 11 and 14 are differentially expressed (Usami et al., 2016) representing several Wnt functions in this tissue. Retroviral-based overexpression of Wnt5a and Wnt5b or Wnt major secreted antagonist Frzb/Frp3/Sfrp3, for instance, induce chondrogenic differentiation of the mesenchymal cells whereas the expression of other Wnt factors like Wnt 1, 4 or 8A suppress this differentiation in chick limb bud in vitro culture or during chick limb development (Usami et al., 2016). Further studies have indicated an inhibitory effect of Wnt1 and Wnt7a over the chondrogenic differentiation of prechondrogenic mesenchyme that was corroborated by the failure of cartilage formation in chick embryos mediated by these Wnt factors (Rudnicki and Brown, 1997). In addition, in chick limbs, whilst β-catenin mutant induces hypertrophic differentiation of chondrocytes, Frzb/Frp3/Sfrp3 and Wnt5a suppress chondrocyte hypertrophy and matrix mineralization revealing diverse functions of these Wnt factors in regulating endochondral ossification (Hartmann and Tabin, 2000, Enomoto-Iwamoto et al., 2002, Tamamura et al., 2005). The Wnt pathway have also been extensively investigated in mice confirming the importance of this signaling pathway in mice skeletal development (Guo et al., 2004, Yuasa et al., 2009, Hill et al., 2005, Day et al., 2005, Mak et al., 2006, Chen et al., 2008, Candela et al., 2014, Cantley et al., 2013).

6.6.2.7. Transcription factor SRY (sex determining region Y)-box 9 (SOX9)

Developmental process of chondrocytes and osteoblasts are precisely controlled by the expression of the specific gene profile (Lefebvre and Smits, 2005, Dy et al., 2012) among which a set of cell-type-specific transcription factors play crucial roles towards cell fate determination (Karsenty et al., 2009, Dy et al., 2012). Several phases of chondrocyte lineage formation from differentiation of mesenchymal stem/progenitor cells to chondrocytes and progression and conversion of proliferative chondrocytes to hypertrophic chondrocytes are regulated by the major transcription factor SOX9 (Lefebvre and de Crombrugghe, 1998, Zhou et al., 1998, Lefebvre and Smits, 2005, Andrade, 2010). The Sox9 mutation affects growth plate structure leading to the severe dwarfism (the disease called campomelic dysplasia) in human (Foster et al., 1994, Wagner et al., 1994).
Mesenchymal cells of the Sox9-/- lineage neither condense nor participate in the subsequent formation of chondrocytes (Bi et al., 1999, Kronenberg, 2003). Furthermore, a selective SOX9 knockout from limb mesenchyme has led to the inhibition of cartilage condensation along with enhanced apoptosis and suppression of the two other SOX family genes Sox5 and Sox6, that are involved in chondrogenesis (Akiyama et al., 2002). The SOX9 is expressed in resting, proliferative and pre-hypertrophic chondrocytes but disappears in hypertrophic chondrocytes both in pre- and postnatal growth plates (Zhao et al., 1997, Hattori et al., 2010) indicating SOX9 role during all sequential stages of chondrocyte differentiation. The absence of SOX9 during chondrocyte differentiation was associated with reduced chondrocyte proliferation and declined extracellular matrix genes (Akiyama et al., 2002, Kronenberg, 2003). On the other hand, misexpression of SOX9 in hypertrophic zone reduced expression of the hypertrophic markers VEGF, MMP-13, and osteopontin thus hinder terminal differentiation and vascularization (Hattori et al., 2010). These investigations have confirmed the necessity of the major transcription factor sox9 for all phases of chondrocyte formation and differentiation to ensure fulfillment of cartilage dependent skeletal growth.

6.6.2.8. Transcription factor Runx2

The Runx2 known previously as cbfa1 is a key transcription factor involved in osteoblast differentiation and bone development (Ducy et al., 1997). The Runx2 expression is required for the osteoblast specification from multipotent mesenchymal cells (Komori, 2002). A homozygous mutation of Runx2 in mice led to the maturational arrest of osteoblasts and complete inhibition of bone formation (Komori et al., 1997). Runx2 also plays a central role during chondrocyte differentiation (Fujita et al., 2004). Runx2 is extensively expressed in pre-hypertrophic and hypertrophic chondrocytes and lack of Runx2 (Runx2 -/- mice) results in severely disturbed differentiation and maturation of chondrocytes (Fujita et al., 2004, Inada et al., 1999). The Runx2 is, however, strongly downregulated in proliferating chondrocytes (Kronenberg, 2003). Mice lacking Runx2 have no or decreased number of hypertrophic chondrocytes, severely hampered matrix mineralization ability, and reduction or suppressed expression of osteopontin and
MMP-13 that are observed in normal late hypertrophic chondrocytes (Kronenberg, 2003). These observations were further corroborated by the effect of Runx2 or dominant negative form of Runx2 in transgenic mice (Takeda et al., 2001, Ueta et al., 2001). The hypertrophic differentiation happening in chondrocytes lacking Runx2 is suggested to be dependent on the function of other genes like Runx3, the other member of Runt-related transcription factors family (Kronenberg, 2003). Furthermore, MADS box transcription factors, mostly Mef2c have been also shown to drive hypertrophic differentiation (Takeda et al., 2001, Arnold et al., 2007). Overall, several lines of evidence substantiated the important role of Runx2 as a central regulator of chondrocytes differentiation.
Aim
7. Aims of the thesis

The osteoblasts, osteoclasts and chondrocytes are three major components of the skeletal system. Investigations by Werenskiold et al. has suggested sST2 as an early marker of osteoblast differentiation expressed concomitantly with early osteoblast differentiation markers collagen I (Col I) and osteonectin (OSN) in mandibular condyle and osteogenic cell lines MCT3T-E1 and KM-1K (Werenskiold et al., 1995). In another study, ST2L dependent regulation of the osteoblast differentiation has been shown in osteosarcoma cells when cultured in a 3-dimensional collagen I matrix (Werenskiold et al., 1999). Moreover, a recent report has demonstrated inhibitory effect of IL-33/ST2 signaling on the osteoclast formation from bone marrow precursor cells both in vitro and in vivo (Schulze et al., 2011).

The expression, regulation and the function of the ST2 protein as a main component of the IL-33/ST2 signaling pathway has not been addressed yet in growth plate, a cartilaginous tissue comprising chondrocytes at different differentiation stages. We hypothesized that ST2 might regulate chondrocytes behavior because the MAPK and NF-KB, the downstream pathways of IL33/ST2 signaling (Schmitz et al., 2005), are known regulators of chondrocyte proliferation, differentiation and cell death (Hutchison, 2012, Murakami et al., 2004, Wu et al., 2007). Therefore, the main goals of this study were to:

- Investigate the ST2 expression pattern during sequential stages of the chondrocyte differentiation in post-natal growth plate.

- Investigate the transcriptional regulation of ST2 in chondrocytes.

- Investigate the ST2 function during chondrocyte differentiation.
Material and Method
8. Material and method
(The chapter below follows (Bonyadi Rad et al., 2017b) publication)

8.1. Cell culture

Murine chondrocyte cell line ATDC5 was purchased from European collection of cell cultures (ECACC, Salisbury, UK). Dulbecco’s Modified Eagle’s Medium/Nutrient F-12 Ham media supplemented with 5% (v/v) fetal bovine serum (FBS) (PPA Pasching Austria), 10 µg/ml human transferrin (Sigma-Aldrich St. Louis, MO) and 3 X 10^-8 M sodium selenite (Sigma Chemical, St Louis, MO, USA) was used to culture/maintain the cells. In differentiation experiments, 10 µg/ml bovine insulin (Sigma-Aldrich St. Louis, MO) and 37,5 µg/ml ascorbic acid (Wako pure chemical industries) were added into the culture/maintenance media according to the previous publication (Altaf et al., 2006). Murine osteogenic cell line MC3T3-E1 was also obtained from European collection of cell cultures (ECACC, Salisbury, UK) and was cultured/maintained in Eagle’s minimum essential medium (Sigma-Aldrich, Vienna, Austria) containing 10% fetal bovine serum and 2mM Glutamine (Invitrogen, CA, USA). Media was changed every 2nd day during the entire study and cells were incubated at 37 °C in a humidified atmosphere with 5% CO2.

