Chapter 3
RUNX2 Transcriptional Regulation in Development and Disease

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Abstract RUNX2, a member of the Runt family of transcription factors, plays important roles in embryonic development to promote osteogenesis and angiogenesis. RUNX2 has been implicated in the promotion of disease, including cleidocranial dysplasia, in cancer progression, and in metastasis of breast and prostate tumors. Its aberrant expression in disease states may be the result of several mechanisms such as haploinsufficiency, mutation, or amplification. In osteogenesis and cancer progression, interactions with core-binding factor-β (Cbf-β) and other cofactors are responsible for the regulation of target gene expression including, but not limited to, VEGF, osteopontin, osteocalcin, MMPs, and BMPs. RUNX2 transcriptional function within cells is regulated by signal transduction events leading to activation of ERK, Smads, cdks, and Akt, which result in phosphorylation, DNA binding, and transcriptional activation or repression of target genes. Constitutive activation of signaling pathways in tumor cells results in aberrant expression and activation of RUNX2. Specific RUNX2 targeting agents, therefore, may bypass the effects of redundant signal transduction pathways within cancer cells and be an effective therapeutic strategy for treatment of RUNX2-positive cancer patients.

Keywords Runx2 • Osteoblast • Cancer • Metastasis • Transcription • Cell cycle • TGF-β • Vitamin D3

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Abbreviations

1,25-OH D3	1,25-OH Vitamin D3
AA	Amino acid
Akt	v-akt murine thymomaviral oncogene homolog 1 (aka: protein kinase B or PKB)
AML	Acute myeloid leukemia
AR	Aldose reductase (Context dependent. Not to be confused with androgen receptor)
AR	Androgen receptor (Context dependent. Not to be confused with aldose reductase)
Atf6	Activating transcription factor 6
BMP	Bone morphogenetic protein
BMP-2	Bone morphogenetic protein-2
BR-DIM	3,3′-diindolylmethane
BSP	Bone sialoprotein (aka: OPN)
C/EBPβ	CCAAT/enhancer binding protein beta
CBFα	Core-binding factor alpha
Cbf-β	Core-binding factor subunit beta
Cbfβ-SMMHC	Core binding factor β – smooth muscle myosin heavy chain
CBP	CREB-binding protein
CCD	Cleidocranial dysplasia
Cdk	Cyclin-dependent kinase
CML	Chronic myeloid leukemia
c-myc	v-myc avian myelocytomatosis viral oncogene homolog
CoAA	Co-activator activator
CSC	Cancer stem cell
CSF2	Colony stimulating factor 2
CST7	Cystatin-7 (aka: cystatin-F or leukocystatin)
CTGF	Connective tissue growth factor
Cx43	Connexin 43
D-ELISA	Deoxyribonucleic acid binding-enzyme-linked immunosorbent assay
DNA	Deoxyribonucleic acid
EC	Endothelial cell
EMT	Epithelial mesenchymal transition
ERK	Extracellular signal-regulated kinase
ERα	Estrogen receptor alpha
EWS-FLI	Ewing's sarcoma breakpoint region 1 t(1122)(q24q12)
FGF2	Fibroblast growth factor 2 (basic)
FGF2R	Fibroblast growth factor 2 receptor
FoxO1	Forkhead box O1
G9a	Histone-lysine N-methyltransferase H3 lysine 9 specific 3 (aka: euchromatic histone-lysine N-methyltransferase)

GPCR	G-protein coupled receptor
HBME	Human bone marrow endothelial cell
HDAC	Histone deacetylase
HG	Hyperglycemia
HIF1-α	Hypoxia inducible factor 1 alpha subunit
HUVEC	Human umbilical vein endothelial cell
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor 1 receptor
Ihh	Indian hedgehog
IL8	Interleukin 8
JNK	c-jun-N-terminal kinase
MAPK	Mitogen-activated protein kinase
MET	Mesenchymal epithelial transition
miRNA	Micro-ribonucleic acid
MLK3	MAP3K mixed-lineage kinase 3
MM	Multiple myeloma
MMP	Matrix metalloproteinase
MORF	Monocyte leukemia zinc finger related factor
MOZ	Monocyte leukemia zinc finger protein
mRNA	Messenger ribonucleic acid
mSin3a	Mammalian transcriptional corepressors Sin3a
NBIF	Neobavaisoflavone
NLS	Nuclear localization signal
NMTS	Nuclear matrix targeting signal
OPN	Osteopontin (aka: BSP)
$p21^{CIP1}$	Cyclin-dependent kinase inhibitor 1A
p27	Cyclin-dependent kinase inhibitor 1B
p300	E1A binding protein p300
p38	p38 mitogen activated protein kinase
PEBP2α	Polyomavirus enhancer-binding protein 2 alpha
PI3K	Phosphoinositide-3-kinase
PIP	Prolactin-induced protein
PKC	Protein kinase C
PlxnA2	Plexin A2
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
PTHrP	Parathyroid hormone-related protein
RANKL	Receptor activator of nuclear factor kappa-B ligand
RNA	Ribonucleic acid
Rorβ	Retinoid-related orphan receptor beta
rRNA	Ribosomal ribonucleic acid
RUNX1/2/3	Runt-related transcription factor 1/2/3
SDC2	Syndecan 2
SDF1	Stromal cell-derived factor 1
SH3PXD2A	SH3 and PX domain-containing protein 2A

shRNA	Small hairpin ribonucleic acid
siRNA	Small interfering ribonucleic acid
Smad	Sma and mad related proteins
Smurf	Smad specific E3 ubiquitin protein ligase
SNAI2	Snail homolog 2/slug
SNP	Single nucleotide polymorphism
Sox9	Sex determining region Y-box 9 transcription factor
Suv39h1	Suppressor of variegation 3–9 homolog 1
TAZ	Transcriptional coactivator with a PDZ-binding motif
TGF-β	Transforming growth factor beta
TLE-1	Transducin-like enhancer protein 1
TLE2/3	Transducin-like enhancer protein 2/3
UV	Ultraviolet
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
Wip1	Protein phosphatase magnesium-dependent 1 delta
WWOX	WW domain containing oxidoreductase
WWP1	WW domaincontaining E3 ubiquitin protein ligase 1
YAP	Yes-associated protein

3.1 Introduction

The *RUNX* genes are a family of transcription factors originally identified in *Drosophila* [1, 2]. There are three mammalian *RUNX* genes encoding the proteins RUNX1, RUNX2, and RUNX3. Historically, the literature contains many different nomenclatures for the mammalian RUNX family proteins. RUNX2(Runt related transcription factor 2) is also AML-3 (acute myeloid leukemia-3), PEBP2α (polyoma enhancer binding protein 2α), and CBFα (core binding factor α) [2–5]. The *RUNX* genes control many normal cellular processes including hematopoiesis (*RUNX1*), osteogenesis (*RUNX2*), and epithelial and neuronal development (*RUNX3*) [1]. *RUNX* genes encode several evolutionarily conserved proteins. There are four *RUNX* genes in zebra fish, four in *drosophila*, one in sea urchins, and one in *C. elegans* to name a few [2]. High conservation of proteins through evolutionary history generally suggests an extremely essential biological function.

