**Role of microRNA-140 in embryonic bone development and cancer**

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**Abbreviations list:** RUNX, runt-related transcription factor; PAX1, paired box 1; SRY, sex determining region Y; SOX, SRY-box; COL2A1, collagen type II alpha 1; Wnt, wingless-type MMTV integration site; LRP, low density lipoprotein receptor-related protein; TGFβ, transforming growth factor beta; COL10A1, collagen type X alpha 1; FN1, fibronectin 1; BMP2, bone morphogenetic protein 2; ZFP521, zinc finger protein 521; IHH, Indian hedgehog; VEGF, vascular endothelial growth factor; HIF1, hypoxia-inducible factor 1; PTHrP, parathyroid hormone related protein; PTHR1, parathyroid hormone receptor 1; HDAC4, histone deacetylase 4; MEF2, myocyte enhancer factor 2; miRNA, microRNA; mRNA, messenger RNA; DGCR8, DiGeorge critical region 8; AGO2, Argonaute 2; RISC, RNA-induced silencing complex; UTR, untranslated region; MRE, miRNA recognition element; circRNA, circular RNA; Dnm3os, dynamin 3 opposite strand; SNAIL1, snail zinc finger 1; TGFBR2, TGFβ receptor II; LIN28A, lin-28 homolog A; Cdc34, cell division cycle 34; E2f5, E2F transcription factor 5; WWP, WW domain containing E3 ubiquitin protein ligase 2; LNA, locked nucleic acid; Cxcl12, chemokine (C-X-C) motif ligand 12; ADAMTS5, ADAM metallopeptidase with thrombospondin type 1 motif 5; DNPEP, aspartyl aminopeptidase; RALA, V-ral simian leukemia viral oncogene homolog A (ras-related); NF-KB, nuclear factor-kappa B; DNMT1, DNA methyltransferase 1; OCT4, octamer-binding transcription factor 4; RAD51AP1, RAD51-associated protein 1; SOST, sclerostin.

**ABSTRACT**

Bone is increasingly viewed as an endocrine organ with key biological functions. The skeleton produces hormones and cytokines, such as FGF23 and osteocalcin, which regulate an extensive list of homeostatic functions. Some of these functions include glucose metabolism, male fertility, blood cell production and calcium/phosphate metabolism. Many of the genes regulating these functions are specific to bone cells. Some of these genes can be wrongly expressed by other malfunctioning cells, driving the generation of disease. MicroRNAs are a class of non-coding RNA molecules that are powerful regulators of gene expression by suppressing and fine-tuning target messenger RNAs. Expression of one such microRNA, miR-140, is ubiquitous in chondrocyte cells during embryonic bone development. Activity in cells found in the adult breast, colon and lung tissue can silence genes required for tumour suppression. The realisation that the same microRNA can be both normal and detrimental depending on the cell, tissue and time point provides a captivating twist to the study of whole-organism functional genomics. With the recent interest of microRNAs in bone biology and RNA-based therapeutics on the horizon, we present a review on the role of miR-140 in the molecular events that govern bone formation in the embryo. Cellular pathways involving miR-140 may be reactivated or inhibited when treating skeletal injury or disorder in adulthood. These pathways may also provide a novel model system when studying cancer biology of other cells and tissues.

**INTRODUCTION**

Bone and cartilage are specific to vertebrates. The first organisms to develop such an endoskeleton, Chondrichthyes, evolved around 422 million years ago. Two million years later and our first ancestor, Osteichthyes, contained an endoskeleton with all the same four types of mineralised tissue (bone, cartilage, enamel and dentin) as modern mammals (1). The tissue classification criteria were considered controversial due to fossils of primitive vertebrates containing other tissues difficult to classify by palaeontologists (1). Further controversy existed regarding whether the four tissue types developed early in vertebrate evolution, were a result of phenotypic plasticity or that cartilage and bone developed analogous to one another. Put to rest in the 2000s using new technology in evolutionary genetics, skeletogenesis is now regarded as involving the four mineralised tissue types expressing specific secretory calcium-binding phosphoprotein genes and other core developmental gene networks essential to vertebrate phylogeny (1). The expression of runt-related transcription factors (RUNX1, RUNX2 and RUNX3) is vital for these criteria. RUNX proteins are critical in regulating skeletogenesis and cartilage development in all vertebrates. They are also important for differentiating the bone cell population from mesenchymal and haematopoietic stem cells (2).