8.2. Primary human growth plate chondrocytes (PHCs) isolation and culture

At the time of surgery, the supernumerary digits of 5 polydactyly patients (3 males and 2 females) from 0.25 to 6 years old were excised by the surgeon and transferred aseptically to the cell culture lab. The cells were collected after the growth plate tissues were digested overnight in 2 mg/ml collagenase B (150 units/mg collagenase B, Worthington Biochemical Corp., Lakewood, NJ, USA) at 37.5 °C in a shaking water bath. The cell suspensions were subsequently passed through two layers of nylon grid (40 µm mesh size) and cultured in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 containing 5 % FBS and 2 mM glutamine. PHCs were maintained at 37 °C, in a humidified atmosphere with 5% CO2. Passage one and passage two of PHCs were used during entire study and the medium was changed every alternate day. The consents were received from the parents or legal representatives. Ethical approval for this investigation received
from Medical University of Graz ethics committee and all experimental procedures were conducted according to the relevant guidelines and regulations.

8.3. Quantitative reverse transcription polymerase chain reaction (qPCR)

The qPCR experiment was carried out according to the previous report (Bonyadi Rad et al., 2016). Total RNA was extracted from ATDC5 chondrocyte, PHCs and M3T3-E1 osteoblast cell line using Rneasy Mini Kit (Qiagen, Hilden, Germany) in accordance with manufacturer’s protocol. During RNA purification and in accordance with the manufacturer’s protocol, the potential DNA contamination was removed using Dnase I (Qiagen, Hilden, Germany). 2µg of total RNA was utilized for the cDNA preparation by the First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). The SYBR Green Master Mix was purchased from Invitrogen and used for PCR amplification reactions. The AB7900 HT TaqMan PCR system (Applied Biosystems) was utilized for detecting PCR products. GAPDH and 18S RNA was used as the internal control throughout knockdown/overexpression and differentiation experiments respectively. The PCR data are represented as relative expression to the respective controls and calculated by the $2^{-\Delta\Delta Ct}$ method. Some of qPCR experiments were performed by Maryam Heidary (Institute Curie, Paris, France).

8.4. Reverse transcription PCR (RT-PCR) and promoter usage analysis by RT-PCR

The procedures of RNA isolation and cDNA preparation explained before. The synthesized cDNAs were subsequently utilized in PCR reactions using phusion high fidelity DNA polymerase (Thermo scientific- Fisher Scientific - Austria GmbH). The following primers assessed the promoter usage analysis for the expression of murine ST2L and sST2:

distal first exon (E1a) forward primer : 5′GAATAAAGATGGCTAGGACCTCTGG3′,
proximal first exon (E1b) forward primer : 5′AATGAGACGAAGGAGCGCCAAGTAG3′,
ST2L reverse primer : 5′GCCACTCAACGGAGCCGCAA3′ and sST2 reverse primer: 5′ACCAATACCAATGTCCCTTGTAGTCGG3′ (Hayakawa et al., 2005).
Promoter usage analysis in human chondrocyte samples was examined by following primers.

Exon1a (E1a) forward primer: 5’GAGAAATTGGCTTCTGAGTTGTGAAACTGTGGGC3’, exon1b (E1b) forward primer: 5’TCACTGACTCAAGTTTCATCCCTCTCTGTTTTCAG3’, ST2L reverse primer: 5’TCAAATCACAGATGCCTTTGCACATCAGCAGGCA3’ and sST2 reverse primer: 5’CTCTTGAACCACAACACTCCATCTGCTTACACTTGC3’ (Iwahana et al., 1999). 1 % ethidium bromide-stained agarose gels were used to observe the PCR products. The qPCR and RT-PCR primers are shown in Table 1.
Table 1. List of qPCR and RT-PCR primers utilized in this study. Table 1 was published elsewhere (Bonyadi Rad et al., 2017b)

<table>
<thead>
<tr>
<th>Primer sequences, qPCR</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Murine ST2L: F5′GCCCGAGCAGTTCTTGAAAATA3′, R5′ATCTCTCTGCTCGTGGAGCACA3′</td>
<td>This study</td>
</tr>
<tr>
<td>Murine ST2 (Total): F5′CCAGCCAGAGTGGAAGACTC3′, R5′CGAGTTAATGTTGACCGAGC3′</td>
<td>This study</td>
</tr>
<tr>
<td>Murine Runx2: F5′TGTTCTCTGATGCGTCTCAGTG3′, R5′CTGCTGGCTTGTCTTCTT3′</td>
<td>(Cho et al., 2013)</td>
</tr>
<tr>
<td>Murine Col II: F5′CCAGCCAGAGTGGAAGACTC3′, R5′CGAGTTAATGTTGACCGAGC3′</td>
<td>(Huang et al., 2012)</td>
</tr>
<tr>
<td>Murine Col X: F5′TGAGAAAACCTGGAGCAAC3′, R5′CTGCTGGCTTGTCTTCTT3′</td>
<td>(Huang et al., 2012)</td>
</tr>
<tr>
<td>Murine MMP-13: F5′TGATGAAACCTGGAGCAAC3′, R5′CTGCTGGCTTGTCTTCTT3′</td>
<td>(Huang et al., 2012)</td>
</tr>
<tr>
<td>Murine OSC: F5′AAGCAGGAGGGAATGGAAGG3′, R5′TAGGAAATGCTTCTCAAGCCATA3′</td>
<td>(Kobayashi et al., 2015)</td>
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<td>Murine Runx3: F5′ACCACAGGAGCAGTCGACC3′, R5′CGATGCTGCTGCGCTTGA3′</td>
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<td>Murine VEGFA: F5′CCAGGGGAGGCTCCGGA3′, R5′ACCACAGGAGCAGTCGACC3′</td>
<td>(Crnkovic et al., 2016)</td>
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<td>Human Runx3: F5′CAGAAGCTGGAGGACCAGAC3′, R5′GTCGGAGAATGGGTTCAGTT3′</td>
<td>(Cheng et al., 2008)</td>
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<td>Human sST2: F5′GGCTTGAGAAGGCACACCGT3′, R5′GGAGTGGGGGAGGACGAAC3′</td>
<td>(Mun et al., 2010)</td>
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<td>Human Aggrecan: F5′GCCAGTTTGTCATGGTGAA3′, R5′TTCTGAGGAGAGGGAGGAA3′</td>
<td>(Li et al., 2011)</td>
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<td>Human Col II: F5′CCAGGCTCCACACCTTCTT3′, R5′CTGCTGGCTTGTCTCAGG3′</td>
<td>(Li et al., 2011)</td>
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<tr>
<td>Human Col X: F5′TGAGAAAACCTGGAGCAAC3′, R5′CTGCTGGCTTGTCTTCTT3′</td>
<td>(Li et al., 2011)</td>
</tr>
<tr>
<td>Murine 18 S: F5′GCCCTCCAGATACTTTTG3′, R5′CTGCTGCTGGCTTGTCTG3′</td>
<td>(Suckow et al., 2012)</td>
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<td>Murine GAPDH: F5′GCCCTCCAGATACTTTTG3′, R5′CTGCTGCTGGCTTGTCTG3′</td>
<td>(Huang et al., 2010)</td>
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<tr>
<td>Human GAPDH: F5′GAAGGTGAAGGTCGGAGTC3′, R5′GAAGATGGGTGTAGGGATTTG3′</td>
<td>(Huang et al., 2010)</td>
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<table>
<thead>
<tr>
<th>Primer sequences, RT-PCR</th>
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<tr>
<td>Murine ST2L: F5′TGCTGGATCAGTTTTACCCTCTCAGGTC3′, R5′GCCCACTAAGGGACGGGAA3′</td>
</tr>
<tr>
<td>Murine sST2: F5′AGCTGCTGCTGGTCATCCGTTTTC3′, R5′ACCAAGCAATTTTGCTGGA3′</td>
</tr>
<tr>
<td>Murine GAPDH: F5′GAAGGGCAGGCTGCCAG3′, R5′GCCAGGAAGCAGTTTTTGCTGGA3′</td>
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<td>Human ST2 forward Primer (common for both ST2L and sST2): 5′AGGCCATTTTCTCAGTCCATA3′</td>
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<tr>
<td>Human ST2L Reverse Primer: 5′GGCCCTCAATCAGGGATTTTTAGGATGATAA3′</td>
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<tr>
<td>Human sST2 Reverse Primer: 5′CAGTGGACAAGAGGAGGGTTTCTGAAAAAGT3′</td>
</tr>
<tr>
<td>Human GAPDH: F5′GAAGGTGAAGGTCGGAGTC3′, R5′GAAGATGGGTGTAGGGATTTG3′</td>
</tr>
<tr>
<td>Human Runx2 (PPH01897C)</td>
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</table>
8.5. siRNA silencing and cDNA overexpression experiments