The RUNX proteins are members of a heterodimeric complex composed of an α and β subunit. The *runt* domain genes contain the DNA binding α subunit [1]. The β subunit, consisting of core-binding factor-β (Cbf-β), binds to the runt domain within RUNX proteins (Fig. 3.1) to help stabilize RUNX-DNA interactions [1]. In addition, Cbf-β protects RUNX proteins from phosphorylation and degradation via the proteasome [1]. The RUNX proteins share many common protein domains (Fig. 3.1). The runt domain, which is responsible for binding DNA, is located in the N-terminus of the protein and is composed of 128 amino acids [3]. The runt domain is the most highly conserved domain among members of the RUNX family including the *Drosophila* ortholog *runt*. Located C-terminal to the runt domain is the nuclear

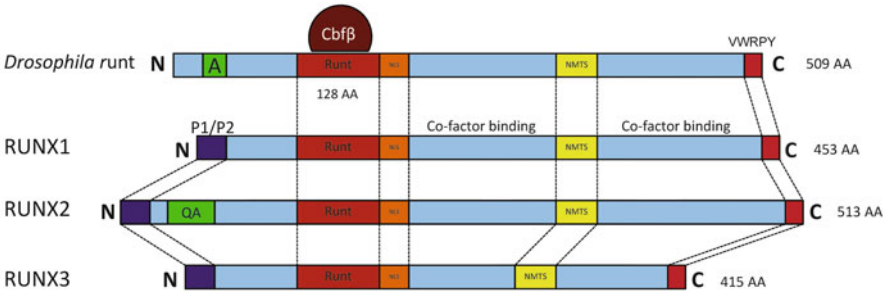

Fig. 3.1 Mammalian RUNX isoforms protein structure. The three mammalian RUNX proteins share domains with *drosophila runt*. N terminal (*purple*) are the P1 and P2 promoters. The Runt domain (*red*) is the DNA binding and Cbf-β interaction domain. C-terminal of the Runt domain (*orange*) is the nuclear localization sequence (NLS) controlling nuclear translocation of RUNX proteins. C-terminus (*light blue*) are a variety of sequences mediating co-factor binding to either activate or repress RUNX transcription of target genes. The nuclear matrix target sequence (NMTS; *yellow*) controls sub-nuclear localization of RUNX proteins

matrix targeting signal (NMTS) which is responsible for sub nuclear localization of RUNX proteins [1, 6]. This domain is comprised of 38 amino acids [5] folding into a loop-turn-loop tertiary structure [6]. Mutations within this domain reveal the essential nature of its function: without a functional NMTS, RUNX proteins cannot be transactivated or localized in foci within the nucleus [5]. Mutations within the second loop of the NMTS have been shown to inhibit RUNX2 interaction with the nuclear matrix [6] leading to compromised gene regulation. The nuclear localization signal/sequence (NLS) is comprised of 9 amino acids [5] and maintains RUNX localization to the nucleus. Furthermore, the C-terminus of RUNX proteins contains binding sites for corepressors and coactivators [1], which modulate RUNX activity. Depending on the cell stimulus or the cell type, different modulators of transcription are able to bind the RUNX proteins and either enhance or repress transcription.

All of the RUNX proteins have been implicated in disease (see Fig. 3.2 for diseases in which RUNX2 has been shown to play a role). Their normal functions are altered via mutations, epigenetic silencing, chromosomal translocation, cellular mislocalization, or by gene amplification. There is evidence that the RUNX proteins function as both tumor suppressors and as oncogenes depending on the disease context.

Although RUNX1 is not the largest protein isoform in mammals, it does encompass the largest genomic coding and regulatory region of 260 kb of DNA, comprised of 11 exons [4] encoding for 453 amino acids [1]. Within cells, RUNX1 functions to maintain normal hematopoiesis [1]. It is the target of numerous mutations and chromosomal translocations in hematological malignancies such as leukemia. In most reported cases of leukemia (specifically acute myeloid leukemia) where RUNX1 translocations are discovered, there appears to be a dominant negative function resulting from the new RUNX1-fusion protein. RUNX1 translocations and mutations are seen in acute myeloid leukemia (AML), blast crisis of chronic myeloid leukemia (CML), and acute lymphoblastic leukemia (ALL) [4]. There have been a few reports of gain of function mutations in RUNX1 as a result of an extra copy of

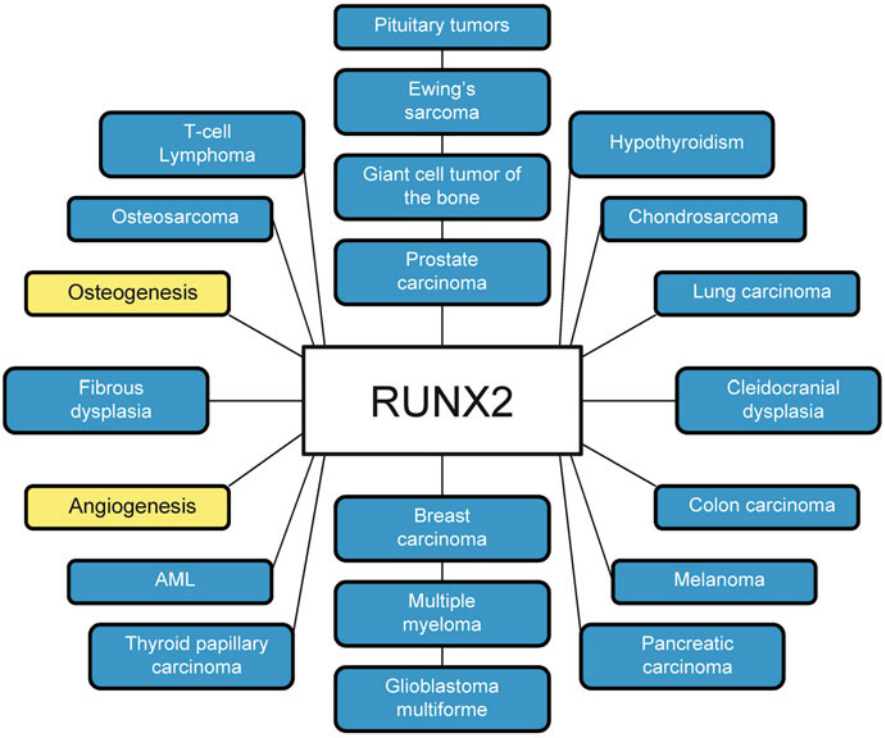

Fig. 3.2 RUNX2 in development and disease. Normal functions of RUNX2 (*yellow*) and diseases in which RUNX2 has been shown to play a role (*blue*)

the RUNX1 gene. This has been reported in Down Syndrome-related acute megakaryoblastic leukemia [4]. Knockout mice display a wide range of defects including, but not limited to, megakaryocyte defects, T-cell defects, myeloproliferative diseases, as well as T-cell lymphomas [2]. New studies have suggested a role for RUNX1 in endochondral ossification to mediate fracture healing in bone [7].

The smallest of the three mammalian RUNX proteins, RUNX3, is also thought to be the most primitive in evolutionary history, spanning 67 kb of DNA and composed of six exons [4] translating to 415 amino acids [1]. Expressed ubiquitously throughout the body, it can be found within the epithelia, mesenchyma, blood cells, dorsal root ganglion neurons, and predominantly in the gut epithelia [4]. RUNX3 has been shown to be essential for proper gut epithelial and neuronal development [8]. RUNX3 is essential for proprioceptive neuron axon path finding in the spinal cord [2] and there have also been reports of RUNX3 regulating CD4 silencing in T-cells [2]. Within the gut epithelia, research demonstrates that inactivation of RUNX3 leads to hyperplasia with a loss of response to transforming growth factor-β (TGF-β) inhibition [4] resulting in gastric cancers [8]. Inactivation has been described to occur through mutation, epigenetic silencing, hemizygous deletion, or cytoplasmic mislocalization.