In a human embryo it takes eight weeks post-conception for the skeleton to begin to form, one of the last organs to develop before becoming a foetus. Within the three developmental lineages that generate the skeleton, the somites produce the axial skeleton (skull, spine and ribs), the lateral plate mesoderm generates the appendicular skeleton (limbs and hips) and the neural crest develops the pharyngeal arches, craniofacial bones and future cartilage (3, 4). With the exception of the neurocranium, parts of the jaw and the medial clavicle where bones are formed de novo, embryonic bone development is regulated by endochondral ossification (5, 6). Conserved amongst vertebrates, endochondral ossification governs the differentiation of mesenchymal progenitors into chondrocytes, where the entire skeleton is first laid out as an intermediate cartilage template before replacement with bone (7). Cell-cell communication is critical for fate determination and anterior-posterior patterning of the developing skeleton.

Paracrine signalling acts on mesenchymal precursor cells to condense and commit to the chondrocyte cell lineage (5). Induction of chondrogenesis begins with production of the transcription factor paired box 1 (PAX1) (4)*.* Cell adhesion molecules such as N-Cadherin bind immature chondrocytes into compact nodules. This structure formation further induces specialisation with the activation of requisite transcription factors for chondrogenesis – RUNX2, sex determining region Y (SRY)-box 9 (SOX9) and collagen type II alpha 1 (COL2A1) (5). These transcription factors are required for driving the proliferation of the chondrocyte population and determination of osteoblast cell fates, as well as overseeing other transcriptional regulation in chondrocyte differentiation. Molecular cues and signalling pathways derived from immature chondrocytes sustain the differentiation process. The wingless-type MMTV integration site (Wnt) signalling pathway is shut down; with reduced levels of pericellular Wnt ligands, intracellular β-catenin and expression of transmembrane receptors low density lipoprotein receptor-related protein (LRP) 5 and 6 (8). The co-activation of transforming growth factor β (TGFβ) signalling thrusts the propagation and patterning of the entire skeletal model as cartilage (8).

Once the model is complete, chondrocytes lying within the central regions of the nascent skeleton undergo a molecular switch. The small, round chondrocytes stop dividing and increase their cell size, by at least twenty-fold, giving rise to hypertrophic chondrocytes (5, 9). The extracellular matrix produced by hypertrophic chondrocytes changes, with increased production of collagen type X alpha 1 (COL10A1) and fibronectin 1 (FN1). This new matrix enables mineralisation with hydroxyapatite, using calcium and phosphate from maternal blood. The formation of bone in this region is termed the primary ossification centre. As the generation of bone gradually replaces the cartilage model from the primary ossification centre, the assembly and maturation of chondrocytes is restricted to the epiphysis. Here, they turnover within their own secondary ossification centre (5, 10) (Figure 1). Blood vessels invade the cartilage model with hypertrophic chondrocytes undergoing programmed cell death. The empty space left behind caused by chondrocyte apoptosis is replaced with bone marrow.

The final phase of skeletogenesis is the differentiation of osteoblasts; RUNX2 interacts with other transcription factors such as bone morphogenetic protein 2 (BMP2) and zinc finger protein 521 (ZFP521) to encourage osteoblast differentiation of mesenchymal cells that have migrated and enveloped the cartilage model (11). In antithesis to earlier in skeletal development, increased paracrine signalling of Wnt ligands and Indian hedgehog (IHH) supports the reduction of the chondrocyte population. These signalling pathways also act to increase osteoblast maturation and bone formation (12).

As greater Wnt and IHH signalling progresses throughout the developing skeleton, it is imperative the chondrocytes are not replaced by osteoblasts in the primitive joints. Chondrocyte survival within the secondary ossification centre at the epiphysis relies on a reduced exposure to Wnt and IHH signalling. Now developing under hypoxia, chondrocytes express vascular endothelial growth factor (VEGF), hypoxia-inducible factor 1 (HIF1) and its oxygen sensitive component HIF1α to mediate the survival response within the joints (13). These molecules are common to any cell where there is a reduction in oxygen availability.

