Bone and Cartilage from Stem Cells:
Growth Optimalization and Stabilization of Cell Phenotypes

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Abstract

Osteochondral cells engineered from progenitor cells to replace damaged bone and cartilage can be obtained through a diversity of single manipulations. However, the selection of type of manipulations, to which these progenitor cells should be subjected, is not straightforward. Here, we describe the recently accomplished progress of osteochondral tissue engineering, trying to envisage a way to establish an algorithm for multifactorial manipulations of progenitor cells. To be taken into consideration is the tissue engineering process per se, but we also try to account for special needs related to the detrimental influence of inflammation on phenotype stability and premature development of senescence.

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1. Introduction

Engineered cells replacing tissues should mimic the three-dimensional (3D) structure and reflect the different cell phenotypes exhibited by the lost or damaged tissue (Raimondi 2006; Keung, Healy et al. 2010). The engineered cells should also demonstrate a certain plasticity, i.e. an ability to adapt to the environment, in which they are deposited, reflecting the minute differences in features necessary to rebuild a functional tissue, which is able to renew itself over time (Grad and Salzmann 2009; Ohishi, Chiusaroli et al. 2009; Tare, Kanczler et al. 2010).

Osteoblastic cells in bone need to be able to involve themselves in a remodelling cycle with osteoclasts (Hanada, Hanada et al. 2010; Trouvin and Goeb 2010), which may be recruited from surrounding bone structures, and/or may be furnished as preosteoclasts within the population of osteoblasts. Furthermore, the osteoblasts should be able to undergo a distinct alteration in terms of life-span defined characteristics (Lian and Stein 2003; Lian, Stein et al. 2006; Gordeladze, Reseland et al. 2009), including the transition to osteocytes. Finally, the newly formed engineered tissue does not survive unless it develops a vascular network (Matsumoto, Kuroda et al. 2008; Grellier, Bordenave et al. 2009) furnishing the bone tissue with oxygen, growth factors and nutrients.

Chondrocytes in engineered cartilage should be able to produce an extracellular matrix reflecting the composition, water-binding capacity and mechanical characteristics of true hyaline cartilage (Knecht, Vanwanseele et al. 2006; Heinegard 2009; Bertrand, Cromme et al. 2010; Goldring and Goldring 2010). This type

of cartilage exhibits certain features, such as hypoxic conditions and chondrocytes demonstrating gradients of gene transcript levels (cell phenotype plasticity) between the juxta-luminal and bone-lining surfaces of a joint (Grimshaw and Mason 2001; Lu, Subramony et al. 2010; Oh, Kim et al. 2010).

The features described above have been extensively described and confirmed in the literature, however, a joint approach to produce well adapting engineered osteoblasts and chondrocytes has hitherto not been the subject of review articles or book chapters. The present outline encompasses a combined literature review of phenomena to take into consideration when engineering such cells from stem cells (SCs): sources of SCs to use (Logeart-Avramoglou, Anagnostou et al. 2005; van Osch, Brittberg et al. 2009), genes or microRNAs to manipulate (Goldring, Tsuchimochi et al. 2006; Betz 2008; Grundberg, Brandstrom et al. 2008; Duggal, Fronsdal et al. 2009; Gordeladze, Djouad et al. 2009; Lin 2009; Granchi, Ochoa et al. 2010; Sun 2010; Herlofsen, Kuchler et al. 2011), selection of gene and microRNA delivery systems (Saraf and Mikos 2006; Phillips, Gersbach et al. 2007), choice of humoral growth factors to facilitate SC differentiation (Shahdadfar, Fronsdal et al. 2005; Boeuf and Richter 2010; van der Kraan, Davidson et al. 2010), selection of appropriate scaffolds to support "asymmetric" SC differentiation" (Vinatier, Bouffi et al. 2009; Oh, Kim et al. 2010; Seidi, Ramalingam et al. 2011), combination of stem cell niches and/or co-cultures to ensure organ mimicry reflecting proper cell-cell interactions (Grad and Salzmann 2009; Grellier, Bordenave et al. 2009; Boeuf and Richter 2010; Tare, Kanczler et al. 2010), mechano-stimulation of cells (Kelly and Jacobs 2010; Nowlan, Sharpe et al. 2010), and three-dimensional (3D) organ printing (Williams 2009; Visconti, Kasyanov et al. 2010).

Furthermore, this review also discusses how to stabilize osteoblasts and chondrocytes obtained by differentiation of SCs, i.e. how we can make the subject cells resilient to the detrimental effects of inflammatory cytokines and T-cells (Gordeladze, Reseland et al. 2009; Gruber 2010; Hanada, Hanada et al. 2010; Pacifici 2010), and exosomes shredded from immune cells (Valadi, Ekstrom et al. 2007; Camussi, Deregibus et al. 2010; Zomer, Vendrig et al. 2010).

2. Sources of stem cells for osteoblast and chondrocyte differentiation

Osteoblast differentiation

Bone marrow, which is the natural repository of osteoblasts, is widely used as source for bone engineering. Under appropriate conditions, bone-derived stem cells (bone mesenchymal stem cells = bone MSCs) can differentiate into osteoblasts, chondrocytes, adipocytes, and stromal cells (Javazon, Beggs et al. 2004; Otto and Rao 2004; Logeart-Avramoglou, Anagnostou et al. 2005). The differentiating potency of bone MSCs was enhanced when embedded in diffusion chambers or organ capsules, however, there is now a plethora of scaffolds securing the development of "proper" osteoblasts to produce bone for tissue replacement purposes. The advantage of using bone MSCs is related to the large number of obtainable osteoblasts, their high number of passages before the differentiating potential is lost, and their ability to be stored frozen for a long period. And the default pathway of bone MSCs is the osteogenic pathway (Logeart-Avramoglou, Anagnostou et al. 2005).

During the past 10-12 years, many other stem cell sources with osteogenic potential have been isolated, including blood, adipose tissue, lung, synovium, skeletal muscle and tooth pulp (for review, (Barry and Murphy 2004; Logeart-Avramoglou, Anagnostou et al. 2005; Gordeladze, Reseland et al. 2009; Bodle, Hanson et al. 2011; Levi and Longaker 2011; Witkowska-Zimny and Walenko 2011)). However, it seems that adipose stem cells (ASCs), provided that they are similar to MSCs in terms of surface receptor molecule profile (STRO-1, CD34, CD45, CD117 negative; CD44, CD49 CD29, CD90, CD105, CD106 positive) (Logeart-Avramoglou, Anagnostou et al. 2005; Niemeyer, Krause et al. 2006), may serve as a good source for bone engineering (Bodle, Hanson et al. 2011; Levi and Longaker 2011; Monaco, Bionaz et al. 2011). Irrespective of whether the source encompasses MSCs or ASCs, it seems that "proper" osteoblasts may be obtained if incubation conditions (i.e. the choice of growth factor source) and appropriate scaffolds are employed (Logeart-Avramoglou, Anagnostou et al. 2005; Kanczler and Oreffo 2008; Kwan, Slater et al. 2008; Gordeladze, Reseland et al. 2009; Tiainen, Lyngstadaas et al. 2010; Rahaman, Day et al. 2011; Sabetrasekh, Tiainen et al. 2011).

Chondrocyte differentiation

As for bone engineering, cartilage engineering relies firmly on the use of MSCs and ASCs (van Osch, Brittberg et al. 2009; Boeuf and Richter 2010; O'Sullivan, D'Arcy et al. 2011; Witkowska-Zimny and Walenko 2011). Some other sources, i.e. ectodermal cells like skin and hair follicles, as well as perinatal tissue and umbilical cord blood (Kuhn and Tuan 2010). One article refers to the use of synovial membrane stem cells (SMSCs) and compares their potency for chondrocyte differentiation with MSCs and ASCs (Segawa, Muneta et al. 2009). The criteria for selection of cell source may vary, but the authors focus on the necessity to analyse chondrocytes differentiated from these stem cells and choose the better source depending on how close they resemble the gene expression

profile of mature chondrocytes isolated from hyaline cartilage (Segawa, Muneta et al. 2009; Vinatier, Bouffi et al. 2009; Vinatier, Mrugala et al. 2009).

Differentiating MSCs and ASCs produce all the components constituting ECM and represent the cells of choice for engineering articular cartilage. However, adult chondrocytes isolated from various sources like articular cartilage, nasal septum, ribs or ear cartilage (Kafienah, Jakob et al. 2002; Isogai, Kusuhara et al. 2006) produce de novo cartilage displaying the characteristics of its original tissue (Isogai, Kusuhara et al. 2006). It is therefore more appropriate to use hyaline cartilage as the preferred source of chondrocytes, and a comparison between different sources of hyaline chondrocytes (nasal, costal, and articular) has shown the superiority of costal and nasal chondrocytes in terms of quantity of cartilage formed after subcutaneous transplantation (Isogai, Kusuhara et al. 2006). One major limit related to the use of chondrocytes, is their instability in monolayer culture resulting in loss of phenotype (i.e. loss of collagen II, aggrecan and superficial zone protein = SZP (Darling and Athanasiou 2005). Loss of the chondrocytic phenotype is accompanied by a phenotypic shift towards fibroblast like cells, which is characterized by an enhanced expression of collagen I (Schnabel, Marlovits et al. 2002). This dedifferentiation process is reversible, and dedifferentiated chondrocytes arranged in a three-dimensional (3D) lattice may retrieve their differentiated phenotype (Domm, Schunke et al. 2002; Malda, van Blitterswijk et al. 2003). This is especially true for dedifferentiated chondrocytes, having been "reversed" some 7-10 days before assuming the fibroblast phenotype (Brinchmann et al., unpublished observations).

