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Review

Concerted stimuli regulating osteo-chondral differentiation from stem cells: phenotype acquisition regulated by microRNAs

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Bone and cartilage are being generated *de novo* through concerted actions of a plethora of signals. These act on stem cells (SCs) recruited for lineage-specific differentiation, with cellular phenotypes representing various functions throughout their life span. The signals are rendered by hormones and growth factors (GFs) and mechanical forces ensuring proper modelling and remodelling of bone and cartilage, due to indigenous and programmed metabolism in SCs, osteoblasts, chondrocytes, as well as osteoclasts and other cell types (*eg* T helper cells).

This review focuses on the concerted action of such signals, as well as the regulatory and/or stabilizing control circuits rendered by a class of small RNAs, designated microRNAs. The impact on cell functions evoked by transcription factors (TFs) via various signalling molecules, also encompassing mechanical stimulation, will be discussed featuring microRNAs as important members of an integrative system. The present approach to cell differentiation *in vitro* may vastly influence cell engineering for *in vivo* tissue repair.

Keywords: growth factors; transcription factors; mechano-stimulation; MicroRNAs; stem cells; osteoblasts; chondrocytes; tissue engineering

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Introduction

Many review articles describe in detail the differentiation of osteoblasts[1–7] and chondrocytes[8–13] from stem cells (SCs) following hormone and growth factor (GF) exposure, as well as mechanical stimulation, both converging towards gene regulation by transcription factors (TFs), co-activators, associated proteins and repressor molecules. However, it seems that it is still difficult to arrive at a consensus as to which parameters are more important to predict/measure true functional features needed for osteoblasts or chondrocytes to function as proper cells maintaining bone or cartilage in a 3D-structure *in vivo*, rendering them able to withstand challenge from immobilization, overuse and/or inflammatory processes. This paper will focus on the role of microRNA in the present enigmatic issue.

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Current status of cell engineering (replacement therapy) of bone and cartilage

The necessity for an approach in order to establish interdisciplinary therapeutic strategies for the treatment of bone defects has been addressed by scientists for many years. This awareness is apparent from the multitude of approaches taken (*eg* GF-based therapy, gene therapy, SC-based therapy, scaffold-based therapy) to engineer tissues, integrating contributions from many medical and technical disciplines, *eg* immunology, biomechanics and material science[14–20]. The ultimate goal will be to use test-engineered cells on scaffolds *in vitro* and *in vivo* to assess the ideal, site- and environmentally adaptable phenotype and resilience of the engineered osteoblastic cells.

The use of mesenchymal stem cells (MSCs) for cartilaginous cell therapy and regeneration comprises at least two approaches. The first pertains to *ex vivo* cartilage tissue engineering, in which a replacement tissue is constructed *in vitro* using MSCs combined with scaffolds under appropriate environmental stimuli. The second is *in vivo* cartilage regeneration via MSC-based therapy using its anti-inflammatory

and immunosuppressive effects[21–25]. MSCs are expanded and injected locally into the affected joint, but can also be applied systemically. Due to their potential regenerative trophic role, they may influence the articular micro-environment and aid in the regeneration of the cartilage. However, there is no consensus as to the optimal cues for cartilage formation *in vitro*, be it the proper MSC source, the make of bioactive scaffolds, environmental factors for differentiation of MSCs, *in vitro* characteristics predictive for *in vivo* functioning and site-related adaptation, long term chondrocyte stabilization etc.

Importance of the osteo-chondro transition area

The process of endochondral ossification, from loose mesenchyme to initial osteoblast differentiation, entails intermediate chondrocyte differentiation. The loose mesenchymal progenitor cells proliferate and organize into the condensed mesenchyme comprised of osteo-chondral progenitor cells[10]. The cells of the condensed mesenchyme differentiate into chondrocytes, and the cells at the centre of the condensation stop proliferating and become hypertrophic[10, 26, 27]. Perichondral cells adjacent to the hypertrophic chondrocytes differentiate into osteoblasts and form the bone collar, as the invasion of blood vessels begins along with continued osteoblast differentiation[10, 28, 29].

Whether or not the endochondral ossification potential is necessary to maintain in engineered chondrocytes, it may be worth while preserving the potential to vascularise, since several attempts to replace chondrocytes have failed, due to a lack of vascularization *in vivo*, thus stimulating the shedding of cartilage containing dead chondrocytes[29–31]. Furthermore, it may well be necessary to produce a fraction of chondrocytes of the hypertrophic type, since they have been shown to be involved in both cartilage and bone remodelling by secreting MMPs, ADAMs, and RANK-L [10, 32, 33].

Signalling systems in developing osteoblasts and chondrocytes

The osteoblast

The differentiation of MSCs towards osteoblasts undergoes several phases including osteoprogenitor cells, their differentiation into pre-osteoblasts and mature osteoblasts. The latter are transformed into osteocytes, which may constitute the mechano-sensing lattice and nervous system junction connecting bone to the outer "environment"[2, 5, 34–40], or they are subjected to apoptosis (Figure 1). A concerted action of both positive and negative regulatory factors determines the developing

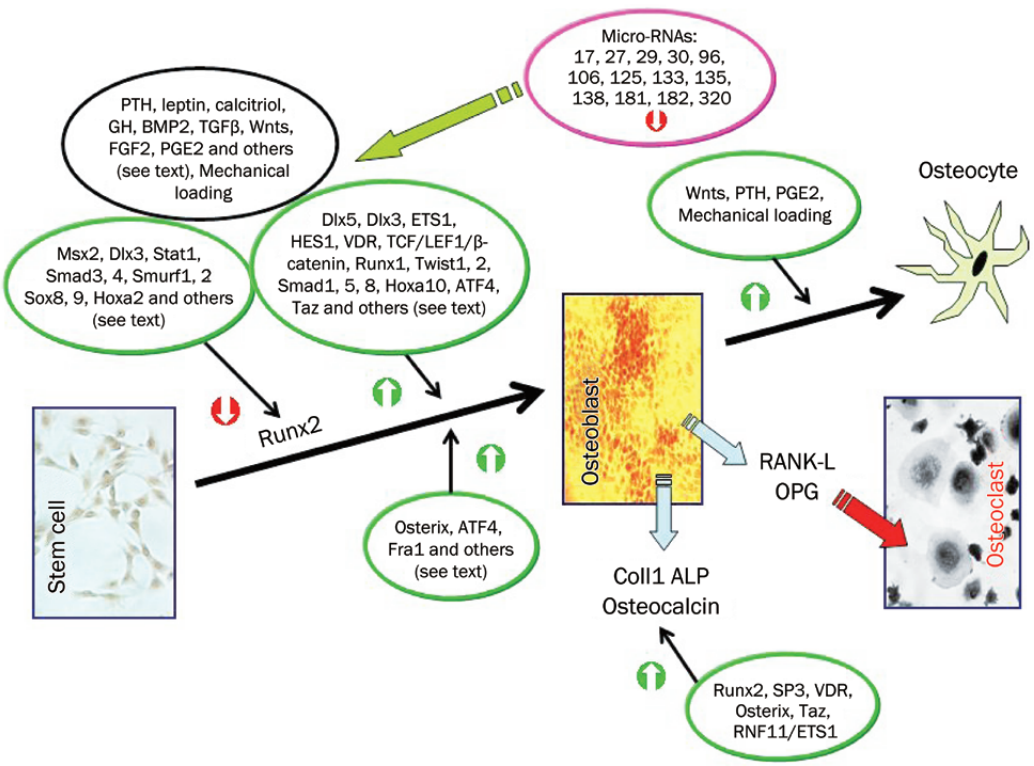

Figure 1. The differentiation of stem cells (SCs) towards osteoblasts. The impact of hormones and growth factors (GFs), mechanical loading, as well as transcription factors (TFs) and TF-modulating proteins are indicated. Some TFs and TF-modulating proteins are negatively or positively influencing the activity of Runx2, while others are involved in the differentiation process independently of Runx2. MicroRNA species negatively affecting the differentiation of SCs elicited by GFs and/or TFs are depicted (see especially microRNAs 29, 125, 133, and 135). The osteoblast secretes matrix proteins, whose gene transcripts are modulated by TFs. It also affects osteoclast differentiation and activation by secreting the opposite acting factors RANK-L and OPG. Finally, the osteoblast might be subjected to apoptosis or acquiring an osteocytic state. Osteocytes serve as a connection between the bone tissue and the nervous system, while also perceiving the mechanical load on the skeleton.