At the end of adolescence when bone modelling has ceased and bone remodelling takes over, immature chondrocytes in the joints secrete parathyroid hormone-related protein (PTHrP) in response to hypoxia and IHH signalling from hypertrophic chondrocytes at the bone-cartilage border (12, 14) (Figure 2). The activation of parathyroid hormone receptor 1 (PTHR1) by PTHrP slows the rate of immature chondrocyte molecular switching to hypertrophic chondrocyte (12). Reports suggests PTHrP regulates the movement of a transcriptional suppressor, histone deacetylase 4 (HDAC4), into the nucleus to negatively regulate the activity of downstream transcription factors by direct binding. These transcription factors include myocyte enhancer factor 2 (MEF2), ZFP521 and RUNX2(15-18).PTHrP is also endogenously present in other cartilaginous sites, for example, in the perichondrium and chondrocyte population adjacent to hyaline cartilage.

Understanding the molecular mechanisms that oversee chondrogenesis, bone formation and cartilage homeostasis allows researchers and physicians to reactivate or modify these pathways with treatments to promote skeletal tissue repair after injury or disease. Early bone development is a highly conserved arrangement across vertebrate species, from fish to humans. In animal models of embryonic development, a number of studies have identified cellular responses to chemical genetics that underlie human bone and cartilage disorders (19-22). Manipulation of ‘transcription dynamics’ - described as the cyclic nature of RNA polymerase assembly, remodelling of chromatin, binding of transcription factors, transcriptional elongation, RNA editing, RNA splicing and post-transcriptional regulation (23) – is a hot topic in regenerative medicine.

**MicroRNA BIOLOGY**

MicroRNAs (miRNAs) are a class of non-coding RNA molecules that are the most well-known components behind the cellular machinery of ‘RNA silencing’ - a mechanism of RNA-mediated gene silencing first discovered in nematode worms (24). Oscillations of messenger RNA (mRNA) transcription and translation are buffered by co-expression of complementary miRNAs with ensuing negative regulation of gene activity. In the canonical biogenesis pathway, miRNA genes are transcribed from the genome by RNA polymerase II to produce pri-miRNA transcripts (25). The transcripts are enzymatically cleaved within the nucleus by the microprocessor complex; a protein assembly composed of Drosha and DiGeorge critical region 8 (DGCR8). The following ~70 nucleotide pre-miRNA forms a characteristic hairpin secondary structure and is transported to the cytoplasm by RanGTP-dependent nuclear envelope-bound Exportin-5 (25, 26). Here, the multi-domain enzyme Dicer processes the pre-miRNA into a ~22 base pair miRNA-5p and miRNA-3p duplex. Dicer achieves this through its possession of a double stranded RNA binding domain for initial capture of the pre-miRNA, two tandem RNase III nucleases for cleavage and an N-terminal ATPase/helicase domain for unwinding the 3p and 5p strands (27). One of the strands is immediately loaded into the Argonaute 2 (AGO2) effector structure of a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC). The other strand, previously known as miRNA star, is usually degraded. RISC uses the mature single stranded miRNA sequence as a guide for targeting complementary sequences in the mRNA 3’ untranslated region (UTR); also known as the miRNA recognition element (MRE) (28). Less commonly the 5’ UTR and mRNA coding sequence also contain MREs (29, 30). The mRNA is silenced via translational suppression or mRNA decay (and very rarely through target cleavage when the complementarity between the miRNA and MRE is near-perfect). There are 2,588 human mature miRNAs recorded in miRBase version 21 (31), collectively believed to regulate as much as 60% of the coding transcriptome (32). The regulation of gene expression at this level is important in many fundamental biological processes, including cellular differentiation, proliferation, migration and extracellular communication.