Human RUNX2, located on the short arm of chromosome 6 at position 21 (6p21) [3], is the largest family member containing 513 amino acids [1] and has unique domains not present in the other mammalian isoforms (RUNX1 and RUNX3); one in the N-terminus and one in the C-terminus (see Fig. 3.1). RUNX2 is expressed early in embryonic development in mesenchymal stem cells [5]. During mouse embryogenesis, RUNX2 mRNA has been detected as early as E11.5 in the limb buds and the condensation of the humerus [5]. There is very weak expression observed as early as E9.5 in the notochord [5]. RUNX2 is the master regulator of osteoblast differentiation and chondrocyte maturation in a process called osteogenesis [1, 5]. RUNX2 controls the commitment of mesenchymal stem cells to the osteoblast lineage and has been shown to be abnormally expressed in adult tissues, leading to disease. Haploinsufficiency of RUNX2 promotes cleidocranial dysplasia (CCD) [9, 10]. In addition, there have been a few reports of RUNX2 mutations occurring within the runt domain which also result in CCD [5]. The oncogenic potential of RUNX2 was first identified from its ability to synergize with c-myc in T-cell lymphoma development [8, 127]. Aberrantly expressed RUNX2, normally at non-detectable to low levels in epithelial tissue, is thought to promote bone metastasis through activation of genes in malignancies such as breast and prostate cancer [8]. These target genes include, but are not limited to, vascular endothelial growth factor (VEGF), osteopontin (OPN), osteocalcin (OC), and matrix metalloproteinases (MMP's) [8]. The rest of this review will focus on RUNX2.

3.2 RUNX2: A Master Transcription Factor

3.2.1 Function in Osteogenesis and Angiogenesis

Osteogenesis consists of intramembranous ossification (bone) and endochondral ossification (cartilage) [5]. Bone homeostasis is an important process that requires a balance between bone formation (osteoblasts) and resorption (osteoclasts). Osteoblasts are responsible for laying down new bone matrix in addition to the mineralization of the new bone matrix [11]. Osteoblasts also stimulate the differentiation of osteoclasts while osteoclasts produce factors which digest the mineralized bone matrix [11]. RUNX2 is the master regulator of osteoblast differentiation and osteogenesis [132]. RUNX2 expression is controlled by two promoters: P1 and P2 early in osteoblast differentiation [12]. As differentiation progresses RUNX2 protein levels do not increase, but rather, the transcriptional activity level increases [11]. Experiments in knockout mice show the essential role of RUNX2 in bone formation: knockout mice die soon after birth because of asphyxiation as a consequence of a lack of skeletal formation [5, 11]. In addition, analyses reveal that these mice lack mature osteoblasts thereby inhibiting the formation of any bone matrix or osteoclast differentiation.

Since osteoblasts lay down bone matrix to form mineralized bone and osteoclasts break down the matrix to resorb bone; RUNX2 indirectly controls osteoclast

differentiation. Receptor activator of nuclear factor kappa-b ligand (RANKL) promotes osteoclast maturation and is also a RUNX2 target gene [5]. Cells lacking RUNX2 express less RANKL and, therefore, there is less osteoclast maturation [5] and less bone resorption. Research has further shown that while endochondral ossification is delayed in RUNX2 knockout mice, it does eventually occur [5, 130, 134]. Therefore, there is redundancy and other factors are able to compensate for the lack of RUNX2 during endochondral ossification.

The RUNX2 transcription factor is also a regulator of angiogenesis in bone development [13, 14], is expressed in vascularizing adult tissues [15], and promotes tumor metastasis [16, 17]. It interacts with its heterodimeric partner, Cbf-β, and with hypoxia-inducible factor 1-α (Hif1α) to activate the major angiogenic factor, VEGF [18]. RUNX2 is a transcriptional activator of specific target genes that promote angiogenesis, such as MMPs [19]. Conversely, it represses the cell cycle inhibitor $p21^{Cip1}$ and increases endothelial cell (EC) or cancer cell proliferation [20, 21]. Our laboratory has found that glucose metabolism, autocrine IGF-1 signaling, and phosphorylation by cyclin-dependent kinases, regulate RUNX2 DNA-binding activity, angiogenic target genes, EC proliferation, tube formation, and wound healing [20, 22–25, 138]. However, exposure of EC to hyperglycemia (HG) activated the aldose reductase (AR) polyol pathway, which increased oxidative stress and inhibited RUNX2 DNA binding [22].

3.2.2 RUNX2 in Disease

Although RUNX2 regulates osteogenesis and angiogenesis [1, 12], aberrant expression of RUNX2 can lead to disease (Fig. 3.2). Cleidocranial dysplasia (CCD) is a disease in which there are abnormalities in bone and dental development [5, 9, 26, 27]. Haploinsufficiency [1, 9] of RUNX2 has been shown to be a leading cause of CCD. In addition, there are mutations in RUNX2 that lead to CCD [26]. A heterozygous single-base deletion resulting in a premature stop codon in the runt domain produces a truncated form of RUNX2 [26] that is unable to bind DNA and control transcription of essential target genes. This mutation was not found in normal individuals or non-CCD subjects.

RUNX2 has been proposed as a biomarker in numerous cancers [1–4, 16, 17, 28, 29, 36–38, 41, 43, 45–49, 53, 54, 61, 94, 127–129, 131, 135] to evaluate the promotion of cancer cell metastasis to the bone. Sase et al. showed that RUNX2 expression was significantly associated with human colon carcinoma progression [28, 29]. In colorectal cancer RUNX2 is not only amplified, but the *RUNX2* gene also contains genetic variations termed single nucleotide polymorphisms or SNPs. In cases of colorectal carcinoma, Slattery et al. found a total of 19 SNPs in *RUNX2* [29].

One tumor in which both mRNA and protein levels of RUNX2 have been shown to be elevated is osteosarcoma [24, 30–35]. Osteosarcoma is a very aggressive pediatric cancer of the bone with a highly heterogeneous phenotype [32, 33, 35]. In osteosarcoma and chondrosarcomas, RUNX2 expression was found to positively correlate with bone morphogenetic protein-2 (BMP-2) mRNA levels [27]. Cell cycle

deregulation of RUNX2 led to osteosarcoma pathogenesis [24]. High levels of RUNX2 in osteosarcoma resulted in high rates of metastasis and a poor survival rate, supporting the notion of RUNX2 as a good prognostic marker [35]. RUNX2 was also the only upregulated marker in osteosarcoma that exhibited a positive correlation with chemotherapeutic resistance [33].

RUNX2 expression correlates with unfavorable prognoses in prostate cancer [36] and has been found to be upregulated in both breast and prostate cancers [8, 16, 25, 37–46, 128, 129]. In breast and prostate cancer cell lines RUNX2 was shown to enhance cell motility [41]. Unfortunately, many of the RUNX2 functions are cell type specific making it difficult to discern a universal function in disease progression. In MDA-MB-231 breast cancer cells, knockdown of RUNX2 had no effect on cell growth and proliferation whereas in MCF7 breast cancer cells RUNX2 enhanced cell proliferation upon growth factor deprivation [41]. In MCF-10A cells RUNX2 disrupts normal mammary acini formation in suspension culture [47]. Using electron microscopy, an absence of lumen formation was noted, possibly due to an increase in cell proliferation, decreased apoptosis, and a loss of basement membrane formation which is dependent on RUNX2 expression [47]. These phenotypes can be reversed in MDA-MB-231 cells using siRNA to deplete RUNX2 [47]. The consensus of data supports an oncogenic function for RUNX2 in breast cancer. However, there is a report suggesting RUNX2 functions as a tumor suppressor in breast cancer [48]. There have been a few reports implicating RUNX2 in promoting the formation of hematological malignancies [131] such as myeloid leukemia [49]. For example, RUNX2 cooperates with the fusion protein, Cbf-β-SMMHC, to promote leukemia development [49] and haploinsufficiency of RUNX2 delays the onset of acute myeloid leukemia [49].