phenotype of the osteoblastic cells, including the processes of bone modelling and remodelling. This involves the concerted action of secreted RANK-L and OPG, acting on osteoclastic cells[2, 4–6, 41, 42]. Major functional features of the osteoblast pertain to sequential synthesis and deposition of matrix proteins and enzymes necessary to complete these processes. Finally, the transition of osteoblasts to osteocytes are also modulated by several factors (Figure 1), yielding a complete and versatile cellular system, being able to adapt to various physiological conditions, when necessary[2, 5, 43].

The osteoblast commitment, differentiation and various functions are governed by several TFs, resulting in expression of phenotypic genes responsible for the acquisition of the plasticity of the osteoblastic phenotype[2, 3]. Runx2 and Runx3 are present in osteoblastic lineage cells. Runx2 controls bone formation during both skeletal development and post-natal life. Expression of Runx2 is both necessary and sufficient for MSC differentiation towards the osteoblastic lineage[3, 4, 6, 44]. The Runx regulatory element can be found in the promoter of all major genes controlling their expression, including collagen Iα1, osteopontin, BSP and osteocalcin. Runx2 can be phosphorylated and activated by the MAPK cascade by the activation of α2β1-integrins on the osteoblast surface[2, 45, 46]. Finally, Runx2 has been found to negatively control osteoblast proliferation by acting on p85 PIK3 and GADD45β, the latter known to be associated with cell cycle G2-M arrest[47]. However, Runx2 is not essential for the maintenance of the expression of major bone matrix protein genes in the mature osteoblast, since over-expression of Runx2 yields osteopenia, due to a reduced number of osteoblasts and an increased number of osteoclasts[48, 49]. Runx2, being considered a crucial factor in osteoblast recruitment and differentiation, is tightly controlled by other TFs, protein-DNA or protein-protein interactions. In early pre-osteoblast development, the factors Hoxa2 and Satb2 regulate Runx2 activity[50]. Some complex mechanisms involving factors like Stat1, Sox9, Sox8, Aj18, MEF, Nrf2, and YAP repress Runx2 expression[2, 51–54], while most of the published literature describes factors (Rb, TAZ, HoxA10, BAPX-1, Smad1&5, CEBP/β&δ, and Menin) actively enhancing the function of Runx2[2, 55–58]. Runx2 protein degradation may be accelerated by Smurf1, however this loss of Runx2 may be counteracted by factors like YAP, TAZ, and WWP1-Schnurri[2, 59]. A compilation of factors known to affect Runx2 regulation is shown in Figure 2A–2C. Of the ones depicted, some deserve further comments.

Osterix (or SP7), a zink-finger TF, acts down-stream of Runx2 and complexes with NFATc1. In turn, this co-activation stimulates the Wnt-pathway, which is considered very important for bone formation, being predictive of bone mass[60–62]. Wnt-protein interaction with Frizzled and LRP5/6 co-receptors enhances β-catenin phosphorylation, nuclear accumulation and enhanced gene expression promoted by the LEF/TCF1 TFs. Wnt-mediated signalling (by Wnt10b) also activates Runx2, osterix, and Dlx5 expression[2, 6, 41, 63, 64].

Cyclic AMP, a long "forgotten" second messenger in this field, also appears to be involved in bone formation. ATF4 (or CREB type 2) interacts with Runx2 to stimulate osteocalcin expression, while also enhancing the amino acid transport into osteoblasts. The latter system stimulates protein synthesis in bone[65, 66], where essential amino acid loading seems to be very important for maximizing peak bone mass[67, 68]. Some homeobox proteins, like Msx1&2, Dlx5&6 play a role in osteoblast differentiation. Msx2 inhibits Runx2-mediated differentiation, while Dlx5 activates the expression of Runx2[69–72]. Furthermore, some helix-loop-helix proteins, like Id and Twist are appearing during osteoblast proliferation, of which Twist controls Runx2 expression[73, 74]. PPARγ2 may interact with Runx2 to induce adipocyte differentiation[2, 75]. PPARγ2-agonists, which are used to enhance insulin secretion and restore insulin sensitivity in diabetic patients, may thus disfavour bone mass maintenance. Other TFs involved in adipocyte differentiation are the C/EBPs, which may also down-regulate the effect of Runx2 on MSC-acquisition of the osteoblast phenotype[76, 77].

In general, several hormones and growth factors (GFs) are

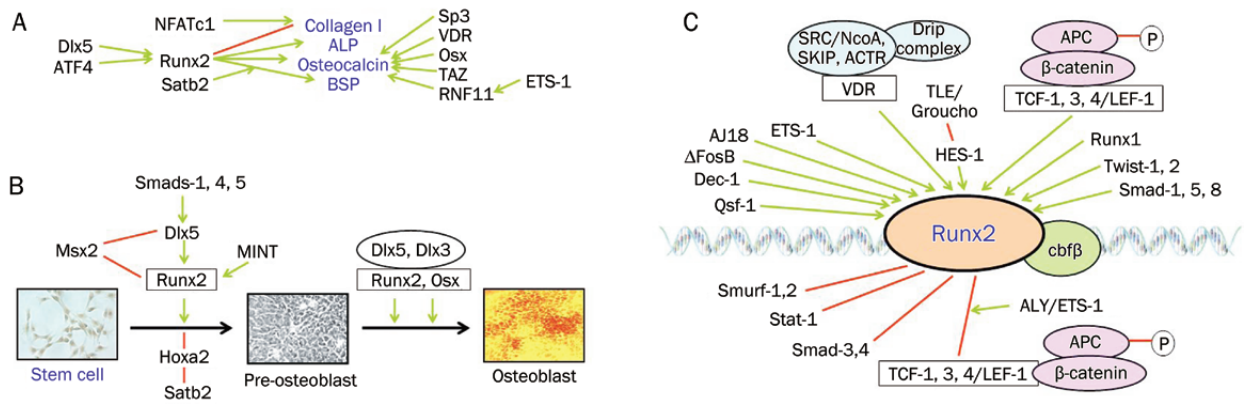

Figure 2. The influence of transcription factors (TFs) and transcriptional modulating molecules on functional gene expression (A), on pre-commitment and differentiation (B), and on Runx2 function in osteoblastic cells (C). Some TFs exert their function on osteoblast pre-commitment and early osteoblast genes, while others are active during later stages of the osteoblast's functional repertoire. However, several TFs are somehow involved in the activity of Runx2, which is deemed important for osteoblast differentiation and function in general.

involved in osteoblast differentiation, and of those, PTH, oestrogen, glucocorticoids, PGE_2, calcitriol, BMP-2, TGFβ, FGF-2, and IGF-1 impinge on signalling cascades converging towards mechanisms of action involving the TFs mentioned above, along with nuclear co-modulators and associated proteins[1, 2, 14, 15, 21, 35, 39, 78, 79]. Certain of these GFs have served as standards in the differentiation of MSCs towards osteoblastic cells for cellular therapy. Some of these signalling systems will be outlined in a later paragraph.