While the core model of miRNA biogenesis and regulation of gene expression is widely known, turnover of miRNAs is less clear. In a similar fashion to mRNA transcripts, miRNA production is differentially controlled through the association of regulatory factors. This can take place both within the biogenesis pathway and post-transcriptionally (33). The first miRNA to be discovered, *lin-4* in *Caenorhabditis elegans*, was primarily described as a ‘small temporal RNA’ owing to its transient pattern of expression (24). When this finding was discovered to be conserved through to humans, it added weight to the concept that miRNAs could be produced in tissue-specific spatiotemporal patterns similar to that of coding genes (34). RNA polymerase II-mediated transcription provides a major regulator for the production of miRNAs (33). Mapping of human miRNA promoters through nucleosome positioning and chromatin immunoprecipitation suggests the promoter structure of miRNA genes, including the frequencies of CpG sites, cis-regulatory elements and histone modifications, is indistinguishable between miRNA and mRNA loci (35, 36). Protein complexes, such as transcription factors, that facilitate mRNA expression are largely those that also modulate miRNA expression. The tumour suppressor protein p53 was recently identified to upregulate miRNA production through the generation of a complex with Drosha and an RNA helicase, DDX5, when there is damage to DNA (37). Similarly, proto-oncogenes such as c-Myc may modulate miRNA expression through binding to enhancer boxes in miRNA cluster promoters (38). Since miRNAs are transcribed in an almost identical manner to mRNAs, the mechanisms of epigenetic control known for protein coding genes are likely to apply to miRNA loci (33). Hypermethylation of tumour suppressor genes is a common occurrence in cancer biology; the same is true for tumour suppressor miRNAs such as miR-9-1, -193a, -137, -342, -203 and -34b/c which are also found to be hypermethylated in various human cancers (39, 40).

Not only are miRNA loci subject to transcriptional and epigenetic control, mature miRNAs are also under post-transcriptional regulation. Circular RNAs (circRNAs) are highly expressed in the human and mouse brain where they act as a molecular sponge to soak up miRNA transcripts, inhibiting their activity (41). The circRNA counterpart for miR-7, or ciRS-7, contains more than 70 conserved miRNA target sites but is entirely resistant to miRNA-mediated destabilisation (41). ciRS-7 strongly suppresses miR-7 activity, resulting in an increase of miR-7 target mRNAs. The testis-determining factor SRY is also a circRNA for miR-138 (41). This finding advocates miRNA sponge effects achieved by circRNA formation is a general phenomenon. It is also the first time natural RNA circles have been shown to regulate mature miRNAs. Expansion of this discovery will provide further insight to biological processes and pathophysiology where miRNAs have been characterised in the aetiology of disease.

**miRNAs IN EMBRYONIC BONE DEVELOPMENT**

Bone formation requires a multifaceted sequence of cellular events to drive progenitor differentiation and assembly of the bone cell population. Extracellular signalling provides the molecular signals to accomplish this process, which is ultimately achieved through targeted gene expression. A key element of this complexity is the tuning of signal-induced genes by miRNAs, as has been demonstrated in Dicer knock out studies in mice. A global reduction of miRNA expression in chondrocytes and limb bud mesenchymal progenitors was previously shown to cause significant growth defects in skeletal development (42). On such a genome-wide scale these effects are typically expected. The biological function of individual miRNAs in skeletal development will provide the key to understanding normal development, while exposing a process that may be manipulated by medical treatments.

In mice, the miR-199a/214 cluster is expressed from the opposite strand of dyanamin 3 (*Dnm3os*) (43). Replacing this locus with the *lacZ* gene in mice caused growth retardation, craniofacial and vertebral hypoplasia and osteopenia (42, 43). This strongly proposes the functional role of this miRNA cluster in developing chondrocytes. Mechanistic insight of other miRNAs promoting chondrogenesis include miR-365, which performs by targeting HDAC4 (44). miR-30a, -30c and -125b enhance the differentiation of tracheal chondrocytes by suppressing a chondrogenesis inhibitor, snail zinc finger 1 (SNAIL1) (45). miR-337 also promotes chondrogenesis in developing rat bone by downregulating TGFβ receptor II (TGFBR2) (46).

Let-7 was recently shown to regulate chondrocyte proliferation specifically in the epiphyseal growth plate, providing further evidence for the biological role of individual miRNAs in chondrogenesis (47). Overexpression of a Let-7 inhibitor, lin-28 homolog A (LIN28A), caused growth impairment through the action of reduced chondrocyte proliferation (47). An upregulation of predicted Let-7 target genes such as cell division cycle 34 (*Cdc34*) and E2F transcription factor 5 (*E2f5*) was also observed. The phenotype of *Lin28a* transgenic mice was mild, however when combined with a reduction of another miRNA, miR-140, the observed phenotype of reduced growth were significantly amplified (47). The findings suggest these miRNAs work in synergy to promote normal skeletal development; or miR-140 has a key role in chondrogenesis and the generation of cartilage.

