RETINOIC ACID-INDUCED MAFB EXPRESSION
DURING MYELOID CELL DIFFERENTIATION IN
CULTURE AND BONE CHONDROGENESIS IN VIVO

A Dissertation in
Nutrition
by
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ABSTRACT

Vitamin A and its active metabolite, all-trans retinoic acid (RA) regulate many physiological processes, such as skeletal development, cell differentiation, and immune functions. RA has been shown to induce monocytic cell differentiation and enhance phagocytic activity. Moreover, RA concentration is higher in hypertrophic chondrocytes, play an essential role in inducing chondrocyte terminal differentiation. MafB, a basic leucine-zipper transcription factor, is involved in development and monocyte-macrophage differentiation. Microarray analysis conducted by our laboratory revealed that MafB was one of the most strongly regulated genes by RA in THP-1 human monocytic cells. Therefore, we have hypothesized that RA-induced MafB is a mediator in the differentiation of monocytic cells and chondrocytes. To address this hypothesis, my dissertation was divided into two parts. The first part was to investigate the role of MafB in RA-induced monocytic cells differentiation using THP-1 cells as a model. There were three aims under this part: 1) to clarify MafB expression under the condition of RA treatment in monocytic THP-1 cells; 2) to determine the downstream genes of MafB during RA- and tumor necrosis factor (TNF) α-induced monocytic differentiation; 3) to determine the requirement of MafB in the RA- and TNFα-induced monocytic differentiation. The second part of my dissertation was to clarify the roles of MafB in RA-induced chondrogenesis using rats as an animal model. Two aims was developed: 1) to investigate the alteration of MafB expression and other bone makers during the rat limb development under different maternal vitamin A status and oral vitamin A and RA supplementation; 2) to determine MafB expression and function during the RA-induced chondrocyte terminal differentiation.
In THP-1 human mononuclear cells, MafB mRNA and protein levels were up-regulated by RA in a dose and time-dependent manner while, additionally, RA and TNFα synergistically regulated MafB expression, mainly at the transcriptional level. SPOCK1, Blimp1 and CCL2 were identified as target genes of MafB. Binding activity of MafB to the promoters of each of the target genes was increased in the RA- and TNFα- treated cells. Conversely, reducing MafB protein by microRNA silencing significantly decreased the expression of SPOCK1, Blimp1 and CCL2. Moreover, the reduction in MafB resulted in decreased cell differentiation and phagocytic activity. Together, RA- and TNFα-induced MafB may play a role in mononuclear cell differentiation through transcriptional regulation of Blimp1, CCL2 and SPOCK1.

In a rat study, maternal dietary vitamin A (VA) intake was represented the range of VA in most human diet: VA marginal, VA adequate, and VA supplemented. Their offspring were studied at birth (P0) and postnatal day 7 (P7). Half of the newborns received an oral supplement of VARA, or oil placebo. Maternal VA did not affect bone mineralization assessed by von Kossa staining; however, significantly differences (P<0.01) in the lengths of pups’ femur and tibia were observed across different maternal VA diets. Only in the VA-marginal group, oral feeding with VARA significantly increased the length of the hypertrophic zone, close to the length in pups from VA-adequate group. Neither type X nor type II collagen mRNA was altered by maternal VA status, although VARA intake significantly enhanced type X collagen mRNA levels. However, one of the most important finding was that VARA-treated pup femurs from VA-supplemented dams decreased aggrecan, a major component of cartilage matrix, and increased matrix metalloproteinase (MMP)13 expression, which catalyzes degradations
of aggregan and collagens. This result suggests that maternal VA supplementation plus neonatal VARA treatment could potentially be unfavorable for early bone development. Moreover, MafB was localized in both proliferative and hypertrophic chondrocytes in the growth plate. The expression in hypertrophic zone was stronger when maternal VA intake was higher. In cultured chondrocytes, RA dose-dependently increased MafB expression. MafB knockdown in chondrocytes resulted in an increase of aggregan and a decrease of RA-induced MMP13. These results implicate RA-induced MafB as a regulator of chondrocyte gene expression and matrix formation via the control of aggregan and MMP13 expression.

In conclusion, my study have provided a strong evidence that MafB was induced by RA, mediating RA-induced cell differentiation in both monocytic cells and primary chondrocytes.
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<th>Description</th>
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<tbody>
<tr>
<td>ADH</td>
<td>alcohol dehydrogenases</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>ARAT</td>
<td>acyl-coA: retinol acyltransferase</td>
</tr>
<tr>
<td>ASK-1</td>
<td>apoptosis-signaling kinase-1</td>
</tr>
<tr>
<td>Blimp1</td>
<td>B lymphocyte-induced maturation protein 1</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>CCL2</td>
<td>chemokine CC motif ligand 2</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer binding protein</td>
</tr>
<tr>
<td>CFU-G</td>
<td>colony-forming unit-granulocyte</td>
</tr>
<tr>
<td>CFU-M</td>
<td>colony-forming unit-monocyte</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>common myeloid progenitor</td>
</tr>
<tr>
<td>CRABP</td>
<td>cellular retinoic acid-binding proteins</td>
</tr>
<tr>
<td>CRBP</td>
<td>cellular retinol-binding protein</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP-responsive element</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>ETS</td>
<td>E-twenty six</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>GADPH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>GMP</td>
<td>granulocyte/monocyte progenitors</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cells</td>
</tr>
<tr>
<td>HZ</td>
<td>hypertrophic zone</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>Ihh</td>
<td>Indian hedge hedgehog</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon-regulatory factor</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>ISH</td>
<td>in situ hybridization</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KD</td>
<td>knock down</td>
</tr>
<tr>
<td>LBD</td>
<td>ligand-binding domain</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LRAT</td>
<td>lecithin: retinol acyltransferase</td>
</tr>
<tr>
<td>MafB</td>
<td>v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B</td>
</tr>
</tbody>
</table>
MARE  maf recognition element
M-CSF  macrophage colony-stimulating factor
miRNA  micro RNA
Mitf  Mi transcription factor
MMP  matrix metalloproteinase
NCoR1  nuclear receptor corepressor 1
NFAT  nuclear factor of activated T cell
NFκB  nuclear factor κB
NK  natural killer
OSCAR  osteoclast associated receptor
PAMP  pathogen associated molecular pattern
PTHrP  parathyroid-hormone-related protein
PZ  proliferating zone
q-PCR  quantitative real-time PCR
RA  all-trans retinoic acid
RALDH  retinal dehydrogenases
RANKL  receptor activator of nuclear factor κB ligand
RAR  retinoic acid nuclear receptor
RARE  retinoic acid response element
RBP  retinol binding protein
RDH  retinol dehydrogenase
RE  retinyl ester
REH  retinyl ester hydrolase
RIP-1  receptor interacting protein-1
RXR  retinoid X receptor
RZ  resting zone
siRNA  small interfering RNA
SMRT  silencing mediator for retinoid and thyroid hormone receptor
SODD  silencer of death domain
SPOCK1  sparc/osteonectin, cwcv and kazal-like domains proteoglycan 1
STRA6  stimulated by retinoic acid 6
TGFβ  transforming growth factor beta
Th  T helper
TLR  toll-like receptor
TNFα  tumor necrosis factor
TNFR  tumor necrosis factor alpha receptor
TRADD  TNF receptor-associated death domain
TRAF2  TNFR-associated factor 2
TRE  phorbol-12-Ο-tetradecanoate-13-acetate (TPA)-responsive element
VA  vitamin A
VAA  vitamin A adequate
VAM  vitamin A marginal
VARA  vitamin A and all-trans retinoic acid
VAS  vitamin A supplemented
WT  wild type
Acknowledgments

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CHAPTER 1 LITERATURE REVIEW

Vitamin A and its active metabolite, all-trans retinoic acid (RA) regulate many physiological processes, such as embryonic development, normal growth, cell differentiation, and immune functions. Retinoids have been used as cancer therapeutic and chemopreventive agents [1]. They exert anti-proliferative, differentiation-inducing, pro-apoptotic, and other biological effects. The v-maf musculoaponeurotic fibrosarcoma oncogene family member, protein B (MafB), is an important transcription factor, involved in embryonic development and cell differentiation [2]. The goal of this dissertation is to clarify the effects of RA on MafB gene expression and the roles of MafB in the process of RA-induced cell differentiation, especially in monocytes and chondrocytes. Current literature about each of these components is reviewed in Chapter 1.
1. Vitamin A Metabolism and Signaling

Vitamin A was discovered in 1910’s [3]. Results of early studies demonstrated that vitamin A plays an important role in the maintenance of epithelial tissues and that vitamin A deficiency causes pathologic changes in the morphology of cells, particularly the keratinization of epithelial cells [4]. Later, the chemistry of vitamin A was delineated and its organic synthesis was developed [5-6]. Further studies elucidated the role of vitamin A in vision [7]. And then later, great progress was made in understanding the transport and metabolism of vitamin A [3]. More recent and ongoing studies have focused on identifying the molecular mechanisms by which vitamin A affects development, reproduction, cell proliferation and differentiation, immunity, and more.

1.1. Retinoid Metabolism

Vitamin A is present in the diet as retinyl esters and as provitamin A carotenoids, among which β-carotene is the most active form. Retinyl ester is hydrolyzed to retinol by intestinal enzymes prior to retinol uptake in the small intestine. Dietary β-carotene usually undergoes central oxidative cleavage and is absorbed as retinal, which later is reduced to retinol by retinol dehydrogenases. Retinol is esterified within the enterocyte and transported as a part of the lipid core of the chylomicron, and metabolized in the liver and other target tissues to various types of retinoids including retinyl ester, retinal, RA, and polar metabolites [8-9]. The main active form of vitamin A is RA. It is involved in immunity and reproduction as well as in growth and development. Retinol seems to have an important role to play in reproduction while retinal is essential for vision [8].
The major storage organ of vitamin A is liver. Under condition of vitamin A adequacy, most mammals, including humans, store more than 90% of their total body vitamin A as retinyl esters in liver stellate cells [8-9]. The transport of retinol from the liver to extrahepatic tissues and retinol’s cycling among these tissues are considered as the major route of vitamin A homeostasis. In the circulation, retinol is associated with retinol-binding protein (RBP) to form the retinol-RBP-transthyretin complex. Recently, a transmembrane protein, simulated by retinoic acid 6 (STRA6), has been identified as a receptor for RBP, mediating cellular uptake of vitamin A [10].

Within cells, retinol binds with cellular retinol-binding proteins (CRBPs); whereas RA binds to cellular retinoic acid-binding proteins (CRABPs) [11-12]. By binding with these retinoid-binding proteins, the concentrations of free cellular retinoids are limited; not only retinoids are protected from non-specific oxidation, but also the cell membrane can be prevented from damaging by excessive retinoids. CRBP directs retinol to the enzyme lecithin: retinol acyltransferase (LRAT) by which retinol undergoes an esterification reaction and is converted into the storage form of retinyl ester [13-14]. Another function of CRBP is to direct retinol to be oxidized to retinaldehyde, and then to RA, the major bioactive form. A large number of enzymes catalyze the reversible oxidation of retinol to retinaldehyde: the alcohol dehydrogenases (ADH) and the retinol dehydrogenase (RDH). The enzyme retinal dehydrogenases (RALDH1, 2, 3, and 4) catalyze irreversibly the oxidation of retinaldehyde to RA [15]. The further oxidation of RA is carried out by three specific members of cytochrome P450s: CYP26A1, CYP26B1, and CYP26C1, which is an important reaction for controlling RA concentration in cell and tissues [16]. RA is catabolized to polar inactive metabolites, such as 4-oxo-RA, 4-
OH-RA, and 18-OH-RA, etc (Figure 1).

RA is known to regulate its own metabolism. Recent studies in our laboratory have investigated two key enzymes in retinol metabolism. Both LRAT and CYP26A1 expression are regulated by RA, especially in liver and lung of rats and mice [16-19]. The role of RA in regulating the expression of LRAT and CYP26A1 enables RA to control vitamin A metabolism under different levels of vitamin A status [16]. During vitamin A deficiency, the availability of RA is low, which down-regulates the expression of LRAT and CYP26A1. On the other hand, the continued presence of RA, resulting from vitamin A supplementation or treatment with RA, is a signal of high vitamin A or retinoid levels in the body. As a result, the expression of LRAT and CYP26A1 is upregulated, which subsequently limits the availability of RA and prevent excess retinol. Therefore, to a certain extent, RA may function as a signal of the body's vitamin A adequacy.
Figure 1. Retinoid metabolic pathway. ADH, alcohol dehydrogenase; ARAT, acyl-coA: retinol acyltransferase; CYP26, enzymes of cytochrome P450 family; LRAT, lecithin:retinol acyltransferase; RA, retinoic acid; RALDH, retinaldehyde dehydrogenase; RE, retinyl ester; REH, retinyl ester hydrolase; RDH, retinol dehydrogenase; RAR/RXR, nuclear retinoic acid and retinoid X receptors.
1.2. Retinoid Receptors and Signaling

It is now known that RA can influence gene expression and protein production in many ways, but in terms of molecular mechanisms, a single predominant, classical pathway has been characterized through two families of nuclear hormone receptors composed of three subtypes each: retinoic acid receptors (RARs: RARα, β, γ) and retinoid X receptors (RXRs: RXRα, β, γ) [20-21]. For each isotype of receptor, there are at least two isoforms generated by differential promoter usage and alternative splicing that differ only in their N-terminal region. The RAR family is activated by both all-trans retinoic acid and 9-cis retinoic acid, whereas the RXR family is activated exclusively by 9-cis retinoic acid.

The retinoid receptor gene family belongs to the superfamily of steroid hormone receptors. As most nuclear hormone receptors, each protein contains several domains that are similarly organized and well conserved within the RAR and RXR subfamilies [21-22]. The amino terminus of RAR or RXR contains a ligand-independent transcriptional transactivation function domain (AF-1), which varies among different receptors. This domain contains several consensus phosphorylation sites, and the phosphorylation of the AF-1 domain helps the ligand-dependent recruitment of coactivators and chromatin modifiers. The DNA-binding domain (DBD) in the center of the protein contains two zinc-finger motifs. The carboxyl terminus harbors the ligand-binding domain (LBD). It is functionally complex as it contains the ligand-binding pocket, the main dimerization domain and the ligand-dependent transactivation function (AF-2). The DBD and LBD of all subtypes are highly conserved.

RARs and RXRs commonly work together, in the form of heterodimers, binding to
DNA and regulating gene expression (Figure 2). The binding site for RAR/RXR heterodimers is a specific DNA sequence known as the retinoic acid response element (RARE) [21, 23]. RAREs consist of a direct repeat of a core hexameric sequence, PuG (G/T) TCA, separated by 1, 2, or 5 base pairs (DR1, DR2, or DR5, respectively). RXR homodimers, on the other hand prefer DR-1, although they bind to DR-1 less efficiently than do RAR/RXR heterodimers. In the absence of ligand, RAR/RXR heterodimers are bound to response elements located in the promoter of target genes, and recruit corepressors complexes, such as nuclear receptor corepressor 1 (NCoR1), and silencing mediator for retinoid and thyroid hormone receptor (SMRT), which recruit histone deacetylase (HDAC), resulting in chromatin condensation and gene silencing. Upon ligand binding, RAR and RXR change conformation, which favors the dissociation of corepressors and the recruitment of coactivators complexes that display histone acetyltransferase (HAT), methyltransferase, and ATP-dependent remodeling activities. This results in decompaction of chromatin, making it accessible to transcriptional machinery to initiate transcription [20-21, 23].

Balmer and Blomhoff [24] have classified over 500 genes reported to be regulated by RA into categories of genes that are either directly regulated through classical RAR/RXR-mediated transcriptional activation or are indirectly regulated by RA through nonconventional pathways. Twenty-seven of these genes had been reported to be unquestionably controlled through the classical pathway. For the other genes, regulation may occur through indirect, or unknown, mechanisms that are still important physiologically.
RARs and RXRs are expressed early in embryonic development, a time at which RA helps to specify body pattern formation, and they are also expressed in a wide variety of adult cell types, in amounts and proportions that differ among tissues [25-26]. RARs and RXRs play an essential role in the skeletal development, which will be discussed in detail.
**Figure 2. Mechanism of retinoid receptor action** [21, 27]. RAR and RXR form heterodimers that bind within the regulatory region of target genes through RARE. (A) In the absence of ligand, the heterodimer are associated with transcriptional corepressors which then recruit HDACs, resulting in chromatin condensation and gene silencing. (B) Binding of ligand induces the release of the HDAC complex and results in the recruitment of coactivators, which lead to decompaction of repressive chromatin. (C) Subsequently, mediator complexes and transcription machinery are recruited, resulting in transcription initiation.
2. Retinol Transport and Metabolism during Prenatal and Postnatal Period

In mammals, vitamin A is indispensable during the early stages of life and must be transferred adequately to the young during gestation and lactation. The absence or excess of retinol and RA can lead to abnormal development of embryonic and placental structures.

2.1. Prenatal Stage

The embryo is unable to synthesize retinol and is strongly dependent on the maternal vitamin A supply for retinol or its precursor (retinyl esters or carotenoids) [28]. In the embryo, retinol can be stored as retinyl ester; however, the principal metabolic event for retinol is its conversion into RA, which controls embryonic morphogenesis and organogenesis. All these placental and embryonic events of retinol transport and metabolism are highly regulated.

Before reaching the embryonic tissue, the retinol or the precursors must pass through the placental structures. The different intracellular-binding proteins for retinol and RA are expressed in the placenta of mouse, rat and human [28]. Both CRBP1 and CRBP2 have been described as being expressed in the placenta [29-30]. Moreover, both fetal as well as maternal CRBP2 are required for adequate delivery of vitamin A when maternal dietary vitamin A is reduced to marginal levels [29]. CRABP1 and CRABP2 are expressed in human and mouse placenta [30-31], regulating the availability of RA to the embryo. Since the RBP complex is unable to cross the placental barrier, retinol is dissociated from the maternal RBP complex, then bound to CRBPs, passes through the cytoplasm of the
trophoblastic cells and enters the fetal circulation, where a new retinol-RBP-transthyretin is formed [32]. However, RBP-bound retinol is not the only source of retinoids for the fetus, as indicated by the accumulation of hepatic retinoids storage in the RBP-deficient fetus [33]. An alternative supply of embryonic retinol is retinyl esters delivered in maternal chylomicrons [33].

The placenta is not only able to mediate the transfer of the retinoids, but also expresses several enzymes involved in retinoid metabolism. ADHs and RDHs, and RALDHs, which catabolize the conversion steps from retinol to RA, have been identified in the placenta [28]. This generation of RA is blocked by the presence of ethanol [34]. This finding links alterations of retinoid metabolism to alcohol abuse and fetal malformations. Moreover, CYP26s are expressed at a high level in placenta [28]. However, the protection for the embryo by CYP26s from overexposure to RA is limited; high levels of RA in maternal blood cause fetal malformation [28].

Many studies have addressed the role of RA in regulating gene expression, in patterning the anteroposterior body axis, morphogenesis and organogenesis in the developing embryo. By the 1940s, it had been shown that both vitamin A deficiency and excess have profound effects on the development of the embryo [35]. RA has been detected very early during development, prior to implantation, in the inner cell mass of the blastocyes [36]. There is a greater RA concentration in the posterior part of the early vertebrate embryo [37]. At later stages of development (~E10.5), RA has been identified in the somites, developing heart, lens, and neural tenia, the endoderm layer of the developing gut, the mesenchyme at base of the developing limb buds, and the cervical and lumbar regions of the developing spinal cord [38]. At even later times (~E14.5), RA
is found in ectoderm between the mandible and maxilla and in the nasal placode, developing ear, skin, and somite-derived tissues, a number of internal organs (stomach, metanephric kidneys, and lung), eye, and developing limbs [36]. Several genes regulated by RA have been identified in embryonic development, including homeobox genes, growth factors, and transcription factors that are crucial for proper morphogenesis [39].