The chondrocyte

Formation of cartilage is initiated by the differentiation of MSCs into chondrocytes, which proceed through chondroprogenitor cells with final terminal differentiation of chondrocytes, vascular invasion and cartilage matrix calcification, and finally ossification[8, 10, 11, 80, 81]. Many signals appear to be stimulatory, however, some are also inhibitory of this process (Figure 3). The chondrocytes synthesize many matrix products, enzymes to degrade them, as well as RANK-L. Stimulation of osteoclasts is confined to hypertrophic chondrocytes, sharing many phenotypical features with the osteoblast[9, 10, 12, 13, 32].

Skeletal elements are prefigured in mesenchymal condensations, and common precursor mesenchymal cells divide into chondrogenic and myogenic lineages. The cartilaginous nodules appear in the middle of the blastema, and simultaneously, cells at the periphery become flattened and elongated to form the perichondrium. The differentiated chondrocytes can then proliferate to undergo the complex process of hypertrophic maturation[10, 82, 83]. The initiation of condensation is associated with increased hyaluronidase activity and the appearance of cell adhesion molecules, neural cadherin and neural adhesion molecules (N-CAM). TGFβ, which is among the earliest signals in chondrogenic condensation, stimulates synthesis of fibronectin, which in turn regulates N-CAM[10, 84, 85]. A current view is that a series of patterning systems functions sequentially over time. FGF, hedgehog, BMPs and the Wnt-pathways coordinate signalling through three axes to secure correct patterning along the dorso-ventral and the anterior-posterior axes[86, 87]. Important signalling molecules in this respect are Wnt2A, Wnt2C, Wnt3A, Wnt7A, FGF-10, and FGF-8. The FGFs induce Wnts, which act through the β-catenin pathway in osteoblasts, while mainly through JUNC and PKC signalling in chondrocytes[88, 89]. These early events involve homeobox transcription factors (TFs) like HoxA, HoxD (especially HoxD11&13) and Gli3[82, 87, 90–92] in a sequential manner. Apart from initiating chondrocyte differentiation, the BMPs (*eg* type 2, 4, and 7) co-ordinately induce chondrocyte hypertrophy in concert with BMP receptors and BMP antagonists, like chordin and noggin[87, 93, 94].

The chondrocyte differentiation process is characterized by deposition of a cartilage matrix consisting of collagens II, IX and X and aggrecan, regulated by a family of SOX-proteins,

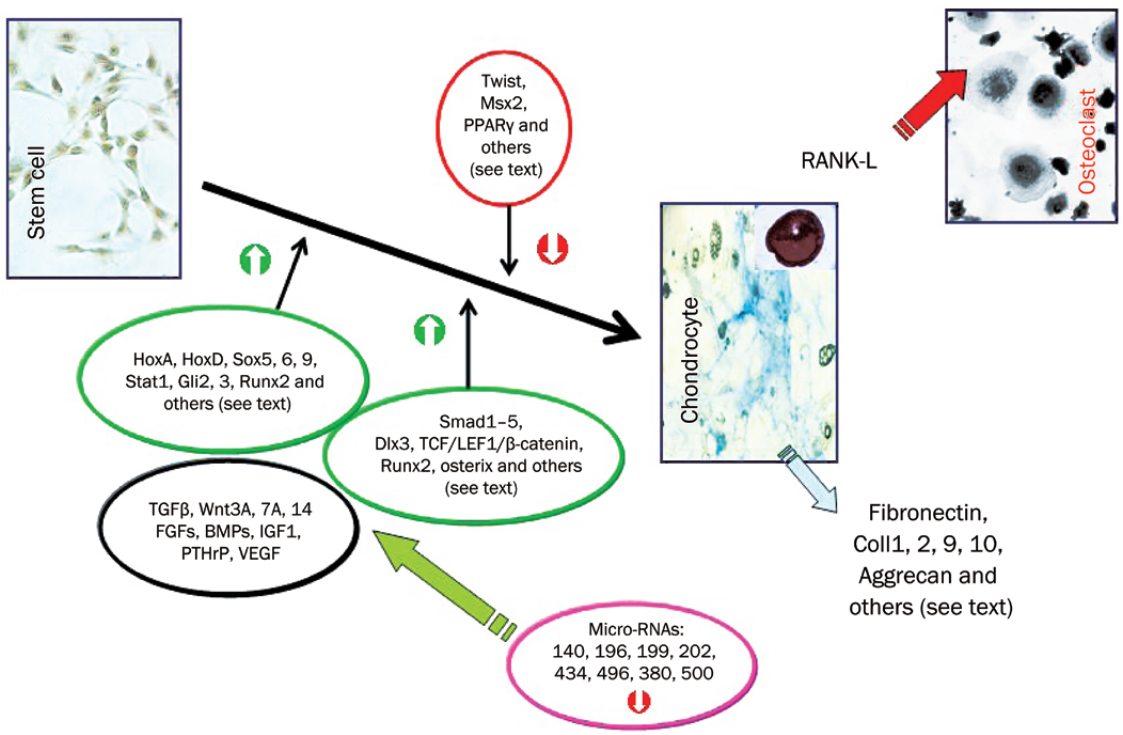

Figure 3. The differentiation of stem cells (SCs) towards chondrocytes. Hormones and growth factors (GFs) responsible for this transition are depicted. Transcription factors (TFs) and TF-modulating proteins are also indicated. Most of the subject GFs and TFs are stimulatory, however some exert negative effects on chondrocyte differentiation. Some of the microRNAs negatively affecting GFs and/or TFs are given (see especially microRNAs 140 and 199). The chondrocyte synthesizes and secretes matrix proteins like collagens, glycosaminoglycans (GAGs) and proteases (MMPs and ADAMs), as well as RANK-L, which will activate osteoclasts.

namely the transcription factors Sox9, L-Sox5, and Sox6[85, 95, 96]. The expression of SOX-proteins is dependent on BMP-signalling via BMPR1A&B, which are actively expressed in condensating chondrocytes[97, 98]. Runx2 expression also appears in all chondrocytes undergoing terminal differentiation, and BMP-induced signalling involves the canonical SMAD pathway, including the Runx2-activating Smads1, 5&8[99, 100].

Throughout chondrogenesis, BMPs and FGFs determine the rate of proliferation, thereby adjusting the pace of differentiation[101]. Proliferation of chondrocytes in the growth plate is regulated by FGFs through signalling converging towards the cyclin D1 gene. This effect is balanced through FGFR3, which inhibits proliferation by Stat1-mediated transcription of the cell cycle inhibitor p21[102, 103]. The proliferation in the lower proliferative and pre-hypertrophic zones is controlled by negative feed-back loops involving PTHrP and Ihh. PTHrP acts through PKA- and PKC-mediated signalling, while Ihh employs a set of Gli transcription factor (Gli1-3) to modulate proliferation, in order to balance the number of chondrocytes being recruited to the hypertrophic stage[92, 104, 105].

Hypertrophic chondrocytes express collagen X, ALP, positively regulated by Runx2, which interacts with Smad1[26, 27]. Runx2 is also instrumental in the synthesis and secretion of MMP-13 (acting synergistically with MMP-9), thus initiating cartilage matrix remodelling and ensuring angiogenesis, which is necessary for the replacement of cartilage by bone[10, 28, 98, 106]. One signal responsible for angiogenesis is VEGF through receptors like Flk, Npn1, and Npn2. Runx2 is also acting in concert with AP-1 family members during cytokine- and PTHrP-induced MMP-13 expression in both chondrocytes and osteoblasts[28, 106, 107]. In the hypertrophic chondrocyte, one may find many of the features of osteoblasts including a complex Runx2 regulated system (Figure 3).