3.3 Transcriptional Regulation: Target Genes and Cofactors

3.3.1 RUNX2 Target Genes

As a DNA-binding factor RUNX2 controls the transcription (through activation and repression) of numerous genes important in normal tissue homeostasis (Table 3.1). Many of these genes are abnormally activated in disease states, including cancers, and enable cancer cells to survive and metastasize to distant niches.

In osteoblasts, RUNX2 activates expression of the essential osteogenesis signaling factor: bone morphogenetic protein-2 (BMP-2) [50]. BMP-2 is a member of the transforming growth factor-β (TGF-β) superfamily of signaling molecules. It binds to TGF-β receptors, leading to activation of intracellular signaling via Smad-dependent and/or Smad-independent pathways ultimately resulting in further RUNX2 activation and promotion of a feed-forward loop. In MC3T3-E1 cells, BMP-2 was shown to enhance RUNX2 association with the promoter of Atf6 [51]. Atf6 is another transcription factor which is known to mediate osteoblast differentiation in a RUNX2 dependent manner [51]. Specifically, BMP-2-stimulated

Table 3.1 Runx2 regulated genes

Gene name	Gene symbol	Basic function	References
Bone morphogenetic protein-2	BMP-2	TGF-beta superfamily protein; cell signaling events; important for development of bone and cartilage	[50]
Snail1	SNAI1	Zinc finger transcription factor; promote and EMT through repression of E-Cadherin transcription	[99]
p27 (cyclin-dependent kinase inhibitor 1B)	CDKN1B	Controls G1 arrest; degradation is required to transition the cell from quiescence to proliferation	[80]
Bone sialoprotein 1 (osteopontin)	BSP1 (OPN)	Extracellular matrix component of the bone	[45, 59, 100]
Ribosomal ribonucleic acid	rRNA	RNA component of the ribosome; functions in protein synthesis	[57]
Matrix metalloproteinase 9 (gelatinase B)	MMP9	Functions in extracellular matrix degradation	[36–38, 44, 100, 101]
Stromal cell-derived factor 1	SDF1/CXCL12	Cytokine/chemokine signaling factor functioning in migration and adhesion	[36, 38, 44]
Cystatin F	CST7	Protease inhibitor; functions in immune regulation; promotes tumor metastasis	[38, 44]
Matrix metalloproteinase 13	MMP13	Functions in extracellular matrix degradation	[36, 37, 44, 52–55]
Prolactin-induced protein	PIP	Water transport; binds immunoglobulins; used as a biomarker in breast cancer; promotes cellular proliferation	[39, 44]
Vascular endothelial growth factor	VEGF	Promotes angiogenesis	[36, 37, 102, 103]
Insulin-like growth factor-binding protein 5	IGFBP5	Prolong the half-life of IGF's and alters IGF interactions with IGFR to promote cell survival, growth, and proliferation	[104]
Collagen type X alpha 1	Col10a1	Extracellular matrix component of the bone	[105]
Transcription factor Sox9	Sox9	Regulates transcription of genes important in osteogenesis	[36, 38]
Lipocalin-2	LCN2	Important for innate immunity; upregulated during inflammation; functions in apoptosis	[36]
Snail2	SNAI2	Zinc finger transcription factor; promotes oncogenic transformation of epithelial cells	[36, 38, 106]
Pepsinogen C (gastricsin)	PGC	Extracellular matrix degradation and invasiveness	[36]
Endothelin 2	EDN2	Important for angiogenesis	[36]
Receptor activator of nuclear factor kappa-b ligand	RANKL	Also called osteoclast differentiation factor; receptor that serves to activate osteoclasts; aids in metastasis to the bone	[36]

Parathyroid hormone-related protein	PTHrP	Regulator of cell growth, differentiation, migration, and survival; aids in bone metastasis	[36, 37, 64]
Interleukin 8	IL8	Cytokine/chemokine signaling; Potent activator of angiogenesis	[36, 37]
Sphingosine kinase 1	SPHK1	Regulates proliferation and survival; aids in metastasis to the bone	[36, 38]
Endothelial differentiation GPCR 3	EDG3	Receptor for sphingosine 1-phosphate; aids in metastasis to the bone	[36]
Kallikrein-2	KLK2	Cleaves PSA to active form; prognostic marker for prostate cancer risk	[36]
Ras small GTPase, Rab type	RAB3B	Modify membrane lipids and small GTPases of the Rab family; secretory pathway	[36]
Ras small GTPase, Rab type	RAB43	Modify membrane lipids and small GTPases of the Rab family; secretory pathway	[36]
Ras small GTPase, Rab type	RAB35	Modify membrane lipids and small GTPases of the Rab family; secretory pathway	[36]
Activating transcription factor 6	ATF6	Involved in the unfolded protein response; activates the transcription of ER chaperones	[51]
Bcl-2-associated X protein	Bax	Promotes apoptosis	[30, 107]
Exostosin-1	Ext1	Tumor suppressor; glycosyltransferase	[72]
Heparanase	HPSE	Helps degrade heparan sulfate; promotes tumor angiogenesis and metastasis	[72]
Bone morphogenetic protein - 3b	BMP-3B	Member of the TGF-beta superfamily of secreted molecules; aid in metastasis to the bone	[108]
Thyroglobulin	Tg	Used by thyroid to make T4 and T3; can be used as a biomarker in thyroid cancers	[109]
Cytochrome P450 11a1	CYP11a1	Converts cholesterol to pregnenolone; aids in cancer progression; sterol/steroid metabolism	[110]
Cytochrome P450 39a1	CYP39a1	Converts cholesterol to bile acids; aids in cancer progression; sterol/steroid metabolism	[110]
Cytochrome P450 51	CYP51	Converts lanosterol to cholesterol; aids in cancer progression; sterol/steroid metabolism	[110]
Lanosterol synthase	Lss	Functions in sterol/steroid metabolism	[110]
7-dehydrocholesterol reductase	Dhcr7	Functions in sterol/steroid metabolism	[110]
MicroRNA cluster	miR23a-27a-24-2	MicroRNA cluster that promotes apoptosis; downregulated during tumor progression	[85]
Regulator of G-protein signaling 2	Rgs2	Functions in signaling; drug resistance; tumor angiogenesis	[111]
Regulator of G-protein signaling 4	Rgs4	Functions in signaling; drug resistance; tumor angiogenesis	[111]
Regulator of G-protein signaling 5	Rgs5	Functions in signaling; drug resistance; tumor angiogenesis	[111]
Regulator of G-protein signaling 16	Rgs16	Functions in signaling; drug resistance; tumor angiogenesis	[111]

(continued)

Table 3.1 (continued)

Gene name	Gene symbol	Basic function	References
G-protein coupled receptor 23	Gpr23	Function in G-protein coupled signaling to activate cAMP signaling and PI3K to promote cell survival, growth, and proliferation	[111]
G-protein coupled receptor 30	Gpr30	Function in G-protein coupled signaling to activate cAMP signaling and PI3K to promote cell survival, growth, and proliferation	[111]
G-protein coupled receptor 54	Gpr54	Function in G-protein coupled signaling to activate cAMP signaling and PI3K to promote cell survival, growth, and proliferation	[111]
G-protein coupled receptor 64	Gpr64	Function in G-protein coupled signaling to activate cAMP signaling and PI3K to promote cell survival, growth, and proliferation	[111]
G-protein subunit alpha-13	Gna13	Function in G-protein coupled signaling to activate cAMP signaling and PI3K to promote cell survival, growth, and proliferation	[111]
Pannexin3	PANX3	Structural component of gap junctions	[112]
Survivin	BIRC5	Member of the inhibitor of apoptosis family; functions in apoptosis; inhibit caspases	[113]
Galectin-3	LGALS3	Functions in cell adhesion, cell activation and chemoattraction, cell growth and differentiation, cell cycle, apoptosis	[114, 115]
Connective tissue growth factor	CTGF	Stimulates extracellular matrix production, chemotaxis, proliferation and integrin expression; angiogenesis	[56]
Lymphoid enhancer-binding factor 1	Lef1	Participates in Wnt signaling; promotes an EMT by repressing E-Cadherin	[116]
Dual specificity protein phosphatase 1	DUSP1	Negative regulator of cell proliferation	[38]
Osteoprotegerin	OPG	Cytokine receptor; decoy RANKL receptor; regulator of inflammation, innate immunity, and cell survival and differentiation	[114]
Aromatase	CYP19a1	Functions in sterol/steroid metabolism	[100]
Osteocalcin	OC	Extracellular matrix component of the bone	[100, 114, 117, 118]
Type 1 collagen	COL1a2	Extracellular matrix component	[100, 117]
Sclerostin	SOST	BMP antagonist; inhibits Wnt signaling; antagonizes bone formation	[42]
Interleukin 11	IL-11	Functions in cytokine/chemokine signaling; promotes bone cell proliferation and differentiation	[42]
Granulocyte-macrophage colony-stimulating factor	GM-CSF	Cytokine/chemokine signaling; regulates growth and differentiation	[36, 38, 44, 77]