RA concentration is tightly controlled by the balancing of its synthesis and degradation. Retinoid biosynthetic and catabolic enzymes are expressed early in embryogenesis. RALDH2 plays a crucial role in the synthesis of RA, and is expressed as early as E7.5 [40]. RALDH2-/- mice die before E10.5 [41]. On the other hand, CYP26 is expressed nearby during the same period, but usually not in the same cells or identical region of the embryo [42]. During the development of the anteroposterior axis in the early embryo, the posterior region, which exhibits high concentrations of RA, expresses RALDH2 [43]. However, the anterior region, which is characterized by low RA concentration, expresses CYP26 [42]. The expression patterns of these biosynthetic and degradative enzymes suggest the induction and suppression of RA biogenesis in waves during morphogenesis.

Multiple fetal anomalies occur in vitamin A-deficient animals as well as in RAR knockout mice. A series of experiments was conducted to describe the large array of congenital defects in the rat attributable to maternal vitamin A deficiency [35]. Congenital abnormalities of the limbs, vertebral column, heart, ocular tissues, respiratory and cardiovascular systems, and abnormal segmentation of the embryo, were observed. Vitamin A-depleted pregnant rats maintained on sufficient oral RA for growth and fertility provided the opportunity to induce deficiency at specific times during
development and thus to study function of RA in specific developmental processes [35]. For example, the fetuses of pregnant rats deprived of RA from E11.5 to E13.5 had neural crest, ocular, and nervous system defects, similar to those seen in mutant mice with deletions of the genes for RXRα and RXRα and RARα, and RARα and RARγ [44]. Even moderate vitamin A deficiency in pregnant rats reduced the number of live births and altered the growth trajectory of fetal organs.

2.2. Postnatal Stage

Birth is a major physiological transition. Vitamin A is important for this transition. However, newborn animals and humans begin life with low reserves of retinol even when the mother has adequate vitamin A [45]. Based on studies in the rat, milk is more effective for transmission of vitamin A to the offspring than the placenta. The vitamin A storage in the neonatal rat liver is low compared to maternal liver reserves [46]. When rats were fed with diets containing either a lower level of retinol (0.6 μg/g) or a higher level (15 μg/g) during pregnancy and lactation, the retinol concentration of the mothers’ liver averaged ~130 and 640 μg retinol/g liver, respectively. However, the concentrations of retinol in the liver of their newborns were only 9 and 15 μg retinol/g, respectively [45].

An adequate transfer of vitamin A from mother to young during lactation is needed to increase the neonate’s vitamin A reserves prior to weaning. In the first 6 months of life in healthy, well-nourished infants, the size of the liver more than doubles and its vitamin A concentration increases 5-fold [47].

Epidemiological and clinical studies in lactating women and experimental studies in rats have shown that dietary vitamin A influences the quantity of vitamin A present in
breast milk [45-46, 48-49]. Milk vitamin A concentration was significantly higher in rats fed a vitamin A-supplemented diet even though plasma retinol concentration was not affected [45]. Moreover, when the rat mothers were fed with different levels of dietary vitamin A, no differences in milk vitamin A concentration on the first day of lactation were observed. However, as lactation progressed, the vitamin A concentration of milk, as well as the liver vitamin A storage of the pups was significantly greater for those mothers fed on a high-vitamin A diet [45]. Moreover, in a rat study [49], when vitamin A intake was switched on day 7 of lactation from 0 to 50 µmol/kg diet, milk vitamin A concentrations increased significantly by day 9 to the same level as in rats administered 50 µmol/kg diet. By contrast, when vitamin A was removed from the diet on day 7, concentrations declined significantly and by day 11 were the same as those in rats given 0 µmol/kg diet. In lactating Indonesian women and their infants studied in a community trial of vitamin A supplementation, the milk retinol concentrations of the vitamin A-supplemented group were higher than those of the placebo group, and significantly fewer of their infants had plasma retinol values (<0.52 µmol/L) [48].

There are two pathways for the uptake of vitamin A into the breast: from circulating holo-RBP and from chylomicron retinyl ester [50-51]. The uptake of retinyl ester from chylomicrons into the lactating mammary gland increases in direct proportion to chylomicron retinyl ester content [50]. Differences in the delivery of chylomicron retinyl ester to the lactating mammary gland may explain the changes in milk vitamin A concentration in response to differences in maternal vitamin A consumption.

Interest in vitamin A requirements in the neonatal period was highlighted by reports that vitamin A supplementation to newborns may improve outcomes in the neonatal
periods. Imdad et al [52] reviewed several studies, and the combined results from six studies showed that neonatal vitamin A supplementation reduced all-cause mortality by 12%. Rahmathullah et al [53] reported the results of a large randomized, placebo-controlled, community-based trial of vitamin A supplementation on day 1 and 2 of life. Supplementation resulted in a 23% reduction in mortality at 6 month. Interestingly, the survival curves began to diverge at ~2 weeks of age and continued to separate until 3 months of age, indicating that vitamin A reduced mortality early in this period, after which the curves were parallel, indicating no further effect after 3 months. The effect of vitamin A supplementation was greatest in low-birth-weight infants.

The mechanisms of vitamin A supplementation protection to the newborn may include beneficial effects on the lungs, intestinal epithelium, and immune system. A placebo-controlled trial among 2067 Indonesian neonates who received either 50,000 IU orally administered vitamin A or placebo on the first day of life revealed that neonatal vitamin A supplementation can reduce the infant mortality rate and the prevalence of severe respiratory infection among young infants [54]. A meta-analysis conducted according to the standards of the Cochrane Neonatal Review Group revealed that supplementing very-low-birth-weight infants with vitamin A is associated with a reduction in death or oxygen requirement at one month of age, and oxygen requirement amongst survivors at 36 weeks post-menstrual age [55]. Moreover, pooled results from several studies showed that vitamin A supplementation reduced diarrhea specific morality by 26% [52]. Mechanisms of vitamin A on the immune system will be discussed in later sections.
3. Vitamin A and Immunity

Vitamin A is required for both innate and adaptive immunity. Vitamin A and RA is essential for maintaining epithelial barriers against infection, for enhancing cytokine and antibody production, for hematopoietic cell differentiation and polarization. These functions may account for the decrease in mortality seen in vitamin A supplementation infants and young children in many areas of the world today [56-57].

3.1. Monocyte/Macrophage Differentiation and Function

Hematopoiesis has been studied for several decades. Hematopoiesis ultimately produces all of the mature blood cell types from rare hematopoietic stem cells (HSC) [58] (Figure 3). Mature blood cells are traditionally categorized into two separate lineages: lymphoid and myeloid. The lymphoid lineage consists of T, B, and natural killer cells. The myeloid lineage includes granulocytes (neutrophils, eosinophils, and basophils), monocytes, macrophages, erythrocytes, megakaryocytes, and mast cells. Dendritic cells have a unique developmental program that can be generated from either the myeloid or the lymphoid pathway. Here, I will focus on macrophages.
Figure 3. A model for hematopoiesis [58-59]. Self-renewing hematopoietic stem cells (HSC) give rise to common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). CLPs produce B, T, natural killer (NK) cells and dendritic cells. CMPs are able to generate granulocytes (neutrophils, eosinophils, and basophils), monocytes, erythrocytes, megakaryocytes, and mast cells. Circulating monocytes give rise to macrophages and dendritic cells.
Circulating monocytes give rise to a variety of tissue resident macrophages, as well as dendritic cells. Monocytes originate in the bone marrow from common myeloid progenitors (CMPs). CMPs differentiate into granulocyte-monocyte progenitor cells, then further to promonocytes, which are then released into the peripheral blood. In the blood, promonocytes differentiate into mature monocytes, where they circulate for hours to several days before entering tissues and replenishing the tissue macrophage populations. Monocytes constitute ~1–6% of peripheral-blood leukocytes in humans [58-59].

In general, the differentiation of a monocyte into a tissue macrophage involves a number of changes: the cell enlarges five- to ten-fold; its intracellular organelles increase in both number and complexity; and it acquires increased phagocytic ability, producing higher levels of hydrolytic enzymes and a variety of soluble factors, such as cytokines. They are present in the liver, lungs, lymphoid organs, gastrointestinal tract, brain, bone and connective tissues [60]. However, tissue microenvironment can markedly influence the phenotype of tissue-resident macrophages. For example, alveolar macrophages function to clear microorganisms, viruses and environmental particles by the high expression of pattern-recognition receptors and scavenger receptors; osteoclasts remodel bones; macrophages in the germinal center clear apoptotic lymphocytes generated during the development of an acquired immune response; and macrophages from the lamina propria in the gut have a unique phenotype that is characterized by high phagocytic and bactericidal activity but weak production of pro-inflammatory cytokines [58, 60].

In addition to tissue-resident macrophages, macrophages represent a major defense system against invasion of a range of microorganisms including bacteria, viruses, fungi, and protozoa [59-60]. They are involved in the recognition, phagocytosis, and destruction
of the organisms. In addition, macrophages are also involved in antigen presentation and secretion of a wide variety of products, including enzymes, cytokines, chemokines, complement components, and coagulation factors [59-60].

Pro-inflammatory, metabolic and immune stimuli all elicit increased recruitment of monocytes to peripheral sites, where monocytes differentiate into macrophages and dendritic cells. Classical activation involves stimulation of macrophages with two signals [59]. The first is interferon (IFN)$\gamma$ and the second is provided by pathogen associated molecular patterns (PAMPs). Early in the immune response, IFN$\gamma$ can be produced by innate immune cells, such as NK or NKT cells, but as the immune response develops, the most important source of IFN$\gamma$ becomes the antigen-specific T helper (Th) 1 cells. The second signal is provided by a microbe that expresses one or more PAMPs, which stimulate macrophage activation through toll-like receptors (TLRs). TLRs recognize a range of microbial products including lipopolysaccharide (LPS) acting on TLR4, bacterial unmethylated CpG DNA acting via TLR9 and viral double-stranded RNA acting via TLR3 [61]. These stimuli generally leads to macrophage increased ability of phagocytosis and stimulation of T cells, production of nitric oxide and reactive oxygen species, production of proinflammatory cytokines, such as tumor necrosis factor (TNF) $\alpha$ and interleukin (IL)-12, and expression of major histocompatibility complex class II and costimulatory molecules which promote antigen presentation and activate B cells to synthesize antigen-specific antibodies that help with opsonisation of the antigen so that the microbe can be better cleared by phagocytosis. Moreover, the combination of TNF$\alpha$ and IFN$\gamma$ results in optimal macrophage activation [62].

Together, macrophages play a variety of roles in immune function. Therefore,
Macrophages have been implicated in a number of disease processes including rheumatoid arthritis, autoimmune, wound healing processes, and atherosclerosis, as well as in tumor biology [60].

### 3.2. Transcriptional Control of Myeloid Development

Differentiation of hematopoietic precursors is associated with stepwise acquisition of a specific lineage identity. Progenitor differentiation through a series of lineage branching points is directed by altering groups of cooperative and counter acting genes which together build a large network of factors that determine cell fate. Transcription factors are key components in this network.

The formation of myeloid cells is orchestrated by numbers of transcription factors. Among them are PU.1, CCAAT/enhancer binding proteins (in particular, C/EBPα, C/EBPβ, C/EBPε) and interferon-regulatory factor 8 (IRF8); as well as other factors, including AP-1, NFκB, and MafB, RARs (Figure 4) [63-64]. C/EBPα has a function in the self-renewal of existing HSCs. Whereas C/EBPα also plays a critical role in the differentiation of CMPs into granulocyte/monocyte progenitors (GMPs). Increased PU.1 activity favors monocytic commitment of the GMPs. Induction of PU.1 by C/EBPα and interaction of PU.1 with c-Jun or c-Fos also contribute to monocyte lineage specification. Macrophage production depends on PU.1 and IRF8, and increased MafB expression. PU.1 induction of Egr-1, 2 facilitates maturation along the monocyte lineage. RARs induced C/EBPε and low levels of PU.1 are required for granulocytic maturation. Gfi-1 and Egr-1,2 repress each other to maintain myeloid lineage fidelity.

PU.1 is a member the large family of E-twenty six (ETS) transcription factors, and
its expression is restricted to blood cells [65], the production of ongene SPI1. PU.1 binds as monomer to the consensus DNA site 5’-AAAG(A/C/G)GGAAG-3’ via its C-terminal domain and activates transcription via its N-terminal glutamine-rich and acidic domain [65]. PU.1 is expressed at varying levels by different haematopoietic lineages. It is detectable in HSCs, CMPs and CLPs at a similar level to that found in B cells, and is present at high levels in mature myeloid cells [63, 66]. High PU.1 levels support the production of macrophages, whereas low PU.1 levels seem to support granulocyte production [67-68]. Genetic analysis also indicates that a higher level of PU.1 favors monocytic over granulocytic development: lack of one PU.1 allele favors neutrophil development from embryonic stem cells in vitro and encourages neutrophil development in vivo in the absence of granulocyte colony stimulating factor (G-CSF) [67]. Increased PU.1 activity favors monocyte over granulocyte lineage commitment. This increase may result from C/EBPα activation of the PU.1 gene or as a result of PU.1 interaction with c-Jun [63].

The C/EBPs homo- and heterodimerize via their C-terminal leucine zipper domains and bind DNA via the adjacent basic regions [69]. C/EBPα in the hematopoietic system is expressed by HSCs, myeloid progenitors and granulocytes, but not by macrophages [70-71]. C/EBPα-deficient mice have normal numbers of CMPs, but lack GMPs and all subsequent granulocytic stages [72]. In addition to its role in myeloid differentiation, C/EBPα also controls stem-cell self-renewal properties [73]. C/EBPα binds and activates the PU.1 promoter and distal enhancer [74]. Moreover, C/EBPα has the potential to cooperate with PU.1 as these proteins directly interact [75]. Another mechanism of facilitation in myeloid differentiation has been proposed that C/EBPα:c-Jun, C/EBPα:c-
Fos or C/EBPα:NF-κB heterodimers may induce PU.1 or a protein that cooperates with PU.1 to favor monocytic commitment [63].

In the myeloid lineage, IRF8 is expressed by progenitors and macrophages, but not by granulocytes [76]. IRF8 expression is strongly induced by IFNγ, suggesting a role of this transcription factor in immunity [77]. Indeed, IRF8-/- mice are highly susceptible to viral infections, a finding that is mechanistically linked to reduced expression of IL12p40 by IRF8-/- macrophages, leading to disrupted Th1 cell function [78]. Compared with wild-type mice, IRF8-/- mice showed a marked increase in the number of granulocytes and their precursors, but had fewer macrophages [79]. Phosphorylation of PU.1 on serine 148 allows interaction with IRF8 [80]. It has been suggested that increased PU.1 facilitate interaction with IRF8 to regulate genes required for monocyte lineage commitment and maturation via Ets/IRF composite elements [63].

MafB, RAR, and NFκB play a role in the myeloid cell differentiation. Their functions will be discussed further.
Figure 4. A model of transcription regulation of myeloid differentiation [63-64]. Self-renewing hematopoietic stem cells (HSC) differentiate into common myeloid progenitors (CMPs). CCAAT/enhancer binding protein (C/EBP)α directs CMPs to granulocyte/monocyte progenitors (GMPs). Increased PU.1 activity by C/EBPα favors monocyte over granulocyte lineage commitment. IRF8, MafB and Egr-1,2 direct monocyte maturation. C/EBPε is required for granulocytic maturation. Gfi-1 and Egr-1,2 repress each other to maintain myeloid lineage fidelity. AP-1 proteins may also have a role during myeloid differentiation via interaction with C/EBPα, PU.1 or MafB. CFU-M, colony-forming unit-monocyte; CFU-G, colony-forming unit-granulocyte.
3.3. Roles of Retinoic acid in Monocyte/Macrophage

During an immune response, enzymes that metabolize vitamin A are induced in certain types of cells such as dendritic and tissue cells for increased production of RA. As a result, induced gradients of RA are formed during the immune response in the body. RA regulates gene expression, differentiation, and function of diverse immune cells. The cells under the influence of RA in terms of differentiation include myeloid cells such as neutrophils, macrophages, and dendritic cells. Also included are lymphoid cells such as effector T cells, regulatory T cells and B cells [56-57, 81]. RA has been shown to alter the Th1-Th2 balance, favor T regulatory differentiation, and induce antibody production by B cells. In this section, I will be focus on its functions in myeloid cells.

Vitamin A deficiency can lead to a significant increase in the total number of macrophages in the secondary lymphoid organs of mice [82], whereas RA treatment can cause a decrease in the number of monocytes found in bone marrow and spleen [83]. In addition to affecting cell numbers, vitamin A deficiency also leads to increased transcription of IL-12 [84]. IL-12 produced by macrophages, which can act as antigen-presenting cells, promotes development of Th1 cells, leading to inducing IFNγ by Th1 cells. Increased IFNγ production can, in turn, lead to increased macrophage activation. Such activation may lead to the higher spontaneous release of nitric oxide by peritoneal macrophages, as observed in vitamin A-deficient mice [85]. Moreover, vitamin A supplementation in patients with low vitamin A levels resulted in increased IL-10 and decreased TNF-α levels, as found in both plasma and monocyte supernatants, possibly favoring anti-inflammatory net effects [86]. Although some macrophage-mediated inflammation is increased by vitamin A deficiency, the phagocytic capacity of
macrophage can be impaired by deficiency. For example, vitamin A deficiency decreases the phagocytic activity and bacteria-killing ability of peritoneal macrophages [87]. Vitamin A supplementation can enhance the phagocytic activity of macrophages [56]. Consistent with these in vivo results, the proliferation of myeloid cells (macrophage-like cell lines P388D1, J774.2, WEHI-265, WEHI-3, and PU-5) are decreased in the presence of RA. And myeloid cells undergo terminal differentiation into fully mature phagocytes in response to RA [88]. The work conducted by our lab further confirmed the effects of RA on monocytic cell differentiation [89] and we explored the mechanisms by using THP-1 cells as a model. The results obtained from this study indicate that RA can rapidly induce alterations in the level or functional status of key cell cycle-related proteins including cyclin E, p27 (p27/Kip-1), retinoblastoma, and the transcription factor E2F, resulting in cell cycle arrest in the G1 phase. Proliferation of THP-1 cells was reduced by RA treatment, whereas the expression of CD11b, a cell surface marker of macrophage differentiation, and phagocytic activity was increased by RA.

RARs are widely expressed, with RARα preferentially found in myeloid cells [90]. The differentiation of stem cells results in two main myeloid lineages: the monocytic and the neutrophilic lineages (Figure 5). RAR is considered to mediate modulation of gene expression controlling the development of neutrophils. A translocation that fuses the RARα gene with a gene known as promyelocytic leukemia is found in patients with the disease acute promyelocytic leukemia [91]. This fusion disrupts normal neutrophil maturation, triggering the proliferation of promyelocytes. RA therapy for acute promyelocytic leukemia patients can cause remission of the disease by inducing maturation of promyelocytes to neutrophils. Moreover, in RARα/γ double knockout mice,
differentiation of bone marrow cells was abnormal in that the hematopoietic cells could not mature beyond the myelocyte/metamyelocyte stage [92]. Regulation of the C/EBP\(\epsilon\) promoter by RAR\(\alpha\) may account in part for the role of RARs in granulopoiesis.

Vitamin A deficiency has two disparate effects on neutrophils; it increases numbers but impairs function. Increased numbers of granulocytes and myeloid precursors have been seen in the peripheral blood of vitamin A-deficient rats [93]. Vitamin A deficiency also disrupts normal neutrophil development and can result in decreased phagocytosis and killing of bacteria [94].

In addition, CD18 forms a heterodimeric complex with CD11b on the surface of granulocytes and monocytes/macrophages that is important in variety of adherence-related activities [95] and as a complement receptor which mediates the phagocytosis of opsonized particles [96]. RARs also activate the CD18 promoter in maturing myeloid cells, and mediate their cell cycle arrest. This may contribute to regulation of RA on phagocytosis of macrophages and neutrophils.