Mechano-stimulation

Mechano-stimulation is important for the net building of bone mass and cartilage on a long term basis[39, 40, 108–110]. The loads which arise from functional activity generate deformation in bone and cartilage tissue through pressure in the intramedullary cavity and within cortices, transient pressure waves, shear forces through canaliculi and even dynamic electric fields as interstitial fluid flows past charged bone crystals[111]. During vigorous activities, peak strain magnitude measured in the load-bearing regions of the skeleton of adult species are ranging from 2000 to 3500 micro-strain ($\mu\varepsilon$)=2000×1/1 000 000 change in cell length/diameter[112].

These mechanical forces render cells in the bone modelling mode. The remodelling and disuse modalities lie below this threshold and yield either zero net bone production or bone loss. It has been shown that both immobilization and overuse will lead to loss of both tissue types[39, 40, 109, 110]. Many cell types display different, but measurable elasticity in cell cultures (*eg* chondrocytes=0.5−8 kpA, depending on where it is situated (endothelial or articular) and osteoblasts=1−2 kpA) indicating that there are certain forces needed to observe a certain indentation in the cells depending on the matrix and orientation of the cells within it[113]. However, these shape alterations are conveyed by external devices (like matrix proteins) and picked up by mechano-receptors linked to the same signalling systems known to regulate cell differentiation and induction of functional characteristics[111]. But pressure may have different effects than strain: chondrocytes subjected to cyclical tension caused an increase in MMP-13 and decreased TIMP-1, while cyclical hydrostatic pressure increased TIMP-1 and decreased MMP-13. Normally, cyclical exposure of cells to pressure or strain may be essentially anabolic, while chronic exposure might lead to loss of both bone and cartilage, even when the level of micro-strain applied is intermediary or low[114].

In the present paragraph, we focus on the signalling systems picking up the mechanical stimuli reaching bone and cartilaginous tissues. In osteoblasts, both shear and strain forces have, since long been known to be implicated in modulating proliferation[115, 116], differentiation (via Runx2, osterix, β-catenin)[116, 117], bone remodelling (via RANK-L osteoprotegerin=OPG)[111, 118, 119], cytokine secretion (via PGE_2, NO)[119–121] and activation of genes related to secretion of matrix proteins (like osteopontin, collagenase-3)[122, 123]. Apparently, several signalling systems are involved in the mechano-stimulation of osteoblasts and chondrocytes, many of which are common to those used by hormones and GFs[111]. MAPK is activated by stretch and shear forces in many cell types, including osteoblasts, stromal cells and osteocytes, as well as chondrocytes and alveoblasts. Stromal cells exposed to mechanical forces secrete RANK-L, which stimulates osteoclastogenesis, however, mature osteoclasts seem to be responding to increased mechano-stimulation by enhancing their bone resorbing potential[118, 124, 125]. Vascular cells and stromal cells in bone release NO (a second messenger activating a soluble guanylate cyclase), which may reach many adjacent cells through its rapid diffusion and high lipid penetrability[119, 126].

Candidate mechano-receptors are ion channels, integrins and integrin-associated proteins, connexins and other membrane based structures[111, 127–129]. The ion channels involved are mechano-sensitive channels susceptive to gadolinium chloride and nifedipine, respectively[111], and the former drug reduced mechanically elevated PGI_2 and NO synthesis[130]. The β1-integrin binds ligands like collagens I & III and fibronectin[131], and fluid flow has been shown to activate MAPK via β1-integrin. In osteoblasts, steady fluid forces up-regulate β1-integrin expression and activate $\alpha_v\beta3$, which co-localizes with src[132–134]. Focal adhesion kinase (FAK) seems to be critical for integrin clustering, while also being a signalling molecule, which is subjected to auto-phosphorylation[135, 136]. Thereafter, FAK contributes to MAPK activation via interaction with c-src, Grb2, and Ras[136–138]. FAK activation also increases the activity of PLCγ1, which is involved in Ca^{2+}-fluxes, since fluid force elicited Ca^{2+}-mobilization requires PLC-mediated IP_3-release[111]. Shear stress has been shown to increase the expression of connexins, making cells more communicative through gap junctions[111]. These channels are often located to dendritic processes, and osteocytes have been shown to increase PGE_2 (an anabolic bone factor), when

mechanically stimulated[111, 129, 139]. Membrane structures, like lipid rafts, have also been shown to integrate mechano-stimulation, conveying the signal to the cell interior[140, 141]. Mechanical forces translocate signalling molecules to the cavaolae, involving activation of signalling molecules like MAPKs, such as ERK1/2, small GTPases, GEFs, RhoA, and Rac1[142, 143].

In general, mechano-stimulation will activate many of the same signalling systems like, for example, VEGF-mediated MAPK-enhancement and cAMP/cGMP- and DAG/IP_3-mediated signalling[118, 144–146] (Figure 4). In osteoblasts, mechano-stimulation increases both cAMP and IP_3 levels. Cyclic AMP may be stimulated by PGE_2-release, however, many G-proteins like $G\alpha_q$ and $G\beta\gamma$ may activate GTP-ases like Ras and Rho GTP-ases. The PGE_2-mediated cAMP increase leads to enhancement of connexin-43 expression through CREBP-activation[111, 139, 145–147]. Calcium spikes in osteoblasts can be obtained from mechano-sensitive ion channels, but also from IP_3-stimulated opening of calcisomes, leading to increased COX_2 activity and c-fos activation[148]. MAPK is involved through stimulation of the cascade $ERK_{1/2}$, p38 MAPK, BMK-1, and JNK. Following the up-regulation of MAPKs activities is a down-regulation of RANK-L secretion and an increase in the expression of eNOS. As a result, eNOS-induced NO-synthesis ensues, which eventually leads to reduced RANK-L expression through activation of the guanylate cyclase, yielding the cells low in RANK-L/OPG-ratio[111, 119, 126, 149].

In terms of cell engineering, one may ask the following questions: which type and application schedule of mechanical forces are producing the better osteoblasts and chondrocytes for cell replacements? Which are the major mechano-transducing molecules in these cells? And which of the signalling pathways are more prone to the detrimental effect of senescence and disease?

The Wnt- and Notch-pathway of signalling

The Wnt-pathway along with the Notch-pathway of signalling[2, 20, 41, 150–152], may serve as switch types of regulators in bone, as well as cartilage remodelling, since they are involved in the stabilization of recruitment of SCs to the different cellular species, as well as the acquisition of precise phenotypic features. The increase of ROS with age antagonizes the skeletal effects of Wnt/β-catenin by diverting β-catenin from TCF- to FoxO-mediated transcription. Activation of FoxO-mediated transcription by ROS via JNK is deemed to lead to the processes of senescence. The adapter protein p66shc is activated by increased intracellular ROS and also generates ROS in the mitochondria. The Wnt/β-catenin signalling cascade is, on the other hand, related to bone remodelling and/or modelling. Activation of the LRP5/6-fz receptor complex by Wnts leads to inactivation of GSK-3β, which prevents the proteosomal degradation of β-catenin and, thereby promotes its accumulation in the cytoplasm. Upon its translocation into the nucleus, β-catenin associates with the TCF/LEF family of transcription factors and regulates the expression of Wnt target genes. With increasing age, increased ROS production diverts the limited pool of β-catenin from TCF/LEF to Foxo-mediated transcription, thus tilting the balance. This shift of the balance may be responsible for the conversion of the beneficial effects of Wnt/β-catenin on bone (*ie* enhanced osteoblastogenesis, and reduced apoptosis of osteoblasts, as well as diminished osteoclastogenesis). FoxO-mediated signalling increases transcription of the superoxide dismutase gene, as well as the genes for catalase and Gadd45, while β-catenin/TCF/LEF enhance the levels of Axin2, OPG, and ALP, of which Axin (along with APC) ensures binding to β-catenin/TCF/LEF. Thereby, the Wnts will ensure transcription of genes leading to bone accrual, also overcoming the problem of diminished osteob-