Syndecan 1	SDC1	Integral cell membrane protein to participate in cell migration, proliferation and cell-matrix interactions	[72]
Syndecan 2	SDC2	Integral cell membrane protein to participate in cell migration, proliferation and cell-matrix interactions	[38, 72]
Syndecan 3	SDC3	Integral cell membrane protein to participate in cell migration, proliferation and cell-matrix interactions	[72]
Twinfilin	TWF	Regulations cytoskeletal dynamics via actin	[38]
SH3 and PX domain-containing protein 2A	SH3PXD2A	Involved in extracellular matrix degradation and cellular invasion	[38]
Alkaline phosphatase	ALP	Dephosphorylate proteins	[117]
Glypican	GPC	Modify signaling pathways and contribute to cell proliferation and tissue growth	[72]
Versican	VCAN	Functions in cell adhesion, migration, and proliferation	[72]
Axin2	AXIN2	Functions within the Wnt signaling pathway to maintain Beta-catenin	[119]
Smad 3	SMAD3	EMT and cell transformation	[38]
Dexamethasone-induced ras-related protein 1	RASD1	Plays a role in altered cell morphology, growth and extracellular matrix interactions	[38]
Fibroblast growth factor receptor 2	FGFR2	Receptor tyrosine kinase receptor that regulates cell signaling controlling cellular proliferation and differentiation	[72]
Fibroblast growth factor receptor 3	FGFR3	Receptor tyrosine kinase receptor that regulates cell signaling controlling cellular proliferation and differentiation	[72]
B-cell lymphoma-2	Bcl-2	Protects cells from apoptosis via maintaining mitochondrial membrane integrity; prevents cytochrome c release from the mitochondria	[8]
Prostate specific antigen	PSA	Protease to dissolve coagulum; serine protease; prostate cancer biomarker	[36]
Membrane type 1 matrix metalloproteinase	MT_1MMP	Promotes tumor growth, tumor invasion, and angiogenesis; extracellular matrix protease	[1]
Cyclin D1	CCND1	Regulate CDK kinases during cell cycle; functions as a regulatory subunit for CDK4 and CDK6 during G1/S transition; contributes to tumorigenesis via altered cell cycle	[1]
Cyclin dependent kinase inhibitor 1A	p21	Regulates cell cycle progression at G1 by inhibiting the activity of CDK4 and CDK2	[1]
Cyclin dependent kinase inhibitor 2A	p14	Induce cell cycle arrest at G1 and G2 phases; acts as a tumor suppressor	[1]

RUNX2 activation of Atf6 enables the bone extracellular matrix, osteocalcin, gene to be expressed. A dominant negative construct of Atf6 was shown to inhibit RUNX2 activation of the osteocalcin promoter [51]. However, by restoring wild type Atf6, osteoblasts were able to differentiate and the expression of osteocalcin was restored.

During cancer progression, cancer cells express many bone specific proteins that mediate metastasis to the bone. Some of these proteins are regulated by RUNX2 transcriptional activity and mediate migration/motility and adhesion. Bone sialoprotein (BSP) and osteopontin (OPN) are two factors which mediate breast cancer metastasis to the bone and are activated by RUNX2 in breast cancer cells [45]. Using siRNA technology, Reufsteck et al. were able to demonstrate that targeting BSP and OPN drastically inhibits migration of MDA-MB-231 breast cancer cells when injected into athymic nude mice [45]. In prostate cancer, RUNX2 has been shown to upregulate genes not only associated with increased migration but genes associated with angiogenesis, epithelial-mesenchymal-transition (EMT), membrane trafficking/secretion, and osteolysis [36]. RUNX2 upregulates secretion factors including PTHrP, IL8, CSF2, and SDF-1 (See Table 3.1 for a description of protein function) [37, 38]. RUNX2 also upregulates MMP9, MMP13, VEGF, osteopontin, CST7, Sox9, SNAI2, Smad3, SDC2, Twinfilin, and SH3PXD2A [37, 38]. RUNX2 promoter occupancy in osteosarcoma was examined to determine potential RUNX2 target genes enabling progression of osteosarcoma. In SAOS-2 osteosarcoma cells, knockdown of RUNX2 resulted in an inhibition of motility [34]. RUNX2 upregulated matrix metalloproteinases during cancer progression to promote cell migration from primary tumor sites. MMP13 and MMP9 are two common MMP's upregulated by RUNX2 to mediate cancer cell invasion and metastasis [1, 52–55].

In addition to epithelial cells, RUNX2 has been shown to modulate gene expression in vascular cells. In human aortic smooth muscle cells and C3H10T1/2 cells RUNX2 inhibited the expression of connective tissue growth factor (CTGF) [56]. This is important for endothelial cells since CTGF has been shown to be a contributing factor to the development of atherosclerosis. Therefore, RUNX2 may protect the vasculature from development of atherosclerosis. Knockdown of RUNX2 enhanced CTGF expression [56] in a TGF-β dependent manner. Further analysis of the CTGF promoter revealed Smad binding elements that were able to interact with RUNX2/Smad3 heterodimers upon stimulation of the TGF-β receptors to inhibit transcription [56].

3.3.2 RUNX2 Cofactors

Regulation of gene expression requires a transcriptional complex composed of RNA polymerase, transcription factors, and corepressors or coactivators. RUNX2 C-terminal domain contains many binding sites for both corepressors and coactivators (Table 3.2).

One group of proteins that function as strong corepressors of RUNX2 are histone deacetylases (HDACs). In osseous cells, HDAC1 has been shown to interact with

Table 3.2 Runx2 cofactors

Cofactor	References
CBP	[50]
p300	[99]
HDAC1	[57, 59]
G9a	[44]
HIF1α	[102]
p38	[62]
SMAD	[56, 62, 63, 83, 111]
CBFβ	[25, 42, 120]
Rorβ	[121]
YAP	[21, 122]
TAZ	[91, 123]
C/EBPβ	[52]
Wip1	[107]
WWP1	[87]
ERα	[100, 129]
FOXO1	[61]
AR	[39, 46, 124]
WWOX	[31, 125]
EWS-FLI	[126]
TLE1	[58, 73]
CoAA	[119]
CBFβ-SMMHC	[49]
Gli2	[64]
HDAC7	[5, 60]
Suv39h1	[108]
mSin3a	[122]
TLE2	[122]
TLE3	[122]
HDAC6	[5, 122]
HDAC4	[5, 122]
HDAC3	[5, 122]
Smurf1	[5]
Schnurri-3	[5]
MOZ	[5]
MORF	[5]
HDAC5	[5]

RUNX2 to inhibit ribosomal RNA (rRNA) gene expression [57] thus inhibiting cellular proliferation and protein synthesis. Knockout of HDAC1 was shown to alleviate the RUNX2-mediated repression of rRNA expression resulting in an increase in cell proliferation and overall protein synthesis [57]. Transducin Like Enhancer-1 (TLE-1), functions to also promote RUNX2 inhibition of rRNA gene synthesis during mitosis [58]. In C3H10T1/2 cells HDAC1 bound to RUNX2 inhibited expression of osteopontin, thus inhibiting both proliferation and differentiation of the osteoblast cell [59]. HDAC7 was also shown to be a potent inhibitor of RUNX2 transcriptional activity [60] and HDAC5 was shown to repress RUNX2 expression [5].