The use of chondrocytes from osteoarthritic (OA) cartilage has also been contemplated. However, OA chondrocytes are subject to metabolic alterations leading to a low response to inductive environmental factors (Fukui, Purple et al. 2001; Sandell and Aigner 2001). Although chondrocytes derived from OA patients seem to be less appropriate for articular cartilage repair, it has been reported that OA chondrocytes may resume a normal chondrocytic phenotype upon 3D-cultivation *in vitro* (Tallheden, Bengtsson et al. 2005).

3. Genes and microRNAs as determinants of bone and cartilage quality

Characteristics of transcriptomes

When tissue replacement with the aid of tissue engineering is the ultimate therapeutic goal, it is vital to understand the differentiation process from precursor cells in terms of gene expression. Hence, it is necessary to identify a transcriptome, which is reflecting the "true" osteoblast and chondrocyte phenotypes pertaining to the function and localization of such cells in the skeleton. Many excellent articles have addressed this task over the past 10 years, of which only some are mentioned here (Kulterer, Friedl et al. 2007; Grundberg, Brandstrom et al. 2008; Duggal, Fronsdal et al. 2009; Morsczeck, Schmalz et al. 2009; Sundelacruz and Kaplan 2009; Bernstein, Sticht et al. 2010; Granchi, Ochoa et al. 2010; Piek, Sleumer et al. 2010; Sun, Mauerhan et al. 2010; van der Zande, Walboomers et al. 2010; Herlofsen, Kuchler et al. 2011). Two transcriptomes featuring putative gene markers of osteoblasts and chondrocytes (two each), respectively will be described in detail.

Grundberg et al. (Grundberg, Brandstrom et al. 2008) used human trabecular osteoblasts stimulated with BMP-2 and dexamethasone for 24 hours. The article refers to genes specific for trabecular bone cells (osteoblasts) and genes up-regulated after 2 and 24 hours incubation with BMP-2 and dexamethasone, as well as genes altered upon 24 hours incubation with dexamethasone alone. Top similarity pathways of cell phenotype modulation (as assessed by Ingenuity®) were the IGF-1, Leptin-, BMP-2- and Wnt-pathways, indicating a good correlation with the bulk of literature on osteoblast differentiation (Gordeladze, Drevon et al. 2002; Komori 2006; Marie 2008; Gordeladze, Reseland et al. 2009). The second paper on osteoblastogenesis (Granchi, Ochoa et al. 2010) was based on incubation of human MSCs in differentiating medium with dexamethasone for 24 hours, and thereafter in mineralizing medium with dexamethasone for 7 days. The main groups for the classifications of up-regulated genes were characterized by: angiogenesis, apoptosis, bone development, cell communication, cell cycle, embryonal development, TGFβ-signalling, and Wnt-signalling. The cumulative gene lists from these reports constituted the osteoblast transcriptome (188 genes) used as osteoblast reference to evaluate osteoblast differentiation (see paragraph 11. Bone and cartilage engineering revisited).

As for the chondrocytic differentiation, it is referred to the papers of Bernstein et al. (Bernstein, Sticht et al. 2010) and Herlofsen et al. (Herlofsen, Kuchler et al. 2011). Bernstein and co-workers used chondrocytes from articular cartilage and MSCs in an intricate array of manipulations. The cells were incubated in a differentiating medium containing TGFβ3 or control medium without growth factor. Transcriptomes obtained from the cells were categorized in gene transcripts up-regulated, down-regulated or "unsteady". The following results were obtained: Genes were classified as belonging to groups designated TGFβ-related, Wnt-pathway, glycans, actin metabolism, integrins/motility, bone development, muscle development, neuronal development, sperm development, and lipid metabolism. Comparisons with published gene ontology (GEO) datasets revealed that 1) MSC differentiation towards the chondrogenic lineage resembled MSC differentiation in mouse embryo limb

buds (endochondral differentiation), and 2) an increasing confluence of proliferating MSCs will resemble the pellet situation in a timely delayed manner (transition from proliferation to differentiation). The paper by Herlofsen et al. describes MSCs differentiated to chondrocytes in alginate beads for 21 days in a standard differentiating medium with BMP-2. The following gene transcripts COL1A1, COL2A1, COL10A1, SOX5, SOX6, SOX9, ACAN, COMP, VCAN, MMP13, ALPL, RUNX2, and SOX8 were analysed by Q-PCR for verification of differentiation. However, a similarity search, where the transcripts for COL2A1 (up-regulated upon differentiation) and CXCL12 (down-regulated upon differentiation) was compared with the time-course of other genes (1072 up-regulated, 898 down-regulated). From these exercises, a joint list was compiled, consisting of 261 genes. This cumulative list of genes was used as chondrocyte reference to evaluate chondrocyte differentiation (see paragraph 11. Bone and cartilage engineering revisited).

These are just some examples of transcriptomes characterizing cell phenotypes subsequent to exposure to differentiating conditions in vitro. Since these experiments have been conducted ex vivo, it is reasonable to anticipate that the ultimate transcriptome can only be revealed if factors and/or conditions like cell sources, growth factors (adapted incubation media), scaffolds or organ printing, mechano-stimulation, gene and/or microRNA manipulations, or gene and microRNA delivery systems are all taken into account when a final "tissue engineering" process or algorithm is selected.

Spectrum of microRNAs expressed

MicroRNAs are short (20-24 nt) non-coding single-stranded RNA molecules that play an important role in regulating cellular gene expression. They function at the post-transcriptional level, by binding mRNA molecules (Gordeladze, Djouad et al. 2009; Lin 2009; Beezhold, Castranova et al. 2010). MicroRNAs have been found to play important roles in mediating fundamental biological processes like proliferation and differentiation in a variety of cells within defined tissues types. Recent reports have suggested that microRNAs may play a significant role in bone and cartilage development (Gordeladze, Djouad et al. 2009; Lin 2009; Sun 2010; Karlsen, Shahdadfar et al. 2011).

MicroRNAs suppress target gene translation by binding to the 3'-untranslated region (3'-UTR) of mRNA, thus repressing translation and/or enhancing mRNA degradation. This requires that the 3'-UTR contains at least one specific 6-7 nt sequence which exhibits at least partial complementarity to a so-called "seed site," located within the 5'-region of the microRNA molecule (Lin 2009; Beezhold, Castranova et al. 2010).

Despite mounting evidence that miRNAs play a significant role in embryonic development and other biological processes, the function of only a handful of miRNAs has been determined thus far. And of these miRNAs, only a small subset has been implicated in cartilage and/or bone development. These are mir-140 (targeting HDAC4), mir-199a and mir-26a (both targeting SMAD1), mir-126 (targeting VCAM1 and HOXA9), mir-125b (targeting ERBB2), mir-145, and mir-146 (Lin 2009). In a recent review article, Gordeladze et. al. summarized reports on microRNA species like mir-29b (targeting HDAC4, TGFβ_3, ACVR2A, CTNNBIP1, and DUSP), mir-125b (target not specified), mir-133b and mir-135a (both targeting SMAD5 and RUNX2), and mir-199b (target not specified) somehow being involved in osteochondral development and skeletogenesis (Gordeladze, Djouad et al. 2009). Other microRNAs involved in TF-interactions belong to the microRNA family of mir-23a-27a-24-2 (targeting APC2, RUNX2 and SATB2) (Hassan, Gordon et al. 2010).

Other studies involving differentiation of human mesenchymal stem cells into osteocytes and chondrocytes implicated a different subset of miRNAs. Mir-638 and mir-663 were found to be up-regulated in chondrocytes, while mir-24, let-7a, let-7b, let-7c, mir-138, and mir-320 were associated with osteocyte maturation (Lakshmipathy, Love et al. 2007). We have also found (Gordeladze et al., unpublished observations) that the microRNA species 638 and 663 are up-regulated in chondrocytes as early as 3 days of differentiation from MSCs, but these microRNAs are also heavily up-regulated in Th-17 cells differentiated from CD4+ naive T-cells after 5 days (Yssel et al., unpublished observations). Mir-638 and mir-663 appear to have the following targets in common (JUN, FOSB, SP3, and MYC, all of which are important for osteoblastogenesis). Finally, a recent survey of the literature revealed that several microRNA species (mir-335-5p, mir-27, and mir-29) directly target molecules involved in the Wnt-signalling pathway (Kapinas, Kessler et al. 2009; Kapinas, Kessler et al. 2010; Wang and Xu 2010; Zhang, Tu et al. 2011).

MicroRNAs directly targeting specific gene markers of bone and cartilage structural ECM molecules have not been indisputably identified, however, many microRNAs have been shown to affect the steady state levels of such molecules, though probably indirectly. These microRNAs are mir-34a, mir-675, mir-21, mir-146a (COL2A1) (Yamasaki, Nakasa et al. 2009; Abouheif, Nakasa et al. 2010; Dudek, Lafont et al. 2010; Kongcharoensombat, Nakasa et al. 2010), mir-140 (COL2A1, ACAN) (Miyaki, Nakasa et al. 2009), and mir-29b (also directly targeting COL1A1, COL5A3, AND COL4A2, as evidenced by the use of 3'UTR reporter assays) (Li, Hassan et al. 2009).