Gene knockdown and overexpression experiments were carried out according to the previous study (Bonyadi Rad et al., 2016). The siRNAs against ST2, Runx2 and Runx3 were obtained from Dharmacon (Thermo Scientific). 2nd additional siRNA sets targeting Runx2, and ST2 were purchased from Santa Caruz and control siRNAs (scRNA) were bought from Qiagen, Vienna, Austria. For the gene knockdown experiments, using Lipofectamine 2000 reagent (Invitrogen), ATDC5 cells were transfected with 150–200 pmol siRNA and 48 to 72 hrs post transfection expression analysis was conducted. For the cDNA overexpression experiments, cDNA clones encoding murine ST2L (NM_001025602), murine sST2 (NM_010743), murine (NM-001145920) and human (NM-001024630) Runx2 were obtained from Origene EU. To generate stable overexpressing ATDC5 clones, ATDC5 cells were transfected with 3µg of either empty vector or Runx2 cDNA plasmids. Approximately 24 hrs after transfection, cells were washed with phosphate-buffered saline (PBS), trypsinized and transferred to 25cm flasks. ATDC5 cells stably overexpressing murine Runx2 were then selected with 400 µg/ml G418 antibiotics treatment for one to two weeks. For experiments of the primary human growth plates chondrocytes, transient human Runx2 transfection was performed and gene expression was analyzed 24 to 48 hrs post-transfection. Lipofectamine 2000 reagent (Invitrogen) was used for the siRNA/cDNA transfection experiments according to the manufacturer’s protocol.

8.6. Immunoblotting

Immunoblotting was carried out according to the previous studies (Bonyadi Rad et al., 2016, Bonyadi Rad et al., 2017a). The whole cell lysates from ATDC5 chondrocytes and PHCs were prepared using RIPA buffer (Sigma-Aldrich St. Louis, MO). The protein concentration of each sample was measured by Bradford Protein Assay (BioRad, Hercules, CA). Protein samples were loaded at 10 µg per lane. After separation on 10% SDS-polyacrylamide gels, the protein was transferred to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica MA). To avoid any negative effect of stripping on the protein levels, before probing with primary antibodies, the membranes were cut based on the molecular weights of proteins of interest. The overnight incubation with primary antibodies was then
performed at 4 °C, and membranes were subsequently incubated with respective peroxidase-conjugated secondary antibodies. To visualize the proteins of interest, enhanced chemiluminescence (ECL) reagent (Amersham) was applied on the membrane followed by the membrane exposure to the X-ray films (Kodak). Protein expressions were normalized to their concerned β-actin levels and protein band intensities were quantified by the Image J software (1.46r). Following antibodies were used during our study: Anti-DDK tag antibody (Origene), anti-Runx2 (D1L7F) and Runx3 (D6E2) antibodies (Cell Signaling) anti-murine ST2 (AP30829PU) antibody (Acris), anti-human ST2 (C-20), β-actin (N21) and secondary antibodies (Santa Cruz).

8.7. Histochemistry and Immunohistochemistry (IHC)

Histochemistry and IHC analysis were conducted according to the previous reports (Musumeci et al., 2012b, Musumeci et al., 2012a). The mice were provided by Akira Lab, Osaka University, Japan and housed in the animal facility of the University of Erlangen-Nuremberg, Germany. The ST2−/− mice generation described elsewhere (Hoshino et al., 1999). The mice with c57bl/6 background were 10 times backcrossed and following primers used to genotype wild-type (WT) and homozygous ST2−/− mice before the experimental procedure: 5′TTGGCTTCTTTTAATAGGCC3′ (WT), 5′CTATCAGGACATACGGTTGGCTACC3′ (ST2−/-) and 5′TGTTGAAGCCAAGAGCTTACC3′ (WT and ST2−/-). The ethical approval for the animal studies was obtained from Mittelfranken government and all experiments were carried out according to the concerned regulations and guidelines. The specimens fixation from 4 WT and 3 homozygous ST2−/− mice (all male at 3 and 5 week-old age) was performed in 10% buffered formalin (for 24 hrs) or 4% paraformaldehyde (for 16 hrs) at room temperature after initial wash with PBS. The specimens were then rinsed overnight and incubated in a decalcifying solution (14% EDTA, PH: 7,2) for 7–10 days. They were subsequently washed for 1 hr followed by a dehydrating and clearance procedures in graded ethanol and xylene respectively. The specimens eventually embedded in paraffin wax. The prepared paraffin blocks were subjected to the machine (rotary microtome (Leica RM2235; Leica Microsystems, Wetzlar, Germany)) cut
generating sections with 3–4µm thickness. The prepared sections mounted on the silane-coated slides (Dako, Glostrup, Denmark) were then exposed to the air to dry. To assess the growth plate structures, the slides were stained with Hematoxylin and Eosin (H&E) after they were dewaxed and hydrated by xylene and graded ethanol respectively. The same procedure of dewaxing and hydrating was performed before IHC staining. In the next step, endogenous peroxidase activity was quenched by 30 min incubation of slides in 0.3% H2O2/methanol. Slides were then rinsed thoroughly for 20 min using PBS, blocked in 5% bovine serum albumin (BSA; Sigma, Milan, Italy) for 1 hr in a humid chamber and incubated overnight at 4 °C with following antibodies: rabbit polyclonal anti-ST2 (ab25877; Abcam, Cambridge, UK), rabbit polyclonal anti-RUNX2 (ab23981; Abcam, Cambridge, UK), rabbit polyclonal anti-OSC (ab93876; Abcam, Cambridge, UK) and rabbit polyclonal anti-Col X (ab58632; Abcam, Cambridge, UK). Antibody dilution is 1:100 in PBS. Primary antibody incubation was followed by the HRP-conjugated anti-rabbit secondary antibodies incubation. To visualize the immunoreactions, section incubation in a 0.1% 3,3′-diaminobenzidine (DAB) and 0.02% hydrogen peroxide solution (DAB substrate Chromogen System; Dako, Denmark) was performed for 2 minutes. A gentle counterstaining (1 min) was performed using Mayer’s hematoxylin (Histolab Products AB, Göteborg, Sweden) and sections were mounted in GVA media (Zymed Laboratories, San Francisco, CA, USA). Axioplan Zeiss light microscope (Carl Zeiss, Oberkochen, Germany) and digital camera (AxioCam MRC5, Carl Zeiss, Oberkochen, Germany) were used for observation and photography of the sections. Histochemistry, Immunohistochemistry and densitometric analysis experiments were performed by Giuseppe Musumeci, Marta Anna Szychlinska and Paola Castrogiovanni (University of Catania, Catania, Italy). Mice genotyping and ST2 -/- and WT sample preparation was performed by Christina Böhm (Universitätsklinikum Erlangen, Erlangen, Germany). Some of WT sample preparation was performed by Sriveena Srinivasaiah (Medical University of Graz).
8.8. Immunohistochemistry evaluation