In prostate cancer the forkhead box O (FoxO1) protein was found to be a corepressor of RUNX2 [61]. Inhibition of RUNX2 by upregulation of PTEN or FoxO1 protein inhibited prostate cancer cell migration and invasion. In prostate cancer specimens, immunohistochemistry revealed an inverse relationship between RUNX2 and FoxO1 nuclear localization [61]. PTEN inactivating mutations are often seen in prostate cancer [61] and therefore would potentiate RUNX2 activity to promote prostate cancer cell migration and invasion.

While some cofactors function as coactivators and corepressors a few are also able to function as both in a gene-dependent manner. One group used a doxycycline-regulated RUNX2 expression system in C4-2B prostate cancer cells to show that G9a (histone methyltransferase) is able to function as a corepressor for RUNX2 target genes MMP9, CSF2, SDF1, and CST7 [44]. However, G9a functions as a coactivator for RUNX2 transcription of MMP13 and PIP in the C4-2B prostate cancer cell line [44].

3.4 Transcriptional Regulation: Activation and Repression

3.4.1 Regulation of RUNX2 Activity

Several intracellular signaling pathways have been shown to modulate RUNX2 activity (Fig. 3.3). When RUNX2 becomes activated it is able to bind DNA and either promote or inhibit the transcription of its target genes (Refer to Table 3.1). Mitogen-activated protein kinase (MAPK) and TGF-β intracellular signaling are both essential to RUNX2 activation to promote osteoblast differentiation or tumor progression. TGF-β/BMP signaling is a highly important signaling axis used by numerous cells to inhibit cell growth and proliferation under normal conditions. In cancer, tumor cells escape the inhibitory effects of TGF-β resulting in unrestricted proliferation. TGF-β/BMP intracellular signaling can occur via canonical Smad-dependent or non-canonical Smad-independent pathways. Upon stimulation, TGF-β receptors initiate intracellular signaling events leading to Smad activation (canonical TGF-β pathway), nuclear translocation, and Smad interaction with RUNX2 [6, 56, 62–64]. In osteogenesis, TGF-β/BMP signaling is responsible for activating RUNX2 to promote osteoblast differentiation [62, 65]. Conversely, in PC3 prostate cancer cells TGF-β cooperates with RUNX2 to promote cellular growth [46].

A potent regulator of RUNX2 activity is the extracellular signal-regulated kinase, ERK [66, 67, 138, 139]. ERK is a classical MAPK activated upstream in response to numerous extracellular factors including growth factors, cytokines, and G-protein coupled receptor ligands. MAPK signaling is essential for normal bone development. In many cancers where RUNX2 is aberrantly expressed, MAPK signaling is also altered generally through inactivating or activating mutations to components within the signaling cascade. In preosteoblast MC3T3-E1 cells it was demonstrated that S301 and S319 [66], two ERK phosphorylation sites on RUNX2, are

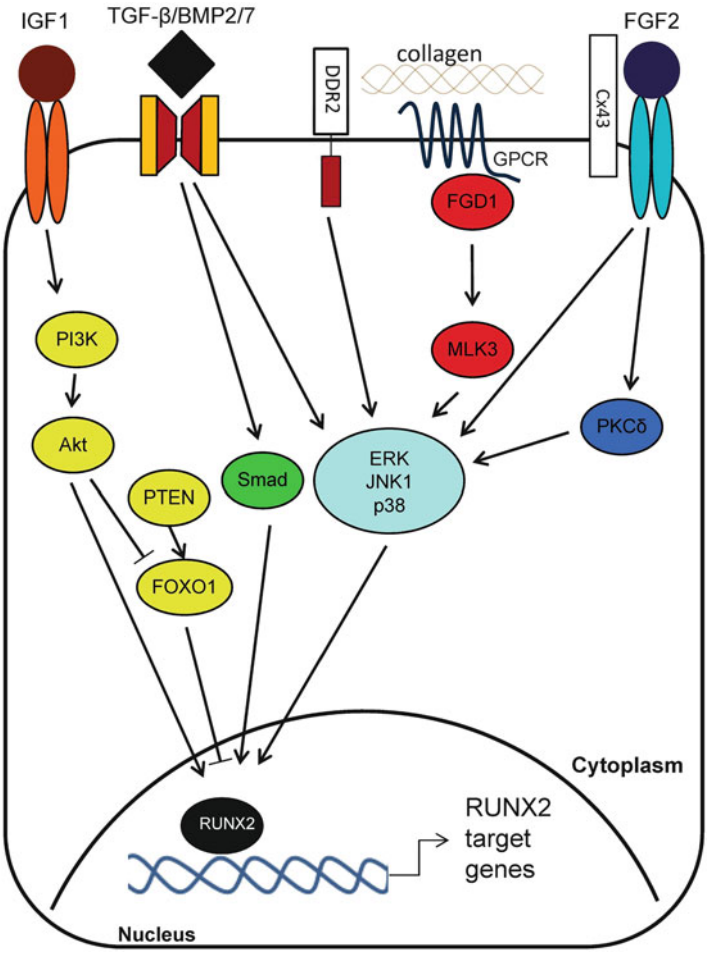

Fig. 3.3 RUNX2 and signal transduction. Many signaling pathways lead to activation or repression of RUNX2 transcriptional activity. These Pathways converge onto the MAPK pathway, the TGF-β/Smad pathway, and the PI3K/Akt pathway

phosphorylated upon nuclear translocation of ERK [67]. MAP3K mixed-lineage kinase 3 (MLK3) has been shown to activate ERK and p38 (another classical MAP kinase). Activation of ERK via MLK3 results in RUNX2 phosphorylation and subsequent osteoblast differentiation [68]. Insulin-like growth factor-1 (IGF-1) binding to IGF-1R (IGF-1 receptor tyrosine kinase) has been shown to be essential for normal skeletal development [69]. IGF-1 regulates the activation of RUNX2 via activation of ERK resulting in phosphorylation of RUNX2 [22, 69, 138]. p38, activated via several extracellular factors, has been implicated in modulating RUNX2 activity levels. p38 activation from TGF-β/BMP signaling (non-canonical TGF-β pathway) has been shown to mediate RUNX2 phosphorylation to promote osteoblast differentiation [62].

Fibroblast growth factor 2 (FGF2) is another extracellular growth factor implicated in promoting osteoblast differentiation through activation of RUNX2 [70–72]. In activating RUNX2, FGF2 functions as a double-edge sword. FGF2 activates protein kinase C (PKC), which in turn increases ERK activity, leading to RUNX2 phosphorylation in MC3T3 osteoblasts [71]. FGF2 also activates ERK through PKC-independent mechanisms (refer to Fig. 3.3). The gap junction protein, connexin-43 (Cx43), enhances this activation via stabilization of FGF2 to FGF2R (refer to Fig. 3.3). An inhibitor of gap junctions, 18β-glycyrrhetinic acid, attenuated the enhancement in RUNX2 transcriptional activity in MC3T3 osteoblasts [71]. In breast cancer MCF7 cells, FGF2 was able to increase BSP expression, which in turn upregulated RUNX2 mRNA [70].