Interestingly, the expression of microRNAs in osteoblasts and chondrocytes seems to be reciprocal, in the sense that the microRNA species highly expressed in chondrocytes are virtually absent in osteoblasts (and vice versa). A series of articles on microRNA expression profiles in osteoblasts grown in a variety of scaffold composite material have recently been published (Annalisa, Furio et al. 2008; Palmieri, Pezzetti et al. 2008; Palmieri, Pezzetti et al. 2008; Palmieri, Pezzetti et al. 2008), however, the microRNA profiles are not overtly compatible with single microRNA studies where proof-of-microRNA-binding has been shown.

The possible role of microRNAs in disease processes like rheumatoid arthritis (RA) and osteoarthritis (OA) has been addressed, and a significant up-regulation of mir-155 and miR-146a in synovial fibroblasts (RASFs) and synovial fluid derived from patients with RA have been documented (Stanczyk, Pedrioli et al. 2008; Duroux-Richard, Presumey et al. 2011). These findings unite the concepts of cell-specific microRNA signatures and microRNA-exchange between cells in the form of exosomes (Valadi, Ekstrom et al. 2007; Zomer, Vendrig et al. 2010). Lastly, predicted polymorphisms in binding sequences for mir-146 in the promoter region of the FGF2 has been have been found (Lei, Papasian et al. 2011), implicating microRNAs even more closely with development and treatment of disease states.

4. Gene and microRNA manipulations and selection of delivery systems

Osteoblasts and bone engineering
Biologists have identified several bioactive factors being able to induce or support bone generation, including BMPs, TGFβ, IGF-1, FGFs, LIM mineralizing protein-1 (LMP-1), VEGF and caALK2 (activin-receptor like kinase-2, mediating BMP-signalling) (for review, see (Betz 2008)). Delivery systems frequently used are viral vectors, adenoviral vectors, retroviral and lentiviral vectors, adeno-associated vectors (AAV), and non-viral vectors (mostly plasmids). Standard transfer procedures comprise electroporation, lipofection and gene-activated matrices (GAM) (Betz 2008). Other osteogenic factors of interest for gene manipulation are RUNX2, SMADs, DLX3, DLX5, AP-1, and SP7 (osterix) (Marie 2008; Gordeladze, Djouad et al. 2009).

Gene therapy may be based on single genes, however, more successful attempts have been made by using combination of genes, such as BMP + VEGF, BMP2 + RUNX2, VEGF + RANKL, IGF-1, BMP-2 + IGF-1, and BMP-2 + SMAD8 (Gersbach, Phillips et al. 2007). Another strategy is to deliver the above mentioned osteoinductive growth factors or hormones in a scaffold material to render a "kick-start" in terms of osteoblast function, cell organization and bone building (Fischer, Kolk et al. 2011). Other applications of gene therapy for osteogenesis, such as for periodontal and craniofacial regeneration, have been described elsewhere (Scheller and Krebsbach 2009; Rios, Lin et al. 2011).

Chondrocytes and cartilage engineering
The concepts of gene therapy for cartilage repair have been thoroughly reviewed by Steinert et al. (Steinert, Noth et al. 2008). Approaches mentioned are stimulation of chondrogenic differentiation (using TGFβs, BMPs, WNT, SMADs, SOX9, Brachyury), stimulation of cartilage matrix synthesis and/or cell proliferation (TGFβs, BMPs, IGF-1, PDGF, type 2 Collagen minigene, COMP, GlcAT-1), inhibition of osteogenesis/hypertrophy growth factors (Noggin, Chordin, PTHrP, SMAD6,7), the use of anti-inflammatory agents (IL-1 blockage, TNFα-inhibition, MMP-inhibition), senescence inhibition, and inhibition of apoptosis (Saraf and Mikos 2006; Steinert, Noth et al. 2008). The delivery systems for chondrogenic genes show many common features to the ones described for enhancing osteoblastogenesis (see above) (Saraf and Mikos 2006; Betz 2008).

5. Choice of humoral factors for differentiation purposes

The differentiation of progenitor cells to osteoblasts or chondrocytes in vitro has been conducted in media containing differentiating factors like Calcitriol, Dexamethasone, BMP2, IGF-1, PDGF, EGF, FGFs, TGFβs, HGF, PTH/PTHrP, (Logeart-Avramoglou, Anagnostou et al. 2005; Steinert, Noth et al. 2008; Tilg, Moschen et al. 2008; Gordeladze, Reseland et al. 2009; Boeuf and Richter 2010; Levi and Longaker 2011; Witkowska-Zimny and Walenko 2011). The choice of such factors, either as a single remedy, or in combinations, most certainly will affect cell phenotype acquisition in different ways (Kulterer, Friedl et al. 2007; Grundberg, Brandstrom et al. 2008; Duggal, Fronsdal et al. 2009; Sundelacruz and Kaplan 2009; Bernstein, Sticht et al. 2010; Granchi, Ochoa et al. 2010; Piek, Sleumer et al. 2010; Herlofsen, Kuchler et al. 2011). Thus, the outcome of the differentiation process is not easy to predict.

In vitro differentiation normally requires fetal bovine serum (FBS), however, FBS rises a concern over infections, possible immunological reactions to xenogenic peptides and inorganic compounds (like non-human sialic acid) (Hattori, Nogami et al. 2008). Hence, the use of serum-free incubation media is warranted. It has been shown that

MSCs grown in serum free-media will acquire both osteoblast and chondrocyte phenotypes when exposed to EGF and bFGF, stimulating the ERK-pathway (Solmesky, Lefler et al. 2010), and similar results have been obtained by others (Gigout, Buschmann et al. 2009; Felka, Schafer et al. 2010). Waese et al. report on a one-step successful generation of chondrocytes in a serum-free monolayer system (with the addition of TGFβ3 or BMP-4) (Waese and Stanford 2011), and several articles underscore the importance of serum source for optimal differentiation and inhibition of senescence in engineered chondrocytes (Shahdadfar, Fronsdal et al. 2005; Dahl, Duggal et al. 2008; Duggal and Brinchmann 2011).

Hence, the combination of factors inducing optimal differentiation and the selection of serum-free media to produce good chondrocytes and osteoblasts for tissue engineering purposes represents a major task to elucidate.

6. Properties of scaffold materials in bone and cartilage engineering

Trauma (including bone fractures and cartilage destruction), cancer metastases, rheumatoid arthritis and osteoarthritis represent a therapeutic challenge, which previously has been approached by implanting autologus tissues (Gordeladze, Reseland et al. 2009; Torroni 2009; Giannoudis and Dinopoulos 2010; Khan, Johnson et al. 2010; Lu, Subramony et al. 2010; Takeda, Nakagawa et al. 2011). The modern approach of using scaffolds as artificial cell- and tissue-supporting material for cell support is promising and has been extensively reviewed by Sundelacruz and Kaplan (Sundelacruz and Kaplan 2009). Basicly, che choice of scaffold biomaterial and biocompatibility is vital for support of cell proliferation, differentiation, and suitability for implantation in vivo. Secondly, the geometry and architecture is important determinants of support of 3D tissue growth, control of morphology of the growing tissue, support of cell proliferation, and favourisation of cell differentiation into particular lineages. Thirdly, the porosity of the scaffold is important for the support of cell differentiation, recruitment, aggregation, and vascularisation. Furthermore, the mechanical properties, degradation rate, and biochemical stimuli are determinant of the scaffold's ability to permit new tissue ingrowth, allow remodelling of the ECM formed, match the healing rate of the new tissue, and stimulate progenitor cells to assume a functional and stable cell phenotype. The following scaffolds have been tested in different ostechondral tissue engineering settings: PET, PLDL, PLA, PGA, PLGA, HA, TCP, and silk fibroin (porosity and pore size), HA, TCP, various synthetic polymers and co-polymers, polymer-ceramic composites (pore inter-connectivity), natural synthetic polymers, including collagen, silk, PLGA, and PCL (degradation), natural synthetic polymers, bioactive glasses and ceramic material (mechanical strength), and finally PLGA, CaP, TCP, chitosan, HA, collagen, and silk fibroin (incorporation of biochemical signalling).

In order to arrive at the very best system for tissue engineering, large experimental permutations of the above mentioned factors including cell sources, humoral factors and gene therapeutic approaches, should be performed to obtain the better cell phenotype for osteochondral tissue replacement.

However, some recent articles featuring the use of polymeric scaffold structures in osteochondral engineering deserve citation here. Hydrogels incorporating agarose, alginate, collagen, hyaluronic acid polymer and gelatine have been successfully applied to support stem cell differentiation and 3D-structuring (Vinatier, Bouffi et al. 2009; Vinatier, Mrugala et al. 2009; Hunt and Grover 2010). MSCs embedded in fibrin hydrogel showed superior differentiation to osteoblasts compared to cells grown in monolayers, however, they did not assume a preferred phenotype after 28 days of incubation. Tiainen and co-workers have reported on an ultra-porous titanium oxide (TiO_2) scaffold with high compressive strength (above 2.5 MPa at 80-90% porosities) (Tiainen, Lyngstadaas et al. 2010), satisfying criteria for mechano-stimulation and pore size favouring cell differentiation, recruitment, aggregation and vascularisation. Another report on TiO_2 confirms its applicability in producing a proper bone replacement material (Sabetrasekh, Tiainen et al. 2011). Rahman et al. used bioactive glass, which, despite its brittleness, showed physical characteristics favouring neo-vascularisation being necessary for the perpetuation of engineered bone, when implanted in vivo (Rahaman, Day et al. 2011). And finally, it should be mentioned that osteoblast-like cells cultured in a bone-mimicking material made of poly-L-lactide + carbon nanotubes + micro-hydroxyapatite differentiated well into proper, bone-forming osteoblasts, as ascertained by genetic profiling (van der Zande, Walboomers et al. 2010).