Light microscopy observation of positive immunostaining was performed through detection of brown chromogen on the cell nucleus edge, in the cytoplasm and plasma membrane. The reaction/expression of Runx2, ST2, Col X and OSC was analyzed along with positive and negative controls. The scale of 0–4 (no detectable staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3, very strong staining = 4) was considered as the Intensity of Staining (IS) for the expression of ST2 and Runx2 in femur and tibial growth plates as well as trabecular bone. In addition, the Extent Score (ES) indicative of the % of immunopositive cells were analyzed and reported as follows: <5% (0); 5–30% (+); 31%–50% (++); 51–75% (+++), and >75% (+++). Zeiss Axioplan light microscope at 200× magnification was used for counting. 2 anatomical morphologists and one histologist have assessed the IHC sections and in case of any interpretation discrepancy, a unanimous agreement was attained after the case revision. To measure the full size of each femur and tibia digital Vernier Caliper was utilized. The length measurement of different growth plate zones (PZ and HZ) was conducted by a software (AxioVision Release 4.8.2 - SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany) for image acquisition and analysis. The software measures the length of determined zones. The average size of PZ and HZ are calculated and plotted on the graph as % relative to the full size of concerned femur and Tibia. For the densitometric analysis of OSC and Col X, software for image acquisition and densitometric analysis (AxioVision Release 4.8.2 - SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany) was used to quantify the immunostained area in the PZ (green) and HZ (red) that determined by us. The densitometric count (pixel²/unit area 1000 µm²) of OSC and Col X in each zone is then plotted on the graphs. Digital micrographs were taken by Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) having a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

8.9. Statistical analysis

Statistical evaluation of two means was performed by Mann Whitney test whilst means from more than two groups were assessed by Kruskal–Wallis test with post Dunn’s test for multiple comparisons. Data were shown as the mean±SD.
GraphPad Instat Biostatistics version 6.0 software (GraphPad Software, Inc. La Jolla, CA, USA) was utilized for the statistical analysis.
Results
ST2 expression pattern during different stages of the chondrocyte differentiation
9. Results
(The chapter below follows the (Bonyadi Rad et al., 2017b) publication)

9.1.1. Robust ST2 expression during hypertrophic stage of chondrocytes differentiation in murine tibial and femur growth plates

IHC analysis of trabecular bone in femur and tibia of three-week-old mice indicated strong endogenous expression of the ST2 protein (Figure 4A, B). The ST2 expression was subsequently assessed in the growth plate chondrocytes. Our results revealed that expression of ST2 is highly differentiation-dependent in the growth plate chondrocytes from tibia and femur. While low or no ST2 was detected in resting and proliferative zones, IHC results indicated enhanced expression of ST2 at the pre-hypertrophic stage and robust persistent expression in hypertrophic chondrocytes (Figure. 4C, D). Evaluation of IS and ES of ST2 immunostaining in both trabecular bone and growth plates verified the IHC observations (Table2). The IHC results of the ST2 expression in mice growth plate revealed peak expression during hypertrophic stage implying a role for ST2 during late stages of the chondrocyte differentiation.
Figure 4. The pattern of ST2 expression in murine tibial and femur trabeculae and growth plate.

Immunohistochemical evaluation of the ST2 expression pattern in the trabecular bone-growth plate of the tibia (A) and femur (B) and tibial (C) and femur growth plates (D) from three-week-old mice. The figures are representative of at least 6 different staining. The scale bars are 100 and 50 µm for the figures with the magnification of 20x and 40x respectively. RZ: resting zone, PZ: proliferative zone, HZ: hypertrophic zone and TB: trabecular bone. Figure 4 was published elsewhere (Bonyadi Rad et al., 2017b)

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<td><strong>TIBIA (ST2)</strong></td>
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Table 2. Table 2 represents a comparison of Intensity of Staining (IS) and Extent Score (ES) of ST2 and Runx2 expression in murine trabecular bone and growth plate (HZ: hypertrophic zone, PZ: proliferative zone and TB: trabecular bone). Table 2 was published elsewhere (Bonyadi Rad et al., 2017b).
9.1.2. Pronounced up-regulation of ST2 isoforms expression during ATDC5 chondrocyte hypertrophy

With an objective to affirm our IHC results, ST2L and sST2 isoforms expression was analyzed in the murine chondrogenic ATDC5 cell line, the pre-osteoblastic cell line MC3T3-E1 as well as during chondrogenic differentiation of the ATDC5 cells. Our RT-PCR results confirmed expression of both ST2L and sST2 isoforms in undifferentiated ATDC5 and MC3T3-E1 cell lines (Figure 5A).

The qPCR quantification of the ST2 isoform expression was performed during sequential stages of the ATDC5 differentiation over a period of twenty eight days. The ATDC5 murine chondrocyte cell line undergoes multi-step sequential differentiation and cellular condensation mimicking the in vivo chondrocyte differentiation process (Yao and Wang, 2013) thus provides an ideal model for the gene signature evaluation associated to the various stages of chondrocyte development. Chondrogenic differentiation of the ATDC5 cells was assessed by qPCR analysis of an early differentiation marker Col II and two hypertrophic markers Col X and MMP-13 (Figure 5B - D). The expression pattern of the early and late phase differentiation markers corroborated time dependent progression in the ATDC5 differentiation. During this process, both ST2L and sST2 expression levels were noticeably though not significantly reduced in the course of early differentiation at day seven. However, during the hypertrophic stage at day 21 and 28 and concomitant with elevated expression of hypertrophic markers Col X and MMP-13, a significant increase in the expression of both isoforms was documented (Figure 5E, F). These results are in line with prominent ST2 upregulation observed in hypertrophic zone of the murine growth plate (Figure 4C, D). The absence of ST2 during early chondrocytes differentiation and strong induction during hypertrophic differentiation suggest that ST2 likely play a role in the process of chondrocyte differentiation. The Runx2 mediates chondrocyte maturation (Fujita et al., 2004, Inada et al., 1999, Zheng et al., 2003, Kim et al., 1999). Consistent with these reports, a concomitant upregulation of Runx2 and ST2 isoforms was documented during hypertrophic differentiation of ATDC5 cells (Figure 5 G). Moreover, strong Runx2 induction during late chondrocytes differentiation in vivo was verified by IHC and IS/ES analysis of the murine tibial
growth plate (Figure 5 I, Table 2) suggesting a potential Runx2 function in the regulation of ST2 expression during late stages of the chondrocyte differentiation.
Figure 5. Expression of ST2 isoforms in undifferentiated and differentiating ATDC5 chondrocyte.

(A) Endogenous mRNA expression of transmembrane (526 bp) and soluble (360 bp) ST2 isoforms assessed in undifferentiated ATDC5 and MC3T3-E1 cells using RT-PCR. 1% agarose gels were used in RT-PCR studies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as the internal control. After ATDC5 cells reached confluence (four days culture) the maintenance media was substituted with differentiation media. The ATDC5 cells were then incubated in differentiation media for 28 days to undergo differentiation. The mRNA expression analysis of Col II (B), Col X (C), MMP-13 (D), ST2L I, sST2 (F) and Runx2 (G) was carried out by the qPCR from total RNA isolated at days 0, 7, 14, 21 and 28. The mRNA expression of sST2 was obtained from ST2/ST2L proportion. (H) A representative picture of negative control staining, in the growth plate of the murine tibia. (I) IHC analysis of Runx2 expression in the growth plate of the murine tibia at three-week-old age. Scale bar of 100µm and 50µm for the pictures with the magnification of 20x and 40x are shown respectively. 18s rRNA was used for normalization of gene expression and graphs represent mean ± S.D. n=5. * P ≤ 0.05, ** P < 0.01, *** P < 0.001. Figure 5 was published elsewhere (Bonyadi Rad et al., 2017b).
Transcriptional regulation of ST2 in chondrocytes
9.2.1. ST2 isoforms undergo post-transcriptionally modification