In summary, RA plays a role in cells differentiation in both branches of myeloid lineages. The function of RA in cell differentiation is complex, and the mechanisms have not been understood completely yet.
Figure 5. Myeloid differentiation [64]. HSC, hematopoietic stem cells; CMP, common myeloid progenitors; GMP, granulocyte/monocyte progenitors.
3.4. Tumor Necrosis Factor Alpha in Monocytic Maturation

TNFα is produced primarily by macrophages, but it is also produced by a variety of cell types including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts, and neuronal tissue. Large amounts of TNFα are released in response to LPS, other bacterial products. TNFα has been shown to be one of the most abundant early mediators in inflamed tissues. Moreover, it is suggested to play a critical role in the development of many chronic inflammatory diseases [59-60].

TNFα signals through two transmembrane receptors, TNFR1 and TNFR2, and regulates a number of critical cell functions, including cell proliferation, survival, differentiation, and apoptosis [97]. TNFR1 is constitutively expressed in most mammalian tissues, whereas the expression of TNFR2 is highly regulated and it is typically found in the cells of the immune system. Depending on the cell type, TNFR1 and TNFR2 may have distinct as well as overlapping roles in signal transduction and gene expression [97]. Upon contact with their ligand, TNF receptors form trimers, their tips fitting into the grooves formed between TNF monomers. This binding causes a conformational change of the receptors, leading to the dissociation of the inhibitory protein, silencer of death domains (SODD), from the intracellular death domain. This dissociation enables the receptors to bind to an adaptor complex, including TNF receptor-associated death domain (TRADD), receptor interacting protein-1 (RIP-1), and TNPR-associated factor 2 (TRAF2) [97]. Then this complex is internalized and activates key signaling pathways. RIP-1 recruitment of MEKK-3 and transforming growth factor-beta (TGFβ)-activated kinase (TAK1) subsequently activates the IKK complex. The IKK complex then phosphorylates IκB, which then leads to the ubiquitination and degradation
of IκB. This then results in the release of NFκB subunits that are bound to IκB under unstimulated conditions. The free NFκB subunits translocate into the nucleus and evoke gene transcription. In addition to RIP-1-MEKK3-TAK1 pathway, other pathways have been identified to be involved in activation of NFκB. For example, TRAF2 has been shown to activate NFκB by binding to the IKK complex [98]. G-protein coupled receptor kinase-2, and -5 are also involved in TNFα-induced NFκB activation [99]. Moreover, stimulation of TNFR also activates a MAP3K called apoptosis-signaling kinase-1 (ASK-1) [100], leading to activation of c-Jun N-terminal kinase (JNK) and p38 MAPK. JNK and p38 subsequently activate transcription of many genes through transcription factors such as AP-1. Furthermore, TNFR also activates the ERK signaling pathway. In addition to these signaling pathways, TNFR activation is also involved in pro-apoptotic signaling via the Fas-associated death domain [97].

TLRs induce the production of TNFα from macrophages. TNFα, in turns, activates macrophages. TNFα and IFNγ exhibit a cross-talk at the level of TNFR to induce activation of macrophages [62]. It has been shown that TNFα induces a stronger activation of NFκB in the presence of IFN-γ. TNFα contributes to prolong macrophage survival in the condition of LPS-induced stepsis [101]. Moreover, TNFα also enhances the production of macrophages in vitro from primitive mouse hematopoietic progenitor cells [102]. In addition, TNFα has macrophage differentiation capabilities [103]. TNFα is expressed during differentiation of bone marrow-derived macrophages [104]. In the absence of TNFα in the differentiating macrophages, the cells followed a proliferative program instead of going through the differentiation program, suggesting that TNFα, working as an autocrine factor, is important in promoting the macrophage differentiation.
Taken together, these results suggest that TNFα plays an important role in the proliferation, differentiation and activation of macrophages.

4. Bone and Chondrogenesis

Bones are rigid organs. They move, support, and protect the various organs of the body, produce blood cells and store minerals. The sizes and shapes of bones need to be carefully coordinated to allow efficient movement of our symmetric bodies. It is not surprising that skeletal development is a complex and tightly-regulated spatio-temporal process.

4.1. Chondrogenesis

Chondrogenesis is the dynamic cellular process that leads to the establishment of various types of cartilage. The process initiates from the condensation of mesenchymal cells; cluster of cells adhere through the expression of adhesion molecules [105]. Cells within these condensations subsequently differentiate into spherical-shape chondroblasts, secreting an extracellular matrix rich in type II collagen and various proteoglycans. Once embedded in matrix, chondroblasts lose cell-cell contacts and become chondrocytes, producing a cartilage template that will ultimately be replaced by bone and marrow [105-106]. During this process, the chondrocytes undergo a process of maturation or terminal differentiation. (Figure 6). Cells in the resting zone serve as precursors for the chondrocytes undergoing this differentiation process. Chondrocytes in the proliferative zone are small in size and synthesize an abundant extracellular matrix consisting
primarily of collagen II, as well as the large proteoglycan aggrecan. As the cells differentiate, their proliferation slows down and their size increases (hypertrophic), concomitant with a change in the composition of the extracellular matrix. Type II collagen expression gradually declines, while type X collagen and matrix metalloproteinase-13 (MMP-13) expression is increased. Proteins that promote mineralization, such as the enzyme alkaline phosphatase, are also found in the hypertrophic zone. The chondrocytes at the lower border of the hypertrophic zone undergo apoptosis, and the matrix eventually becomes mineralized, and this mineralized matrix serves as a template for deposition of trabecular bone. This process of chondrocyte maturation is responsible for most longitudinal bone growth, both during embryonic development and in the postnatal long bone growth plates.
Figure 6. A schematic model of chondrocyte terminal differentiation. Illustration of zones of growth plate and important matrix component, and distribution of RA and its receptors (RAR). AP, alkaline phosphatase; HZ, hypertrophic zone; MMP13, matrix metalloproteinase 13; PZ, proliferating zone; PHZ, pre-hypertrophic zone; RZ, resting zone.
Numerous signaling molecules have been implicated in regulation of chondrogenesis, including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), Indian hedgehog (Ihh), parathyroid-hormone-related protein (PTHrP), Sox9, Wnt, and RAR.

PTHrP acts primarily to keep chondrocytes in the proliferative pool. PTHrP works with Ihh to form a negative-feedback loop, controlling chondrocyte proliferation [107]. PTHrP is secreted from perichondrial cells and chondrocytes at the ends of long bones. PTHrP acts on receptors on proliferating chondrocytes to keep the chondrocyte proliferating and thereby, to delay the production of Ihh. When the sources of PTHrP production are distant and the concentration of PTHrP is relative low in the prehypertrophic zone, then Ihh is produced. Ihh acts on its receptor on chondrocytes at the ends of bones to stimulate the production of PTHrP and increase the proliferation rate. Ihh knockout results in chondrocytes leaving the pool of proliferating chondrocytes prematurely, leading to an increase of post-mitotic hypertrophic chondrocytes [108-109].

The transcription factor SOX9 is essential for all phases of chondrocytes lineage development from early condensations to the conversion of proliferating chondrocytes to hypertropic chondrocytes. SOX9 is expressed in cells of mesenchymal condensations and in proliferating chondrocytes, but not in hypertropic chondrocytes. In culture cells, SOX9 stimulates transcription of many cartilage matrix genes, such as type II collagen and aggrecan [110-111]. When SOX9 was knocked out from the early limb mesenchyme by a Cre-loxP strategy, no cartilage condensation formed, and increased apoptosis in the mesenchyme was observed [112]. When SOX9 was deleted from chondrocytes at later stages of development, chondrocytes displayed decreased proliferation, decreased
expression of matrix genes, and decreased expression of elements in the Ihh-PTHrP signaling pathway.

BMPs are growth and differentiation factors of the TGFβ superfamily. BMPs act at all stages of chondrocyte terminal differentiation [113]. For example, treatment of prehypertrophic chondrocytes with BMPs induces type X collagen expression and alkaline phosphatase activity. BMP signals classically go through the Smad pathway, which has been demonstrated to be essential for endochondral bone formation. Runx2 is an essential transcription factor in chondrocyte differentiation, supported by the fact that chondrocyte terminal differentiation is totally blocked in Runx2-/- mice and these animals show no bone formation [114]. Smads physically interact with Runx2, leading to activation of Runx2 function in stimulating terminal differentiation of chondrocytes [115].

Wnt signaling pathway is considered as a powerful regulator of cell fate. It can strongly affect bone matrix anabolic and catabolic metabolize [116]. Acute activation of Wnt signaling strongly inhibits gene expression of aggrecan and type II collagen, stimulates gene expression and activity of matrix proteases, resulting in matrix loss in vivo and in vitro [116-117].

In addition, FGFs act to decrease chondrocyte proliferation, to increase the production of Ihh, and to accelerate the differentiation of hypertrophic chondrocyte into terminally differentiated chondrocytes [105].

4.2. Roles of Retinoids in Chondrogenesis

The importance of vitamin A in embryonic development was originally
demonstrated by studies on the effects of hyper and hypo-vitamin A. Bone lesion in vitamin A deficient calves were first described by Moore et al in 1935. Additional studies were designed to explore the histological effects and mechanism of vitamin A in bone development. Comparing vitamin A deficient guinea pigs with controls, an increase in sulfur uptake into glycoaminoglycans of the bone organic matrix and a decreased calcium content in newly formed bone was observed by Harris et al in 1977 [118]. The histologic appearance of nylon-tube implanted bone samples in vitamin A deficient and vitamin A adequate animals was conducted by the same group [119]. Tissue within the tube contained numerous spicules of new bone surrounded by fibrous connective tissue. Osseous tissue in implants from vitamin A deficient pigs contained less mature bone spicules, whereas the maturation of bone spicules was reversed by RA treatment, closely resembling those from controls [119]. More recent studies have reported that chronic vitamin A deficiency causes embryonic skeleton hypoplasia, including retardation of ossification and the reduced size of skeleton extending from axial to appendicular skeleton [120].

On the other hand, the effects of the administration of an excessive amount of vitamin A upon the growth of bone have been described for several species. The effects include the acceleration of all processes of bone growth-epiphyseal cartilage sequences concerned, remodeling sequences involving resorption of bone and appositional bone formation and compact-bone formation [121]. Moreover, in hypervitaminosis A, it was observed that the zone of proliferation was less clearly defined than in the normal, and the zone of growth was narrowed. Furthermore, the effects of high maternal vitamin A dose on development of offspring were examined [122]. A single dose (10,000IU) of
vitamin A given to impregnated females 9(1/3) days after conception caused more severe abnormalities than those treated earlier. The anomalies of the skin, face, mouse, teeth, the underlying soft tissues, the cartilaginous and osseous skeletons were observed, including massive maxillomandibular ankylosis; reduction in length of the mandible due to foreshortening and absent or defective rami; heterotopic cartilage; lateral ankyloglossia; absent, fused, supernumerary, and ectopic teeth; absent masseter muscle; absent parotid, sublingual, and submaxillary glands and ducts; ectopic submaxillary glands, etc. Taken together these results suggested that vitamin A plays an essential role in skeletal development.

Following the initial discovery that animals with either high or low vitamin A status produce offspring with a large array of congenital malformations including skeletons, it was shown that an imbalance of RA had similar effects. For example, the skeletal defects were most pronounced when RA was administered during the condensation and differentiation stages of chondroprogenitors [123], highlighting a need for precise RA levels during formation of the cartilage template. Numerous studies carried out in vivo and in vitro have shown a failure of cells to differentiate into chondroblasts in response to excess RA [124].

In addition to inhibitory effects of RA on the early stages of chondroblast differentiation, RA also regulates later stages of chondrocyte differentiation and maturation. A transgenic mouse expressing β-galactosidase under the control of an RA-responsive promoter displays expression only in the hypertrophic zone of development cartilages [125]. In accordance with this, RA induced maturation of cultured prehypertrophic chondrocytes of chick embryo tibia, as indicated by RA induction of type
Collagen X has also been shown to regulate other genes characteristic of hypertrophic chondrocytes, such as alkaline phosphatase and MMP13, and inhibit expression of aggrecan and type II collagen [117, 128]. RA dose-dependently reduces Sox9 mRNA and protein levels, and reduces the rate of Sox9 transcription in fully differentiated cartilage from the newborn mouse [129]. In addition to direct regulation of gene expression characteristic of hypertrophic chondrocytes, RA is likely to have indirect effects as well. RA has been indicated to influence BMP and Wnt signaling pathways during chondrocyte maturation. For example, RA stimulates BMP signaling though upregulation of Smad1 and Smad5 proteins [130]. RA increases Wnt protein and its receptor, consequently enhancing matrix loss triggered by Wnt signaling [117]. In summary, RA has a dual role in chondrogenesis: precise RA is required for the control of condensation and early chondroblast differentiation; and RA functions the terminal maturation and replacement by bone at later stage.

Unlike the distribution of RA in growth plate, RARγ, the most strongly expressed of the RARs in growth plate, appears in proliferative, prehypertrophic and hypertrophic chondrocytes, prior to the appearance of type X collagen [131-132]. Retinoid-dependent and RAR-mediated mechanisms are required for completion of the chondrocyte maturation process and endochondral ossification in the developing limb. A RAR conditional gene knockout in cartilage of mice was used for studying function of RARs in skeletal development and growth plate functioning [132]. Mice deficient in RARα and RARγ (or RARβ and RARγ) exhibited severe growth retardation that was obvious by about 3 weeks postnatally. Their growth plates were defective and displayed a major drop in aggrecan expression and content. Mice deficient in RARα and RARβ, however, were
virtually normal, suggesting that RARγ is essential for bone development. As discussed earlier, RA increases Wnt protein and its receptor, consequently enhancing matrix loss triggered by Wnt signaling, and as a result, inducing chondrocytes to undergo terminal maturation. RARγ strongly inhibited Wnt signaling in retinoid-free cultures. Moreover, RARγ interacts with β-catenin via its N-terminal domain, inhibiting β-catenin activity [117]. These results coordinate the distribution of RARγ in the proliferative chondrocytes, suggesting roles of unligand RARγ in the maintenance the proliferation of chondrocytes.

5. Roles of MafB

MafB (V-maf musculoaponeurotic fibrosarcoma oncogene family, protein B) is a transcription factor, and belongs to the Maf (musculoaponeurotic fibrosarcoma) family [2]. Since the identification of the v-maf oncogene in an avian tumor virus, the knowledge of the Maf protein family has grown rapidly. Maf proteins have been identified to be involved in transcriptional control of gene expression during development, differentiation and oncogenesis.

5.1. Maf Family

All Maf family members have similar structures, including a characteristic basic region linked to a basic leucine zipper (b-Zip) domain which mediates DNA binding and subunit dimerization [2, 133]. Members of the Maf family are divided into two subgroups: the large Maf proteins, c-Maf, MafB, MafA, and NRL, containing a distinctive acidic domain that enables transcriptional activation, and the small Maf proteins, MafK, MafF and MafG, all of which lack activation domains. The Maf proteins form homodimers or
heterodimers with other b-Zip proteins present in the cell and bind to Maf recognition elements (MARE) in DNA. Consensus Maf binding sequences for binding of Maf homodimers or heterodimers of Maf and related b-Zip proteins are TRE [phorbol-12-O-tetradecanoate-13-acetate (TPA)-responsive element]-type MARE (T-MARE): TGCTGACTCAGCA and CRE (cAMP-responsive element)-type MARE (C-MARE): TGCTGACGTCAGCA [2]. Moreover, Maf can also bind to 5’-AT-rich MARE half-site [134]. Maf family can bind to homodimers or heterodimers with Maf and related b-Zip proteins. MafB is able to form homodimers, and to bind with c-Fos forming a heterodimer [133].

Human MafB gene (also called KRML) within chromosome 20 was cloned by Wang et al in 1999 [135]. The protein contains 323 amino acids and has a calculated molecular mass of 35.8 kD (without posttranslational modification). MafB has a Pro-Ser-Thr-rich transcriptional activation domain at its N terminus, followed by 2 histidine repeats, an extended homology region, a basic DNA-binding domain, and a C-terminal leucine zipper domain (LLLLYL) (Figure 7). MafB is highly expressed in spleen, blood leukocytes, heart and skeletal muscle. Moue MafB gene (also called Kreisler) was cloned in 2000 [136]. Human MafB protein shares 84% amino acid identity with its murine homolog.
Figure 7. MafB protein sequences. Open box from amino acid (AA) 1-110; Pro-Ser-Thr-rich transcriptional activation domain; shaded AA 131-138 and 162-167: two histidine repeats; shaded AA 213-237, extended homology region, contributing to the DNA-binding specificity; yellow highlighted AA 237-265, basic DNA-binding domain; pink highlighted AA 266-301, leucine zipper domain (LLLLYL).
5.2. Roles of MafB in Hematopoiesis

During hematopoietic cell differentiation, MafB is not detected in multipotent progenitors, but expressed at moderate levels in myeloblasts and strongly up-regulated in monocytes and macrophages [137]. MafB in the myelomonocyte subsets interact with a transcription factor Ets-1 to inhibit erythroid-specific gene expression, suggesting increased MafB expression during monocytic differentiation prevents inappropriate erythroid gene expression in this lineage [137]. Kelly et al first stated MafB is an inducer of monocytic differentiation [138]. They reported that MafB favors myeloid colony formation of transformed chicken hematopoietic progenitors, and overexpression of MafB in transformed myeloblasts drives formation of macrophages; on the other hand, dominant negative MafB inhibits both processes. Later, expression MafB in human CD34+ hematopoietic progenitor cells by viral transduction results in the induction of a monomacrophage differentiation [139]. As I introduced previously, circulation monocytes give a rise to tissue resident macrophages and dendritic cells. Balancing the expression levels of MafB and PU.1 may determine the cell fate from monocyte to macrophage or dendritic cells [140]. During the differentiation to monocytes and macrophages, both MafB and PU.1 expression increase, in which moderate levels of PU.1 cooperate MafB to direct macrophage differentiation. However, differentiation from monocytes to dendritic cells leads to high expression of PU.1. PU.1 inhibit MafB expression and directly binds to MafB to inhibitor MafB transcription activity, preventing macrophage-specific gene expression. Consistent with this, constitutive MafB expression inhibits dendritic cell differentiation. Taken together, these results strongly suggest a critical function of MafB in macrophage differentiation.
On the other hand, MafB-/- macrophages show increased responsiveness to macrophage colony-stimulating factor (M-CSF)-induced morphological changes [141]. Moreover, MafB deficient HSCs are more sensitive to M-CSF and MafB deficiency in HSCs causes activation of PU.1, resulting in population shifting toward myelomonocytic division. Therefore, MafB expression level, at this point, can balance HSC commitment divisions [142].

In addition, MafB is considered as a negative regulator for receptor activator of nuclear factor κB ligand (RANKL)-mediated osteoclast differentiation [143]. RANKL is an essential cytokine to stimulate the differentiation of osteoclast from hematopoietic progenitor cells. Binding of RANKL to its receptors activates transcription factors including c-Fos, Mi transcription factor (Mitf) and nuclear factor of activated T cell (NFAT) c1, which are important for osteoclast differentiation. and then NFATc1 expression is further enhanced by binding of c-Fos and NFATc1 to its promoter. Increased NFATc1 induce expression of osteoclast-associated receptor (OSCAR), which is essential for osteoclastogenesis, by activating the promoter of OSCAR. Overexpression of MafB inhibits osteoclastogenesis by interfering with the DNA-binding domains of c-Fos, Mitf, and NFATc1, thereby inhibiting the activation of NFATc1 and OSCAR. Moreover, an increase of long transactivating form of C/EBPβ inhibits osteoclastogenesis through increasing expression of MafB by affecting its promoter activity [144]. Retinoids inhibit differentiation of osteoclast progenitors partially via increasing MafB expression [145].