Sabetrasekh and co-workers showed that Hydroxylprolyl-methyl Cellulose Hydrogel (Histocare™) (Sabetrasekh 2011) supported the differentiation of MSCs and preosteoblasts and cell clusters forming an artificial tissue favouring cell-cell interactions. Duggal et al. (Duggal, Fronsdal et al. 2009) showed that MSCs exposed to high-guluronic tripeptide arginine-glycine-aspartic acid (RDG) alginate scaffolds, facilitating binding to integrin, differentiated well into chondrocytes in the absence of any growth factors. Integrins are extracellular receptors conveying mechano-stimulation to the interior of the cell (Liu, Calderwood et al. 2000; Weyts, Li et al. 2002; Kapur, Baylink et al. 2003; Gordeladze, Reseland et al. 2009), and the use of RDG alginate scaffolds makes the addition of growth factors less critical for chondrocyte phenotype acquisition (as shown by transcriptome

analyses). Hyalouronan (HYAFF-11®) scaffolds have been shown to produce useful cells for osteochondral tissue replacement, provided that MSCs were applied instead of ASCs in the presence of TGFβ1 (Loken, Jakobsen et al. 2008; Jakobsen, Shahdadfar et al. 2010). Finally, it should be mentioned that scaffold material (e.g. polycaprolactone, PLGA/Hap/, Collagen/Hap, agarose/gelatine hydrogel, polyacryl-amide hydrogel, PLGA nanofiber, agarose gel, polyacryl-amide gel, poly(2-hydroxyetylmethylmethacrylate) micro-porous gel, and silk fibroin) made with a gradient in pore size (Sundelacruz and Kaplan 2009; Oh, Kim et al. 2010; Seidi, Ramalingam et al. 2011) is especially well suitable for interface (i.e. ligament-to-bone, tendon-to-bone and cartilage-to-bone) tissue engineering.

The concept of scaffolds/biomaterial presently extends to include biopolymers, self assembled systems, nanoparticles, carbon nanotubes and quantum dots (Williams 2009). This definition also includes micro-structured surfaces (Kolind, Dolatshahi-Pirouz et al. 2010), shown to inhibit cell proliferation and favour differentiation, as well as UV-bioimprinting of single cell surfaces (Muys, Alkaisi et al. 2006), favouring propagation of surface-cell-cell interactions, ensuring proper development of a defined cell phenotype in a 3D-structure. Application of the scaffolds principle to create a functional 3D-tissue structure can also be refined to what is called organ printing. Organ printing is a process which is scaffold free or involving hydrogels, and is defined as layer-by-layer additive robotic bio-fabrication of 3D-functional living macro-tissues and organ constructs using tissue spheroids as building blocks. These spheroids are subject to tissue fusion, constituting the final 3D-structure of living tissue (Mironov, Visconti et al. 2009). The principles consists of three steps, including a) the production of homo-cellular aggregates, b) building hetero-cellular aggregates, and finally c) the assembly of organ-mimetics containing a 3D-vascular bed (Mironov, Visconti et al. 2009; Visconti, Kasyanov et al. 2010).

Organ print design of tissues may solve some of the problems encountered in osteochondral tissue engineering, namely vascularisation of bone tissue and gradient expression of genes from the luminal space to the bone interface of chondrocytes in hyaline cartilage, due to a lack of blood-born delivery of nutritional substances and oxygen (Salim, Nacamuli et al. 2004; Gibson, Milner et al. 2008). Transient changes in oxygen tension inhibit osteogenic differentiation, as demonstrated by reduced transcription of gene classes related to angiogenesis, family of matrix proteins, HIF-1α, as well as RUNX2, osteocalcin, and COL1A1 (Salim, Nacamuli et al. 2004). As for chondrocytes, it has been shown that high O_2 tension makes them shift from producing normal articular isoforms of collagen (types II, IX, and XI) to collagen types I, III, and V (Gibson, Milner et al. 2008). High O_2 levels also suppress the expression of SOX9, necessary for chondrocytic differentiation and Aggrecan synthesis (Murphy and Polak 2004). Interestingly, the microRNA species mir-210 has been shown to be enhanced by HIF-1α, thus improving tissue tolerance to low O_2 levels (Huang, Le et al. 2010). This is consistent with the fact that mir-210 is down-regulated in dedifferentiated human articular chondrocytes assuming a more fibroblast/stem cell like phenotype (Karlsen, Shahdadfar et al. 2011).

Hence, there exists a plethora of scaffold materials to be considered, when optimal osteochondral tissue engineering conditions are to be defined.

7. Selection of stem cell niches and/or cell co-cultures

MSCs can be obtained from various tissues (Aicher, Buhring et al. 2010). Today the main source for isolation of MSCs in mammals is the bone marrow. However, bone marrow and other sources including placenta and adipose tissue contain MSCs displaying heterogeneous cell populations. Only a restricted number of appropriate stem cell markers have been explored so far, and it seems that the expression profile of CD-molecules differ on MSCs isolated from bone marrow, trabecular bone, dental pulp, articular cartilage, synovial membrane, adipose tissue, perivascular sites, term placenta, amnionic fluid, umbilical cord and pancreas (Bartholomew, Sturgeon et al. 2002; Dean and Bishop 2003; Le Blanc, Tammik et al. 2003; Niemeyer, Krause et al. 2006; Drosse, Volkmer et al. 2008; Gordeladze, Reseland et al. 2009; Aicher, Buhring et al. 2010). Knowledge of the phenotypical characteristics and the functional consequences of such subsets of MSCs might allow the development of improved regimens for regenerative medicine. MSCs, which express the specific cell adhesion molecule CD146, also known as MCAM, are well suited for bone repair. MSCs expressing CD56, CD146 and/or CD271 seem to be adaptable for the regeneration of bone, cartilage and intervertebral disks (Ohishi, Chiusaroli et al. 2009; Aicher, Buhring et al. 2010).

Using two or more MSC niches may thus prove beneficial for the generation of bone tissue (Matsumoto, Kuroda et al. 2008; Grellier, Bordenave et al. 2009). CD34-positive, VEGF-secreting endothelial/skeletal progenitor cells have been shown to enhance the vascularisation and speed up fracture healing (Matsumoto, Kuroda et al. 2008). Such progenitor cells are normally recruited to the bone-forming site by the CXR4/SDF-1 pathway (Otsuru, Tamai et al. 2008). Grellier and co-workers have reviewed the literature as to the use of co-cultures of osteogenic and endothelial cells (Grellier, Bordenave et al. 2009). They describe combinations of osteogenic cells and endothelial cells like osteoblasts, osteoprogenitor cells, umbilical vein endothelial cells, endothelial progenitor

cells, saphenous vein endothelial cells, outgrowth endothelial cells, and dermal vascular endothelial cells cultured in 2D- or 3D-structures of various scaffold materials (Villars, Bordenave et al. 2000; Wenger, Stahl et al. 2004; Kaigler, Krebsbach et al. 2005; Stahl, Wu et al. 2005; Kaigler, Krebsbach et al. 2006; Clarkin, Emery et al. 2008; Guillotin, Bareille et al. 2008; Grellier, Ferreira-Tojais et al. 2009; Grellier, Granja et al. 2009) where the endothelial cells form a tubular structure surrounded by ECM-producing and mineralizing osteoblasts (Grellier, Bordenave et al. 2009).

In conclusion, co-cultures of niches of MSCs and/or vascularisation of appropriate scaffolds (e.g. scaffolds supporting "asymmetric" differentiation of tissue-generating cells) might secure a better functional and long-lasting engineered tissue.

8. Mechano-stimulation of progenitor cells during differentiation

Mechano-biology is a relatively new research field, where most of the insight related to osteochondral tissue engineering comes from embryonic skeletal development (Nowlan, Sharpe et al. 2010). However, the "mechanostat" principle was launched several decades ago by Frost and colleagues (Frost 2003; Skerry 2006; Mulvihill and Prendergast 2008). Genetic lesions or immobilization (surgical or drug-induced) lead to muscle less limbs, reduced muscle fibre size/number, or non-contractile muscles, and to underdeveloped joints and bones, mostly due to a lack of mechano-stimulation (Gomez, David et al. 2007; Kahn, Shwartz et al. 2009; Nowlan, Bourdon et al. 2010; Nowlan, Sharpe et al. 2010).

Several humoral factors growth factors and receptors/ECM-protein/anchoring proteins share important signalling pathways, thus eventually leading to osteochondral differentiation of progenitor cells, for review, see (Gordeladze, Reseland et al. 2009; Kelly and Jacobs 2010; Potier, Noailly et al. 2010). Osteochondral progenitor cells may be subjected to shear stress (by fluid flow), compressive load (scaffold compression, hydrostatic pressure), or stretching (uni-, bi-, or equi-axial) leading to both proliferation and differentiation (Potier, Noailly et al. 2010). Several mechano-modulatory regimens (featuring detailed molecular mechanisms, type of mechano-stimulation, mechanical load applied, static or intermittent load, frequencies, as well as time frame during osteoprogenitor cell differentiation) using both 2D- and 3D-incubation systems, have extensively been described elsewhere (Angele, Schumann et al. 2004; Huang, Hagar et al. 2004; Woods, Wang et al. 2005; Campbell, Lee et al. 2006; Miyanishi, Trindade et al. 2006; Sumanasinghe, Bernacki et al. 2006; Mauck, Byers et al. 2007; McMahon, Campbell et al. 2008; McMahon, Reid et al. 2008; Thorpe, Buckley et al. 2008; Wagner, Lindsey et al. 2008; Arnsdorf, Tummala et al. 2009; Arnsdorf, Tummala et al. 2009; Gordeladze, Reseland et al. 2009; Haudenschild, Hsieh et al. 2009; Li, Kupcsik et al. 2010). However, the permutation of various factors enlisted above, yielding the optimal osteochondral cells for further studies in vivo, is difficult to envisage.