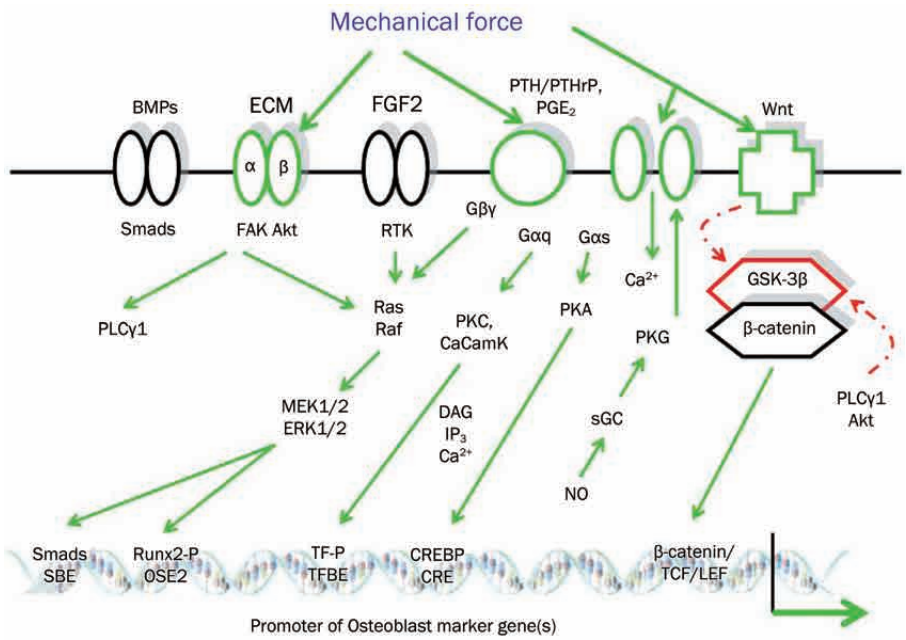

Figure 4. Mechano-stimulation of osteoblasts (and chondrocytes). Signalling molecules like BMPs, FGFs, G-protein activating hormones, the extracellular matrix (ECM), and Ca^{2+}-channels are impinging on intracellular signalling mediators converging towards transcription factors (TFs) and TF-modulating proteins determining gene transcriptional activities, here exemplified by TF-binding elements in osteoblasts associating with Smads, Runx2, CREBP, and β-catenin.

last differentiation and maintenance of bone tissue due to the onset of senescence[39, 151] .

The Notch-signalling mechanism leads to suppressed osteoblast differentiation. Direct interaction of Notch with its ligands, Delta or Jagged, leads to the cleavage, release, and nuclear translocation of its intracellular domain (NICD). In the nucleus, NICD forms a complex with CSL proteins and masterminds the regulation of gene transcription. This complex or the product of the NICD target gene, Hey1, binds to Runx-2 to inhibit osteoblastogenesis. NICD also inhibits Wnt signalling mediated by β-catenin and, either directly or through its interactions with FoxO1, has the potential to inhibit NFATc1 signalling. The down-regulation of NFATc1 eventually leads to the inhibition of osteoblastogenesis. In addition, the abundance of RANK-L is decreased by Notch signalling, whereas that of OPG is increased, which leads to impaired osteoclastogenesis. Hence, the Notch pathway interferes with the Wnt-pathway by inhibiting its effect on osteoblastogenesis. However, the direction of the overall switch mechanism (between the anabolic and catabolic stages) depends on the levels of FoxO1, Runx2, APC/axin, β-catenin, as well as other factors[151, 152].

The Wnt/β-catenin pathway in osteocytes (as well as osteoblasts and to a certain extent chondrocytes) responds to mechanical loading[39, 111]. Mechanical load applied to bone is perceived mostly by the osteocyte through an unknown mechanism, although induced fluid flow through the lacunar-canalicular system may be a critical component. Perception of load (strain) triggers a number of intracellular responses including the release of PGE_2 through a poorly understood mechanism into the lacunar-canalicular fluid, where it can act in an autocrine and/or paracrine fashion. In this, connexin-43 hemi-channels (Cx43HC), PGE_2 and integrin proteins appear to be involved. Binding of PGE_2 to its EP2 and/or EP4 receptor, leads to a downstream inhibition of GSK-3β, (likely mediated by Akt) and the intracellular accumulation of free β-catenin, but integrin stimulation can also lead to Akt activation and GSK-3β inhibition[39, 41]. New evidence suggests that the endoplasmic reticulum may participate in the nuclear translocation of β-catenin, which leads to changes in the expression of a number of key target genes. One of the apparent consequences is the reduction in sclerostin and Dkk1 (Wnt inhibitors), with ensuing increased expression of Wnt. The net result of these changes is to create a permissive environment for the binding of Wnt to Lrp5-Fz and an amplification of the load-related signalling[151, 152].

Micro-RNA as phenotype controllers

Scrutinizing the above mentioned mechanisms responsible for pre-commitment of either osteoblasts or chondrocytes and the many factors responsible for the sequential acquisition of phenotypic characteristics, it seems mandatory to look for ways the cells may control the developmental processes and secure minimal or non-existent de-differentiation or trans-differentiation between them. Such a system may be constituted by a species of small RNAs, designated microRNAs[153–155].

MicroRNAs are small RNAs, 21–25 nt in length, encoded in the genome, and exert important regulatory roles[156–158]. Most of the microRNAs more or less perfectly match the sequence of mRNAs and result in their degradation. These small RNAs reside within the introns of other genes or, more commonly, they can be located outside genes. They may be found in polycistronic clusters or exist individually[159, 160]. Hence, some may be regulated in parallel with other genes and some may not. However, most microRNAs are transcribed by polymerase II from flanking promoters and contain caps. Firstly, a pre-miRNA is processed into a stem-loop structure (60−80 nt) by the RNA endonuclease Drosha. These pre-miRNAs are exported into the cytoplasm, where the hair-pin is cleaved by Dicer, releasing a miRNA duplex. The two strands separate and release one 21−25 nt mature microRNA. This microRNA is incorporated into a protein complex (RISC), which interacts with its target mRNA by base-pairing, preferentially in its 3'UTR[159, 160]. MicroRNAs act through two mechanisms; mRNA cleavage or translational repression of mRNA without cleavage. However, most animal microRNAs suppress gene expression by repressing protein translation (Figure 5A) and/or enhance mRNA degradation[161]. Presently, more than 1200 microRNAs encoded by the human genome have been published. Furthermore, genomic sequences of putative microRNAs have been reported.

Each microRNA species may target hundreds of mRNAs and some targets are combinatorially affected by multiple microRNAs. In this context, it should be noted that the wiring of most microRNA target pairs depends on minimally complementary "seed" matches that are experimentally insufficient to confer overt repression. Hence, it should be expected that focussing on single species of microRNAs in loss-of and/or gain-of experiments may not yield significant findings, while a group or cluster approach may do so[157, 158, 162]. However, the latter approach is both tedious and costly to perform.