The predicted molecular weights of protein for ST2L isoform is 63 and for sST2 is 37 kDa. However, ST2 isoforms appeared to undergo post-transcriptional alterations mainly glycosylation leading to the various molecular weights (Shimizu et al., 2005, Kakkar and Lee, 2008). To analyze the actual molecular weight of ST2L and sST2 isoforms in ADTC5, cells were transiently transfected with either ST2L or sST2 cDNA vectors (pcmv6-ST2L and pmcv6-sST2) and then subjected to immunoblotting. As detected by anti-DDK tag antibody, two bands at approximately 70 and 85 kDa (Figure 6, black arrows) in ATDC5 cells ectopically expressing ST2L cDNA were observed. Furthermore, two 38 and 60 kDa bands were detected in cells transfected with sST2 cDNA (Figure 6, gray arrows). As suggested in previous reports, the 85 and 60 kDa bands indicate post-transcriptionally modified (PTM) transmembrane and soluble forms respectively (Shimizu et al., 2005, Kakkar and Lee, 2008). These results revealing ST2L and sST2 post-transcriptionally modification in ATDC5 cells contributes to the better understanding and analysis of ST2 expression on the protein level.

![Figure 6. Post-transcriptional modification of ST2L and sST2 isoforms.](image)

By Immunoblotting, anti-DDK antibodies detected ST2L (black arrows) and sST2 (gray arrows) bands in ATDC5 protein lysates approximately 24 hrs post-transfection with pCMV6, murine ST2L (PCMV6-ST2L) or murine sST2 (PCMV6-sST2) cDNA vectors. Figure 6 was published elsewhere (Bonyadi Rad et al., 2017b).
9.2.2. Runx2 mediated induction of ST2 and Runx3 expression in ATDC5 chondrocyte

Using transient knockdown or stable overexpression of Runx2 in ADTC5 cells, we tested whether or not Runx2 regulates ST2 expression. Runx2 down-regulation (siRunx2) suppressed ST2L and sST2 mRNA expression in ATDC5 cells as compared to the scrambled RNA transfected control cells (Figure 7 A-C). Accordingly, stable transfection (S.T) of ATDC5 chondrocytes with mouse Runx2 cDNA (pcmv6-Runx2) markedly induced mRNA expression level of both ST2L and sST2 isoforms compared to the empty vector transfected control cells (pcmv6) demonstrating that Runx2 is a potential transcriptional activator of ST2 (Figure 7 D-F).

Immunoblotting analysis and quantification of the protein expression level established a distinct siRunx2 mediated down-regulation of Runx2 protein (Figure 7 G, H). Runx2 down-regulation resulted in a reduction of the protein expression of ST2L and sST2 isoforms (Figure 7 G, H). Conversely, an increase of ST2L and sST2 protein expression was observed when Runx2 was over-expressed (Figure 7 I, J). It should be noted though that ST2 isoforms present in untreated or treated ATDC5 chondrocytes are mainly in the unmodified form since PTM signals were negligibly detected. Moreover, the suppression of Runx3 another transcription factor from the runt-related family was detected after the Runx2 knockdown in ATDC5 cells (Figure 7 K, M, N). Accordingly, a significant induction of Runx3 was observed in Runx2 overexpressing cells both on the mRNA and protein levels (Figure 7 L, O, P) demonstrating Runx3 to be a new Runx2 target in ATDC5 cells. The ST2 expression was not however influenced by Runx3 silencing (Figure 7 Q-S) leading to the conclusion that Runx2 is the main inducer of ST2 expression in ATDC5 chondrocytes.
Figure 7. Runx2 regulation of ST2 and Runx3 expression in ATDC5 chondrocytes.

The qPCR analysis of Runx2 (A), ST2L (B) and sST2 (C) mRNA expression level in siRunx2 ATDC5 or Runx2 (D), ST2L (E) and sST2 (F) in ATDC5 chondrocytes stably transfected with Runx2 cDNA (pcmv6-Runx2). (G and H) Representative immunoblotting and quantification of the protein expression level of Runx2 (silenced by two different sets of siRNA), ST2L, sST2 (PTM), sST2 and Actin in ATDC5 chondrocytes. Silencing effect was analysed approximately after 72 hrs post-transfection. (I and J) Representative Immunoblotting and quantification of the protein expression level of Runx2, ST2L, sST2 (PTM), sST2 and Actin in ATDC5 chondrocytes stably overexpressing empty vector (pCMV6) or mouse Runx2 (pCMV6-Runx2) (n=5-6). (K and L) The mRNA expression level of Runx3 in siRunx2 (K) or Runx2 overexpressing ATDC5 cells (L) analysed by the qPCR (n=5-6). (M and N) Representative immunoblotting and quantification of the protein expression level of Runx2 (silenced by two different sets of siRNA), Runx3 and Actin in ATDC5 chondrocytes. (O and P) Representative immunoblotting and quantification of the protein expression level of Runx2, Runx3 and Actin in Runx2 overexpressing ATDC5 cells. Results were analysed 48 to 72 hrs post transfection with siRNA or cDNA. (Q-S) The mRNA expression level of Runx3, ST2L, and sST2 in Runx3 knockdown ATDC5 cells analysed by qPCR. In qPCR experiments, GAPDH was used as a normalizing gene. The protein expression levels (quantification) are normalized to the concerned β-actin levels. *P≤0.05, **P<0.01. Figure 7 was published elsewhere (Bonyadi Rad et al., 2017b).
9.2.3. Runx2 mediated induction of ST2 and Runx3 expression in primary human growth plate chondrocytes

In the next step, endogenous expression of Runx2, ST2 isoforms and Runx3 were studied in human chondrocyte samples. The endogenous expression of the specific chondrocyte markers, Col II, aggrecan and Col X confirmed the chondrogenic phenotype of isolated PHCs (Figure 8). Whilst sST2 and sST2 associated signals showing post-transcriptionally modification (PTM) were found to be expressed in all five PHC samples, only one PHC sample (PHC5) expressed low endogenous level of ST2L mRNA and protein (Figure 9 A, B). Runx2 was differentially expressed in PHC samples and the highest expression was observed in PHC5 (Figure 9 B). In all PHCs, a noticeable correlation between Runx2 and sST2 expression level were observed. However, among all PHCs, only PHC5 expressed low ST2L mRNA and protein level in which Runx2 expression was high (Figure 9 B). Moreover, a correlation between Runx2 and Runx3 expression was detected in PHC1, PHC3 and PHC5 samples (Figure 9 B). To find out the effect of Runx2 on the expression level of ST2 isoforms, PHCs were subjected to the transient overexpression of human Runx2 (Figure 9 C). The Runx2 cDNA transfection strongly induced sST2 mRNA expression in all PHCs. (Figure 9 D, E). Furthermore, ST2L transcripts were detected post Runx2 cDNA transfection in PHCs lacking ST2L mRNA (Figure 9 D). The ST2L mRNA induction was accompanied with a negligible protein expression in PHC1 and PHC5 only and in other PHC samples ST2L mRNA upregulation was not associated to the protein expression (Figure 9 G). On the other hand, whilst Runx2 overexpression remarkably induced unmodified sST2 isoform in all PHCs, the PTM signals were only detected in PHC1, PHC3 and PHC5 samples (Figure 9 G). Moreover, Runx2 overexpression was associated to the strong up-regulation of Runx3 mRNA and protein in all PHC samples (Figure 9 F, G) confirming the results obtained in ATDC5 chondrocyte.
Figure 8. The chondrogenic phenotype assessment of PHCs.

The qPCR indicating expression of Col II, aggrecan and Col X in 5 different PHCs. Figure 8 was published elsewhere (Bonyadi Rad et al., 2017b).
Figure 9. Runx2 dependent activation of ST2 and Runx3 expression in primary human growth plate chondrocytes.