Cell shape, determined by the RhoA-Rho kinase = ROCK (influencing the actin cytoskeleton), has received much attention as a controller of cell development (McBeath, Pirone et al. 2004; Arnsdorf, Tummala et al. 2009; Kelly and Jacobs 2010). This has renewed the interest in scaffold material made by nanotechnology, which is able to deliver 2D- and 3D-surfaces mimicking the ultimate surface pattern of osteoblasts and chondrocytes encountered in live tissues (Muys, Alkaisi et al. 2006; Kolind, Dolatshahi-Pirouz et al. 2010).

9. Stabilization of the osteoblast and chondrocyte cell phenotypes

In order to succeed replacing tissues like bone and cartilage, it is vital that the differentiated cells, whether pre-embedded in scaffolds or not, do not develop tumours or alter phenotype within a short period after implantation. The preferable phenotype should not lose acquired features or assume new ones. However, it has been speculated that engineered osteoblasts may be subject to premature senescence, acquire "drag-over" adipocyte characteristics, lose their ECM-synthesizing and mineralizing ability, while also enhancing osteoclast-mediated resorption yielding negative bone mass through multiple remodelling cycles. Furthermore, engineered hyaline cartilage chondrocytes may possibly shift their collagen-synthesizing and non-collagenous ECM producing profile towards hypertrophic and mineralizing chondrocytes. And chondrocytes may also recruit, activate and over-stimulate osteoclasts to resorb adjoining bone structures. Finally, it should be mentioned that engineered cartilage to replace hyaline articular cartilage also will be subject to remodelling, e.g. via the IL-1 induced Syndecan4-ERK-MMP3-ADAMT5 cleavage of Aggrecan, which is up-regulated in osteoarthritic joints (Bertrand, Cromme et al. 2010). Finally, it should be mentioned that immune cells (e.g Th-17 cells) secrete interleukins known to differentiate and activate osteoclasts from monocytes (Weitzmann and Pacifici 2007; Adamopoulos and Bowman 2008; Tilg, Moschen et al. 2008; Hanada, Hanada et al. 2010; Pacifici 2010), and that chondrocytes exposed to exosome-like structures or certain microRNA antago- or pre-mirs (e.g. antagomir-222), are detrimental to the chondrocyte phenotype (Gordeladze et al., unpublished observations).

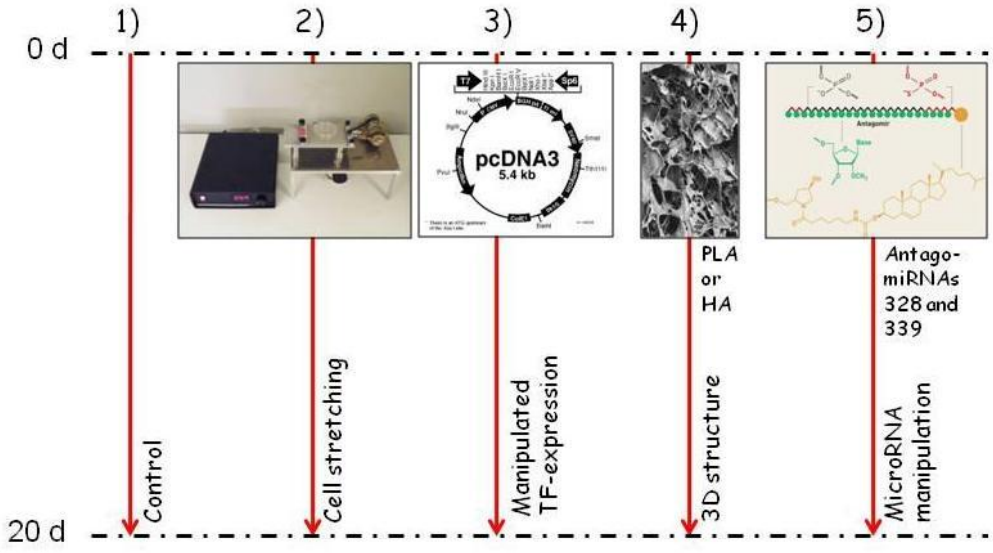

Figure 1A. Osteoblast differentiating scheme. Human mesenchymal stem cells (hMSCs) were incubated for 20 days in standard differentiating medium (containing dexamethasone), subjected to mechanical loading, transfected with the pcDNA3-Runx2 containing plasmid, grown in a 3D-lattice (PLA- or HA-based scaffolds), or transfected with antagomiRNAs corresponding to mir-328 and mir-339.

It is therefore suggested that gene manipulations (at least temporal transcription control) should be considered as part of a strategy to create and stabilize in vivo engineered bone or cartilage for tissue replacement. Potentially, one should consider the transient manipulations of microRNAs, since these short RNA-molecules are known to interfere with a plethora of cell specific transcriptions factors (Gordeladze, Djouad et al. 2009). MicroRNAs are also targeting epigenetic factors (Roach and Aigner 2007; Dahl, Duggal et al. 2008; Haberland, Montgomery et al. 2009; Lee, Jung et al. 2011; McGee-Lawrence and Westendorf 2011) like HDACs involved in the differentiation of stem cells and stabilization of various cell phenotypes (Li, Xie et al. 2009; Li, Hassan et al. 2009; Lee, Jung et al. 2011).

10. Bone and cartilage engineering revisited

Permutation of factors influencing cell phenotypes

There are numerous reports in the literature featuring the results of manipulations of single or a few variables known to affect the result of cell engineering based on stem cell or progenitor cell differentiation towards osteoblasts or chondrocytes to be implanted to heal osteochondral tissue lesions. These factors relate to cell source(s), application of growth factors, the use of gene therapy, application of mechano-stimulation and the selection of scaffold material (Isogai, Kusuhara et al. 2006; Gordeladze, Reseland et al. 2009; Aicher, Buhring et al. 2010; Granchi, Ochoa et al. 2010).

To find the combination of factors rendering engineered cells functional enough to assume a "proper" phenotype, generating tissues not deviating from their original counterparts with given characteristics, represents a painstaking task. It seems insurmountable, since the number of permutations necessary to explore all possible additive or synergistic interactions are numerous. It is therefore probably a good approach to define a set of measurable end-point characteristics for osteochondral tissues to evaluate the experimental steps taken, when going from bench to patient. Osteochondral tissues represent certain geometrical and mechanical properties (Knecht, Vanwanseele et al. 2006; Gordeladze, Reseland et al. 2009), as well as gene expression profiles (Grundberg, Brandstrom et al. 2008; Duggal, Fronsdal et al. 2009; Granchi, Ochoa et al. 2010; Herlofsen, Kuchler et al. 2011), which may guide the selection of major combinations of treatments, as envisaged by the permutation process. To shed light on this exercise, some bioinformatics exercises have been conducted, and some selected experiments have been described.

Permutations encompassing mechano-stimulation, 3D-growth, and manipulations of genes and microRNAs
MSCs were differentiated in standard media towards osteoblasts or chondrocytes, by subjecting them to cyclical mechano-stimulation (uni-axial stretch), gene manipulations, growth in 3D-lattices, and finally to manipulations of microRNA levels. The following test battery was used: Q-PCR analyses of osteoblast and chondrocyte "specific" transcription factors (TFs) and marker genes (e.g. RUNX2, OSTERIX = SP7, VDR, RANK-L, OPG, SOX9, GLI3, FOXO3A, WNT5A, ALPL, COL1A1, OSTEOCALCIN, OSTEOPONTIN, COL2A1, COL10A1, AGGRECAN); Q-PCR of mir-326, mir-339, mir-24, and mir-149; immunohistochemistry of COL2a1 and AGGRECAN; cell staining using Alizarin Red S and Alcian Blue; mineralization (radiology and histology) in SCID mice (m. tibialis); GAG/DNA-ratio, clinical score for micropellets and alginate beads; osteoclast resorption assay (using PBMCs + RANK-L/CSF-U on dentine slices). Some of the results obtained with MSCs differentiated towards osteoblasts are referred to in Figures 1 and 2, panels A and B.