5.3. Roles of MafB in Early Development

MafB is best known as a regulator of early embryonic development [146-149]. The
recessive MafB (or Kreisler/kr in mice) mutant was identified in a large X-array irradiation-induced chromosomal inversion encompassing the MafB locus [149]. The kr mutant mice have defective inner ear development, lose hearing, cannot swim, and run in circles, which results from an earlier segmentation defect in the hindbrain [149]. MafB/kr is identified in the rhombomeres (r) 5 and 6, and the mutation causes elimination of r5 and altered r6 identity [149]. RA signaling is critical for acquisition of all hindbrain fates posterior to r3 [150]. FGFs are produced by r4. Both RA and FGFs activate MafB expression in r5 and r6 to render them fully develop [147]. Moreover, MafB knockout mice die at birth mainly due to the defects in respiratory rhythmogenesis and central apnea [151].

MafB, as well as MafA, has been reported to be involved in islet β-cell differentiation. MafA and MafB show a dynamic expression pattern in developing and adult β-cells [152]. MafB is expressed in about 90% of insulin+ cells at E15.5, but at the same stage, MafA is expressed in only 54% of insulin+ cells. However, soon after birth, only MafA is produced by β-cells. MafB is expressed in α-cells. MafB can directly activate expression of insulin and glucagon [153]. Moreover, comparison the gene profile of MafB-/ β-cells with the wild type reveals that MafB is involved in ion binding and transport, signal transduction, and hormone secretion [152].

MafB and c-Maf have been identified in rat chondrocytes [154]. C-Maf has been shown to strongly induce connective tissue growth factor [155], which regulates the differentiation of chondrocytes and osteoblasts. But, so far, the function of MafB in chondrocytes is not clear.
CHAPTER 2 STATEMENT OF HYPOTHESIS

The hypothesis of this dissertation was first developed during the course of our studies regarding RA regulation of THP-1 cell differentiation. Results of studies conducted by our laboratory suggested that RA regulated cell cycle progression and cell differentiation in human monocytic THP-1 cells [89]. To clarify the mechanism of RA-induced monocytic cell differentiation, a microarray analysis was conducted in THP-1 cells. It revealed that MafB was one of genes strongly upregulated by RA during monocytic differentiation of THP-1 cells. Therefore, we hypothesize that MafB is a mediator in RA-induced monocytic differentiation.

As reviewed in Chapter 1, MafB is involved in cell differentiation in several processes. MafB expression has been reported in chondrocytes. But the distribution and function has not been clarified. RA stimulates the terminal differentiation of chondrocytes. Thus, we were interested in understanding RA regulation of MafB expression in chondrocytes, and the distribution and function of MafB in the growth plate.

Therefore, the hypothesis is that RA-induced MafB expression is a mediator in myeloid cell differentiation and bone chondrogenesis.

Accordingly, five specific aims were designed to test the hypothesis:

1. To clarify MafB expression under the condition of RA treatment in monocytic cells. This could be tested by examining MafB expression in response to RA treatment at mRNA and protein levels, with different doses of RA for different time points, in the presence or absence of TNFα, using THP-1 cells as a model.
2. To determine the downstream genes of MafB during RA- and TNFα-induced monocytic differentiation. This could be tested by predicting MafB target genes using bioinformatic approach based on consensus Maf binding motif sequences and verifying selected putative targets by testing MafB binding ability to their promoters.

3. To determine the requirement of MafB in the RA- and TNFα-induced monocytic differentiation. This could be tested by establishing MafB-knockdown THP-1 cells and examining cell differentiation and phagocytic activity.

4. To investigate the alteration of MafB expression during the rat limb development under different maternal vitamin A status and oral vitamin A and RA supplementation. This could be tested by measuring mRNA levels of MafB and other chondrocyte markers under different maternal vitamin A status and oral vitamin A and RA supplementation, and localizing MafB in the growth plate by testing its mRNA and protein by in situ hybridization and immunohistochemistry staining.

5. To determine MafB expression and function during the RA-induced chondrocyte terminal differentiation. This could be tested by examining MafB mRNA in response to RA treatment in the isolated primary chondrocytes, and comparing chondrocyte markers in MafB-knockdown cells with wild-type cells.

Chapter 3 is focused on monocytic cells (Aims 1, 2 and 3) and Chapter 4 on chondrocytes (Aims 4 and 5).
CHAPTER 3  MAFB IS A MEDIATOR OF RETINOIC ACID AND TUMOR NECROSIS FACTOR ALPHA INDUCED MONOCYTIC CELL DIFFERENTIATION

Adapted from

Zhang Y, Chen Q and Ross AC (2011) MafB is a mediator of retinoic acid- and tumor necrosis factor alpha- induced monocytic cell differentiation. (Manuscript in preparation)
1. Abstract

All-trans-retinoic acid (RA), the major active metabolite of vitamin A, is a regulator of monocytic cell cycle and differentiation. In the present study, we asked whether MafB, a basic leucine-zipper transcription factor involved in development, hematopoiesis and monocyte-macrophage differentiation, is a mediator of RA-induced differentiation. However, little is known about the regulation and targets of MafB. In THP-1 human monocytic cells, MafB mRNA and protein levels were up-regulated by RA dose and time-dependently while, additionally, RA and tumor necrosis factor (TNF) α synergistically regulated MafB expression, mainly at the transcriptional level. MafB target genes were screened and SPOCK1, CCL2 and Blimp1 were identified as potential targets, due to the presence of Maf recognition elements (MARE motifs) in their promoter regions. These genes are related to cell communication, recruitment and differentiation. SPOCK1, Blimp1 and CCL2 mRNA levels were highly correlated ($P<0.001$) with MafB mRNA, across cell treatments. ChIP assays demonstrated increased MafB protein binding to MARE elements in the promoter regions of SPOCK1, Blimp1 and CCL2 in RA and TNFα treated cells, as well as acetylation of histone-H4 in MARE containing regions, measured as an indictor of chromatin activation. Conversely, reducing MafB protein by microRNA silencing significantly decreased the expression of SPOCK1, Blimp1 and CCL2 ($P<0.01$). Moreover, the reduction in MafB resulted in decreased cell differentiation as determined by cell-surface CD11b expression and phagocytic activity. We conclude that MafB functions in the RA and TNFα-induced differentiation of monocytic cells in part through regulation of SPOCK1, CCL2 and Blimp1.
2. Introduction

All-trans-retinoic acid (RA), an active natural metabolite of vitamin A, is well known as a potent regulator of gene expression, cell cycle regulation, and cell differentiation. RA acts through nuclear retinoic acid receptors (RARs) to transcriptionally activate target genes [21, 156]. It is well established that retinoids are required for normal innate and adaptive immune responses to agents of infectious diseases [57, 157-158]. In human monocytic THP-1 cells, a useful model of monocytic cell differentiation [159], RA has been shown to regulate cell cycle progression and the expression of CD11b, a cell surface marker of macrophage differentiation, and to increase phagocytic activity, supporting a role for RA in promoting the differentiation of monocytic cells into macrophages [89]. This could be one of the pathways by which RA enhances innate and adaptive immune responses. However, mechanisms through which RA acts to promote differentiation are incompletely understood.

MafB is a transcription factor, belonging to the Maf family [2]. Similar to other so-called large Maf proteins, MafB contains a basic leucine zipper structure at the carboxy-terminal portion, which mediates DNA binding and subunit dimerization, involved in the regulation of gene expression. Maf proteins bind to a specific DNA sequence, called Maf recognition elements (MAREs). MafB is best known as a regulator of early embryonic development [146-148], but it has also been reported to be involved in islet beta-cell differentiation [160-163] and to be expressed in monocytes and macrophages where it is thought to be an important regulator of macrophage differentiation [138, 140, 164]. Overexpression of MafB was shown to limit myeloid progenitor proliferation while
promoting functional macrophage differentiation [138-139]. Moreover, a deficiency of 
MafB inhibited monocyte/macrophage differentiation in avian myeloid progenitors [138].
Together, these results strongly suggest a critical function of MafB in monocytic 
differentiation.

The regulation of MafB by RA and the potential role of MafB in RA-induced 
monocyte differentiation was first suggested to us by results of microarray studies in 
THP-1 cells, in which we identified the MafB gene as one of the more strongly retinoid-
regulated genes. Therefore, in the present study we hypothesized that MafB may be a 
significant mediator of the effects of RA on the regulation of downstream genes that are 
involved in monocytic cell differentiation and macrophage-like functions. Additionally,
TNFα, which is well known as one of the key mediators of inflammation that induces 
monocytic cell maturation and activation [103], synergistically upregulated MafB 
expression when combined with RA. However, the downstream effectors of RA and 
TNFα-induced MafB expression are not known. Therefore, we used a bioinformatics 
approach to screen for genes that were significantly regulated by RA and TNFα, in order 
to identify potential genes that may be mediated by the activity of MafB. Several genes 
were identified that contained a putative MARE and that exhibited strong co-regulation 
with the level of MafB, and three of these genes were selected for further study due to 
their potential involvement in monocyte immune functions: sparc/osteonectin, cwcv and 
kazal-like domains proteoglycan 1 (SPOCK1) [165-167], B lymphocyte-induced 
maturity protein 1 (BLIMP1; gene PRDM1) [168], and chemokine CC motif ligand 2 
(CCL2) [169-171]. Our results show that reducing MafB expression via transduction with 
a MafB-specific miRNA resulted in decreased RA and TNFα-induced THP-1 cell
differentiation and phagocytic activity. Overall, RA- and TNFα-induced MafB may be a mediator of some of the actions of these agents on monocytic cell differentiation, through transcriptional regulation of MafB, and hence its targets including SPOCK1, Blimp1, and CCL2.

3. Experimental Procedures

Cell and culture conditions—THP-1 cells from the ATCC (Rockville, MD) were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS), 500 μM β-mercaptoethanol, and 100 units/ml penicillin and streptomycin, at 37°C in a humidified 5% CO₂-air incubator, and used within 15 passages [172]. For experiments, the cells were grown in the same medium with 3% FBS, and the desired final concentration of all-trans-RA (Sigma-Aldrich, St. Louis, MO) and recombinant human TNFα (R&D Systems, Minneapolis, MN) were added. The vehicle control, ethanol, did not exceed 0.01%. For inhibition of certain phosphorylation pathways, cells were pretreated with inhibitors, PD98059 or SB20358 (Promega, Madison, WI), 30 min before addition of RA and TNFα. After treatment, the cells were harvested and subjected to RNA or protein analysis. Cell viability was monitored by addition of 0.4% Trypan Blue dye (Sigma-Aldrich) and was consistently > 95%.

Microarray analysis—Microarray analysis was used as a starting point for these studies and global results will be reported elsewhere. Briefly, THP-1 cells were treated with 20 nM RA at 0 h and with a second dose of RA plus TNFα (5 ng/ml) at 16 h, and cells were harvested after 40 h for analysis. Total RNA was prepared and sent to the
Microarray Facility of the National Cancer Institute, Frederick MD, for analysis, as described earlier [173]. These data have been deposited under GEO accession number GSE28995. We sorted genes from the full database (54,675 gene targets) that were upregulated by both RA and TNFα and showed significant regulation as detected by one-way ANOVA using a criterion of a \( P \) value <0.001. Putative promoter sequences (2000 bps upstream of the transcription starting site for each gene) of these RA- and TNFα-upregulated genes were downloaded from the UCSC genome browser website (http://genome.ucsc.edu). A Python script (http://www.python.org) was used to scan these sequences for the presence of MARE sequences (TGCTGACTCAGC and TGCTGACGTCAGCA) and for 5′-AT-rich MARE half-sites, on both the sense and anti-sense strands of the promoter sequences.

*Quantitative real-time PCR (q-PCR)*– Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Valencia, CA). For reverse transcription, as described before [174], 1 µg of total RNA was subjected to reverse transcription using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The diluted reaction product (1/20th of the reaction products) was applied for quantitative real-time PCR analysis using iQ SYBR Green Supermix from BioRad (Emory, CA) in a final volume of 20 µl. 18S RNA was amplified at the same time as an internal control. Primers used for MafB were: 5′-CCGCTGGCCATGGAGTATGT-3′ (forward), 5′-GGCGACGCTTGGGTATG-3′ (reverse); for SPOCK1: 5′-GACCGGGACCTGACTCAGC and TGCTGACGTCAGCA-3′ (forward), 5′-TCCTTTCTGCCTTGTGCTTT-3′ (reverse); for Blimp1: 5′-TGGACATGGA GGACGCGGTATG-3′ (forward), 5′-GGTTGGCAGGGATA
GGCTTAATAG-3’ (reverse); for CCL2: 5’-CCCAGTCACCTGCTGGTTAT-3’ (forward), 5’-TGGAATCCTGAACCCACTTC-3’ (reverse).

**Immunoblotting**—As described previously [89], briefly, whole cell lysates (50 µg) were denatured and separated by polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membranes, which were sequentially incubated in primary antibody, anti-MafB (Santa Cruz Biotechnology, Santa Cruz, CA), and horseradish peroxidase (HRP)–conjugated secondary antibody (Santa Cruz Biotechnology). Detection of the HRP conjugate was done using the ECL system (Pierce Biotechnology, Rockford, IL). For equal loading controls, the membranes were blotted with an anti-ß-actin antibody (Santa Cruz Biotechnology) or stained by Ponceau S.

For the dephosphorylation assay, THP-1 cells after a 24-h treatment with RA and TNFα were lysed in RIPA buffer with proteinase inhibitors, and the cell lysate was incubated in calf intestinal phosphatase (1.0 unit/µg protein) (New England Biolabs, Ipswich, MA) at 37°C for 60 min, then subjected to Western blotting.

**ChIP Assay**—Cell nuclei were subjected to ChIP assays similar to as described [174]. Antibodies specific to MafB and acetylated histone H4 were used for immunoprecipitation. Anti-acetylated histone H4 (Millipore, Billerica, MA) was used as chromatin activation indicator, and nonspecific IgG (Santa Cruz Biotechnology) as a negative control. The immunoprecipitated DNA (~500bps) was subjected to quantitative (q)-PCR with oligonucleotide primer pairs designed for the MARE region on the promoters of SPOCK1, Blimp1 and CCL2, using iQ SYBR Green SuperMix, as above. Primers for the MARE-containing regions in the promoter were, SPOCK1: 5’-GGATGGGCAGTGAGATATT-3’ (forward), 5’-CACCTGGGTGGA GAGAGAAG-
3’ (reverse); Blimp1: 5’-CAGCAGTTGCATGATGGTGT-3’ (forward), 5’-AGAGAAATCCAGCCTGCTCA-3’ (reverse); and CCL2: 5’-CGCTGGAAAGTATGTCAGCA-3’ (forward), 5’-AAATGCATGGGGTTTCTTGA-3’ (reverse).

Micro RNA (miRNA) silencing—MiRNAs were introduced into THP-1 cells by using a lentiviral Pol II miR RNAi expression system (Invitrogen Corp., Madison, WI) according to the manufacturer’s protocol. Four miRNAs targeting different area of the MafB mRNA (Figure 8) were tested, and position 2 giving the greatest reduction in MafB protein expression in RA plus TNFα-treated cells was selected for further experiments. As a negative control we used a miRNA (Invitrogen Corp., Madison, WI), which is predicted not to target any known vertebrate gene. After transduction, the cells were cultured in complete medium with 5 ng/ml blasticidin for 10 days to eliminate untransfected cells, after which cells were treated with vehicle or the combination of RA and TNFα for 24 h.
Figure 8. MicroRNA targeting positions on MafB mRNA. Four microRNAs targets are highlighted.
Flow cytometry-- For determination of CD11b expression, THP-1 cells were treated with vehicle or RA- and TNFα for 24 h. Then cells were stained with phycoerythrin (PE)-conjugated anti-CD11b antibody (BD Biosciences, San Jose, CA) fixed, and quantified by flow cytometry, as described [89].

Phagocytosis assay-- THP-1 cells were treated with RA and TNFα or vehicle as control for 24 h. At the end of treatment, *E. coli* FITC-labeled bioparticles (Molecular Probes) was added (E. coli to cell ratio of 10:1) for 2 h at 37°C [89]. The cells were pelleted and resuspended in 0.25% Trypan Blue for 1 min to quench the surface-bound fluorescence, washed with PBS twice, and subjected to staining with PE-labeled anti-CD11b, followed by two-color flow cytometric analysis.

Statistical analysis-- Results are shown as the mean ± SE of at least 3 experiments each. Prism 5 software (GraphPad Software, La Jolla, CA) was used for analysis. A *P* value less than 0.05 was considered statistically significant.

4. Results

*MafB expression is upregulated synergistically by RA and TNFα--* First, we tested the effect of RA on MafB expression. After a 6-h incubation, MafB mRNA was significantly increased by RA dose-dependently, at as low as 10 nM RA (Figure 9A). Based on this dose response, we chose 20 nM RA for later treatments. MafB expression continued to increase with time, up to 7-fold at 48 h (Figure 9B).
Figure 9. MafB mRNA is upregulated by RA in a time- and dose-dependent manner. (A) Q-PCR analysis of MafB mRNA in THP-1 cells. Cells were treated with different concentrations of RA from 0 to 1000 nM for 6 h. * P < 0.05 compared with vehicle control, n≥3. (B) Q-PCR analysis of MafB mRNA. Cells were treated with 20 nM RA for 2, 6, 16, or 48 h. * P < 0.05 compared with vehicle control; n≥3, expected 48 h (n=1).
We also tested TNFα (5 ng/ml) alone and combined with RA (20 nM) on MafB mRNA and protein levels. Whereas either agent alone increased MafB mRNA and protein time-dependently, a strong synergistic induction of MafB expression was produced when cells were treated with RA and TNFα in combination (Figure 10A and B). Furthermore, lipopolysaccharide (LPS), which can induce elaboration of TNFα from monocytes, such as THP-1 cells [175], also induced MafB expression, and RA enhanced LPS-induced MafB expression, similar to that observed with RA and TNFα (Figure 11).