(A) The endogenous mRNA expression of ST2L (454 bp) and sST2 (659 bp) in five PHCs assessed by RT-PCR. The GAPDH was used as loading control. (B) The immunoblotting of the ST2L, sST2, Runx3 and Runx2 protein level endogenously expressed in PHCs. (C) The expression of Runx2 in five PHCs post human Runx2 cDNA vector transient transfection assessed by RT-PCR. (D) The RT-PCR evaluation of ST2L (454) and sST2 (659) mRNA 24 hrs post human Runx2 cDNA transfection. (E and F) The qPCR evaluation of sST2 and Runx3 expression in 5 PHCs 24 hrs after Runx2 overexpression. (G) Immunoblotting of Runx2, Runx3, ST2L, sST2 and sST2 (PTM) in Runx2 (Runx2) and empty vector (pcmv6) overexpressing PHCs. The numbers indicating % of the protein expression are relative to the concerned control that were normalized to the β-actin level as normalizing gene/loading control. Figure 9 was published elsewhere (Bonyadi Rad et al., 2017b).
9.2.4. Proximal promoter is the dominant promoter expressing ST2 isoforms in ATDC5 and PHCs

The distal or proximal promoter associated transcription of ST2 isoforms initiating from either of the concomitant non-coding E1a or E1b, is highly cell type-dependent process (Iwahana et al., 1999, Gachter et al., 1998, Hayakawa et al., 2005, Bergers et al., 1994). However, regardless of which promoter is involved in regulating ST2 gene expression, translation starts from common E2. The distal and proximal promoters associated ST2 isoforms expression in ATDC5 chondrocytes was examined by RT-PCR primers which have been shown to specifically bind to the murine E1a or E1b as per Hayakawa M. et al. (Hayakawa et al., 2005). Our results exhibited transcription initiation of both transmembrane and soluble isoforms from the proximal promoter in control ATDC5 and Runx2 overexpressing ATDC5 cells (Figure. 10 A). As shown by the E1a associated signals, sST2 expression was also induced in Runx2 overexpressing cells, indicating the transcription factor Runx2 ability in regulating distal promoter (Figure. 10 A). In addition, the PHCs promoter analysis indicated that proximal promoter is mainly used for the sST2 transcription in these cells (Figure. 10 B). Overall, these results demonstrate the proximal promoter-dependent transcription of the ST2 isoforms as the dominant promoter in chondrocytes.
Figure 10. Promoter usage analysis in ATDC5 and PHCs.

(A) The RT-PCR evaluation of the promoter usage in ATDC5 chondrocytes after empty vector (pCMV6) and mouse Runx2 cDNA vector (pCMV6-Runx2) stable transfection. The PCR primers binding specifically to the E1b resulted in the generation of ST2L (1863 bp) and sST2 (1754 bp) fragments. The E1a associated sST2 fragment representing distal promoter function was 1718 bp (Hayakawa et al., 2005). (B) The promoter analysis by RT-PCR indicated E1b dependent sST2 expression (1237 bp fragment) in 3 different PHCs (Iwahana et al., 1999). Figure 10 was published elsewhere (Bonyadi Rad et al., 2017b).
The role of ST2 in chondrocyte differentiation
9.3.1 Hypertrophic zone reduction in femur and tibial growth plates of ST2 knockout (ST2 -/-) mice

The in vivo functional relevance of the ST2 expression was appraised by the analysis of the growth plates of femur and tibia isolated from WT and ST2 -/- mice at age of 3 and 5 weeks. H & E staining revealed no detectable effect on the PZ of ST2 -/- growth plates as compared to WT controls (Figure 11 A-C). On the other hand, the size measurement indicated a perceptible HZ reduction in ST2 -/- mice growth plates in both femur and tibia (Figure 11 A-C). To examine the possible effect of the ST2 ablation on the expression of the hypertrophic and terminal differentiation markers, IHC and densitometric analysis of Col X and OSC (Zhang et al., 2004) expression was performed. Our results illustrated significant diminished Col X and OSC expression throughout the HZ of the growth plates in ST2 -/- mice (Figure 12 A-P). Overall, HZ size reduction as well as decreased expression of the hypertrophic markers Col X and OSC in ST2 -/- mice, suggests likely the ST2 function in regulating the hypertrophic differentiation in vivo.
Figure 11. Reduced hypertrophic zone in ST2 -/- mice.

The H&E staining of WT (A upper panels) and ST2 -/- (A lower panels) femur and WT (B upper panels) and ST2 -/- (B lower panels) tibial bones of 5-week-old mice. The magnifications of 2.5X (left panels) and 10X (right panels) with the scale bar of 500 and 100 µm are shown respectively. (C) The graph illustrates % of PZ and HZ relative to the full length of the concerned femur and tibia in 3- and 5-week-old WT and ST2−/- mice. The size evaluation was performed by the measurement of several randomly selected fields in PZ and HZ from 3 WT (6 tibias and 6 femurs) and 3 ST2−/- (5 tibias and 5 femurs) mice. PZ: proliferative zone, HZ: hypertrophic zone. Figure 11 was published elsewhere (Bonyadi Rad et al., 2017b).
Figure 12. Diminished Col X and OSC expression in ST2 -/- mice.

(A and C) IHC assessment of Col X expression in WT femur and tibia. (B and D) IHC assessment of Col X expression in ST2 -/- femur and tibia. (E and G) Densitometric analysis (pixel²/unit area 1000 µm²) of Col X expression in WT femur and tibia. (F and H) Densitometric analysis (pixel²/unit area 1000 µm²) of Col X expression in ST2 -/- femur and tibia. (I and K) IHC analysis of OSC expression in WT femur and tibia. (J and L) IHC analysis of OSC expression in ST2 -/- femur and tibia. (M and O) Densitometric assessment (pixel²/unit area 1000 µm²) of OSC expression in WT femur and tibia. (N and P) Densitometric analysis (pixel²/unit area 1000 µm²) of OSC expression in ST2 -/- femur and tibia. * P ≤ 0.05. Figure 12 was published elsewhere (Bonyadi Rad et al., 2017b).
9.3.2. ST2 and Runx3 dependent modulation of proliferative and hypertrophic markers in ATDC5

To examine the effect of ST2 on the regulation of chondrocyte differentiation the siRNA mediated ST2 knockdown was performed in ATDC5 chondrocytes. The ST2 silencing in these cells slightly reduced Col X expression. Furthermore, ST2 knockdown resulted in a significant suppression of OSC corroborating the IHC and densitometry observations in ST2 -/- mice (Figure 13 A-C). In addition to hypertrophic markers Col X and OSC, the terminal differentiation markers, VEGFA and MMP-13 were further examined. The ST2 silenced ATDC5 cells showed significant reduction of VEGFA whilst MMP-13 was not influenced (Figure 13 D, E). The Runx2 transcript level was not also affected by the ST2 silencing in ATDC5 cells (Figure 13 F). On the other hand, two proliferative stage markers Col II and Sox9 were significantly upregulated after ST2 suppression (Figure 13 G, H). Runx3 silencing was associated with the enhancement of the Col II expression (Figure 13 I). The Runx3 knockdown, however, repressed hypertrophic markers Col X and OSC in ATDC5 cells (Figure 7 Q; Figure 13 J, K). Furthermore, terminal differentiation markers VEGFA and MMP-13 were repressed (Figure 13 L, M) by Runx3 knockdown indicating ST2 and Runx3 to substantially regulate expression of early and late differentiation markers in ATDC5 chondrocytes. To test the cooperative regulation of hypertrophic differentiation through Runx2 mediated activation of ST2 and Runx3 in ATDC5 chondrocytes, ST2 and Runx3 were separately silenced in Runx2 overexpressing cells (Figure 13 O, P). Up-regulation of the mRNA expression of Runx2, Total ST2, Runx3, Col X, OSC, VEGFA, and MMP-13 were documented in Runx2 cDNA transfected ATDC5 cells (Figure 13 N-T). Whereas the siRNA induced ST2 suppression had a minimal effect on the enhanced Col X expression, augmented expression of OSC and VEGFA were decreased (Figure 13 O, Q-S). The ST2 silencing, however, did not alter the enhanced expression level of MMP-13 in these cells (Figure 13 T). Furthermore, the Runx3 knockdown downregulated Col X to approximately basal level and led to the decreased expression of OSC, VEGFA, and MMP-13 in Runx2 transfected cells (Figure 13 Q-T). In addition, Runx3 but not ST2 slightly diminished Runx2 level in Runx2 overexpressing cells (Figure 13 N). Taken together, these findings suggest that ST2 and Runx3 as two novel targets of Runx2 appose proliferative
differentiation and act towards induction of hypertrophic differentiation in ATDC5 chondrocyte.
Figure 13. ST2 and Runx3 dependent regulation of proliferative and hypertrophic markers in ATDC5 chondrocytes.