Not all intracellular signaling pathways that lead to RUNX2 phosphorylation result in RUNX2 activation. The c-jun-N-terminal kinase (JNK) is activated by BMP-2 signaling in both C2C12 multipotent cells and MC3T3-E1 preosteoblastic cells [73]. JNK is able to phosphorylate RUNX2 at S104 [73] resulting in inhibition of RUNX2 activity and thus preventing osteoblast differentiation. Inhibition of JNK via a dominant negative JNK1, JNK knockdown, or treatment with a JNK inhibitor counteracted this inhibition to enable osteoblast differentiation.

RUNX2 has also been shown to play a role in endothelial signaling and cell cycle progression in response to physiological levels of glucose [22]. Euglycemic conditions were able to restore RUNX2 DNA binding through autocrine IGF1/IGFR signaling to promote endothelial cell migration, proliferation, and angiogenesis [22] indicating glucose has a function in modulating RUNX2 activity. Hyperglycemic conditions, however, inhibited RUNX2 activity through the aldose reductase polyol pathway [22]. Inhibition of RUNX2 by hyperglycemia inhibited endothelial cell migration, proliferation, and angiogenesis [20, 22]. Treatment with 2-deoxyglucose (inhibitor of glucose metabolism) under euglycemic growth conditions in endothelial cells resulted in a delayed exit from G1/S into G2 phases of the cell cycle with subsequent lower levels of RUNX2 DNA binding activity [20]. Similarly, nutrient and serum deprivation blocked endothelial cell exit from G1 [20]. Using shRNA lentiviral knockdown to reduce RUNX2 levels, exit from G1 and progression through the cell cycle was found to be dependent upon RUNX2 [20].

3.4.2 Regulation of RUNX2 Expression

RUNX2 expression in osteoblasts promotes differentiation through tight control of RUNX2 activity to maintain the balance of bone formation and remodeling. Similarly, during tumorigenesis many of the negative regulators of RUNX2 are inhibited, thus allowing sustained expression and activity. In general, RUNX2 expression and DNA-binding activity depend on transcriptional mechanisms of activation versus repression and on post-translational protein modifications that include phosphorylation, acetylation, and ubiquitination [74].

3.4.2.1 Transcriptional Activation and Repression

Osteoblast differentiation requires activation of RUNX2, but what regulates RUNX2 expression in pre-osteoblasts has remained an enigma for quite some time. Tu et al. determined that activation of RUNX2 expression is dependent upon Indian hedgehog (Ihh) signaling [75]. In Ihh null mice even the forced expression of RUNX2 failed to induce osteoblast differentiation [75] confirming that Ihh is not only important for activation of RUNX2 expression but also for RUNX2 activity. In osteoblasts, it was found that BMP-2 increased RUNX2 induction and expression [76]. Shu et al. generated BMP-2 knockout mice and found a dramatic reduction in RUNX2 expression resulting in severe chondrodysplasia [76]. BMP-2 was also able to upregulate PlexinA2 (PlxnA2) in pre-osteoblastic cells [77]. The upregulation of PlxnA2 was associated with increased RUNX2 expression, osteoblast differentiation, and bone mineralization [77]. This upregulation of RUNX2 was thought to be a result of PlxnA2 stabilization of BMP-2 binding to BMP-2 receptors.

TWIST, a transcription factor implicated in epithelial-mesenchymal-transition (EMT) and metastasis in many types of cancers is also an essential transcription factor in development. TWIST was shown to be an inhibitor of RUNX2 expression in bone marrow-derived mesenchymal stem cells [78]. Under low oxygen conditions (hypoxia), Hif1α upregulated expression of TWIST, which directly inhibited RUNX2 expression [78] and osteoblast differentiation. Conversely, studies in PC3 prostate cancer cells showed that TWIST enhances RUNX2 expression levels to promote metastasis to the bone [79]. Glucocorticoid receptor binding to the P2 promoter of RUNX2 was shown to inhibit RUNX2 expression [80]. This resulted in adipocyte differentiation [80] instead of osteoblast differentiation and reveals how signaling events can lead to cell fate determination by regulating RUNX2 expression.

RUNX2 expression is increased in breast tumors where it has been implicated in mediating metastasis to the bone. How RUNX2 expression is enhanced in breast cancer tissue is poorly understood. Using breast cancer cells, one group was able to show that serotonin induced parathyroid hormone related protein (PTHrP), which in turn increased transcription of RUNX2 [40]. Since PTHrP is also a RUNX2 target gene, it fuels a feed forward loop potentiating maximal RUNX2 expression to promote progression of breast cancer to a metastatic stage. In multiple myeloma (MM) it has been shown that Gfi1 is upregulated and represses RUNX2 expression [81]. This inhibition of RUNX2 then results in inhibition of osteoblast differentiation.

3.4.2.2 Post-translational Regulation

Intracellular signaling is able to modulate RUNX2 post-translational modifications to regulate DNA-binding activity and RUNX2 levels. Using antibodies to detect phosphoserine sites on RUNX2 in endothelial cells, it was shown that RUNX2 is phosphorylated under euglycemic growth conditions by cyclin dependent kinase 4 (cdk4). This phosphorylation was abrogated by mutation of the cdk site S451 [20].

A RUNX2-S451A mutant showed inhibition of DNA binding in endothelial cells as well as a reduction in wound healing activity [20]. Inhibition of cdk4 produced similar results demonstrating that cdk4 can activate RUNX2 through phosphorylation of S451 in response to glucose. In addition to glucose modulation, RUNX2 activity was cell cycle regulated [136, 137]. RUNX2 was associated with DNA when cells were proliferating but was sequestered to subnuclear loci when cells were quiescent [20, 23, 136]. RUNX2 protein levels were maximal in endothelial cells in late G2 and M phases of the cell cycle [23]. Using RNA interference to knockdown RUNX2, endothelial cell exit from G2/M phases of the cell cycle was delayed [23] resulting in a decrease in cell proliferation. In vitro kinase assays showed S451 must be phosphorylated to allow RUNX2 to function in promoting progression through the cell cycle [23]. However, in osteoblasts, RUNX2 inhibited osteoblast proliferation and RUNX2 protein levels were maximal in G1 [24], suggesting that RUNX2 regulation may be cell type dependent.

BMP2 is able to regulate RUNX2 protein levels via inhibition of cdk4 [76]. This inhibition leads to protection from proteasomal degradation thus maintaining cellular protein levels. Recent research has focused on how micro-RNA's (miRNAs) regulate RUNX2 protein levels. MiRNAs modulate protein levels through an RNA interference pathway ultimately leading to mRNA degradation or reduced protein translation. MiRNAs are 18–25 nucleotide RNAs that repress translational activity of mRNAs [82]. Wu et al. have suggested that the miR-30 family of miRNAs may play an essential role in osteogenesis. Their data show that miR-30 was able to negatively regulate both Smad1 and RUNX2 [82]. Alkaline phosphatase (marker of osteoblast differentiation) was shown to be dramatically decreased after exogenous miR-30 expression [82]. Furthermore, miR-30 family miRNAs were able to bind to the 3′-untranslated region of both Smad1 and RUNX2 mRNA [82] thus inhibiting the effects of BMP-2-stimulated osteoblast differentiation pathways. MiR-203 is a known tumor suppressor miRNA which is downregulated in prostate cancer [83, 84]. It has been shown to bind RUNX2 mRNA resulting in a mesenchymal to epithelial transition (MET), inhibition of cell proliferation, and inhibition of cell migration and invasion [83, 84]. In addition to the miRNAs already described, it has been shown that miR-23a, miR-34 cluster, miR-133a, miR-135a, miR-137, miR-204, miR-205, miR-217, miR-218, and miR-338 all regulate RUNX2 expression [85, 86]. In addition to miRNA regulation of RUNX2, the proteasomal degradation pathway is implicated in regulating RUNX2 levels in the cell. For example, WWP1 (WW domain-containing E3 ubiquitin protein ligase 1) has been shown to function as the E3 ubiquitin ligase responsible for ubiquitinating RUNX2 and targeting it for proteasomal degradation [87].