To determine whether regulation of MafB by RA and TNFα takes place at the transcriptional level, THP-1 cells were treated with actinomycin D, an inhibitor of new RNA synthesis, prior to the treatment of RA and TNFα. The increase in MafB expression was completely inhibited by actinomycin D (Figure 10C), suggesting that RA and TNFα increased MafB expression at the transcriptional level. When the cells were pretreated with cycloheximide, an inhibitor of protein biosynthesis, RA- and TNFα-induced MafB expression was not inhibited (Figure 10C). However, the induction of MafB expression by LPS was abolished or attenuated by both actinomycin D and cycloheximide (Figure 12), further confirming the importance of mediators, such as TNFα, in LPS-induced MafB expression.
Figure 10. RA and TNFα synergize in inducing MafB mRNA and protein. (A) Q-PCR analysis of MafB mRNA in THP-1 cells. Cells were treated with 20 nM RA, or 5 ng/ml TNFα, or both for 1, 3, 6, or 24 h. * P < 0.05 compared with vehicle control; # P < 0.05 compared with RA treatment and TNFα treatment; n≥3. (B) Immunoblot analysis of MafB protein. Cells were treated with 20 nM RA, or 5 ng/ml TNFα, or both for 6 h. (C) Q-PCR analysis of MafB mRNA of THP-1 cells were treated with 20 nM RA, or 5 ng/ml TNFα, or both in the absence or presence of actinomycin D (ActD) or cycloheximide (CHX) for 6 h. n=2.
Figure 11. MafB mRNA is induced by RA and LPS. Q-PCR analysis of MafB mRNA in THP-1 cells. Cells were treated with RA (20nM), or LPS (100ng/ml), or both for 24 hours. * $P<0.05$ compared with vehicle control treatment. # $P<0.05$ compared with RA or LPS treatment. $n=3$. 
Figure 12. LPS induction on MafB mRNA is abolished by addition of actinomycin D and cycloheximide. Q-PCR analysis of MafB expression under the inhibitors. THP-1 cells were treated with RA (20nM), or LPS (100ng/ml), or both in the absence or presence of actinomycin D (ActD, 5μg/ml) or cycloheximide (CHX, 5μg/ml) for 6 hours. n=2.
SPOCK1, Blimp1 and CCL2 are predicted as MafB target genes—To identify MafB target genes, we first sorted genes from microarray data that were upregulated by both RA and TNFα (P < 0.001). Then, upstream sequences of transcription start sites of those genes were screened for a potential MafB binding site, MARE (TGCTGACTCAGC and TGCTGACGTCAGCA) [2] and a 5’-AT-rich MARE half-site [134]. Among the 637 genes that were responsive to treatment with RA and TNFα in THP1 cells (see Materials and Methods), 64 genes contain potential binding sites in their promoter regions (Table 1), including SPOCK1, Blimp1, and CCL2. These three genes were selected for further study due to their potential involvement in different aspects of monocytic cell differentiation.
**Table 1. RA- and TNFα-upregulated genes containing putative MARE in their promoter regions**

<table>
<thead>
<tr>
<th>REFSEQ</th>
<th>Gene Annotation</th>
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<tr>
<td>NM_001561</td>
<td>Homo sapiens tumor necrosis factor receptor superfamily, member 9 (TNFRSF9)</td>
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<tr>
<td>NM_006142</td>
<td>Homo sapiens stratifin (SFN)</td>
</tr>
<tr>
<td>NM_020365</td>
<td>Homo sapiens eukaryotic translation initiation factor 2B, subunit 3 gamma,(EIF2B3)</td>
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<tr>
<td>NM_02053</td>
<td>Homo sapiens guanylate binding protein 1, interferon-inducible, 67kDa (GBP1)</td>
</tr>
<tr>
<td>NM_004833</td>
<td>Homo sapiens absent in melanoma 2 (AIM2)</td>
</tr>
<tr>
<td>NM_012394</td>
<td>Homo sapiens prefoldin subunit 2 (PFDN2)</td>
</tr>
<tr>
<td>NM_032174</td>
<td>Homo sapiens translocase of outer mitochondrial membrane 40 homolog (yeast)-like (TOMM40L), nuclear gene encoding mitochondrial protein,</td>
</tr>
<tr>
<td>NM_003101</td>
<td>Homo sapiens sterol O-acyltransferase (acyl-Coenzyme A: cholesterol acyltransferase) 1 (SOAT1)</td>
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<td>NM_002928</td>
<td>Homo sapiens regulator of G-protein signalling 16 (RGS16)</td>
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<tr>
<td>NM_030769</td>
<td>Homo sapiens N-acetylneuraminic pyruvate lyase (dihydridopicolinate synthase) (NPL)</td>
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<td>Homo sapiens ubiquitin-conjugating enzyme E2D 1 (UBC4/5 homolog, yeast) (UBE2D1)</td>
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Q-PCR results were first obtained to validate the microarray data. For each of SPOCK1, Blimp1 and CCL2, mRNA expression was increased by RA and TNFα alone (Figure 13A, B and C, respectively), and the effect of the combination treatment was more than additive. Moreover, correlation analysis based on multiple experiments showed that expression of SPOCK1, Blimp1, and CCL2 were each highly correlated with expression of MafB ($P < 0.001$) (Figure 13D, E and F, respectively).

To further test MafB binding ability to the promoter of these genes, ChIP assays were conducted. The results showed that binding events of MafB protein to each promoter were increased 3-4 times by the treatment of THP-1 cells with RA plus TNFα (Figure 14). Moreover, acetylation of histone H4 in the MARE containing regions, measured as an indicator of chromatin activation, was also increased by the treatment of THP-1 cells with RA plus TNFα.
Figure 13. Expression of SPOCK1, Blimp1 and CCL2 are correlated with MafB. (A-C) Q-PCR analysis of SPOCK1 (A), Blimp1 (B) and CCL2 (C) in THP-1 cells treated with 20 nM RA, or 5 ng/ml TNFα, or both combined for 24 h. * P < 0.05 compared with vehicle control; n=3. (D-F) Correlation analysis of SPOCK1 mRNA (D), Blimp1 mRNA (E), and CCL2 mRNA (F) with MafB mRNA over several independent experiments.
Figure 14. MafB binding events are enhanced by RA and TNFα. Anti-MafB antibody (A) and anti-acetylated histone 4 (Anti-AcH4) antibody (B) were used in ChIP assays. MARE-containing regions were amplified by q-PCR using primers illustrated previously in experimental procedures. Y-axis represents the fold changes of MafB binding events to MARE-containing regions for cells treated with 20 nM RA plus 5 ng/ml TNFα compared with vehicle-treated control cells. Results represented four independent experiments. * \( P < 0.05 \) vs fold change =1; \( n=4 \).
Inhibition of MafB phosphorylation reduces RA and TNFα-induced gene expression

The activity of many transcription factors is regulated in a rapid and reversible manner by phosphorylation. Protein phosphorylation on MafA, another Maf family member, has been reported and phosphorylation of MafA was suggested to be important for its transactivation function [176-177]. Moreover, Sii-Felice et al. [177] also showed MafB was phosphorylated by p38 mitogen-activated protein kinase. Analysis of the protein sequence of MafB revealed that there are multiple threonine and serine residues in the transactivation domain. To examine the phosphorylation of MafB protein, the lysate from RA- and TNFα-treated cells was incubated with phosphatase for 1 h at 37°C. The upper band of MafB protein disappeared after incubation with phosphatase (Figure 15A). To further clarify the phosphorylation pathway, THP-1 cells were treated with PD98059 or SB203580, which are inhibitors for the ERK and p38 pathways, respectively. These inhibitors did not change MafB mRNA levels in response to RA and TNFα (Figure 16). Both inhibitors, however, partially reduced the phosphorylation level of MafB protein, and the inhibition lasted 24 h (Figure 15B). This result suggested that the p38 and ERK pathways may be involved in the modification of MafB protein in response to treatment of cells with RA and TNFα. This modification may enhance the transactivation activity of MafB and consequently alter its target gene expression. To test this hypothesis, THP-1 cells were pretreated with PD98059 or SB20358, and q-PCR analysis of SPOCK1, Blimp1, and CCL2 was performed. The results (Figure 15C) showed that PD98059 blocked more than 90% of RA- and TNFα-induced increase in SPOCK1 expression and about 80% of the increase in CCL2 expression, and SB203580 blocked more than 50% the RA-and TNFα-induced increase in Blimp1 expression. Overall the results suggest
that reduced levels of SPOCK1, Blimp1 and CCL2 mRNA might result from the decrease in phospho-MafB protein due to inhibition of cellular phosphorylation pathways.
Figure 15. Inhibition of MafB phosphorylation reduces RA and TNFα-induced gene expression. (A) Immunoblot analysis of MafB protein. Cells were treated with 20 nM RA plus 5 ng/ml TNFα for 24 h. The cell lysates were incubated with phosphatase for 1 h at 37°C or buffer as mock, and then subjected to immunoblotting. (B) THP-1 cells were treated with Vehicle (C) or RA plus TNFα (RT) for 1, 6, or 24 h in the absence or presence of PD98059 (PD) or SB203580 (SB), which are inhibitors for the ERK and p38 pathways, respectively, and then subjected to immunoblotting. For loading controls, membranes were stained with Ponceau S. (C) Q-PCR analysis of gene expression in the presence of the inhibitors. Cells were treated with RA plus TNFα for 24 h in the absence or presence of PD or SB inhibitors. Y-axis represents mRNA level changes of each gene that were relative to vehicle treatment (DMSO). n=2.
Figure 16. MafB mRNA level is not affected by the phosphorylation inhibitors. Q-PCR analysis of MafB expression in THP-1 cells which were treated with or without RA (20nM) plus TNFα (5ng/ml) for 24 hours in the absence or presence of PD98059 (PD, 25μM) or SB203580 (SB, 10μM). n=2.
MafB is a mediator in the RA-induced monocytic differentiation—To determine the requirement of MafB in the RA-induced monocytic differentiation, we established a MafB knockdown model by introducing micro RNA (miRNA) into THP-1 cells. A vector-based miRNA construct was packed into lentivirus, and then transduced into the cells. MafB knockdown was verified by testing mRNA and protein levels (Figure 17). Moreover, in MafB knockdown (MafB\textsuperscript{KD}) cells, the responsiveness of SPOCK1, Blimp1 and CCL2 to RA and TNF\(\alpha\) were all significantly decreased compared with wild-type (MafB\textsuperscript{WT}) cells (Figure 17C-E).

Next, we examined cell differentiation and functional activity of MafB\textsuperscript{KD} cells. The expression of cell surface CD11b is known to be increased during monocyte differentiation and maturation, with the highest level on mature macrophages. To determine the state of MafB\textsuperscript{KD} cell maturation, the cells treated with 20 nM RA and 5 ng/ml TNF\(\alpha\) for 24 h was subjected to flow cytometry to determine the CD11b expression level per cell (Figure 18). The percentage of CD11b\(^+\) cells was moderately but significantly reduced in MafB\textsuperscript{KD} cells compared with MafB\textsuperscript{WT} cells, with or without RA plus TNF\(\alpha\) treatment (Figure 18A). Moreover, the mean fluorescence intensity (MFI) of MafB\textsuperscript{KD} cells was significantly lower than MafB\textsuperscript{WT} cells when treated with RA plus TNF\(\alpha\) (Figure 18B), indicating fewer CD11b molecules per cell. Although the percentage of CD11b-positive cells increased with RA and TNF\(\alpha\) independent of MafB level, the MFI increased only in MafB\textsuperscript{WT} cells and not in MafB\textsuperscript{KD} cells. Overall, the total CD11b expression (combination of percentage and MFI) in MafB\textsuperscript{KD} cells was nearly 40\% lower than in control cells (Figure 18C).
Figure 17. Expression of MafB, SPOCK1, Blimp1 and CCL2 is reduced in MafB\textsuperscript{KD} cells. MafB knock down (KD) cells were prepared by introducing a specific miRNA targeting MafB mRNA (see Materials and Methods). As a negative control, designated MafB\textsuperscript{WT}, a miRNA that is predicted not to target any known vertebrate gene was used. The MafB knock down was first verified at the mRNA (A) and protein (B) levels. Cells were treated with vehicle or 20 nM RA plus 5 ng/ml TNF\textalpha for 24 h. Then SPOCK1 (C), Blimp1 (D), and CCL2 (E) in MafB\textsuperscript{WT} and MafB\textsuperscript{KD} cells were analysis by q-PCR. * $P < 0.01$ compared with control treatment within each genotype; # $P < 0.01$ compared between genotype; $n=3$. 
Figure 18. Differentiation and function are decreased in MafB<sup>KD</sup> cells. (A) MafB<sup>WT</sup> and MafB<sup>KD</sup> cells treated with vehicle or 20 nM RA plus 5 ng/ml TNFα for 24 h were subjected to flow cytometry to determine the percentage of CD11b positive cells. (B) Mean fluorescence intensity (MFI) of CD11b positive cells. (C) The total CD11b expression was calculated by multiplying the percentage positive cells and MFI. (D) After treatment with RA plus TNFα, MafB<sup>WT</sup> and MafB<sup>KD</sup> cells were incubated with FITC- tagged E. coli bioparticles, and then subjected to flow cytometry to determine FITC positive and CD11b positive cells. * P < 0.01 compared with control treatment within each genotype; # P < 0.01 compared between genotype; n=3.
To evaluate the functional activity of THP-1 cells, phagocytosis was assessed using fluorescein-tagged E. coli bioparticles to detect the uptake of opsonized particles by the cells. Since phagocytic capability is associated with cell differentiation [89], the percentage of CD11b+ and FITC+ cells was increased by RA and TNFα in both MafB WT and MafB KD cells, but was lower in both the control and RA- and TNFα-induced MafB KD cells (Figure 18D), suggesting that differentiated MafB-knockdown cells are also moderately less phagocytic. These results further support a significant role of MafB in RA- and TNFα- alterations of monocytic cell function.

5. Discussion

The present study provides evidence that the transcription factor MafB functions in the induction of monocytic cell differentiation caused by exposure of cells to RA, TNFα, and especially to both RA and TNFα in combination. Although MafB expression was detectable in untreated THP-1 cells, the level of expression was increased in a time and dose-dependent manner by a physiological concentration of RA, while RA also synergized with TNFα to increase expression to a much higher level for both MafB mRNA and protein. The same conditions that produced a much higher expression of the MafB gene led to an increased expression of numerous other genes, suggesting that the response of MafB is part of a program of cell differentiation induced by RA, with and without TNFα. Relatively little is known about MafB target genes, and thus to assess if any of the RA- and TNFα-regulated genes may be regulated in part through in the increased expression of MafB, we first examined the promoter regions of putative target
genes for the presence of MARE sequences, the consensus binding region for large Maf proteins [2, 134]. Several candidates were identified and we focused on three of them – SPOCK1, Blimp1 and CCL2 – due to their known or potential involvement in monocyte-macrophage cell differentiation and/or function. ChIP assays demonstrated increased binding activity of MafB to the promoters of each of target genes, while moreover, MafB\textsuperscript{KD} cells exhibited decreased the expression levels of Blimp1, CCL2 and SPOCK1 mRNA during treatment, and a decrease in RA- and TNF\(\alpha\)-induced cell differentiation and phagocytic activity. Together, RA and TNF\(\alpha\)-induced MafB may play a role in monocytic cell differentiation through transcriptional regulation of Blimp1, CCL2 and SPOCK1.

RA is a ligand for nuclear receptors RAR\(\alpha\), \(\beta\), and \(\gamma\), which dimerize with retinoid X receptors (RXR\(\alpha\), \(\beta\), \(\gamma\)) and bind to specific DNA sites, known as retinoic acid response elements (RARE) [20-21]. Analysis of the MafB promoter did not result in finding a classical DR2 or DR5, however, there are two hexameric motifs separated by 7 nucleotides, which might be RAR/RXR binding sites, since RAR/RXR hererodimers are able to bind to motifs for which the spacing nucleotides are not limited to 2 or 5 [178]. Our promoter analysis revealed that there is an potential NF-\(\kappa\)B binding site in the 5’-untranslated region in the MafB gene, which may explain the rapid increase of MafB expression by TNF\(\alpha\). Whether these putative sites could interact to account for the synergistic effect of RA and TNF\(\alpha\) on the induction of MafB, as observed in our study, is currently unknown. However, it is known that both RA and TNF\(\alpha\) can influence gene expression and protein production in many ways besides through nuclear receptors. For example, RA and TNF\(\alpha\) have been indicated to rapidly induce the MAPK
phosphorylation cascade [179-181], increase acetylation of histone H4, and increase the recruitment of polymerase II[174, 182]. The p300 and CBP [cAMP-response-element-binding protein (CREB)-binding protein)] are transcriptional co-activators, and both have intrinsic histone-acetyltransferase activity, and may act directly on chromatin-associated histones to facilitate transcription [183]. Recruitment of CBP/p300 was enhanced by RA and TNFα [182, 184]. Moreover, CBP/p300 protein levels were induced by RA [184]. Thus, synergistic effects of RA and TNFα may exert through recruitment of CBP/p300. Therefore, RA and TNFα may potentially coordinate several pathways to enhance MafB expression.

From our initial microarray data, 64 genes, which were initially identified as being significantly upregulated by RA and TNFα at the $P < 0.001$ level, were identified to contain potential MafB binding sites in their promoter regions. We selected SPOCK1, Blimp1 and CCL2 for further study. SPOCK1 has been reported to have calcium binding affinity and to be involved in cell adhesion [165-167], and RA and TNFα-treated THP-1 cells are noticeably more attached and spread in appearance compared to untreated cells, which grow in suspension. Thus, adhesion appears to be an important part of the differentiation program initiated by RA and TNFα, and SPOCK1 could potentially participate in this process. Blimp1, a transcriptional repressor, has been shown to be a trigger for monocytic differentiation [168]. Blimp1 was originally identified as a master regulator of B cell plasmacytic differentiation [185], but it also is involved in several other cell differentiation processes. Over-expression of Blimp1 was shown to drive macrophage differentiation of U937 cells [168]. CCL2/monocyte chemotactic protein-1 (MCP1) is well known as a chemoattractant for monocytes, with roles in cell
differentiation and cell trafficking [169]. Furthermore, increased expression of CCL2 in monocytes during their differentiation, associated with its receptors, provides a feedback mechanism in the control of macrophage recruitment and activation [170-171]. In the present study, we showed that, for each of these genes, SPOCK1, Blimp1 and CCL2, expression was increased after treatment of cells with RA and TNFα. Binding events of MafB to each promoter were enhanced by the treatment of RA plus TNFα, and shown by ChIP assays. Moreover, lower responses of THP-1 cells to treatment with RA plus TNFα were observed in the condition of decreased phospho-MafB and in MafB KD cells. Together, these results strongly suggest that MafB modulates cell behavior through targeting SPOCK1, Blimp1 and CCL2. Other pathways may also be involved, as suggested by the presence of putative MARE sequences in other genes regulated by RA and TNFα in THP-1 cells (Supporting Table S1), which we have not yet explored. Thus MafB and RA- and TNFα-induced MafB levels may have more widespread downstream effects on monocyte biology than has been revealed in our present study.

It is worthy of note that c-maf, another large Maf family member, is also involved in monocytic differentiation [186]. We tested c-maf in THP-1 cells and found that c-maf mRNA was increased 2-fold by RA and TNFα (data not shown), however, the induction level was much lower than that of MafB (~20 fold). MafB knockdown did not affect c-maf expression in THP-1 cells (data not shown). This suggests that c-maf could act together with but independently of MafB in the process of RA-induced monocytic differentiation and function.

To date, the MafB gene has not been deleted in vivo, but an X-ray mutagenesis induced mutation, known as the kreisler mutation, affects MafB expression in
rhombomeres 5 and 6 and results in defects in oto-neurogenic development in \( kr/kr \) mutant mice [164, 187]. Only recently has MafB been implicated in broader functions including hematopoiesis [138, 140, 164] and islet beta-cell maturation [160-163]. The current study adds to what is known about MafB in monocytic cells by showing that MafB expression is itself highly regulated by the cell’s environment, and by identifying new potential targets regulated by MafB.

In summary, this present work has demonstrated a regulatory pathway affecting MafB, through which RA and TNF\( \alpha \) may modulate monocytic cell function. Both of these agents are important regulators in many normal aspects of immunity and cell differentiation, both are important therapeutic targets in clinical medicine, and both have the potential for unwanted side effects when present at high levels. Our studies using RA at a physiological level, similar to that in plasma, and TNF\( \alpha \) at a relatively low dose, provide evidence that both of these agents, individually and especially in combination, positively regulate the levels and activity of MafB, which can then mediate effects through MARE sites on downstream target genes including SPOCK1, Blimp1, and CCL2. Thus RA- and TNF\( \alpha \)-regulated changes in MafB expression could potentially be important in regulating the morphology, function and migration characteristics of monocytic cells.
CHAPTER 4  CHONDROCYTE MARKERS AND MAFB EXPRESSION IN NEONATAL BONE: RESPONSES TO DIFFERENT LEVELS OF MATERNAL DIETARY VITAMIN A AND DIRECT NEONATAL SUPPLEMENTATION WITH VITAMIN A AND RETINOIC ACID
1. Abstract

Vitamin A (VA) and its active form, retinoic acid (RA), are regulators of skeletal development and chondrogenesis. The present study was carried out to determine if maternal VA intake during pregnancy and lactation, and direct oral supplementation of neonates with VA+RA (VARA) in early life, alter neonatal bone formation and chondrocyte gene expression. The pups of rats fed 3 levels of VA (marginal, adequate, and supplemented) for 10 weeks were studied at birth (P0) and postnatal day 7 (P7). Half of the newborns received an oral supplement of VARA, or oil placebo, on P1, P4 and P7. Tissues were collected 6 h after the last dose on P7. Tissue VA and VA homeostatic genes were increased by both maternal VA intake and VARA ($P<0.01$). Maternal VA did not affect bone mineralization assessed by von Kossa staining, but significantly altered femur and tibia lengths ($P<0.01$). Only in the VA-marginal group, oral feeding with VARA significantly increased the length of the hypertropic zone, suggesting VARA caused a catching up of growth. VARA-treated pup femurs from VA-supplemented dams decreased aggrecan, and increased matrix metalloproteinase (MMP)13 expression. The transcription factor MafB was localized to both proliferative and hypertrophic chondrocytes. MafB mRNA increased with maternal VA intake and VARA treatment. MafB protein levels in the hypertrophic zone were stronger in pups from VA supplemented dams.