The mRNA analysis of total ST2 (A), Col X (B), OSC (C), VEGFA (D), MMP-13 (E), Runx2 (F), Col II (G) and Sox9 (H) in ST2 silenced or Col II (I), Col X (J), OSC (K), VEGFA (L) and MMP-13 (M) in Runx3 silenced ATDC5 chondrocytes using qPCR. The mRNA expression of Runx2 (N), total ST2 (O), Runx3 (P), Col X (Q), OSC (R), VEGFA (S) and MMP-13 (T) in ATDC5 chondrocytes stably overexpressing Runx2 (pcmv6-Runx2), stably overexpressing Runx2 with silenced Runx3 (pcmv6-Runx2, siRunx3) and stably overexpressing Runx2 with silenced ST2 (pcmv6-Runx2, siST2). The GAPDH was used as internal control and graphs show mean±S.D. n=5-6. (VEGFA experiments: n=7-8). *P≤0.05, **P<0.01. Figure 13 was published elsewhere (Bonyadi Rad et al., 2017b).
Discussion
10. Discussion

(The chapter below follows the (Bonyadi Rad et al., 2017b) publication)

In the proliferative zone of the growth plates no or low ST2 expression was detected; on the other hand, pronounced cytoplasmic ST2 expression was observed in the pre-hypertrophic and hypertrophic zones. In the zone of chondrocyte hypertrophy, during in vitro culture of mandibular condyle, Werenskiold et al. (Werenskiold et al., 1995) documented a distinct cytoplasmic staining of sST2. Our result is thus consistent with this report indicating that ST2 expression is a differentiation-dependent reaching to the peak expression during late stages of chondrocyte differentiation. This observation was further substantiated by the expression of ST2 isoforms during differentiation course of ATDC5 chondrocytes. In differentiating ATDC5 cells, ST2L and sST2 isoforms were progressively enhanced on days 21 and 28 when ATDC5 cells underwent hypertrophic differentiation. During MC3T3-E1 and KM-1K osteoblastogenesis differentiation course, ST2L has been undetectable and sST2 expression was detected along with early differentiation markers Col I and OSN that precedes late differentiation markers ALP and OSC (Werenskiold et al., 1995). Moreover, the expression of ST2L was shown to be decreased during differentiation course of calvarial osteoblasts (Schulze et al., 2011) indicating that ST2 isoforms are differentially expressed during osteoblast and chondrocyte differentiation. This difference consequently suggests a contrasting differentiation-dependent role of ST2 in osteoblast and chondrocyte cells.

In vivo expression of Runx2 the key regulator of chondrocyte hypertrophic differentiation has been reported to initiate in pre-hypertrophic phase and retained throughout the hypertrophic stage (Fujita et al., 2004, Inada et al., 1999, Kim et al., 1999). Furthermore, Runx2 expression is augmented during ATDC5 chondrogenic differentiation as previously shown in several studies (Altaf et al., 2006, Temu et al., 2010, Caron et al., 2012, Enomoto et al., 2000, Zhang et al., 2011b, Guo et al., 2015, Zhang et al., 2015). As noted, the ST2L and sST2 isoforms analysis demonstrated upregulation during late ATDC5 chondrogenic differentiation concomitant with the increased expression of known hypertrophic markers Runx2, Col X and MMP-13. Runx2, the key regulator of hypertrophic differentiation, promotes chondrocyte hypertrophy by activating target genes such as Col X
through binding and activating their promoters (Zheng et al., 2003). ST2L and sST2 transcript and protein interference were observed after siRNA induced Runx2 suppression in ATDC5 cells. In accordance with this observation, Runx2 overexpression activated ST2L and sST2 isoforms at both mRNA and protein levels. In human growth plate chondrocytes, whilst a pronounced sST2 expression was observed in all PHCs, ST2L was detected to a lesser extent and in one of five human samples only. In PHCs, transient overexpression of Runx2 induced an increase of sST2 transcript and protein expression. Runx2 transfection however induced ST2L mRNA expression in all human chondrocyte samples even though this induction was accompanied by no or slight increase of ST2L protein. It should be noted though that a low endogenous ST2L protein expression was observed in the PHC sample expressing high Runx2 level. The lack of ST2L protein in human chondrocyte samples might have different causes. One of the reasons can be ascribed to the detection limit of immunoblotting technique since Runx2 dependent activation of ST2L transcript was apparently weak in comparison to sST2 mRNA level induced by Runx2 transfection. More importantly, the highest expression level of ST2L was observed in PHC5 where strong Runx2 expression was noted. In addition, ST2L reaches the peak level during hypertrophic differentiation in ATDC5 chondrocytes. Therefore, a differentiation-dependent ST2L expression, that is, during chondrocyte hypertrophy is highly conceivable. It should also be taken into account that Polydactyly associated autosomal mutations dominantly influencing genes or cis-regulatory elements in Hoxa- or Hoxd clusters or Wnt and Notch signaling pathways (Lange et al., 2014) might affect ST2L level in these cells. Genetic differences between ATDC5 and PHCs are thus another considerable reason for the absence of ST2L expression in human chondrocyte samples. In both cell systems, however, Runx2 induced ST2L mRNA and sST2 mRNA and protein revealing ST2 as a novel Runx2 target during hypertrophic differentiation of chondrocyte.

In mice embryos, co-expression of transcription factors Runx2 and Runx3 was reported during cartilage development (Stricker et al., 2002). The Runx3 is dominantly expressed in the pre-hypertrophic and hypertrophic zone and decreased during terminal differentiation of chondrocytes (Yoshida et al., 2004) giving rise to a similar expression pattern as Runx2. Consistent with these reports, gain and loss of Runx2 expression experiments confirmed Runx3 to be another
novel target of transcription factor Runx2 in both ATDC5 and PHC cells. Runx3 expression is regulated by dual promoter regions (termed P1 and P2) (Bangsow et al., 2001). Considering two adjacent consensus Runx binding sites in the vicinity to the transcription start site (TSS) on the P1 promoter that is conserved in mice and human (Bangsow et al., 2001), direct Runx2-Runx3 prompter interaction to induce Runx3 expression is highly conceivable in chondrocytes. Our study concluded that Runx2 is the main ST2 inducer since Runx3 silencing had a minimal effect on the expression level of ST2 in ATDC5 chondrocytes.

Regulation of ST2 gene through distal and proximal promoters was shown to be in a cell type-specific manner (Iwahana et al., 1999, Gachter et al., 1998, Hayakawa et al., 2005, Bergers et al., 1994). Whilst murine and human fibroblasts, for instance, utilize proximal promoter to express both soluble and transmembrane ST2, murine mast cells and thymoma cell line EL-4, use distal promoter to induce ST2 expression (Iwahana et al., 1999, Gachter et al., 1998, Hayakawa et al., 2005). In the human leukemic cell line UT-7, although distal promoter is the dominant promoter generating both ST2L and sST2 isoforms, the proximal promoter is also capable of ST2 transcription (Iwahana et al., 1999). The rat homologue of ST2 (known as FIT-1) however exhibits promoter-specific production of ST2 isoforms, expressing transmembrane form from the distal promoter and soluble form from the proximal promoter (Bergers et al., 1994). Our results indicated that proximal promoter mediates transcription of ST2L and sST2 isoforms in ATDC5 chondrocytes and PHCs, even though the sST2 can be also transcribed from the distal promoter in ATDC5 cells. Overall, it can be concluded that the proximal promoter is the main promoter expressing ST2 isoforms in chondrocyte lineage.