The role of microRNAs in gene regulation may be regarded as feed-forward loops to safeguard the complete repression of certain gene transcripts related to a given cell phenotype[153–155], with reference to the cell cycle ("beyond the point of no return"). In a coherent model, TFs repress transcription of a certain target gene, while also stimulating the expression of microRNA(s) responsible for nullifying the translation of "leaking" mRNAs of the same target gene (coherent FFL). Alternatively, the TFs may enhance the expression of a certain target gene, while also inhibiting the expression of microRNA(s) repressing the same target gene (coherent FFL). This gives the opposite result, *ie* a continuous exposure of the cellular environment to the gene product in question. A third variant (incoherent FFL) is that a set of TFs augment the transcription of a certain target gene, while simultaneously inhibit the expression of microRNA(s) repressing the same target gene. This arrangement yields an unstable, or a transition state for the expression of the target gene in question. This mental modelling exercise for elucidation of the action of microRNAs may be extended to include co-activators of TFs, associated proteins, and repressors (Figure 5B).

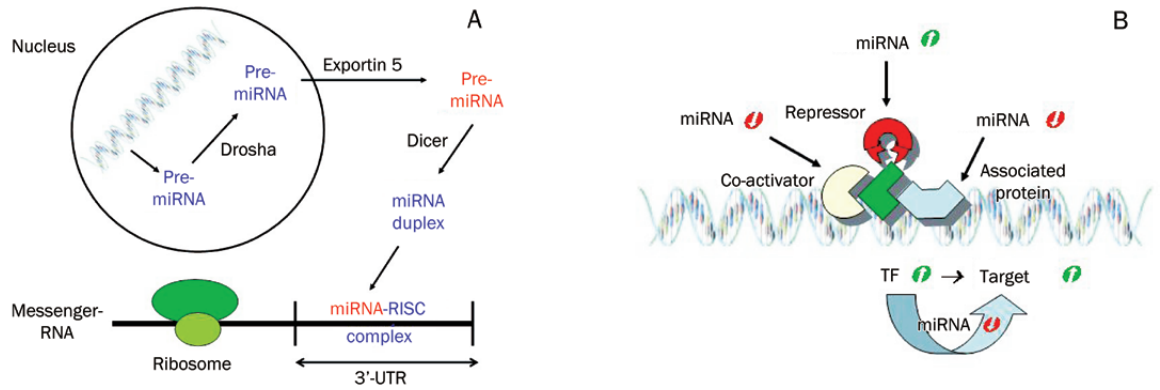

Figure 5. Production of microRNA-RISC complexes (A) and the effect of microRNA species on target gene transcripts related to the integrated effect of transcription factors (TFs) and TF-modulating proteins (B). Pri-miRNAs are transcribed, trimmed by Drosha to pre-miRNAs, exported to the cytoplasm, made into duplexes by Dicer, and incorporated into a miRNA-RISC complex. This construction will bind to mRNA species and block the translational process and/or facilitate degradation of mRNAs. The microRNA species may target TFs, however, one should also take into consideration which of several known repressors, co-activators or associated proteins might be affected by the action of other microRNA species. Together, several microRNAs will ensure a proper time-related expression of genes characterizing a given cell phenotype.

Hence, clusters or small families of microRNAs may appear important to identify and follow through the entire differentiation process to be able to identify the minimal group of microRNAs to ensure proper differentiation from stem cells (SCs) or parent cells[157, 158, 163]. It is therefore not straightforward to assert that a microRNA species exerts a static role in terms of gene translation control. The impact of microRNAs may be classified in several ways in terms of targets, level of target repression and time-course of their presence in a certain cell phenotype[153–155]. One model of microRNA classification action depends on whether their major effect is mediated through one or a few targets or through many targets (tens or hundreds). All known genetic switches concern cases of one or a few important microRNA targets, although it is theoretically possible for one microRNA to have many genetically important targets. In a setting, in which hundreds of targets are simultaneously repressed by a given microRNA, it may be that one of the individual regulatory events is particularly important, but that the system collapses when all the regulatory links are broken. In such a setting, a microRNA might serve as a global enforcer of a cell or organ identity[154].

Nevertheless, the microRNA system may represent the controlling "switch" in the transition state between two different cellular phenotypes, *eg* the transition of MSCs into osteoblasts or chondrocytes. A further consequence of this concept is that, in the transition phase between phenotypes, there is a co-expression between certain microRNAs and their target genes, while before and after, there is an inverse relationship between microRNAs and their target genes.

Some mammalian microRNA species appear to be ubiquitously expressed, but most have been found to exhibit developmentally regulated expression patterns in a variety of normal and cancer cells and tissues[159, 164–166], as delineated in the above described conceptual model systems. Many microRNAs are specifically expressed during embryonic stem cell (ESC) differentiation and embryogenesis, as well as during brain development, neuronal differentiation and differentiation of haematopoietic lineages[167–176]. Most of these microRNAs are more or less unique to hESCs, however, some might also be present at a lower amount (*cfr* the incoherent pathway of regulation). This indicates that most hESC microRNAs are subject to a regulation of the type 1 coherent FFL, indicating that one might look amongst genes that will be switched on during differentiation to incorporate them into such FFLs.

In this review, however, we will only concentrate on microRNAs reported to exist in MSCs and which microRNAs have been shown to exert important functions in osteoblasts/osteocytes and chondrocytes. The following microRNA species have been found to exist in hESCs: miRNAs Let-7a, 15b, 16, 17, 18ab, 19b, 20, 21, 29a, 92, 93, 106a, 127, 130b, 134, 143, 154, 200c, 222, 290, 291, 292, 293, 294, 295, 296, 302abcd, 367, 368, 371, 372, 373, 494 [168–171]. Many of these microRNAs have been demonstrated to be involved in cancer development[159, 165, 166], however, most of them are definitively involved in the regulation of gene-related pathways being important for self-renewal[167, 177–180]. Among the genes highly expressed in different hESC lines and tissues, were TFs like Oct3/4, FoxD3, Sox2, and a DNA methyl-transferase DNMT3B. In addition, genes involved in the Wnt/β-catenin signalling pathway, such as Frizzled 7, Frizzled 8, and Tcf3 were also highly expressed. Furthermore, also all 4 variants of the FGF family were expressed in hESCs[177–180]. Finally, it was also shown that, in six different hESC lines, the genes for Oct3/4, Nanog, GTCM-1, connexin 43/GJA1, TDGF1 and Galanin were highly expressed[167, 177–180]. A cross-comparison of the gene lists generated by these efforts shows that Oct3/4, Nanog, Sox2, Rex1, DNMT3B, Lin28, TDGF1, and GDF3 are commonly expressed in all hESCs.

When it comes to haematopoietic stem cells (HSCs), a cross-comparison among efforts to demonstrate components evolutionally conserved and developmentally prominent regulated pathways, the following list emerged: Wnt pathway (Lef1,

Tcf4, Dsh), the TGFβ super-family (BMP4, Activin C, serine, and threonine kinases NIK and Ski), the Sonic hedgehog (Shh) pathway (Smoothened, SMO), the Notch family (Notch 1 and Manic Fringe), members of the homeobox regulatory cascade (Hoxa9, Meis-1, TGIF, and Enx-1) and Bmi-1[167, 177–180].

Using different search engines available (MirnaViewer, Sanger, PicTar, and others) for some of the human MSCs deemed necessary for self-renewal, it is possible (according to the concept of coherent FFLs) to arrive at some predicted microRNAs responsible for the arrest of the stem cell phenotype, *ie* the identification of microRNAs putatively important for the onset of differentiation. The following result was achieved for the "disappearance" of the first member of the different gene families, *ie* Lef1, BMP4, NIK, SMO, Notch1, and Hoxa9 (Table 1).

Table 1. *In silico* search for microRNA species targeting transcripts of family members of evolutionally conserved and developmental prominent genes shown to be important for the self-renewal and/or pluripotency of stem cells (SCs).