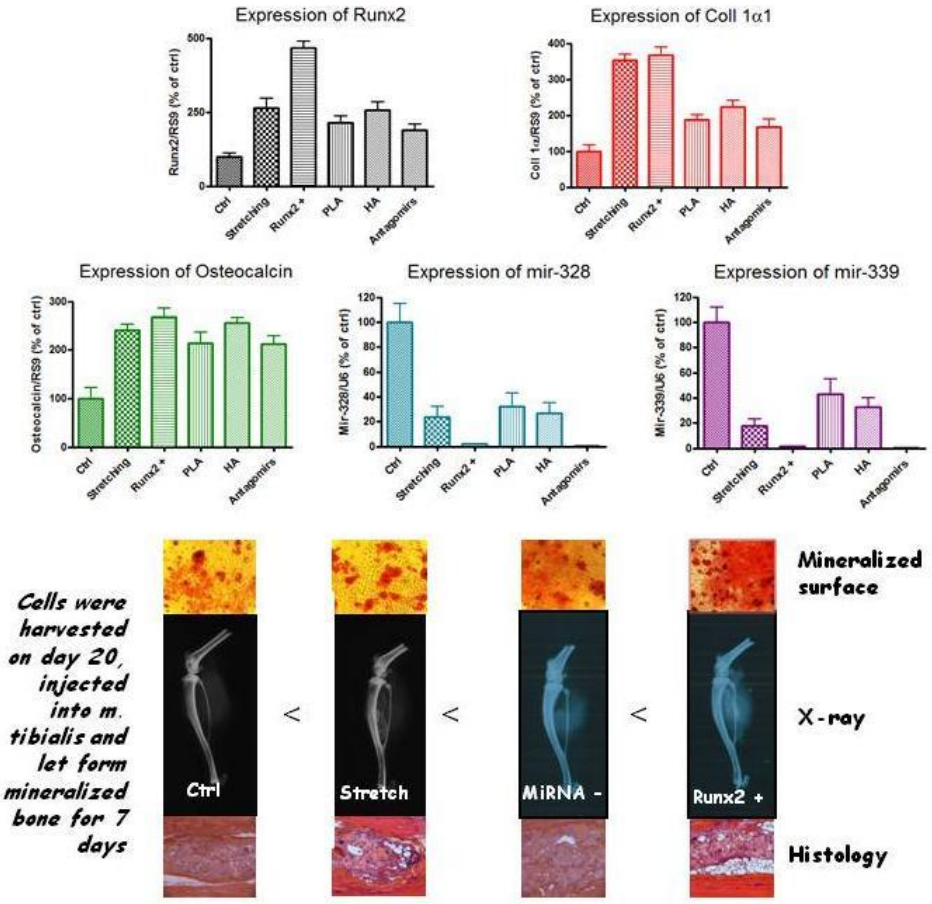

Figure 1B. Selected results of the experiment described in Figure 1A. Mechanical loading was performed in monolayers using uni-axial cell diameter alteration by 1000 μE (1E = 1 micro-strain = 1/1,000,000 alteration of the cell's diameter) for 30 min every other day. The antago-miRNAs were transfected (by lipofection) into cells in monolayers every 5 days. Expression of genes like Runx2, Collagen-1, Osteocalcin and the microRNAs 328 and 339, were performed using Q-PCR. Furhtermore, Alizarin Red staining (indicating mineralized surface) was performed at day 20, and cells being deposited (for an additional 7 days) in the tibial muscle of SCID mice were X-rayed, harvested and subjectd to histological analyses

Figure 1A indicates the manipulation of MSCs grown in: 1) osteoblast differentiating medium 2) in mono-layers, 3) exposed to mechano-stimulation, 4) subject to up/down-regulation of TFs, and 5) grown in PLA- or HA-scaffolds (cylinders), or 5) transfected with pre- or antago-microRNAs. Figure 1B features some of the results of these single manipulations, indicating that RUNX2 over-expression is superior in terms of osteoblast differentiation, however, mechano-stimulation, and suppression of mir-328 and mir-339 also give promising osteoblasts for in vivo implantation.

Figure 2A describes the manipulation of MSCs grown in chondrocyte differentiating medium 1) in mono-layers, 2) exposed to mechano-stimulation, 3) subject to up/down-regulation of TFs, 4) grown in alginate beads or micropellets, or 5) transfected with pre- or antago-microRNAs. Figure 2B summarizes selected results of these single manipulations, indicating that suppression of RUNX2 is no better than incubation in micropellets or alginate beads, or transfecting the cells with premir-24 and premir-149. All in all, manipulating the microRNA species seem to give superior results.

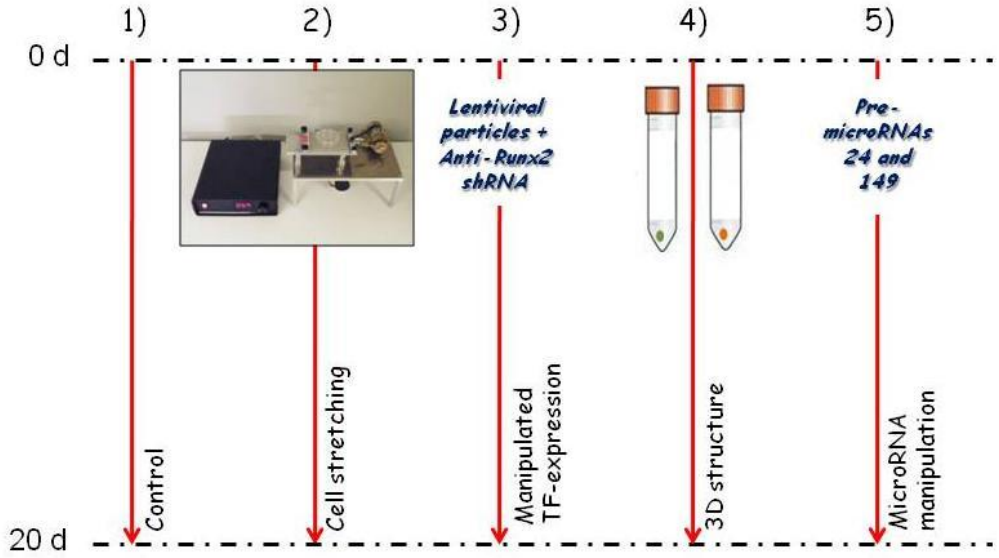

Figure 2A. Chondrocyte differentiation scheme. Human mesenchymal stem cells (hMSCs) were incubated for 20 days in standard differentiating medium (containing TGFβ3), subjected to mechanical loading (1000 μE), infected with anti-Runx2-shRNA (contained within a lentiviral construct), grown in a 3D-lattice (micropellet or alginate), or transfected with premiRNAs corresponding to mir-24 and mir-149.

From the above experiments, a 20 day differentiating scheme was envisaged, where gene- and microRNA-manipulated MSCs, grown in standard differentiating media, were mechano-stimulated for 10 days and thereafter incubated for another 10 days in a 3D-structure (HA-scaffold for osteoblast, and alginate beads for chondrocytes). These incubation schemes are shown in Figure 3A, while results of the experiments are summarized in Figure 3B. By combining the different manipulations, it was shown that osteoblast and chondrocyte "specific" markers were enhanced some 3-4 fold over control MSCs differentiated in mono-layers compared to 2-3 fold for single condition manipulations. To assess the influence of inflammation (using incubation media containing interleukins and TNFα) on osteoblast or chondrocyte phenotype stability and osteoclast activation, cells were exposed to IL-1β, IL-6, IL-17 and TNFα for 14 days. Osteoclasts differentiated from PBMCs for 7 days were than co-cultured the osteoblasts or chondrocytes, and resorption pit surface was assessed. It became quite clear that inflammatory cytokines were detrimental to the ostechondral cell phenotypes and microRNA profile, and they also enhanced their ability to stimulate bone resorption through activated osteoclasts. From these experiments, it seems that one might chose transient microRNA manipulations in combination with either cell stretching or growth in scaffold/hydrogel, if a permanent gene manipulation (e.g. alteration of RUNX2- and possibly also SOX9-expression) may render the cells less prone to negative influence encountered within their new environment.

Bioinformatics networking using micro-arrays of translated RNAs and non-translated microRNAs

To elucidate the concept of permutations of variables pertaining to the differentiation of stem cells (SCs) to become preferred osteoblasts or chondrocytes for tissue replacement, we will present an interesting exercise with transcriptomes, microRNA micro-arrays and a literature survey. Based on osteoblast derived transcriptomes (Grundberg, Brandstrom et al. 2008; Granchi, Ochoa et al. 2010), featuring gene transcripts from cells in human trabecular hip bone explants, and differentiating human mesenchymal stem cells (MSCs) undergoing differentiation and mineralization phases, respectively, a combined transcriptome of 188 genes were constructed. This transcriptome was run against two microRNA micro-arrays obtained from a) human MSCs differentiated to osteoblasts within a hydroxyapatite (HA) scaffold for 28 days, and from b) human MSCs differentiated to

osteoblasts in monolayers for 3 days only, using a bioinformatics program designated Mir@nt@n (Le Bechec, Portales-Casamar et al. 2011). Furthermore, a transcriptome of genes pertinent to the chondocyte phenotype, consisting of 261 genes, was compiled by Brinchmann et al. (Duggal, Fronsdal et al. 2009; Herlofsen, Kuchler et al. 2011). These MSCs, grown in PRONOVA-LVG alginate for 21 days, represented genes displaying either the same time course over 21 days as did COL2A1 or CXCL12. The present transcriptome was run against two microRNA micro-arrays obtained from a) human chondrocytes embedded in hyaline cartilage and dedifferentiated for 28 days, and b) human MSCs differentiated to chondrocytes in micropellets for 3 days.