3.5 RUNX2 as a Therapeutic Target

RUNX2 is a transcriptional regulator of gene expression. Mutations, amplification, or inappropriate expression of RUNX2 has the potential to amplify the expression or repression of a variety of target genes. This could regulate global changes in gene

regulatory networks and lead to a process called transformation amplification. Therefore, RUNX2 may be a prime target for therapeutic intervention [88, 128] to treat disease because multiple transformation pathways could be inhibited. Many cancers develop resistance to therapies due to signaling pathway redundancy allowing signaling switches to occur. Being able to target transcription factors allows therapies to bypass the redundancy of signaling.

Prostate cancer bone metastases form osteolytic lesions before the development of osteoblastic lesions. Li et al. found that isoflavone and 3, 3′-diindolylmethone (BR-DIM) are able to multifunctionally inhibit these metastases from forming [88]. This combination therapy may inhibit not only osteoblast differentiation but also osteoclast differentiation. Their research showed that one of the ways in which this combination treatment functions is to inhibit signaling of RANKL [88]. However, further cellular analysis revealed isoflavone and BR-DIM combination therapy inhibited signaling from the Akt, AR (androgen receptor), PSA, and p27 signaling axis as well as blocking the RNA interference pathway by inhibiting miR-92a which is associated with RANKL signaling [88]. This is one example of inhibiting the effects of RUNX2 through targeting of its target genes.

Natural compounds have been shown to have potent medicinal benefits. Astragaloside II a compound from the plant, *Radix astragalus*, was tested on rat primary osteoblasts to determine its effects on viability, proliferation, differentiation and maturation [89]. Astragaloside II promoted proliferation, differentiation and mineralization of primary rat osteoblasts [89]. The effects of this drug on post-menopausal women could potentially prevent osteoporosis. One benefit in cancer patients could be prevention of bone fractures associated with osteolytic metastases by promoting osteoblast differentiation to prevent bone degradation caused by cancer cells. However, use of this drug for patients with metastatic cancers could increase the incidence of bone metastases and lead to early death by stimulating RUNX2 expression within tumor cells. A second compound, Neobavaisoflavone (NBIF), was isolated from the plant *Psoralea corylifolia L* and was shown to have a similar effect on RUNX2 in osteoblasts as Astragaloside II [90]. NBIF was shown to upregulate RUNX2 expression in MC3T3-E1 cells while also activating its gene regulatory functions [90]. NBIF was shown to upregulate osteocalcin, bone sialoprotein, and type 1 collagen [90]. While this drug may have pro-bone forming functions and could potentially be a way to restore bone loss as a result of bone degradative diseases, one must also take into consideration the dosing regimen that would make this drug specific for osteoblasts while not further stabilizing RUNX2 positive cancer cells.

An understanding of upstream signaling pathways that activate RUNX2 and how cofactors regulate RUNX2 activity in disease would improve the development of therapeutics. In MM it was shown that the Gfi1 targeted drug, Trichostatin-A, was able to block the inhibition that Gfi1 imposes on RUNX2 [81]. Bortezomib (Velcade) is a proteasomal inhibitor and is also used in the treatment of MM because it was shown to induce osteoblast differentiation [91]. Bortezomib inhibited FGF2 induced TAZ (a RUNX2 binding coactivator) protein degradation [91], thus allowing TAZ to interact with RUNX2 and promote osteoblast differentiation in MC3T3-E1 cells. Therefore, restoration of RUNX2 expression enabled osteoblast differentiation and disabled MM progression in the bone microenvironment.

Studies that show targeting upstream signaling pathways that activate RUNX2 or cofactors have been reported, but the research for direct RUNX2 inhibition is limited. Our laboratory showed for the first time that cholecalciferol (inactive Vitamin D3 precursor) directly modulates RUNX2 DNA-binding activity [25]. Cholecalciferol is produced in the skin as a result of exposure to UV or it can be absorbed in the digestive system through the diet [25]. Cholecalciferol is normally converted to active Vitamin D3 in the body to 1,25OH-D3, which interacts with Vitamin D Receptor (VDR) to promote calcium absorption in the gut and increase bone formation. However, Vitamin D3 also exhibits paracrine and autocrine activity by regulating epithelial cell differentiation and modulating immune system function [25]. Using a quantitative DNA binding assay (D-ELISA) it was shown that cholecalciferol was able to modulate RUNX2 DNA binding in a VDR-independent manner. Analysis of RUNX2-positive breast tumor cells (MCF7), endothelial cells (HBME), and osteosarcoma cells (SaOs2) showed that cholecalciferol was able to inhibit cellular proliferation [25], suggesting a RUNX2-specific function. Further research needs to be conducted to study the effects in animal models before this strategy could be modified for clinical trials in the treatment of RUNX2 positive tumors.

Direct targeting of epithelial or bone cell RUNX2 pathways and cofactors that modulate RUNX2 expression or activity is another therapeutic strategy. However, targeting the microenvironment is also another option. Angiogenesis is an essential process that must occur for many solid tumors to metastasize to bone. Endothelial cells express RUNX2, which mediates wound healing by stimulating new blood vessel formation. However, tumors use angiogenesis to vascularize and provide nutrients to tumors that also allow cancer cells to intravasate. Inhibition of angiogenesis using VEGF inhibitors has been a therapeutic strategy for many years. In theory if one starves tumors of their nutrient supply, it should lead to tumor regression or necrosis. Alternatively, by stabilizing blood vessels feeding the tumors then in theory chemotherapeutic agents would be able to get to sites of tumor growth more effectively. However, these therapies have been less successful in the clinic. LGD1069 is a selective retinoid X receptor ligand used to treat T-cell lymphoma but has also been shown to inhibit angiogenesis in lung cancer [92]. LGD1069 inhibited activation of the TGF-β/Smad pathway thus reducing both activation and expression of RUNX2 in human umbilical vein endothelial cells (HUVECs) [92]. However, endothelial cells are not the only cells that use the TGF-β/Smad pathway to activate the expression and activity of RUNX2. Therefore, LGD1069 activity in other cell lines (breast, prostate, and osteosarcoma) should be tested to determine whether it decreases RUNX2 protein or activity. In melanoma, it was shown that SD-208 (a TGF-β receptor I kinase inhibitor) blocked expression of RUNX2 through downregulation of TGF-β/Smad signaling [93], supporting the notion that targeting of the TGF-β/Smad axis could have therapeutic benefit in RUNX2 positive tumors by inhibiting expression within the tumor itself or by preventing angiogenesis.

3.6 Future Directions

The biological understanding of disease will be essential to creating new therapeutics to treat disease. Signaling pathways are difficult to ablate because of pathway redundancy. Therefore, it is essential to target upstream signaling as well as downstream signaling, especially of transcription factors. Understanding the role that RUNX2 plays in cancer progression will be essential to be able to use it as a therapeutic target to inhibit metastasis to the bone. RUNX2 has been shown to be highly upregulated in the cancer stem cell (CSC) populations of prostate cancer, breast cancer, and osteosarcoma [94–98]. Therefore, with the emergence of the CSC as a key contributor to cancer development, progression, and resistance to modern therapies, it will be important to understand the role that RUNX2 is playing in CSC regulation so that appropriate therapeutic strategies can be developed.

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