In the primary chondrocytes, isolated from neonatal rat ribs, RA dose-dependently increased MafB, and reduced aggrecan and increased MMP13 mRNA levels. After MafB was knocked down by siRNA transfection, aggrecan expression was increased, and RA-induced changes in MMP13 expression were attenuated ($P<0.05$). Our results implicate
RA-induced MafB as a regulator of chondrocyte gene expression. Maternal VA supplementation and VARA treatment both altered extracellular matrix composition. The reduction in aggrecan and elevation in MMP13 expression by the combination of maternal VA supplementation and neonatal VARA treatment could potentially be unfavorable for early bone development.

2. Introduction

Prenatal and postnatal skeletal growth is a complex and tightly-controlled process [105, 188]. Growth plate is responsible for the bone elongation and formation of most elements. Cartilage growth occurs through the proliferation of chondrocytes, and matrix production, which primarily consist of type II collagen and the large proteoglycan aggrecan. As chondrocytes differentiate, cells enlarge into pre-hypertrophic and hypertrophic chondrocytes, extracellular matrix is replaced by type X collagen, and MMP13, which catalyze degradation of collagen and aggrecan, is increased [189-190].

The chondrocytes at the lower border of the hypertrophic zone undergo apoptosis. The matrix eventually becomes mineralized, and replaced by bone and marrow.

Maternal vitamin A (VA) status affects the embryonic development, including the growth of bone [35, 191]. Studies on VA deficiency in pregnant animals revealed a large array of congenital malformation in the embryos. Defects were found in the development of the eye, heart, lung, kidney, central nervous system, genitourinary tract, and the skull, skeleton and limbs [35, 191]. On the other hand, administration of excessive vitamin A during pregnancy also results in congenital anomalies, such as to the external surface of the head, cranial deformity, ectopic teeth, heterotopic cartilage, and so on [122, 192]. All
These studies establish a critical role of vitamin A in skeletal development. RA, a major bioactive metabolite of vitamin A, has been detected in a higher concentration in hypertrophic chondrocytes [125], where it stimulates chondrocyte terminal differentiation and alters cartilage matrix synthesis [117, 126-127, 193]. RA can directly alter the expression of genes involved in extracellular matrix, such as type X collagen, MMP13, and aggrecan [117, 126-127, 194]. RA regulates gene expression through its nuclear receptors (RARs), consisting of three subtypes, RARα, RARβ, and RARγ [21]. RARγ is the most strongly expressed of the RARs in the growth plate, prior to the expression of type X collagen [131-132]. RARγ functions in the both unliganded- and liganded forms along with the chondrocyte proliferation and differentiation [117, 132].

As noted in Chapter 1, MafB is a transcription factor, belonging to the Maf (musculoaponeurotic fibrosarcoma) family [2]. Similar to other so-called large Maf proteins, MafB contains a basic leucine zipper structure at the carboxy-terminal portion, which mediates DNA binding and subunit dimerization, involved in the regulation of gene expression. MafB is best known as a regulator of various developmental processes, such as segmentation in the hindbrain [146-148], and islet beta-cell differentiation [160-163]. MafB is also an important regulator of monocytic cell differentiation function [138-140, 164]. MafB has also been identified in the chondrocytes [154]; however, the function of MafB during chondrocyte differentiation has not been studied. Our previous study showed that MafB expression was highly induced by RA in a time- and dose-dependent manner in THP-1 monocytic cells. However, it is not certain whether MafB is responsive to RA signaling in the chondrocytes, and if so, how it functions in this process.

Therefore, in the present study, we investigated whether maternal VA intake and
direct oral supplementation of neonates with VA+RA (VARA) alter neonatal bone formation. Moreover, we were interested in clarifying the distribution and regulation of MafB in the bone in vivo and in a model of chondrocyte differentiation in vitro. Previous studies conducted in our laboratory indicated that VARA increased retinol uptake and retinyl ester formation in lung. The increase by VARA was higher than that produced by VA or RA alone [195-196]. Therefore, in this current study, VA with RA was given orally to the neonates to enhance the effects of vitamin A supplementation. Maternal VA did not affect bone mineralization assessed by von Kossa staining; however, significantly differences (P<0.01) in the lengths of pups’ femur and tibia were observed across different maternal VA diets. Only in the VA-marginal group, oral feeding with VARA significantly increased the length of the hypertrophic zone, close to the length in pups from VA-adequate group. Neither type X nor type II collagen mRNA was altered by maternal VA status, although VARA intake significantly enhanced type X collagen mRNA levels. However, one of the most important finding was that VARA-treated pup femurs from VA-supplemented dams decreased aggrecan, and increased matrix metalloproteinase (MMP)13 expression. Moreover, MafB was localized in both proliferative and hypertrophic chondrocytes in the growth plate. The expression in hypertrophic zone was stronger when maternal VA intake was higher. In cultured chondrocytes, RA dose-dependently increased MafB expression. MafB knockdown in chondrocytes resulted in an increase of aggrecan and a decrease of RA-induced MMP13, These results implicate RA-induced MafB as a regulator of chondrocyte gene expression and matrix formation via the control of aggrecan and MMP13 expression.
3. Animals and Methods

*Animals*- Animal procedures were approved by the Institutional Animal Use and Care Committee of the Pennsylvania State University. Female Sprague-Dawley rats with one-week-old female pups were purchased from Charles River (Wilmington, MA). The mother rats were fed with vitamin A deficient diet after they arrived. To achieve differences in maternal VA status, weaning female rats were fed three levels of VA in a standard AIN-93G purified diet for 10 weeks before mating: VA marginal (0.4 μg retinol/g diet, VAM), VA adequate (7.67 μg retinol/g diet, VAA), or VA supplemented (104 μg retinol/g diet, VAS) diet. Their offspring were studied at birth (P0) and on postnatal day 7 (P7). Additionally, half of the newborns were orally supplemented with VA+RA (VARA), or oil as placebo, on P1, P4 and P7. Tissues were collected 6 h after the last oral dose (Figure 19). Body weights of pups were measured (Table 2). Except that only one litter from VA-supplemented group had significant higher body weight, body weights of all other pups at the same age were comparable. VA (all-trans-retinyl palmitate) and RA were purchased from Sigma-Aldrich (St. Louis, MO). VA was dissolved in oil and given in a concentration of 11 μg retinol palmitate/g pup; RA in 0.6 μg retinoic acid/g pup. In the VA-marginal litters, one more treatment group of high RA dose (3 μg retinoic acid/g pup) was added to achieve stronger effects. Diets were selected to represent the range of VA in most human diets. The VARA supplement represented the dose of VA given to newborns and young children in VA supplementation programs. The amount of RA in VARA, equal to ~0.5 mg/kg body weight, represented a clinical dose, as has been given previously to neonatal rats and noted to promote lung maturation [197].
Figure 19. Illustration of treatments for rats. VAM, vitamin A marginal (0.4 μg retinol/g diet); VAA, vitamin A adequate (7.67 μg retinol/g diet); VAS, vitamin A supplemented (104 μg retinol/g diet); VARA, 11 μg retinol palmitate and 0.6 μg retinoic acid/g pup; RA (high), 3 μg retinoic acid/g pup. P0, date of birth; P1, P4, P7, postnatal day 1, day 4 and day 7.
Table 2. Body weight of pups (g).

<table>
<thead>
<tr>
<th>Maternal diets</th>
<th>P0</th>
<th>P1</th>
<th>P4</th>
<th>P7</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAM</td>
<td>5.70 ± 0.28</td>
<td>6.53 ± 0.39</td>
<td>9.93 ± 0.82</td>
<td>14.51 ± 1.23</td>
</tr>
<tr>
<td>VAA</td>
<td>6.01 ± 0.45</td>
<td>6.18 ± 0.77</td>
<td>9.72 ± 1.11</td>
<td>14.68 ± 1.24</td>
</tr>
<tr>
<td>VAS</td>
<td>6.06 ± 0.40</td>
<td>7.13 ± 0.48*</td>
<td>11.03 ± 1.40*</td>
<td>16.58 ± 2.61*</td>
</tr>
<tr>
<td>VAS ‡</td>
<td>6.00 ± 0.36</td>
<td>6.85 ± 0.48</td>
<td>10.13 ± 0.68</td>
<td>15.02 ± 1.64</td>
</tr>
</tbody>
</table>

Pups body weight was measured at the day of birth (P0), and postnatal day (P) 1, 4, and 7. Values are means ± SD, n ≥9. Rats were fed as previous figure described. VAM, vitamin A marginal; VAA, vitamin A adequate; VAS, vitamin A supplemented. *, body weights of pups from VAS dams are statistically significant higher than others at the same age (at P1, P4, and P7), P<0.05. However, only one litter from VAS group contributes to the significant higher body weight. ‡, Body weights of pups excluded pups from the litter with significant higher body weight are comparable to all other pups at the same age.
Primary chondrocyte culture- Chondrocytes were prepared from 1- to 7-day old rats from mothers with normal chow diets [198]. Briefly, the rib cage was dissected and rinsed in PBS, incubated at 37°C for 30 min in 2 mg/ml proteinase K (Sigma-Aldrich, St. Louis, MO) in PBS, then incubated at 37°C for 30 min in 3 mg/ml collagenase type II (Worthington, Lakewood, NJ) in DMEM (Invitrogen, Madison, WI). Soft tissues were detached from the cartilage. The cartilage was washed with PBS several times by repeated gently pipetting, and separated from soft tissues by sedimentation. The cartilage was cut into small pieces and then further digested with collagenase for 3-4 hours. After digestion, the cell suspension was filtered though a 45μm cell sieve, rinsed in DMEM, then cultured in DMEM with 10% fetal bovine serum (FBS), and 100 units/ml of penicillin and streptomycin, at 37°C in a humidified 5% CO₂-air incubator.

High performance liquid chromatography (HPLC)- Total retinol concentration in plasma, and pup’s stomach milk (indicative of maternal milk retinol concentration), and total retinol storage in the liver and lung of the pups were determined by HPLC as described previously [199]. Briefly, portions of the liver and lung were weighed and extracted overnight in 20 or more volumes of chloroform-methanol (2:1, v/v). The tissue extracts were filtered, washed, and dried under argon. Then the samples underwent hydrolysis by a saponification procedure. Trimethylmethoxyphenyl-retinol (TMMP) was added as an internal standard. Then the samples were dried again and reconstituted in methanol, ready for HPLC analysis. Plasma and milk samples directly underwent hydrolysis without prior extraction.

Anatomical measurement and histology staining- Femur and tibia were dissected out and length was measured with digital calipers (1/100 mm). Femurs were fixed in 4%
paraformaldehyde and imbedded in paraffin. Paraffin-embedded tissue blocks were cut at 8 μm thickness on a microtome and floated in a 40°C water bath containing distilled water. Then sections were transferred onto Probe-On Plus microscope slides (Fisher Scientific, Pittsburgh, PA), and then the slides were dried on a 37°C plate overnight and stored at room temperature. For staining, sections were deparaffinized and rehydrated through xylenes and graded alcohol series, and then stained with 1% Alcian blue (pH 2.5) for 20 min, and counterstained with 0.1% nuclear fast red for 5 min. The hypertrophic zone in the growth plate was measured by SPOT (Diagnostic Instruments, Sterling Heights, MI). For von Kossa staining, sections were incubated in 1% silver nitrate for 20 min, and unreacted silver was removed by 5% sodium thiosulfate for 5 min, and slides were counterstained with nuclear fast red.

**Immunohistochemistry (IHC)-** For IHC demonstration of MafB, an ABC-Elite kit, (Vector Laboratories, Burlingame, CA) was used, following the standard procedure. Briefly, sections were deparaffinized and rehydrated through xylenes and graded alcohol series, and then endogenous peroxidase activity was quenched by incubation in 3% H2O2 in methanol for 10 min, then washed in PBS for 5 min. Non-specific binding was blocked using diluted horse serum in PBS for 30 min at room temperature. The sections were incubated in 1:250 dilution of anti-MafB antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After washing with PBS, the sections were incubated with biotinylated secondary antibody for 30-60 min at room temperature followed by an ABC process. Finally, the sections were developed to 5 mg of 3,3′-diaminobenzidine (DAB, Promega, Madison, WI) in 10 ml TBS (20mM Tris, 150mM NaCl) with 1μl of 30% H2O2. For negative control in the IHC procedures performed, PBS replaced the primary
In situ hybridization (ISH)- Femurs were imbedded in optimal cutting temperature (OCT) (VWR LabShop, Batavia, IL) and frozen on dry ice, and stored at -80°C. Sections were cut at 10 µm thickness in a -20°C chamber of Shandon Cryostat, and transferred onto Probe-On Plus microscope slides (Fisher Scientific, Pittsburgh, PA), and then sections were dried briefly on a 37°C plate and stored at -80°C. ISH was carried out with digoxigenin (DIG)-labeled probes. MafB, type II collagen (Col2a1), and type X collagen (Col10a1) probes were prepared by using DIG RNA labeling kit (Roche Diagnostics, Indianapolis, IN). Briefly, MafB, Col2a1 and Col10a1 were amplified by standard PCR and cloned into T easy-vector (Promega, Madison, WI). Primers were used: MafB: 5’-CAGAGCTTTCGACGGCTTC-3’ (forward), 5’-GGTCCTCCACACTACGGTTG-3’ (reverse); 5’-CGGACTCCCTATCTCGTCTGTC-3’ (forward), 5’-TCAGCTTTGCTGCTACCTTC-3’ (reverse); Col2a1: 5’-ACCTGGTACCCCTGGAAATC-3’ (forward), 5’-GGATCCCTTACTGGGAAACATC-3’ (reverse); Col10a1: 5’-ATCCATGTGAAGGGGACTCA-3’ (forward), 5’-CCAGTGAATAGAAGGCGAA-3’ (reverse). The plasmids were linearized with 5’-overhang end by restriction enzymes. DIG-labeled RNAs (anti-sense and sense RNAs) were synthesized by DIG RNA Labeling Kit using the linearized DNA as templates, then hydrolyzed into 100-150 bp for use.

For detection, slides were incubated in cold acetone for 10 min, then air-dry. The slices were fixed in 4% paraformaldehyde for 15 min at 4°C, rinsed with PBS twice, incubated in 0.5% (v/v) acetic anhydride in 0.1 M triethanolamine buffer (pH 8.0) for 10 min, washed with 2X SSC (20XSSC: 3M NaCl, 0.3M Na3Citrate) twice, incubated in
50% formamide in 1X SSC at 60°C for 20 min, then passed through cold alcohol gradient solution, and air dried. For hybridization, slides were incubated with pre-heated (80°C for 2 min) probes in the buffer (1mg/ml tRNA, 50% formamide, 0.3M NaCl, 10mM TrisCl, pH 6.8, 10mM NaPO4, 5mM EDTA, 1X Denhardt’s, 10% Dextran sulphate) at 42°C overnight. Unhybridized probes were washed with 0.2X SSC twice at 55°C for 1 hour, NTE buffer (500nM NaCl, 10mM TrisCl, pH 7.5, 1mM EDTA) for 30 min, then incubated in NTE containing 20 μg/ml RNase at 37°C for 30 min, rinsed with NTE twice, and finally washed with 0.2X SSC at 55°C for 1 hour. For signal detection, DIG Wash and Block buffer set (Roche Diagnostics, Indianapolis, IN) was used. Slides were incubated in wash buffer for 5 min and then blocking buffer for 30 min, followed by incubation with alkaline phosphatase-conjugated anti-DIG antibody (1:200 dilution, Roche Diagnostics, Indianapolis, IN) overnight. Then the slides were washed in detection buffer for 10 min, then exposed to the substrate (BCIP/NBT, Roche Diagnostics, Indianapolis, IN) with 240 μg/ml levamisole under dark for 2-4 days. The color reaction was stopped by incubation in TE buffer.

Quantitative-PCR (q-PCR)- Femur and tibia were broken and the bone marrow was flushed out. Extraction of RNA from bone was done using Trizol (Invitrogen, Carlsbad, CA). Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA was quantified by spectrophotometry and analyzed for mRNA transcript levels by real-time q-PCR, with 18S rRNA or GADPH as internal control [174]. Primers for MafB: 5’-CAGAGCTTCCACGGCTTC-3’ (forward), 5’-GGTCCTCCACACTACCGTTG-3’ (reverse); Col2a1: 5’-GAGTCAAGGGGTGATCGTGGT-3’ (forward), 5’-TCTGGTTGTTCAGCGACTTG-3’ (reverse); Col10a1: 5’-
CAGGTTACCAGGACAAAA-3’ (forward), 5’-AGCCACACCTGGTCATTTC-3’ (reverse); aggrecan: 5’-CAAACCTCGGTTGTAAGGA-3’ (forward), 5’-GCTTTGCA GTGAGGATCACA-3’ (reverse); MMP13: 5’-CCCTCGAACA CTAATGGT-3’ (forward), 5’-GAGCTGCTTTGAGGCTTTC-3’ (reverse); SPOCK1: 5’-TCTCCCTGTTGCTCTGTG-3’ (forward), 5’-GAGAGGGTCTTTGACGCTTG-3’ (reverse); MMP3: 5’-ACCCCACTCACATTCTCCAG-3’ (forward), 5’-CTGACTGCAATCGAAGGACAAA-3’ (reverse); GAPDH: 5’-ATGGGAAGCTGGTCATCAAC-3’ (forward), 5’-GGATGCAGGGATGATGTTCT-3’ (reverse) RARγ: 5’-TCTTCGCGCGGGCAG-3’ (forward), 5’-GCCCGGTTTCCTGGCTCC-3’ (reverse).

Small interfering RNA (SiRNA) silencing- siRNA targeting MafB (Thermo Scientific, Lafayette, CO) was transfected into cultured primary neonatal rat chondrocytes to knock down MafB expression using DharmaFECT tranfection reagents (Thermo Scientific, Lafayette, CO) according to the manufacturer’s protocol. In the preliminary study, DharmaFECT transfection reagent 1 (one of four supplied in the kit) gave the best knockdown results and was selected for use. siRNA targeting GADPH (Thermo Scientific, Lafayette, CO) was used as a positive control. As a negative control we used a siRNA (Thermo Scientific, Lafayette, CO), which is predicted not to target any known vertebrate gene. The cells were plated in 96-well plates at 10,000 cells per well on day 1. After transfection (day 2), medium were changed to completely culture medium with antibiotics and the cells was treated with 20nM RA on day 3, then harvested on day 4 (48 hours post-transfection) or day 5 (72 hours post-transfection). To evaluate chondrocyte differentiation, Alcian blue staining was conducted. Cultured cells were fixed with 4%
(w/v) paraformaldehyde (or 10% formalin) for 10 min, then incubated in 70% ethanol for 10 min, 5% acetic acid for 5 min, stained with 1% Alcian blue (pH 1.0) for 2 hours. Excess stain was washed out. Then, the stain was quantified by solubilizing the stain with 6 M guanidine hydrochloride overnight at room temperature. Absorbance at OD 620 nm was measured using a spectrophotometer.