The bioinformatics procedure featuring some comprehensive examples is given in Figure 4A. Twelve genes involved in WNT- and NOTCH-mediated signalling (according to KEGG's pathways) and a set of fourteen transcription factors (TFs) known to be important for osteoblastogenesis (Komori 2006; Marie 2008; Gordeladze, Djouad et al. 2009) were loaded into Mir@nt@n and two small networks emerged. All the microRNAs 16, 22, 24, 93, 125b, 141, 149, 200a and 206 have been shown to be down-regulated in osteoblastic cells (Gordeladze, Djouad et al. 2009; Lin 2009), which would be consistent with an up-regulation of TFs (SATB2, ETS1, and RNF11), WNT (signalling molecule binding to FRIZZLED-LRP5/6) (Gordeladze, Reseland et al. 2009). However, NOTCH3 (known to inhibit osteoblastogenesis through interactions with the canonical WNT-pathway and Runx2) (Gordeladze, Reseland et al. 2009) would also be up-regulated. Interestingly, ETS1 seems to be involved in a regulatory network involving NOTCH3, RNF11, and six microRNA species, where mir-206 is reciprocally interacting with ETS1. Mir-206 is marginally down-regulated in osteoblasts, however, significant over-expression of this microRNA species in mice leads to bone loss (Inose, Ochi et al. 2009). Finally DKK2 (an inhibitor of LPR5/6) would be up-regulated, and the present prediction cannot be given a straight-forward, simple interpretation

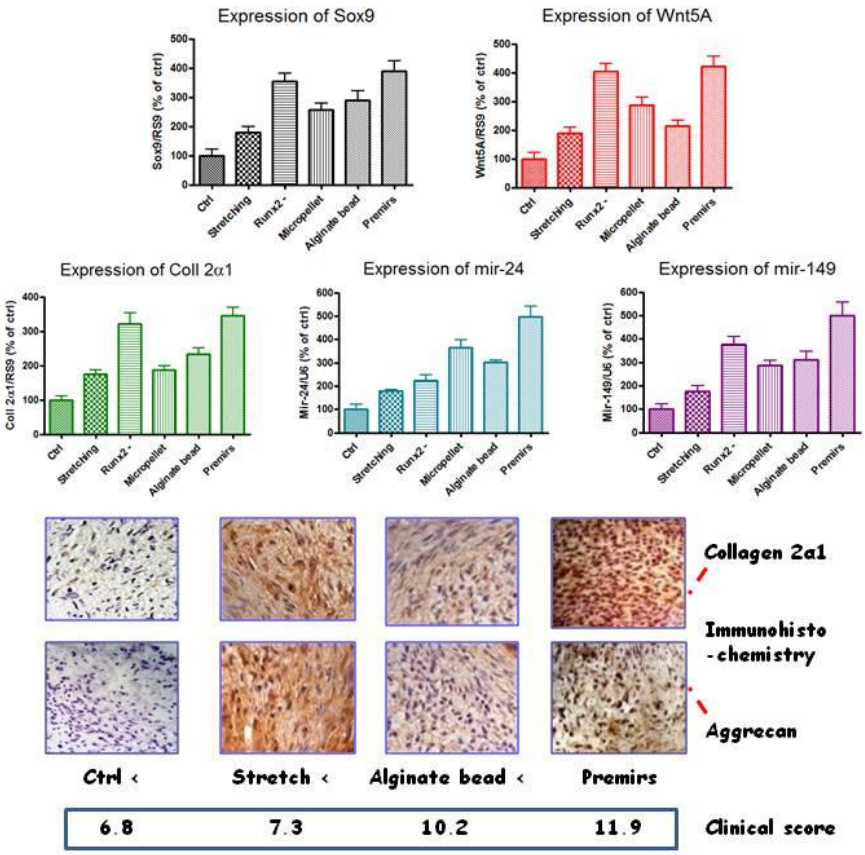

Figure 2B. Selected results of the experiment described in Figure 2A. Mechanical loading of MSCs was performed in monolayers using uni-axial cell diameter alteration (1000 μE) for 30 min every other day. The pre-miRNAs were transfected into cells in monolayers every 5 days. Expression of genes like Sox9, Wnt5A, Collagen-2, and the microRNAs 24 and 149, were performed using Q-PCR. Furhtermore, immunohistochemistry of Collagen 2 and Aggrecan was performed, and clinical scoring (featuring GAG/DNA-ratio, immuno-histochemistry, and distance between cells in micropellets and alginate beads) were also measured.

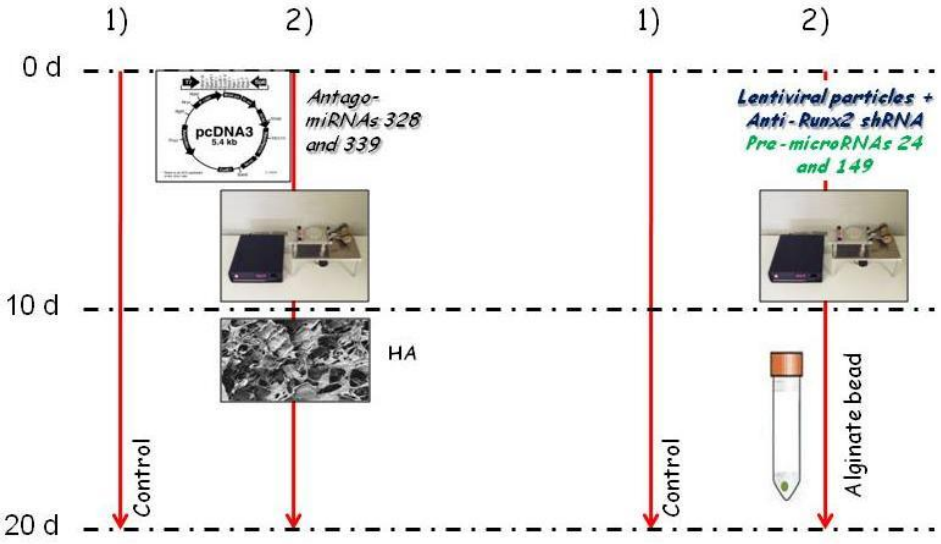

Figure 3A. Multipurpose differentiation scheme. The following experimental settings were selected: Human MSCs were incubated in standard differentiating medium containing dexamethasone or TGFβ3 alone, respectively, or manipulated for 10 days interfering with gene expression, microRNA levels, and cell shape, and thereafter incubated in a 3D-stucture (HA-scaffold or alginate hydrogel, respectively) for another 10 days.

Out of seventeen putative interactions (reciprocal or not) between microRNAs known to be down-regulated in osteoblasts, eight are compatible with a direct inhibitory effect on translation, yielding 47% consistency according to the concept of microRNA-TF interactions (Zhou, Ferguson et al. 2007; Aguda, Kim et al. 2008; Hobert 2008; Do and Scholer 2009). The conclusion to be drawn from this example is that the list of marker genes and microRNAs describing the differentiation of MSCs to osteoblasts is too slim to warrant its use as a predictor of the acquisition of a proper osteoblast phenotype to be employed in bone replacement therapy. But, after all, the marker genes and microRNAs were all just picked from various, independent articles on osteoblast differentiation and from two KEGG's pathways charts.

More interestingly, Figure 4B describes the results of the use of the Mir@nt@n networking algorhitm, where the applied lists of target genes are based on cells derived from healthy human bone and cartilage, and the microRNA species are retrieved from micro-arrays obtained from the tabulated experiments. The four experimental conditions summarized here (involving on average of some 30 microRNA species and 225 genes per experiment) clearly indicate that cell manipulations performed in a 3D-structure, and over a prolonged time frame of 28 days, yield a preferred osteoblast or chondrocyte phenotype, since the per cent compatibility demonstrated by microRNA – target gene interactions were 76% versus 19% (osteoblasts) and 88% versus 16% (chondrocytes), respectively.

Some examples of expected regulation patterns are given underneath: MSCs differentiated into osteoblasts in a HA scaffold show an up-regulated level of mir-143. In parallel, transcripts of putative target genes like DUSP2 (inactivates the MAPK pathway used by TNFs and TGFβs), BMP1 (involved in chondrogenesis), ID1 (belongs to the TGFβ pathway involved in chondrogenesis), TNFAIP6 (TNFα-induced protein 6), and FBN1 (sequestering TGFβ within ECM) were up-regulated. The first five interactions are expected, the last one is not (giving a per cent compatibility of 83%). Furthermore, the mir-29 family (MIR-29a,b,c) of microRNAs was down-regulated, and putative target transcripts of genes like COL1A1, COL4A1, COL4A5, COL5A1, COL21A1, BMP1, ID1, TNFAIP6, and FBN1 should be up-regulated. According to their alleged function, the interaction of the 29-family of microRNAs is expected when the collagens are concerned (Eyre 2002; Almarza and Athanasiou 2004; Goldring, Tsuchimochi et al. 2006; Davies, Chang et al. 2007; Shahdadfar, Loken et al. 2008; Heinegard 2009; Van Agtmael and Bruckner-Tuderman 2010), but do not comply with the expected down-regulation of BMP1, ID1, and TNFAIP6. Cumulative compatibility score is now down to 73%. According to the literature (Li, Hassan et al. 2009), mir-29b does de facto bind to the 3'-UTR of the COL1A1, COL5A3 and COL4A2. Furthermore, the mir-29 family of microRNAs has also been shown to be involved in the regulation of Wnt-signalling through a positive feed-back loop (Kapinas, Kessler et al. 2010) and via suppression of SPARC (osteonectin) (Kapinas, Kessler et al. 2009). Scrutinizing the effect of mir-376c (being down-regulated) reveals that putative targets are DLX1 and RUNX2 (important TFs ensuring osteoblastogenesis), SPP1 (involved in bio-mineral tissue development and

ossification) and PAFAH1B1 (involved in cell cycle adaptation to differentiation). Now, the cumulative compatibility score is 80%. The final cumulative score for this experiment (MSCs to osteoblasts in HA scaffold for 28 days) converged towards 76%. As expected, the 3 days incubation of MSCs seeded in culture flasks in osteogenic medium yielded a compatibility score of only 18%, where some of the microRNAs being modulated in the 28 days experiments with MSCs seeded into HA scaffolds did not appear as significantly altered (e.g. mir-376c).