**miR-140 in embryonic bone development**

Several miRNAs have been discovered and proposed to play a crucial role in modulating oscillations of gene expression during embryonic development (48). One such miRNA, miR-140, was first reported in zebrafish. Its expression was later linked with mesenchymal stem cell frequency and chondrogenesis (49-52). miR-140 is evolutionarily conserved amongst vertebrates and is almost exclusively specific to chondrocytes where it supports cellular homeostasis and identity (53, 54). The pri-miR-140 gene is housed within an intron of WW domain containing E3 ubiquitin protein ligase 2 (*WWP2*) – known for its role in mediating the TGFβ signalling pathway through interaction with SMAD proteins. Expression of miR-140 has an overlapping pattern with *WWP2*, *SOX9* and *COL2A1*; known regulators of chondrogenesis (42). Previous research in chondrocyte cell cultures have confirmed that SOX9 is a promotor for miR-140 expression (55). Studies in mice have confirmed the association between SOX9 and miR-140. These studies show a proximal upstream region of the pri-miR-140 gene possesses a chondrocyte-specific promoter, which is directly regulated by SOX9 (56).

miR-140 is highly expressed during the early stages of endochondral ossification, before the formation of articular cartilage at the epiphysis (57). Timing of expression is crucial as premature miR-140 activity may result in craniofacial defects through modulation of palatogenesis (58). Two independent studies in mice lacking miR-140 identified growth defects but with two different phenotypes (51, 59). In the first study, miR-140-/- mice presented with early onset age-related osteoarthritis, supporting the concept that miR-140 has roles in the preservation and homeostasis of articular cartilage. The second study showed proliferative growth defects of endochondral bones – resulting in dwarfism and craniofacial deformities. This resulted in the hypothesis that miR-140 may play a newly defined role in embryonic bone development.

Locked nucleic acid (LNA) probes in whole-embryo in situ hybridisation were used to detect miR-140 at stages of development not tested in previous studies (57). Using the bones of the digits in chicken embryos as a model, the presence of miR-140 was detected as early as day six in embryonic development (57). At this stage, the limbs were shown to have developed a toe plate with a pointed contour and expression of miR-140 in two rudimentary digit bones. High magnification showed no distinct or well-shaped bones. These results indicated that miR-140 first appears at the early stage of bone development when the condensed mesenchyme is forming a hyaline cartilage template for the future bone (57). In later stages of development when the bones of the digits are distinctly formed, miR-140 expression is seen in the whole body of the bone, including the upper region where future articular cartilage is formed. The shift of miR-140 expression was also observed along the bones of the metatarsus and phalanges of the digits. The last bone of the phalanx showed a homogenous distribution of miR-140 through the whole bone. The other three bones of the phalanx showed intermediate movement of miR-140 to the tops and centre of the bone, leaving two clear zones in between. The central zone where miR-140 appeared in bones three and four and remained well-defined in bone five, is the same location of primary ossification during endochondral ossification (57). This demonstrates that miR-140 is present during early stages of bone development when the hyaline cartilage template of the bone is formed. For a short period later, miR-140 is expressed in the primary ossification centre (57). Lack of miR-140 in mutant mice at these early stages, therefore lacking chondrocyte identity, could lead to premature chondrocyte hypertrophy that explains the observed dwarfism in the mouse experiment discussed previously (57).