Statistics- Results are shown as the mean ± SE of at least 3 experiments. Prism 5 software (GraphPad Software, La Jolla, CA) was used for analysis. Student’s t-test was used for comparison between two treatments. One-way or two-way ANOVA followed by Boneferroni’s post-hoc multiple comparison test were used to determine statistical significance between multiple groups. A $P$ value less than 0.05 was considered statistically significant.

4. Results

Neonatal VA status and VA homeostatic genes were regulated by both maternal VA status and neonatal treatment with VARA.

After 10 weeks of feeding rat dams with VA controlled diets, plasma retinol concentration in VA-supplemented dams was more than 2-fold higher than in VA-adequate dams, whereas plasma retinol levels were comparable between VA-adequate dams and VA-marginal dams (Figure 20A). Since milk is the major source for vitamin A accumulation in the offspring, and retinol content in the milk is related to the mother’s dietary vitamin A [45], retinol levels in milk from the pups’ stomachs were measured. Concentrations were significantly greater (8-fold) in milk from VA-supplemented dams.
than that from VA-adequate dams, whereas retinol in milk produced by VA-marginal
dams was only 50% of retinol by VA-adequate (Figure 20B). Moreover, the oral
supplementation with VARA dramatically increased the retinol levels in the stomach of
the pups, regardless of maternal diet, which reflects the direct dose rather than transfer of
vitamin A in milk.

As expected, tissue VA of newborns was altered by maternal VA intake (Figure 21).
Plasma retinol concentrations of newborns were slightly but significantly different among
dams with different VA diets. At P7, the plasma retinol concentrations were still higher in
the pups from VA-supplemented dams, whereas they were comparable between the pups
from VA-adequate dams and VA-marginal dams. The liver VA storage was related to
maternal VA intake: VA-marginal <VA-adequate <<VA-supplemented at both P0 and P7.
For lungs, although VA storage was comparable between pups from VA-adequate and
VA-marginal dams, the total retinol levels from VA-supplemented dams were much
greater. Moreover, direct oral feeding with VARA increased neonatal plasma retinol,
liver and lung total retinol regardless of maternal VA intake.
Figure 20. Maternal retinol levels. Retinol concentration in maternal plasma (A), and milk in the pups’ stomachs (indicative of maternal milk retinol concentration) (B), were determined by HPLC. As treatments illustrated in Figure 19, weaning female rats was fed with 3 levels of vitamin A diets for 10 weeks before mating and the same diets were continuous during pregnancy and lactation stages: VAM, vitamin A marginal (0.4 μg retinol/g diet); VAA, vitamin A adequate (7.67 μg retinol/g diet); VAS, vitamin A supplemented (104 μg retinol/g diet). Plasma retinol concentrations of mothers were determined before mating, n≥3. Their offspring were orally supplemented with VARA (11 μg retinol palmitate and 0.6 μg retinoic acid/g pup), or oil as placebo on P1, P4, and P7. Stomach contents were collected 6 hours after last oral dose at postnatal day 7 (P7), n≥5. One-way ANOVA followed by Boneferroni’s post-hoc multiple comparison test was used to determine statistical significance. a>b>c>d>e, P<0.05.
Figure 21. Retinol levels in the tissue of pups. Retinol concentration in plasma (A-B) liver (C-D), and lungs (E-F) were determined by HPLC. Animal treatments were the same as described previously. VAM, vitamin A marginal; VAA, vitamin A adequate; VAS, vitamin A supplemented; VARA, vitamin A and retinoic acid (oral supplementation), and oil as placebo. Samples from (A, C, E) were collected at the day of birth (P0). Samples from (B, D, F) were collected 6 hours after last oral dose at postnatal day 7 (P7). Plasma samples: \( n \geq 5 \), liver samples: \( n \geq 6 \); lung samples: \( n \geq 3 \). One-way ANOVA followed by Boneferroni’s post-hoc multiple comparison test was used to determine statistical significance. \( a > b > c > d, P < 0.05 \).
VA homeostatic genes (CYP26A1, CYP26B1 and LRAT mRNA) in liver (Figure 22) were not affected in newborns, whereas CYP26A1 and CYP26B1 in lungs (Figure 23) were higher in pups from VA-supplemented mothers. At P7, CYP26B1 and LRAT were higher in the liver of pups from VA-adequate and VA-supplemented mothers compared to VA-marginal mothers. VARA significantly increased CYP26A1, CYP26B1 and LRAT in both liver and lung regardless of maternal VA intake (Figures 22 and 23). RA is known to regulate its own metabolism [16]; therefore, oral administration of high-dose RA increased expression of CYP26A1, CYP26B1 and LRAT in liver (Figure 22G-I), leading to a decrease of plasma retinol (Figure 21B).

All these results suggested that our design was appropriate to alter vitamin A homeostasis of offspring by maternal diet and direct oral supplementation. Maternal VA supplementation affected the offspring’s VA status at P0 and P7, and direct supplementation with VARA further altered VA status in P7 neonates from all maternal groups.
Figure 22. Expression of VA-homeostasis related genes in liver. mRNA levels of CYP26A1 (A-B), CYP26B1 (C-D), and LRAT (E-F) were determined by q-PCR. Animal treatments were the same described previously. VAM, vitamin A marginal; VAA, vitamin A adequate; VAS, vitamin A supplemented; VARA, vitamin A and retinoic acid (oral supplementation), and oil as placebo. Tissues were collected at day of birth (P0) ($n \geq 4$), or 6 hours after last oral dose at postnatal day 7 (P7) ($n \geq 3$). Two-way ANOVA followed by Boneferroni’s post-hoc multiple comparison test was used to determine statistical significance * P<0.05, ** P<0.01, *** P<0.001.
Figure 22. Expression of VA-homeostasis related genes in liver (continued). mRNA levels of CYP26A1 (G), CYP26B1 (H), and LRAT (I) were determined by q-PCR. Pups from VA-marginal (VAM) dams were orally supplemented with VA+RA (VARA, 11 μg retinol palmitate and 0.6 μg retinoic acid/g pup), or RA (3 μg retinoic acid/g pup), or oil as placebo, on P1, P4 and P7. Tissues were collected 6 hours after last oral dose at P7, n≥3. One-way ANOVA followed by Boneferroni’s post-hoc multiple comparison test was used to determine statistical significance. * P<0.05, ** P<0.01, *** P<0.001.
Figure 23. Expression of VA-homeostasis related genes in lungs. mRNA levels of CYP26A1 (A-B), CYP26B1 (C-D), and LRAT (E-F) were determined by q-PCR. Animal treatments were the same as described previously. VAM, vitamin A marginal; VAA, vitamin A adequate; VAS, vitamin A supplemented; VARA, vitamin A and retinoic acid (oral supplementation), and oil as placebo. Tissues were collected at day of born (P0) ($n \geq 5$), or 6 hours after last oral dose at postnatal day 7 (P7) ($n \geq 3$). Two-way ANOVA followed by Boneferroni’s post-hoc multiple comparison test was used to determine statistical significance. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. 
Maternal VA status and oral feeding altered bone formation.

Because maternal VA intake and oral VARA feeding altered systemic VA homeostasis, we then asked whether bone formation was also changed. First, bone length was determined. At P0, the femur length was slightly but significantly greater in pups of VA-supplemented mothers (P<0.05) (Figure 24A); the length of tibia in pups of VA-marginal dams was shorter than the other two groups, although not statistically significant. Note that, at P7 (Figure 24B and D), maternal vitamin A intake significantly affected the length of both femur and tibia (P<0.01).

Growth plate activity contributes to the skeletal growth; therefore, the histology of growth plate was examined. Sections of femurs were stained with 1% Alcian blue (Figure 25). Then, the hypertrophic zone was quantified on the images by software, called SPOT. The hypertrophic zone of femur chondrocytes was significantly shorter in newborns from VA-marginal mothers (P<0.05) (Figure 26A). Similar to the observation of total bone length, at P7, the formation of hypertrophic zone was changed by maternal VA diet (P<0.001). Only in the VA-marginal group, oral feeding with VARA significantly increased the length of the hypertrophic zone (Figure 26B), close to the length observed in pups from VA-adequate dams, suggesting VARA caused a catching up of growth. In addition, however, bone formation (mineralization) assessed by von Kossa staining did not appear significantly different among all groups (Figure 27), which might be due to the sensitivity of this method.
Figure 24. Length of bone of pups is altered by maternal diet and oral VARA supplementation. At P0 and P7, the length of femur (A and B) and tibia (C and D) was measured by a digital caliper, *n*≥6. Animal treatments were the same as described previously. VAM, vitamin A marginal; VAA, vitamin A adequate; VAS, vitamin A supplemented; VARA, vitamin A and retinoic acid (oral supplementation), and oil as placebo. One-way ANOVA followed by Boneferroni’s post-hoc multiple comparison test was used to determine statistical significance. * P<0.05, ** P<0.01, *** P<0.001. Note that, at P7 (B and D), maternal vitamin A intake affected the length of both femur and tibia significantly (P<0.01, by two-way ANOVA).
Figure 25. Histology analysis of femur growth plate stained by Alcian blue. Sections of femurs of newborn pups (A-C) and 7-day old pups (D-J) were stained with 1% Alcian blue, counterstained with nuclear fast red. Animal treatments were the same as described previously. VAM, vitamin A marginal; VAA, vitamin A adequate; VAS, vitamin A supplemented; VARA, vitamin A and RA. (A, D, G, J) Pups from VAM dams, (B, E, H) pups from VAA dams, (C, F, I) pups from VAS dams. (D-F) pups orally fed with oil, (G-I) pups orally fed with VARA, (J) pups fed with high dose of RA. (K) Illustration of zones of growth plate. BM, bone and marrow; HZ, hypertrophic zone; PZ, proliferating zone; RZ, resting zone. All images are under the same magnification (40x).
Figure 26. Hypertrophic zone of femurs in pups from VAM dams is narrower. Hypertrophic zone of growth plate in femurs was measured at P0 (A) and P7 (B and C) on the Alcian blue staining images by the software, SPOT; n=6. * P<0.05, *** P<0.001; a>b, P<0.05. Note that, at P7 (B), maternal vitamin A intake significantly altered the length of hypertrophic zone (P<0.001, by two-way ANOVA). VAM, vitamin A marginal; VAA, vitamin A adequate; VAS, vitamin A supplemented; VARA, vitamin A and retinoic acid.
Figure 27. Calcification analysis of femurs stained by von Kossa. Sections of femurs of newborn (P0) pups (A-C) and 7-day old (P7) pups (D-I) were stained with 1% Alcian blue, counterstained with nuclear fast red. Animal treatments were the same as described previously. VAM, vitamin A marginal; VAA, vitamin A adequate; VAS, vitamin A supplemented; VARA, vitamin A and RA. (A, D, G, J) Pups from VAM dams, (B, E, H) pups from VAA dams, (C, F, I) pups from VAS dams. (D-F) pups orally fed with oil, (G-I) pups orally fed with VARA, (J) pups fed with high dose of RA. (K) Illustration of growth plate stained by von Kossa. Bones are stained in brown; cell nuclei are stained in red. HZ, hypertrophic zone; PZ, proliferating zone. All images are under the same magnification (40x).
Maternal VA status and oral feeding altered gene expression in bone.

Another parameter to evaluate bone growth is extracellular matrix. We were interested in whether genes involved in matrix synthesis and accumulation were altered by maternal VA status and oral feeding. Gene expression (mRNA) in the bone was examined by q-PCR. At P0 and P7, neither type II collagen (Figure 28A) nor type X collagen (Figure 28D) was affected by maternal VA intake. At P7, type X collagen was significantly increased by oral VARA (Figure 28B). Moreover, type X collagen expression was induced by high-dose RA administration, which was consistent with the increased length of the hypertrophic zone (Figure 26C). Aggrecan and MMP13, related to proteoglycan synthesis and degradation, respectively, have been shown to be reduced and increased by RA treatment in vitro [117, 126-127, 194]. In our study, aggrecan expression did not differ with maternal dietary VA. Oral dosing with VARA significantly decreased aggrecan expression only in pups from VA-supplemented dams (Figure 29B). However, MMP13 expression was increased by both maternal diet and by VARA at P7. Moreover, concordant with aggrecan, MMP13 expression was more strongly increased by oral VARA in pups from VA-supplemented dams. Taken together, not only the lengths of bone and hypertropic zone were changed by diet, but also extracellular matrix.

In addition, RARγ is a predominant RAR in the growth plate [131-132]. Thus, RARγ expression was determined under different VA conditions. The expression of RARγ was not affected by either maternal VA intake or oral VARA supplementation (Figure 30), although high-dose RA significantly increased RARγ expression. However, CYP26A1 expression in bone was strongly increased by oral VARA and high-dose RA in order to control the availability of RA in the growth plate (Figure 30E and F).
Figure 28. Collagen expression in bone. Type II (A-C) and type X (E-F) collagen mRNA levels in bone under the different VA conditions were tested by q-PCR. Animal treatments were the same described previously. VAM, vitamin A marginal; VAA, vitamin A adequate; VAS, vitamin A supplemented; VARA, vitamin A and retinoic acid. Effects of two factors, maternal VA intake and oral supplementation or maternal VA intake and postnatal stages (time), on gene expression were analyzed by two-way ANOVA, * \( P<0.05; n=6 \). Col2, type II collagen; ColX, type X collagen.
Figure 29. Aggrecan and MMP13 expression in bone. Aggrecan (A-C) and MMP13 (E-F) mRNA levels in bone under the different VA conditions were tested by q-PCR. Animal treatments were the same described previously. VAM, vitamin A marginal; VAA, vitamin A adequate; VAS, vitamin A supplemented; VARA, vitamin A and retinoic acid. Effects of two factors, maternal VA intake and oral supplementation or maternal VA intake and postnatal stages (time), on gene expression were analyzed by two-way ANOVA, * P<0.05, *** P<0.001; a>b, P<0.05; n=6.
Figure 30. RARγ and CYP26A1 expression in bone. RARγ (A-C) and CYP26A1 (E-F) mRNA levels in bone under the different VA conditions were tested by q-PCR. Animal treatments were the same described previously. VAM, vitamin A marginal; VAA, vitamin A adequate; VAS, vitamin A supplemented; VARA, vitamin A and retinoic acid. Effects of two factors, maternal VA intake and oral supplementation or maternal VA intake and postnatal stages (time), on gene expression were analyzed by two-way ANOVA, * P<0.05, ** P<0.01, *** P<0.001; a>b>c, P<0.05; n=6.
MafB is expressed in bone and may play a role in chondrocyte differentiation.

MafB, as a transcription factor, is best known as a regulator of early embryonic development and cell differentiation [138, 148-149]. MafB is highly induced by RA during monocytic differentiation. MafB has also been identified in the chondrocytes [154]. Therefore, we were interested in clarifying its distribution, as well as function in RA-induced chondrocyte differentiation. First, to localize MafB expression in the growth plate, its mRNA and protein were detected by ISH and IHC, respectively. Type II collagen was predominant in proliferative zones, whereas type X collagen was induced in the hypertrophic zone (Figure 31). MafB mRNA and protein were expressed in both proliferative and hypertrophic chondrocytes. Next, effects of VA diets on MafB were examined. MafB mRNA was altered by both maternal diet and oral VARA intake (Figure 32), suggesting MafB is responsive to vitamin A regulation in chondrocyte lineage cells. MafB mRNA in pups from VA-supplemented dams was significantly higher than that in pups from VA-marginal dams (Figure 32A), thus protein levels were compared between these two groups by IHC. MafB protein in the hypertrophic zone of pups from VA-supplemented group was much stronger (Figure 31G and H). In addition, MafB expression was higher in P7 bones than P0 bones (Figure 32A).
Figure 31. MafB localization in the growth plate. Type II collagen (A), type X collagen (C), and MafB (E) mRNA were detected by *in situ* hybridization (ISH). (B, D and F) Sense probes were used as negative controls for ISH. (A and B) The slides were counterstained with methyl green. (G and H) MafB protein was detected by Immunohistochemistry (IHC). Positive staining (MafB protein, arrows) is brown. (G) Pups fed with oil from VAS dams. (H) Pups fed with oil from VAM dams. (I) Negative control for IHC; non-specific staining is in the region of bone marrow. All images are under the same magnification (100x). PZ, proliferative zone; HZ, hypertrophic zone; BM, bone and marrow.
Figure 32. MafB expression in bone under the different conditions of vitamin A. MafB mRNA levels in bone were tested by q-PCR. Animal treatments were the same described previously. VAM, vitamin A marginal; VAA, vitamin A adequate; VAS, vitamin A supplemented; VARA, vitamin A and retinoic acid. Effects of two factors, maternal VA intake and oral supplementation or maternal VA intake and postnatal stages (time), on gene expression were analyzed by two-way ANOVA, * P<0.05, ** P<0.01; a>b, P<0.05; n=6.
MafB may play a role in chondrocyte differentiation

To further explore mechanisms, cell culture experiments were performed using primary chondrocytes isolated from neonatal rat ribs, and then treated with different doses of RA for 24 hours. MafB mRNA was significantly increased by RA dose-dependently, at as low as 20 nM RA (Figure 33A). In a kinetic study, after a 6-hour incubation, MafB mRNA was significantly increased by RA, reaching a peak at 24 hours (Figure 33B). Matrix-related genes were tested further to evaluate chondrocyte function during RA treatment. RA inhibited gene expression of aggrecan (Figure 34A) and strongly upregulated expression of MMP13, starting at low concentration of 20 nM RA. However, the effects of RA of 24-hour treatment on type II and type X collagen were weak. To further clarify RA effects, chondrocytes were treated with 1μ RA and harvested after 6, 24 or 48 hours. Type X collagen was only induced at the early time (Figure 33F), and the stimulation effect was not lasting after 24 hours. Contradictory effects of RA on this gene have also been reported by other groups [126, 200]. Effects of RA on type II collagen was time-dependent; longer treatment, stronger inhibition (Figure 33E).
Figure 33. MafB mRNA is upregulated by RA time- and dose-dependently in primary chondrocytes. (A) Q-PCR analysis of MafB mRNA in primary chondrocytes. Cells were treated with different concentrations of RA for 24 h. (B) Q-PCR analysis of MafB mRNA. Cells were treated with 1μM RA for 6, 24, or 48 h. * P < 0.05, ** P < 0.01, *** P < 0.001 compared with vehicle control. n=3.
Figure 34. Effects of RA on matrix-related genes. Aggrecan (A), MMP13 (B), type II collagen (C), and type X collagen (D) mRNA levels in primary chondrocytes treated with different concentrations of RA for 24 h were tested by q-PCR analysis. (E-F) Q-PCR analysis of collagen mRNA. Cells were treated with 1μM RA for 6, 24, or 48 h. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with vehicle control. $n=3$. 
To determine the function of MafB in RA-induced chondrocyte differentiation, we established a MafB knockdown with siRNA specific targeting MafB mRNA. siRNA targeting GADPH was used as a positive control, and siRNA without targets as a negative control. MafB knockdown was verified by testing mRNA. GADPH mRNA was markedly decreased by its siRNA, although the inhibition declined with time (Figure 35A). MafB expression was decreased by its siRNA regardless of RA treatment (Figure 35B). Neither type II nor type X collagen expression was altered by MafB knockdown (Figure 36A, B). However, in untreated chondrocytes, MafB knockdown resulted in increased aggrecan expression (Figure 36C). Moreover, MafB knockdown also attenuated RA-induced expression of MMP13 (Figure 36D). In the previous work, SPOCK1 was predicted as a target gene of MafB, and it has been identified in growth plate. Thus, SPOCK1 was tested in MafB-knockdown chondrocytes. MafB knockdown abolished RA induction on SPOCK1 (Figure 36E), which confirmed our previous prediction.