Gene	MicroRNA (according to MiRNA Viewer and PicTar)
Lef1	22, 24, 26ab, 34abc, 93, 145, 149, 193, 302abcd, 320, 370, 372, 373
BMP4	206, 337
NIK=MAP3K14	17-5p, 19ab, 20, 27ab, 93, 106ab, 130ab, 155, 204, 211, 214, 301, 302abcd, 326, 331, 345, 370, 372, 373
SMO	326, 346, 370
Notch1	15a, 15b, 32, 34abc, 125a, 125b, 139, 195, 223
Hoxa9	Let-7abcefgi, 19b, 26ab, 32, 96, 98, 99, 101, 126, 128ab, 139, 144, 145, 147, 182, 186, 196ab, 199, 205, 301

If one compares the cluster of predicted microRNAs affecting the expression of the above mentioned genes responsible for stem cell renewal with published lists of microRNAs being associated with hESCs, one will notice that Lef1 may be heavily affected by the observed microRNAs 93, the 302-series and the 367–374 series; NIK expression may be blocked by a cluster of microRNAs consisting of 17-5p, 19, 93, 106a, 130b, 301, the 302-series, and the 367–374 series; and Hoxa9 might be down-regulated by microRNAs 19b, 26ab, and 301. Interestingly, one paper recently published *in silico* and *in vivo* studies corroborating these speculations[181].

This yields 3 out of 6 genes, which may be affected directly by known microRNAs expressed in hESCs, and thus, these microRNAs may serve the purpose of "priming" the mRNAs for repression until some crucial, other microRNAs come along. These could be up-regulated during differentiation processes and may be found within the group consisting of, amongst others: Lef1 (microRNAs 22, 24, 34a, 145, 149); NIK (microRNAs 20, 211, 214, 326, 331, 345); Hoxa9 (microRNAs 128, 139, 147, 196, 205). Indeed, many of the microRNAs listed immediately above, like microRNAs 17-5p, 22, 24, 34ac, 125ab, 128b, 149, 193, 326, and 337 actually are putative targets of most of the transcription factors which are listed in Figure 3. Hence, many switch microRNAs suppressing osteoblast differentiation may be found amongst these microRNA species.

However, only a few articles published over the past 3 years have dealt with microRNA expression and function in osteoblasts and chondrocytes[182–186]. Some interesting findings deserve mentioning. Mir-125b has been shown to down-regulate osteoblastic cell differentiation, by down-regulating cell proliferation, where it targets VDR and possibly also ErbB2 and osterix[184, 186]. BMP2-induced osteoblast differentiation involves mir-135 and mir-133, which target Smad5, a mediator of the BMP-2 signalling, as well as Runx2. Several other microRNAs are also mentioned (miRNAs 9, 17, 27, 29, 30, 96, 106, 133, 138, 181, 182, 320, and 326)[183]. Recently, mir-29b has been shown to contribute to the positive regulation of osteoblast differentiation[185], targeting inhibitors of osteoblastogenesis, like the transcripts and/or proteins encoded by the HDAC4, TGFβ$_3$, ACVR2A, CTNNBIP1, and DUSP genes. Furthermore, silicate-based synthetic bone up-regulated miRNAs 26a, 30b, and 92, while reduced miRNAs 337, 377, 25, 200b, 129, 373, 133b, and 489, while finally, bone prosthesis material (Anatase®) appear to exert biological effects on bone cells, since miRNAs 1, 34c and 210 exhibit a significant up-regulation, while miRNAs 17-5p, 22, 23, 24, 93, 130, and 377 are diminished[187–190]. In chondrocytes from growth plates, 30 microRNAs were preferentially expressed, however, these were scarcely expressed in osteoblastic cells (*ie* miRNAs 1, 10, 22, 122, 127, 134, 196, 202, 206, 299, 300, 322, 329, 362, 376, 377, 380, 381, 409, 410, 431, 433, 434, 495, 496, and 500)[182]. Interestingly, some of these microRNAs were virtually absent in osteoblasts (*ie* miRNAs 196, 202, 380, 434, 496, and 500). It should also be mentioned that miRNA 140 has been demonstrated to target histone deacetylase 4 (HDAC4) in chondrocytes, apparently allowing an increase of Runx2 functioning during the phase of hypertrophia and endochondral ossification[191]. Finally, it was reported that mir-199b, a BMP-2 responsive microRNA, regulates chondrogenesis in a time-dependent fashion via direct targeting the Smad1 transcript[192].

At the moment, no other microRNAs than mir-125b, mir-133b, and mir-135a distinguish themselves due to experimentally proven effects and target-analysis, as one singular microRNA, to play the role as a switch between osteoblast and chondrocyte commitment and/or phenotype acquisition and stability. When searching through lists of genes important for early phase chondrogenesis derived from the literature[10] and/or the GenoStem array[193], miRNAs 125b, 133b and 135a appear (to a major extent) to leave such genes unperturbed. Mir-125b is expressed in very large quantities in chondrocytes[194], so is also the case for mir-135b, while mir-133a seems to much less abundant. Hence, these microRNA species may either introduce a collapse of the system maintaining all the important cellular functions constituting the chondrocytic phenotype, or also serve as a switch away from osteoblastic development (*ie* pre-commitment). Mir-135a targets BMPR1a, BMPR2, Jak2, Msx2, Smad5, and Stat6, while mir-133b targets Runx2, FGFR1, and TCF7[183], signifying that

they possess powerful abilities to knock down osteoblastogenesis in their own right. Mir-125b, which overtly hampers the expression of genes responsible for perpetuating the cell cycle[184, 186], may be more ubiquitous in its nature, and thus affect many more cell types than osteoblasts and chondrocytes.

However, according to the newly proposed concept of transcription factor (TF) and microRNA interactions[156], where TF-TF and microRNA-microRNA regulatory loops are favoured over TF-microRNA loops, one may speculate whether or not clusters of microRNAs targeting a certain set of TF transcripts may play a more important role than regulatory loops consisting of only two elements. Hence, we performed a literature search for TFs important for osteoblastogenesis (Figure 2C) and an *in silico* analysis for putative microRNAs targeting two or more of 14 transcriptional modulators selected (APC2, Runx2, RNF11, TAZ, Osterix, SP3, Satb2, VDR, ATF4, Dlx5, ETS1, NFATc1, HES1, and LEF1). From a list of putative microRNAs, we selected six (miRNAs 16, 24, 125b, 149, 328, and 339) for further analysis. It appeared that these microRNAs were virtually non-existent in differentiating osteoblasts, while abundantly expressed both in MSCs and differentiating chondrocytes[182]. Their putative targets were also confirmed using double luciferase-containing psiCHECK2 vector constructs with different 3'UTR sequences [194]. Interestingly, the 14 transcriptional modulators seem to be part of a cluster of genes (located by the search engine Ingenuity) inter-connected to TNFα and to p38 MAPK (Figure 6A). When searching for inter-connections between the transcriptional modulators and other proteins (using the PINA search engine), 25 gene products emerge, of which 23 are transcriptional modulators, one is a protein kinase C type (PRKCA), and one is a histone acetyl transferase (EP300) (Figure 6B). Hence, it is quite clear that the transcriptional modulators chosen as targets for osteoblastogenesis are heavily involved in complex transcriptional processes. Targeting the chosen microRNA species with specific antago-miRNAs did block the $TGF\beta_3$- and BMP-2 induced differentiation of MSCs towards chondrocytes. The subject microRNA species act synergistically and apparently serve as "switches" between the osteoblastic and the chondrocytic phenotypes[194]. This is in line with the concept that one microRNA species may reduce the "activity" of a given mRNA by at most 50%, and that more microRNA species, acting synergistically, may reduce the EC_{50} from some 200−250 pmol/L for one microRNA acting alone, down to almost zero, when more than 6−7 work together[195]. Interestingly, these microRNAs (miRNAs 16, 24, 125b, 149, 328, and 339) appear to be both necessary and sufficient to fully modulate *in vitro* dexamethasone-induced osteoblastogenesis, but only partly for TGFβ-induced chondrogenesis using human MSCs[194] during a differentiation period of 21 days.