The in vivo effect of the ST2 protein was evaluated by the comparison of growth plate structures from femur and tibia of ST2 -/- mice and WT controls. The growth plate structures indicated an apparent comparable PZ morphology in ST2 -/- and WT mice with almost no size difference in both femur and tibia from 3 and 5-week old mice. On the other hand, a perceptible size reduction in the HZ of the growth plates in both femur and tibia of the ST2 -/- mice was observed suggesting that consistent with the upregulation of ST2 during the hypertrophic stage, ST2 likely play a role during hypertrophic differentiation of chondrocytes. However, given the
fact that, hypertrophic differentiation occurs in the growth plates in ST2 knockout mice, the ST2 function is seemingly contributive to the late stages of chondrocyte differentiation. Moreover, we found a down-regulation of the expression of hypertrophic markers Col X and OSC in the growth plates of ST2 -/- mice. Overall, these results suggest that ST2 most likely contribute to the further induction of chondrocyte hypertrophic differentiation in vivo. Despite the fact that this investigation indicates an ST2 function in vivo, growth plate analysis in developing ST2 -/- mice embryos would further support the ST2 regulatory impact on the in vivo progression of the late stages of chondrocyte differentiation. In vitro effect of ST2 knockdown in ATDC5 chondrocytes verified the in vivo influence of this protein on the chondrocyte hypertrophic differentiation. The ST2 suppression was accompanied by the significant repression of OSC and VEGF A, the terminal differentiation markers and a moderate decline of Col X expression in ATDC5 cells. The expression level of the other terminal differentiation marker MMP-13, which is involved in the extracellular matrix (ECM) turnover (Yamamoto et al., 2016), was not however changed. On the other hand, proliferative stage markers Col II and Sox9 were significantly enhanced by ST2 suppression, representing the positive and negative effect on hypertrophic and proliferative differentiation stages respectively. In ATDC5 cells, sST2 slightly decreased Col X whilst in ST2 -/- mice Col X was significantly repressed. This slight discrepancy thus can be as a result of the full ST2 ablation that leads to the stronger effect in knockout mice. As per our investigations and in line with Werenskiold, A et. al. report (Werenskiold et al., 1999), ST2 does not alter the Runx2 transcript level broaching a demand for further evaluation for the ST2 mechanism of action towards hypertrophic differentiation induction. IL-33 is a potent ligand of ST2L receptor and upon binding leads to the activation of downstream pathways MAPK and NFkB (Schmitz et al., 2005). Importantly, MAPK and NFkB pathways are two known signaling pathways crucially modulating chondrocyte responses including differentiation, proliferation, and apoptosis (Hutchison, 2012, Murakami et al., 2004, Wu et al., 2007). A possible mechanism likely leading to ST2L mediated regulation of chondrocyte differentiation is thus IL-33 binding to its receptor and stimulating downstream pathways. Furthermore, sST2 functions as the decoy receptor to inhibit IL-33-ST2L binding and attenuate downstream effect (Hayakawa et al., 2007, Kakkar and Lee, 2008). Further experimental examinations are thus
required to precisely decipher each receptor’s role during hypertrophic differentiation. In this view, the ST2L/sST2 ratio might therefore, determine IL-33/ST2L stimulation level and subsequent downstream biological activity.

Runx3 expression is promoted and maintained during hypertrophic differentiation of chondrocytes (Yoshida et al., 2004). The Runx3 functional effect has been observed by a moderate delay in the hypertrophic initiation and vascular invasion into cartilage in Runx3 knockout mice embryos (Yoshida et al., 2004). Furthermore, a complete inhibition of chondrocytes maturation has been seen in Runx2-/-Runx3-/- mice while that impact was observed to a lesser extent in Runx2 -/- mice (Yoshida et al., 2004). In an investigation by Soung do et al (Soung do et al., 2007), Runx3 was shown to regulate the expression of Col II and Col X in limb bud-derived cell line MLB13MYC. In support of these studies, our results indicate a function for Runx3 as a novel target of Runx2 on the regulation of hypertrophic differentiation in ATDC5 chondrocytes. The hypertrophic and terminal differentiation markers Col X, OSC, VEGFA, and MMP-13 were markedly suppressed in Runx3 silenced cells. Although more prominent, these results resembled ST2 knockdown influence on the expression level of hypertrophic markers in ATDC5 cells. Furthermore, and in a similar fashion, Col II expression was induced in Runx3 silenced cells pointing out that both Runx3 and ST2 function similarly to oppose proliferative and promote hypertrophic differentiation in chondrocytes. To conclude the role of ST2 and Runx3 overexpression in chondrocytes, the expression level of Col X, OSC, VEGFA, and MMP-13 were inspected following ST2 or Runx3 knockdown in Runx2 transfected ATDC5 cells. Whereas Runx2 mediated enhancement of Col X was slightly reduced by the ST2 knockdown, Runx3 silencing strongly suppressed it to the approximately basal level. However, the ST2 and Runx3 silencing decreased the augmented level of OSC and VEGFA almost to a similar extent. On the other hand, Runx2 associated MMP-13 increase was not influenced by the ST2 suppression while that observed in Runx3 silenced cells was noticeable suggesting Runx3 but not ST2 controls MMP-13 level in terminally differentiated chondrocytes. Considering the ST2 regulatory impact on Col X, OSC and VEGFA and not MMP-13, it seems that ST2 selectively target genes involved in the hypertrophic differentiation of ATDC5 chondrocytes. Therefore, our study indicates the ST2 and Runx3 involvement in the promotion of hypertrophic differentiation as two novel targets of key
transcription factor Runx2. It should be also taken into account that siRNA mediated Runx3 suppression slightly decreased Runx2 level in ATDC5 cells. This might consequently result in a further downregulation of Runx2 target genes in ATDC5 chondrocytes. This effect, however, can be cell-type specific or redundant considering that Runx3 -/- mice show the milder effect on the hypertrophic phenotype in comparison with more prominent effect associated to the Runx2 deficiency (Yoshida et al., 2004).
Conclusion
11. Conclusion

(The chapter below follows the (Bonyadi Rad et al., 2017b) publication)

The ST2L dependent regulation of osteoblast differentiation markers Col I, OSC and ALP and thus the osteogenic potential of osteosarcoma cells was documented previously (Werenskiold et al., 1999). In addition, the inhibitory effect of IL-33/ST2L signaling on the osteoclast formation was reported before in both in vitro and in vivo systems (Schulze et al., 2011). Our investigation provides a first evidence for the ST2 involvement in the regulation of chondrocytes differentiation. In our suggested model, key transcription factor Runx2 mediates induction of novel targets ST2 and Runx3 during chondrocytes hypertrophy. Whereas these differentiation regulators halt proliferative differentiation through inhibition of Sox9 and Col II markers, they promote chondrocyte hypertrophy and terminal differentiation by inducing Col X, OSC, VEGFA, and MMP-13 markers leading to cooperative regulation of hypertrophic differentiation in ATDC5 chondrocytes (Figure 14). The process of the growth in long bones is under the control of complex local and systemic factors that are not fully known. This study provides an important new insight into the role of ST2 in growth plate chondrocyte function, thereby allowing to further comprehension of the highly orchestrated process of the longitudinal bone growth.

Figure 14. Schematic representation of our suggested model for the ST2 and Runx3-promoted hypertrophic differentiation of ATDC5 chondrocyte under the regulation of key transcription factor Runx2. Figure 14 was published elsewhere (Bonyadi Rad et al., 2017b).
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13. Appendix

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