Aggrecan and MMP13 are important factors for extracellular proteoglycan synthesis and degradation [189, 201]. Therefore, we determined the chondrocyte phenotype, monitoring proteoglycan accumulation by quantification of Alcian blue staining. RA induces chondrocyte differentiation and decreased extracellular matrix, thereby the staining was decreased (Figure 37). However, knocking down MafB resulted in an increase of the staining (Figure 37), consistent with the gene regulation (Figure 36C and D). Therefore, these results suggested a role of MafB in the regulation of cartilage matrix formation.
Figure 35. Knocking down MafB by siRNA in chondrocytes. (A) Q-PCR analysis in untreated chondrocytes for GADPH mRNA, as a positive control in knockdown assay, the times were indicated the time after transfection. ** $P < 0.01$, *** $P < 0.001$ compared with negative control siRNA. (B) Q-PCR analysis of MafB mRNA. Cells were treated with 20μM RA on one-day post-transfection, and harvested 1 or 2 days after treatment. Note that siRNA significantly inhibited MafB mRNA regardless of RA treatment ($P<0.01$). $n=3$. 
Figure 36. Gene expression in MafB knockdown chondrocytes. Q-PCR analysis of type II collagen (A), type X collagen (B), aggrecan (C), MMP13 (D), and SPOCK1 (E) in MafB-knockdown primary chondrocytes treated with 20nM RA for 2 days, starting from the day after tranfection. * $P < 0.05$, ** $P < 0.01$. $n=3$. 
Figure 37. Proteoglycan staining in MafB knockdown chondrocytes. MafB-knockdown primary chondrocytes treated with 20M RA for 48 h were stained with 1% Alcian blue, which was quantified by solubilizing with guanidine hydrochloride. Absorbance of OD 620 was measured using a spectrophotometer. * $P < 0.05$. $n=6$. 
5. Discussion

In this study, we studied the effects of maternal dietary VA intake or direct oral supplementation with VARA, or both in combination on neonatal bone and chondrocyte biology. In pups from VA-marginal dams, the hypertrophic zone was shorter; and oral VARA supplementation significantly increased the length. In pups from VA-supplemented mothers, VARA supplementation resulted in reduction in aggrecan and increase in MMP13 gene expression. Moreover, we found that MafB was induced by RA in a dose- and time-dependent manner in primary chondrocytes, and MafB knockdown resulted in increased aggrecan expression and attenuated RA-induced expression of MMP13, suggesting it is a regulatory transcription factor in neonatal cartilage formation.

In the rat model, vitamin A homeostasis of pups was affected by maternal vitamin A intake through placental transfer of vitamin A during gestation and through milk during lactation. In the pups, plasma retinol and tissue vitamin A storage were increased by both maternal vitamin A intake and oral VARA supplementation, consistent with previous results [45, 196]. Too much or too little vitamin A alters skeletal growth. One recent study by Li et al. reported that vitamin A deficiency caused embryonic skeleton hypoplasia including retardation of ossification and the reduced size of skeleton, and dysostosis of limb [120]. In our study, reduced size of limbs was observed in pups from VA-marginal dams whereas obvious severe dysostosis was not observed. This may be due to the fact that VA-marginal diet in our study was not extremely low in vitamin A; 5 times more than what Li et al. provided [120]. Our diets were selected to represent the range of VA in most human diets. The VARA supplement represented the dose of vitamin A, per body size, given to newborns and young children in VA supplementation programs.
Moreover, we showed that the hypertropic zone of femur chondrocytes was significant shorter in pups from VA-marginal dams. Only in the VA-marginal group, oral feeding with VARA significantly increased the length of the hypertropic zone, suggesting VARA caused a catching up of growth that was slowed due to the VA-marginal diet.

Aggrecan, a large proteoglycan, forms a major structural component of cartilage [105, 188]. It is responsible for hydrating cartilage, rendering the bone capable of resisting the physical loading, thereby playing a major role in maintaining cartilage integrity and normal functions [202]. The depletion of aggrecan leads to a decrease of compressibility and resilience during joint loading. Over time, this process leads to irreversible cartilage damage. The increased catabolism of aggrecan is a key pathological mechanism in arthritis [203-204]. MMP13 catalyzes degradation of collagen and aggrecan, and is induced in terminally differentiated chondrocytes in the growth plate [189-190]. Our results indicated that the alteration of aggrecan and MMP13 expression by short-term oral VARA supplementation depended on maternal VA status. Only in the pups from VA-supplemented dams, oral dosing with VARA significantly decreased aggrecan expression, along with a great increase of MMP13 expression. This findings suggests that combination of maternal VA supplementation and neonatal VARA treatment could potentially be unfavorable for early bone development.

MafB is best known as a regulator of early embryonic development and cell differentiation [138, 148-149]. In the present study, MafB was localized from proliferative to hypertrophic zone. Its expression in bone was affected by both maternal VA diet and oral VARA supplement. Moreover, protein expression in the hypertrophic zone of pups from VA-supplemented mothers was much greater than that of pups from
VA-marginal mothers. In my earlier study, MafB was found to be highly induced by RA in THP-1 cells. Here, MafB expression in primary chondrocytes was also increased by RA in a dose- and time-dependent manner. Meanwhile, our results, as well as previous studies done by other groups [117, 194, 205], indicated that RA can reduce aggrecan and increase MMP13 in cell culture. MafB gene knock down resulted in an increase of aggrecan in untreated chondrocytes, whereas the inhibition of RA on aggrecan was not reversed by MafB knockdown. These results suggest that MafB is a negative regulator of aggrecan synthesis, and that the RA inhibitory effects may go partially through MafB; however, there may be some redundant pathways mediating strong inhibitory effects of RA on aggrecan expression. Moreover, we showed that MafB knock down attenuated RA-induced MMP13 expression. Analysis of the MMP13 promoter by the Transcription Element Search Software reveals that, although there is no Maf consensus response elements (MAREs), there are multiple putative AP-1 binding sites in the upstream of MMP13 gene. MAREs are related to the AP-1 site [133]. There is an AP-1 binding site at -73bp in the promoter of human MMP13, which has been characterized, where c-Fos and c-Maf can bind in the inflammatory conditions [206-207]. In addition, the region containing the AP-1 site is conserved between human and rat. Thus, the regulation of MMP13 expression by MafB may also go through this binding site. Together with changes of MafB in vivo, these results suggest that MafB is involved in extracellular matrix formation and RA-induced chondrocyte differentiation.

MafB can form a heterodimer with c-Fos [133]. C-Fos plays an important role in chondrogenesis. Overexpression of c-Fos in ATDC5 cells inhibited chondroprogenitors differentiation in vitro [208]. Ectopic expression of Fos in developing chicken limb buds
caused truncation of the cartilage in the long bones; this was due to chondrodysplasia caused by delay in precartilagenous condensation and severely retarded terminal differentiation into hypertrophic chondrocytes [209]. Parathyroid hormone-related peptide (PTHrP) is known to be a critical regulator for maintaining chondrocyte proliferation and suppressing maturation [105]. PTHrP modulates chondrocyte differentiation through AP-1 by induction of c-Fos protein expression [210]. In the presence of dominant negative inhibitors of c-Fos, PTHrP effects are suppressed, and chondrocyte maturation is increased. Moreover, the inhibition of PTHrP on expression of type X is via c-fos [208, 211]. During differentiation of osteoclasts, MafB is considered as a negative regulator; MafB interferes with the DNA-binding ability of c-Fos by dimerization, as well as other factors, inhibiting their transactivation of nuclear factor of activated T cells (NFAT) c1 and osteoclast-associated receptor (OSCAR), which are key factor in osteoclastogenesis. Moreover, RA inhibits differentiation of osteoclasts by inducing MafB and inhibiting c-Fos [145]. It can be speculated that a similar regulation on MafB exists in chondrocytes. Therefore, we propose that RA induces chondrocyte differentiation though inducing MafB and inhibiting c-Fos, and increased MafB may interfere with the c-Fos function (e.g. in PTHrP signaling).

In conclusion, our results for the first time demonstrated RA-induced MafB as a regulator of chondrocyte gene expression and cartilage matrix homeostasis. Several aspects of neonatal bone and chondrocyte biology were affected by maternal dietary VA intake or direct oral supplementation with VARA, or both in combination. A strength of our study is that it used dietary VA intakes designed to be similar to the range present in most human diets. The combination of high maternal dietary VA intake and direct
neonatal VARA supplementation may provide too much VA, as suggested by the reduction in aggrecan and increase in MMP13 gene expression in this group. It can be speculated from our study that neonatal VA supplementation may only be beneficial if the mother’s VA status is low.
1. How MafB functions in RA-induced monocytic differentiation

In Chapter 3, MafB expression in RA-induced monocytic differentiation was discussed. The present study provides evidence that the transcription factor MafB functions in the induction of monocytic cell differentiation caused by exposure of cells to RA, TNFα, and especially to both RA and TNFα in combination (Figure 10 and Figure 38). To assess if any of the RA- and TNFα-regulated genes may be regulated in part through increased expression of MafB, we first examined the promoter regions of putative target genes for the presence of MARE sequences, the consensus binding region for large Maf proteins [2, 134]. From our initial microarray data, 64 genes, which were initially identified as being significantly upregulated by RA and TNFα at the $P < 0.001$ level, were identified to contain potential MafB binding sites in their promoter regions. We focused on three of them: SPOCK1, Blimp1 and CCL2, due to their known or potential involvement in monocyte-macrophage cell differentiation and/or function. The putative Maf binding motifs on their promoter is illustrated in Figure 39. ChIP assays demonstrated increased binding activity of MafB to the promoters of each of target genes, while moreover, MafB$^{KD}$ cells exhibited decreased the expression levels of Blimp1, CCL2 and SPOCK1 mRNA during treatment, and a decrease in RA- and TNFα-induced cell differentiation and phagocytic activity. Overexpression of MafB in CD34+ hematopoietic progenitor cells induced a remarkable monocytic differentiation [139]. During this process, CCL2 expression was increased by MafB transduction in CD34+
cells, which is consistent with our findings. Other genes, such as MMP2 and MMP3 are predicted as downstream genes of MafB by their study and ours.

In summary, the function of MafB in RA- and TNFα-induced monocytic differentiation is highlighted in Figure 40. Retinoic acid with TNFα induced MafB expression, leading to an increase of binding of MafB to the promoters of its target genes, such Blimp1, CCL2 and SPOCK1, so that expressions of these targets are increased. In addition, they exert their functions. As a result, the cells start to undergo differentiation and activation. Overall, RA and TNFα-induced MafB may play a role in monocytic cell differentiation through transcriptional regulation of Blimp1, CCL2 and SPOCK1.
Figure 38. MafB protein is increased synergistically by RA and TNFα. Immunoblot analysis of MafB protein. THP-1 cells were treated with 20 nM RA, or 5 ng/ml TNFα, or both for 2 h.

Figure 39. Putative MafB binding sites on the promoters of SPOCK1, Blimp1 and CCL2. Numbers are relative to transcription start sites.
Previously, our laboratory found that RA can rapidly induce alterations in the level or functional status of key cell cycle-related proteins, such as cyclin E, p27, retinoblastoma, resulting in cell cycle arrest in the G1 phase. MafB is considered as an inducer of monocyte differentiation. PU.1 interacting with MafB controls myeloid lineage commitment. In the present study (highlighted in gray), RA with TNFα transcriptionally induced MafB expression. Transactivation of MafB protein is enhanced by phosphorylation via ERK and p38 pathways. Increased MafB protein leads to an increase of binding of MafB to the promoters of its target genes, such Blimp1, CCL2 and SPOCK1, so that expressions of these targets are increased. In addition, they exert their functions. As a result, the cells start to undergo differentiation and activation. Therefore, RA- and TNFα-induced MafB may play a role in monocyctic cell differentiation through transcriptional regulation of SPOCK1, Blimp1 and CCL2.
2. How MafB functions in RA-induced chondrocyte differentiation

In Chapter 4, MafB expression in RA-induced chondrocyte differentiation was discussed. This study provides evidence that MafB is involved in RA-induced chondrocyte differentiation as a regulator of cartilage matrix formation. MafB was localized to both proliferative and hypertrophic chondrocytes. MafB was increased with maternal VA intake and VARA treatment. In cultured chondrocytes, RA dose-dependently increased MafB and reduced aggrecan, and increased MMP13 expression. After MafB knockdown by siRNA transfection, aggrecan expression was increased, whereas RA-induced changes in MMP13 expression were attenuated.

FGF signaling has been addressed to enhance the RA-vhnfl induction on MafB expression during the hindbrain segmentation [147]. Variant hepatocyte nuclear factor 1 (vhnfl) is a homeodomain transcription factor expressed throughout the posterior hindbrain and anterior spinal cord, was identified in a genetic screen in the zebrafish as a positive regulator of MafB [212]. Although FGF signaling itself has not been shown to increase the level of MafB, over expression of FGF3 with vhnfl dramatically increased MafB expression. Moreover, constitutively active MAPK/ERK kinase, which functions in signaling downstream of the FGF receptor, cooperated with vhnfl to drive MafB expression in a manner similar to that of FGF. On the other hand, the regulation of Maf by FGF signaling is also observed at the posttranslational level. MAPK pathway is activated upon interaction of FGF receptor and ligand, leading to the activation of ERK1/2 kinases, which can phosphorylate MafA [176]. The activity of many transcription factors is regulated in a rapid and reversible manner by phosphorylation. Phosphorylation of MafA was suggested to be important for its transactivation function.
Analysis of the protein sequence of MafB revealed that there are multiple threonine and serine residues in the transactivation domain (Figure 41). Incubation with phosphatase decreased the protein phosphorylation level. Moreover, ERK and p38 pathways may mediate the phosphorylation of MafB, as the results shown in Chapter 3. The function of FGF signaling in chondrocytes is to decrease chondrocyte proliferation, to increase the production of Ihh, and to accelerate the differentiation of hypertrophic chondrocytes into terminally differentiated chondrocytes [105]. It can be speculated that a similar FGF regulation on MafB exists in chondrocytes, which facilitates the RA induction on MafB.
Figure 41. Predicted phosphorylation sites of MafB protein. Potential phosphorylation sites are predicted by NetPhos 2.0. Brown stars indicate the predicted phosphorylated site with the prediction score higher than 0.99. Yellow stars indicate the predicted phosphorylated site with the prediction score lower than 0.99. (P) indicates the residues may be phosphorylated via p38 or ERK pathways, which are conserved phosphorylated residues on MafA [176-177].
Figure 42. Gene expression in chondrocytes. (A) Q-PCR analysis of MafB expression in response of RA and TNFα treatment. Primary chondrocytes were treated with RA (1μM), or 5 ng/ml TNFα, or both for 24 h. * P < 0.05, *** P < 0.001 compared with vehicle control.  (B) Q-PCR analysis of MMP3 expression in MafB-knockdown primary chondrocytes treated with 20nM RA for 2 days, starting from the day after tranfection. *** P < 0.001.
Figure 43. A model for the functions of MafB in RA-induced chondrocyte differentiation. RA-induced MafB plays a role in chondrocyte differentiation and cartilage matrix formation via increasing MMP13 and decreasing aggrecan. This present study is highlighted in gray. FGF signaling may enhance RA-induced MafB activity through activation of MAPK pathway. Moreover, MafB may counteract with c-Fos to promote chondrocyte differentiation.
As I discussed earlier, RA-induced MafB may interfere with c-Fos during chondrocyte differentiation. In addition to the regulation of chondrocyte differentiation and cartilage development, it has been suggested that AP-1 may regulate destruction of arthritic cartilage. Overexpression of c-Fos resulted in inhibition of proteoglycan synthesis, with a significant increase in transcription of MMP3 and with the suppressed transcription of aggrecan and tissue inhibitor of metalloproteinase (TIMP)-1 [213]. Moreover, another AP-1 protein, c-Jun, which can dimerize with c-Fos, has been considered as a mediator of IL-1β-and TNFα-induced cartilage destruction-associated processes in conditions of inflammation [214-216]. C-Jun activity is sustained by stimulation of IL-1β and TNFα, leading to upregulation of MMP genes, alteration of chondrocyte synthesis, and degradation of extracellular matrix. However, in response to TNFα in chondrocyte culture, MafB was decreased, and TNFα attenuated induction of MafB by RA (Figure 42A). In normal chondrocyte differentiation, the MafB knock down led to increased aggrecan and decreased RA-induced MMP3 (Figure 42B), similar with effects of overexpression of c-Fos on MMP3 and aggrecan [213]. Moreover, IL-1β increases MMP13 expression through enhancing c-Fos binding to the AP-1 site on the promoter of MMP13 [206], which site may be a MafB binding site. Therefore, we propose that, in normal conditions, MafB may interfere with c-Fos, regulating normal chondrocyte differentiation by predominantly controlling aggrecan and MMPs in a normal turnover rate. However, in the inflammatory condition, MafB expression in chondrocytes is decreased, the normal differentiation process is disrupted, and c-Fos/c-Jun activity is sustained, and cartilage matrix homeostasis turns to inflammatory catabolic stage.
In summary (Figure 43), RA-induced MafB plays a role in chondrocyte differentiation and cartilage matrix formation via regulation of MMP13 and aggrecan. FGF signaling may facilitate RA-induced MafB activity through activation of MAPK pathway, enhancing MafB expression and transactivation activity. Moreover, RA-induced MafB may counteract with c-Fos to promote chondrocyte differentiation.

3. Significances and future directions

Previously, our laboratory found that RA can rapidly induce alterations in the level or functional status of key cell cycle-related proteins, such as cyclin E, p27, retinoblastoma, resulting in cell cycle arrest in the G1 phase [89] (summarized in Figure 40). MafB is considered as an inducer of monocyte differentiation [138-139]. PU.1 interacting with MafB controls myeloid lineage commitment [140]. The present work has filled in the gap between RA and cell differentiation via bringing MafB in. We tested the regulation of MafB by RA and TNFα at both protein and mRNA levels, and predicted and validated MafB target genes including Blimp1, CCL2 and SPOCK1. Overall, we have provided evidence that RA and TNFα-induced MafB may play a role in monocytic cell differentiation through transcriptional regulation of Blimp1, CCL2 and SPOCK1 (highlighted in gray in Figure 40).

For the future study, firstly, it is important to clarify the function of SPOCK1 in monocyte activation. SPOCK1 has been reported to have calcium binding affinity and to be involved in cell adhesion [165-167]. However, it is not clear how SPOCK1 functions in monocytes. Secondly, it would be worthwhile to further explore MafB target genes that were predicted such as matrix metalloproteinases, which are important for cell
adhesion and migration. Moreover, it would be interesting to understand how RA regulates matrix metalloproteinases in monocytes, which may link RA to atherosclerosis.

In my rat study, our results for the first time demonstrated RA-induced MafB as a regulator of chondrocyte gene expression and cartilage matrix homeostasis though the control of MMP13, and aggrecan (highlighted in gray in Figure 43). Moreover, a strength of our study is that it used dietary VA intakes designed to be similar to the range present in most human diets. As expected, no severe defect in bone development was observed on P0 and P7. Neither type X nor type II collagen was altered by maternal vitamin A status. However, the differences in the lengths of bone and hypertrophic zone in the growth plate were observed across groups with different maternal vitamin A intake. Only in the VA-marginal group, oral feeding with VARA significantly increased the length of the hypertrophic zone, close to the length in pups from VA-adequate group, suggesting catching up of growth by VARA supplementation. Moreover, one of the most important finding in this study was that VARA-treated pup femurs from VA-supplemented dams decreased aggrecan, and MMP13 expression. Therefore, it can be speculated from our study that neonatal VA supplementation may only be beneficial if the mother’s VA status is low.

For future study, firstly, it would be interesting to conduct a long-term study to test bone growth and activity behaviors of pups from dams with different vitamin A status at weaning time or even later stages. Secondly, since c-Fos is a negative factor for chondrocyte differentiation [208-209], and MafB could dimerize with c-Fos [133], it would be also interesting to test regulation of c-Fos by RA in chondrocytes and clarify
how MafB and c-Fos functions in RA-induced chondrocytes differentiation. Moreover, it would be worthwhile to establish MafB knockout chondrocytes to clarify the roles of MafB in chondrocyte.
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