**miR-140 in chondrogenesis and adult cartilage homeostasis**

In the adult skeleton, chondrocytes are restricted to the ends of bones, or epiphysis, where they are the only cell type present in cartilage. They are responsible for the synthesis and turnover of the extracellular matrix crucial to their function of bone protection at the joints. Increased miR-140 expression is correlated with increased expression of *SOX9* and *COL2A1* during human chondrogenesis (49). Healthy chondrocytes steadily express miR-140 throughout their lifetime (60). Degenerative diseases such as osteoarthritis show significantly decreased expression of miR-140 as well as dysregulation of other miRNAs, such as miR-455 (19). Target genes of miR-140 provide an insight to the homeostasis of chondrocyte maintenance. However, target prediction of miRNAs in animals is computationally difficult due to the low complementary annealing efficiency between the miRNA and target mRNA. In animals, perfect base pairing is commonly restricted to the miRNA seed sequence (nucleotide positions 2-8). Overexpressing and antagonising miR-140 in mouse fibroblast cells previously revealed a cross section of forty-nine co-differentially expressed mRNAs. Therefore, correlating miRNA manipulation and mRNA expression provides a general experimental approach to target prediction (61). One of the potential targets – chemokine (C-X-C motif) ligand 12 (*Cxcl12*) known for its role in skeletogenesis, metastasis and tumour angiogenesis - was experimentally validated as a real miR-140 target by luciferase assay (61).

In an extension to the experimental identification of miR-140 target study, the mRNA profiling datasets were used to show *Smad3* is a target at the protein level (62). SMAD3 is a key negative regulator of proliferation in the TGFβ signalling pathway. When *Smad3* mRNA is silenced by miR-140, the cell cannot achieve G1 cell cycle arrest. As bone is one of the richest sources of TGFβ ligands in the human body, this pathway is attractive as a model for studying cancer development where TGFβ signalling is a driver for both proliferation and tumour suppression. It could be hypothesised that miR-140 is a candidate molecule to investigate in the development of malignant chondrocytes. Studies of chondrosarcoma have so far identified other miRNAs that are more likely contenders (63, 64). The favoured position of miR-140 at present is as a key regulator of chondrocyte homeostasis, loss of which results in cartilage breakdown and progressive diseases such as osteoarthritis. Other experimentally validated miR-140 targets include ADAM metallopeptidase with thrombospondin type 1 motif 5 (ADAMTS5), aspartyl aminopeptidase (DNPEP) and V-ral simian leukemia viral oncogene homolog A (ras-related) (RALA) (49, 59, 65).

**miR-140 IN CANCER: A DUAL ROLE AS AN ONCOGENE AND TUMOUR SUPPRESSOR**

**miR-140 in colorectal and hepatic cancer**

miR-140 expression is a marker of chondrocyte identity. Dysregulation and inappropriate expression in other cells has been shown to drive tumourigenesis. In colorectal tumours, miRNA-network analysis has shown miR-140 was amongst the most differentially expressed miRNAs (66). Though target genes of miR-140 were not studied, the authors suggest the downregulation of miR-140 in colorectal cancer is tissue-specific. Mechanistic insight of miR-140’s role could include analysis of cellular pathways known to be perturbed in colorectal cancer such as the Wnt signalling pathway. Loss of miR-140 has also been linked to hepatocellular carcinoma (67). DDX20 is an RNA helicase that regulates the function of miR-140 in the liver. Here, miR-140 has two confirmed targets - nuclear factor-kappa B (NF-KB) and DNA methyltransferase 1 (DNMT1). It was previously known that loss of DDX20 enhances the activity of NF-KB in driving carcinogenesis of hepatocytes. Increased expression of DNMT1 in DDX20-deficient cells resulted in hypermethylation of metallothionein gene promoters. Decreased expression is linked with increased NF-KB activity (67). The action of miR-140 here is twofold; without upstream regulation it cannot prevent the downstream methylation and subsequent bioactivity of NF-KB.

**miR-140 in breast cancer stem cells**

Cancer stem cells are the key drivers of tumourigenesis, malignancy, multiple drug resistance and treatment failure (68). Whether they are hyperactive stem cells or senescent cells that have recovered stem-like behaviours is still a topic of debate. They are, however, much like embryonic stem cells in their physiology and behaviour. They are capable of self-renewal and express totipotency-associated transcription factors such as SOX2 and octamer-binding transcription factor 4 (OCT4) (69). Oestrogen, a contributor to hormone-sensitive breast cancer, promotes the vigour of breast cancer stem cells (70). In studying the mechanistic driver of breast cancers overexpressing the oestrogen receptor, it was demonstrated that activation of the receptor inhibits transcription of miR-140 (69). As miR-140 has been reported as a tumour suppressor in breast cancer, downregulation could initiate cancer stem cell activity. Computational prediction of miR-140 mRNA targets using TargetScan 6.0 revealed SOX2 as a potential target of miR-140. A luciferase reporter assay confirmed the *SOX2* 3’ UTR is targeted by miR-140 (69). Loss of miR-140 through oestrogen receptor activation upregulates SOX2 prompting self-renewal of cancer stem cells.