Finally, it should be mentioned that cells are able to shed micro-vesicles (exosomes) containing many types of signalling molecules, as well as microRNA species, which may be taken up into adjacent cells of different phenotypes[196, 197]. This phenomenon adds to the complexity of microRNA and TF interactions in tissues containing multiple cell types. For example, activated immune cells (*ie* Th-1>Th-2=Th-17 cells) appear to express very high levels of mir-24, mir-34a, and mir-296, which putatively target the transcriptional modulators APC2, ETS1, LEF1, Satb2, VDR, and Sox9. The subject microRNA species may thus serve as a "switch" tilt the chondrocyte and/or MSC phenotypes towards the osteoblast phenotype, as seen in hypertrophic and senescent articular chondrocytes[194, 198]. Other microRNAs (like mir-16 and mir-125b) known to be highly expressed in chondrocytes also appear much more abundantly in blood from patients with rheumatoid arthritis (RA)[194, 199]. It is therefore possible to use various microRNA species as diagnostic and/or prognostic biomarkers[196, 197] for both severity and/or drug responsiveness in inflammatory processes leading to destruction of bone and cartilage due to rheumatoid conditions.

Concluding remarks

Many qualities may be deemed necessary for engineered cells to exhibit, some of which are: *in vitro* functionality, ability to interact with the local environment *in vivo*, ability to arrange themselves in a real-time 3D-structure, self-correction of phenotype according to their position and exposure of O_2, nutrients, hormonal and GF gradients, and phenotype resilience. The latter characteristic is construed as the ability to withstand wide variations in mechano-stimulation, short- and long-term alterations in access to certain nutritional elements, as well as robustness to counteract local inflammatory processes. Probably, the understanding of the links between important signalling and especially TFs and microRNAs involved in regulatory loops evolving throughout the life-span of osteo-chondral cells, will enable the cells to acquire the above mentioned qualities for a successful cell replacement therapy.

In this respect, a successful approach to obtain engineered cells for replacement of bone and cartilage maybe target a set of microRNAs using pre-miRNAs and/or antago-miRNAs and siRNA modulating selected TFs into polycistronic vector constructs to ensure acquisition of proper characteristics of engineered osteoblasts or chondrocytes. One may also exploit the fact that cells may communicate with each other through micro-vesicles (exosomes) containing microRNAs, thus enabling the subject cells to withstand alteration of phenotype, when being confined to a multi-cellular micro-environment (*eg* in an inflamed joint invaded by immune cells like Th-cells). MicroRNAs from immune cell signatures or osteoblast/chondrocyte signatures may therefore serve as biomarkers to characterize the severity of inflammatory processes in bone and cartilage and/or their responsiveness to drug therapy.

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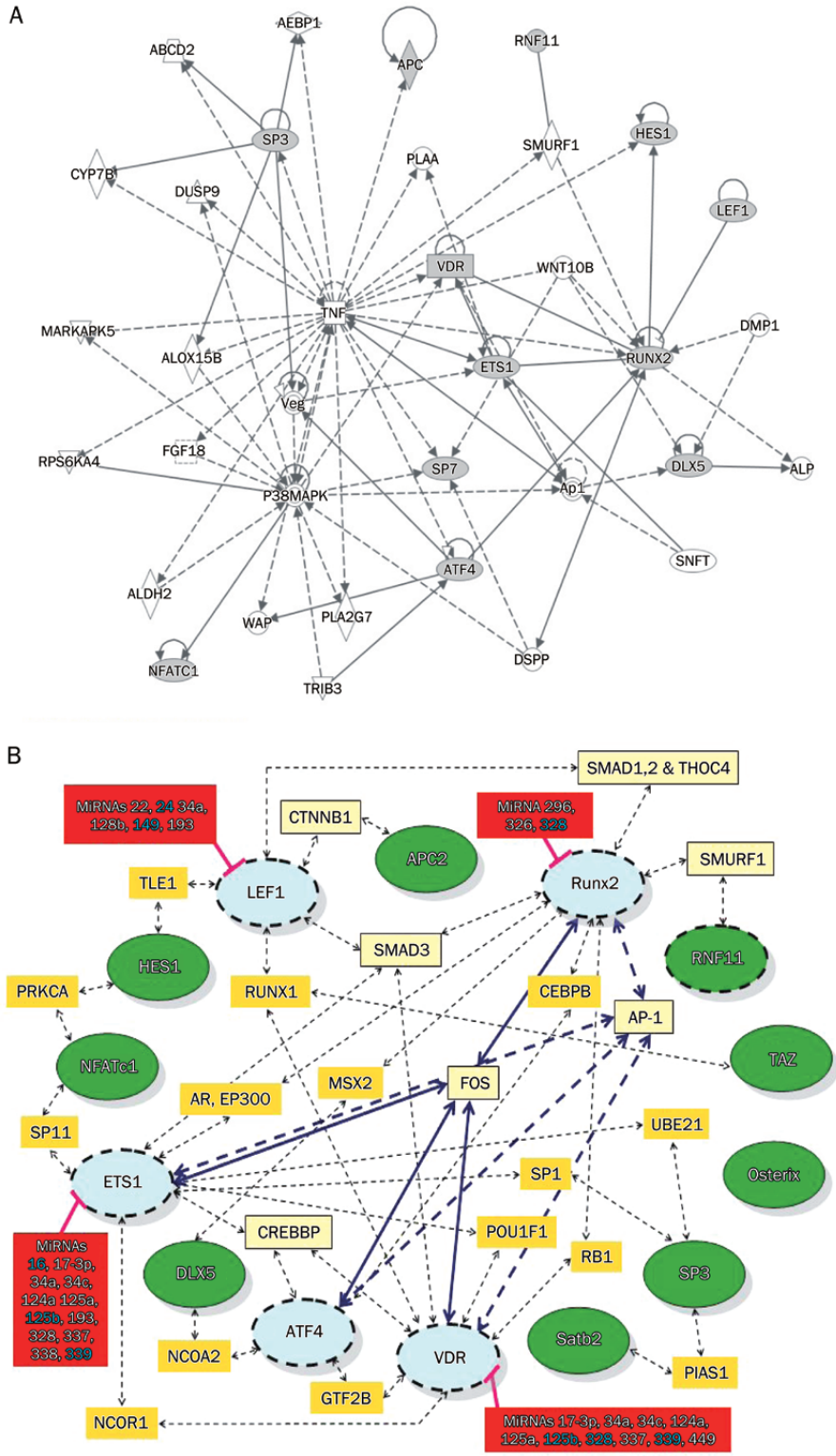

Figure 6. Transcription factors (TFs) deemed important for osteoblastic development were modelled using the Ingenuity® (A) and the PINA® (B) algorithms. The gene-gene and the protein-protein interactions of fourteen TFs with other genes and proteins were analysed, and the following information emerged: Gene-gene interactions gave a *P*-value $<5\times10^{-13}$, signifying an extremely strong relation to osteoblasts, and two "junction" genes (p38 MAPK and TNF) seem to be especially important (A). Furthermore, the protein-protein interaction (each TF with more than one interacting protein are shown) indicated that most of these interacting proteins are TFs (23 out of 25). The microRNAs indicated in blue have been verified (using psiCHECK2® vector-constructs) as to their targeting of four of the five TFs being heavily associated with other proteins (B).

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