**Duplication of the miR-140 gene in a head and neck tumour**

Copy number variations or gene duplications are common to most cancers. Consequently, gene dosage is significantly increased. If a proto-oncogene is duplicated, the increased gene expression can be key to cellular transformation and malignancy. A DNA microarray to detect not only genome-wide DNA copy number changes but also transcriptome alterations, revealed a duplication of the miR-140 loci in human oral squamous cell carcinoma (71). An over-representation of cellular miR-140 transcripts resulted in the suppression of genes involved in normal cell cycling, connective tissue homeostasis, cellular function and cell-cell signalling (71). The suppressed mRNAs included those for p53, NF-KB and HDAC1, consequently driving lymph node metastasis (71).

**Downregulation of miR-140 and upregulation of RAD51AP1 in ovarian cancer**

Application of a novel method to detect conserved MRE-seed sequence matching and significant ‘anti-correlation’ of miRNA and mRNA expression revealed a downregulation of miR-140 in ovarian serous cystadenocarcinoma (72). Using mRNA profiling data from The Cancer Genome Atlas, the upregulated mRNA corresponding to a reduction of miR-140 was RAD51-associated protein 1 (*RAD51AP1*); which is involved in double stranded break repair of DNA (72). Overexpression of RAD51AP1 is a tactic utilised by tumours to evade the impact of platinum-based chemotherapy agents, such as cisplatin, that specifically function by triggering DNA damage in cancer cells.

miR-140 has also been found to be downregulated in basal cell carcinoma (73), lung adenocarcinoma (74) and upregulated in salivary gland adenoma (75).

**Chemoresistance of osteosarcoma, colorectal and gastric cancer is influenced by miR-140 expression**

High-throughput miRNA expression analysis in osteosarcoma and colorectal cancer cells revealed miR-140 expression was associated with chemosensitivity (76). Tumour cells in vitro were transfected with a pre-miR-140 mimic and exhibited resistance to methotrexate and 5-fluorouracil; two commonly used cancer chemotherapy agents. Overexpression of miR-140 inhibited cell proliferation and encouraged G1 and G2 cell cycle arrest in p53-positive cell lines but less so in cell lines with a mutant p53 (76). This is an important finding as there is a p53-associated mutation in half of all human cancers. As first reported in embryonic development of bone (53), this study supports the contention that *HDAC4* is a target of miR-140. The study also revealed endogenous miR-140 upregulation in CD133 (+hi) CD44 (+hi) colorectal cancer stem cells exhibiting chemoresistance. Antagonising endogenous miR-140 with LNA inhibitors partially sensitised the cancer cells to 5-fluorouracil treatment. The study summarises that miR-140 is involved in chemoresistance by reduced cell proliferation through G1 and G2 phase arrest, mediated in part by the suppression of *HDAC4* (76). The reverse is true in stomach cancer where miR-140 was present within a list of miRNAs linked to increased chemosensitivity (77). Taken together, the results highlight the importance of recognising one miRNA may have very different roles contingent on the cell and tissue origin. Therapeutics involving miRNA delivery as demonstrated by the use of LNA inhibitors, must be organ-targeted.

**SUMMARY AND FUTURE DIRECTIONS**

In the chondrocyte, miR-140 is a highly expressed non-coding RNA and could be used as a marker of chondrocyte differentiation. Genes known to interact with miR-140 are accepted markers of chondrocyte proliferation and homeostasis – these include *SOX9*, *COL2A1* and *COL10A1*. Key mRNA targets of miR-140 gene silencing include *HDAC4, CXCL12*, *SMAD3,* *DNMT1* and NF-KB (Figure 3). Targets within the TGFβ signalling pathway are crucial to regulating cellular proliferation in skeletogenesis (Figure 4). They are central to endochondral ossification and cartilage homeostasis.

Loss of miR-140 expression can lead to degenerative diseases such as osteoarthritis, though dysregulation is not thought to be involved in the tumourigenesis of chondrocytes. Conversely, in other cell types, differential expression of miR-140 can play a key role in malignant neoplasia. However, this is strictly determined by the tissue type. In some cancers, such as colorectal and oral, miR-140 plays the role of a tumour suppressor. Here, it silences proto-oncogene transcription where loss of function drives tumourigenesis. In other tumours such as liver, breast, skin and lung, miR-140 is an oncogene or ‘oncomiR’ where gain of function drives tumourigenesis.

**miR-140 and the Wnt signalling pathway**

Expression of miR-140 promotes the proliferation of chondrocytes and sustains chondrogenesis in the developing embryo. Once the cartilage skeletal model is complete, a molecular switch rapidly escalates Wnt ligand production and its complement signalling molecules β-catenin, LRP5 and LRP6. Chondrogenesis is inhibited and restricted to the epiphysis, before osteoblast differentiation and proliferation becomes the primary developmental process. In adulthood, miR-140 regulates cartilage homeostasis with Wnt signalling regulating the homeostasis of bone turnover. Activating mutations in the Wnt signalling pathway can cause high bone mass (78). In contrast, decline of Wnt signalling can cause bone density disorders such as osteoporosis (79).

Wnt signalling is also employed in the colon for intestinal barrier turnover. Inhibition of miR-140 with overexpression of Wnt signalling has been shown to drive the bulk of colorectal cancer cases (80). Though miR-140 has yet to be experimentally linked to the Wnt signalling pathway, there is a clear paradigm between this gene network and these two organs. If there is an interaction between Wnt signalling and miR-140, embryonic bone development may provide an exceptional model for studying colorectal cancer progression. One potential avenue of investigation is the newly-discovered Wnt signalling antagonist molecule sclerostin, or SOST. If *SOST* mRNA is a real target of miR-140, LNA inhibitors for upregulated miR-140 may rescue SOST in a novel treatment for colorectal cancer. Intriguingly, targeting SOST through immunotherapy has been shown to reverse osteoporosis (81).

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**FIGURE LEGENDS**

Figure 1. The skeleton is first formed as a cartilage model. Once entirely patterned, immature chondrocytes lying within central zones undergo a molecular switch to enlarge in size. They begin the transformation which drives the immature chondrocytes towards the end of the bones. Once they become hypertrophic chondrocytes, the matrix changes which allows mineralisation with calcium and phosphate. Wnt signalling is activated in the primary ossification centre where osteoblasts gradually replace chondrocytes and bone is formed. Remaining immature chondrocytes reside at the end of the bone maintaining the cartilage in the joints within the secondary ossification centre, arising from a separate progenitor population.

Figure 2. Hypoxia drives the secretion of PTHrP from both periarticular and perichondral chondrocytes. The PTHrP acts on proliferating chondrocytes, promoting the continuation of cell division and migration towards the bone. PTHrP further acts to block and delay IHH signalling from initiating hypertrophy and maturity. Once the concentration of PTHrP is reduced by proximity, the chondrocytes increase in size and begin to express IHH. IHH promotes the expression of PTHrP at the end of the bone and subsequent chondrocyte proliferation. IHH also maintains the bone-cartilage border, preventing the migration of osteoblasts.

Figure 3. Summary of miR-140 targets. SOX9 induces transcription of the pri-miR-140 loci, subsequently driving the regulation of genes involved in various biological processes in embryonic bone development and cancer.

Figure 4. TGFβ ligands bind to a membrane-bound type II receptor dimer, a serine/threonine kinase. Binding of the receptor catalyses the phosphorylation of a type I receptor dimer, forming a heterotetrameric complex with the TGFβ ligand. The type I receptor phosphorylates the serine residue of SMAD2/3, inducing a conformational change that enables co-binding with SMAD4. The SMAD2/3/4 transcription factor moves into the nucleus to induce gene expression. Both proliferative and apoptotic genes are regulated by this pathway. miR-140 plays a key role in negatively regulating SMAD3. In other pathways, miR-140 also negatively regulates translation of NF-KB, HDAC4 and RUNX2. Loss of miR-140 in chondrocytes leads to dysregulated gene expression and can lead to degenerative diseases such as osteoarthritis.