Chondrocytes embedded within hyaline cartilage were dedifferentiated within their native matrix for 28 days, and a micro-RNA micro-array was obtained. Running these micro-RNA species using the Mir@nt@n algorithm together with the transcriptome of 261 genes gave a compatibility score of 88% (see Figure 4B). The following microRNAs and putative target gene transcripts should be mentioned: Mir-143 was up-regulated upon dedifferentiation, and putatively interact with gene transcripts like SMO (involved in hedgehog = Hh activation of Gli1/2/3-mediated chondrogenesis) (Bale 2002; Takebe, Harris et al. 2011), COL1A1 (serves as bone matrix protein), WNT10B (involved in osteoblast differentiation), ADAMTSL1 (exhibits metalloproteinase activity), and HAS3 (synthesizes hyaluran). The mir-143 mediated suppression of all the above listed genes are expected when chondrocytes are dedifferentiated. Mir-140-3p was down-regulated and coupled to the modulation of gene transcripts like KLF4 (transcription factor activated by the Wnt-pathway) (Saulnier, Puglisi et al. 2011) , FOXQ1 (serves as a down-stream mediator of TGFβ1 signalling) (Feuerborn, Srivastava et al. 2011), CITED4 (serves as a co-activator of CEP/p300, TFAP2, and SMAD4 transcription factors involved in stem cell differentiation) (Braganca, Swingler et al. 2002), and PTCH4 (receptor activated by Hh, thus stimulating the SMO-GLI pathway of gene transcription) (Takebe, Harris et al. 2011).

Characteristics obtained with combined differentiation strategies (based on rank scores)	Osteo Control	Osteo Combined	Chondro Control	Chondro Combined
Effect on osteoblast differentiation (Q-PCR of TFs and marker genes)	100	326		
Effect on chondrocyte differentiation (Q-PCR of TFs and marker genes)			100	365
Effect on osteoblast differentiation (Mineralization/ALP-positive surface, *in vivo* mineralization and histology)	100	287		
Effect on chondrocyte differentiation (Alcian blue surface, GAG/DNA-ratio, immunohistochemistry, histological score)			100	345
Effect on engineered osteoblasts to "reverse" detrimental biological effect of inflammatory cytokines	100	388		
Effect on engineered chondrocytes to "reverse" detrimental biological effect of inflammatory cytokines			100	276
Effect on engineered osteoblasts to resist "increase" in pertinent microRNAs (i.e. MiR-328, -339)	100	445		
Effect on engineered chondrocytes to resist "loss" of pertinent microRNAs (i.e.MiR-24, -149)			100	412

Figure 3B. Selected results obtained in the experiment described in Figure 3A showing synergism of the single manipulations used in combination. The following parameters were analysed: effect on cell differentiation, as estimated by Q-PCR of transcription factors (TFs) and marker genes, or as mineralized/ALP positive surface, in vivo mineralization in SCID mice and de novo bone tissue production (histology), and proteoglycan positive surface (Alcian blue colouration). Furthermore, the impact on cell phenotype stability upon exposure to cytokines (IL-1β, IL-6, IL-17, and TNFα) in terms of osteoclast activation and microRNA stability, was determined

A fall in mir-140-3p is compatible with an up-regulation of the four above mentioned gene transcripts and loss of chondrocyte phenotype. So far, the compatibility score is 100%. Another microRNA up-regulated in dedifferentiated chondrocytes is mir-382, which putatively targets the transcripts of the following genes: PLCG2 (PLCγ2 activates NF-κB, AP-1, and NFATc1 induced gene expression important for osteoblastogenesis) (Chen, Wang et al. 2008; Marie 2008; Gordeladze, Reseland et al. 2009), DKK2 (serves as an inhibitor of the Wnt-signalling pathway), and RUNX3 (cooperates with Runx2 to induce chondrogenesis through Hh synthesis) (Komori 2005). Mir-15b proved to be up-regulated upon dedifferentiation of the mature, hyaline cartilage-embedded chondrocytes, and putatively targets the following gene transcripts: FGF2 (involved in chondrogenesis) (Goldring, Tsuchimochi et al. 2006), CCND1, LRP6, FZD4 (Katoh 2007). All genes targeted by

mir-382 and mir-15b are associated with the osteochondral phenotype and therefore, the compatibility score remains at 100%. Lastly, mir-21 and mir-495 were up-regulated upon dedifferentiation of the hyaline cartilage chondrocytes. Putative targets were SOX5 (transcription factor favouring chondrogenesis), MEF2C (necessary factor for collagen X transcription, and interacting with Dlx5/6 to enhance Runx2 expression) (Solomon, Berube et al. 2008), and SOX6 (transcription factor favouring chondrogenesis, FGF7 (involved in chondrogenesis), CDH13 (predisposing factor along with TGFβ3, PTHR1, and PRG1 in ossification of ligaments of the spine) (Furushima, Shimo-Onoda et al. 2002), GLI3 (early transcription factor appearing during chondrogenesis) (Bale 2002; Takebe, Harris et al. 2011), respectively. This completes the random selection of microRNAs, however, at this point the compatibility score was still a staggering 100%. Subsequent to the analysis of all putative interactions, the score fell to 88%. The same exercise performed on microRNA-arrays from MSCs differentiated in micropellets for 3 days revealed a compatibility score of some 16% only, despite a similar number of microRNAs and gene transcripts significantly modulated compared to controls.

In conclusion, the more in vivo like incubation conditions, the more tissue-adapted osteoblasts and chondrocytes will be obtained when performing in vitro cell engineering. This exercise does not take into considerations all possible favourable factors (like stem cell source, differentiation media, optimal scaffolds, mechano-stimulation, gene-manipulations including phenotype protection by microRNAs etc.), but it is reasonable to believe that a permutation of selected conditions will aid in arriving at osteoblasts and chondrocytes highly suitable for long-lasting tissue replacements. Finally, it should be emphasized that one must improve on the selection of genes (and microRNAs) to constitute the preferred profile of proper osteoblasts and chondrocytes for successful tissue replacements.

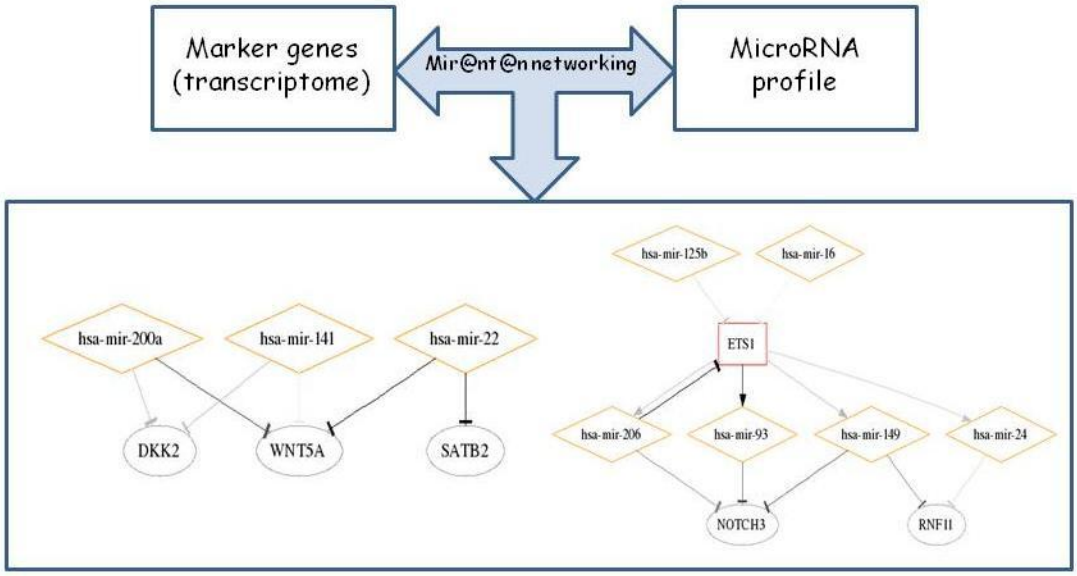

Figure 4A. Bioinformatics-based marker gene and microRNA networking. A list consisting of marker genes taken from the canonical WNT- and the NOTCH-pathways (see KEGG's pathways), as well as transcription factors (TFs) and microRNAs demonstrated to be involved in differentiation of osteoblasts (Gordeladze, Djouad et al. 2009; Hassan, Gordon et al. 2010; Kapinas, Kessler et al. 2010) was loaded into the Mir@nt@n algorithm, searching for interaction networks. Within the complicated network obtained, two types of interactions emerged: 1) microRNAs target several gene transcripts (putatively binding to the 3'-UTR region of the subject mRNAs) (left-hand chart), and 2) microRNAs may be involved in regulatory loops with TFs (right-hand chart).

Of special interest are the observations that the use of microRNA manipulations seems to protect the engineered osteoblasts and chondrocytes from losing their phenotypic characteristics in an environment where inflammation still is active, as well as protecting them from over-activating osteoclasts within the space (i.e. articular space) where they might be replacing damaged tissue.

	Experiment conducted	MirGntGn networking		
		MicroRNA species	Gene transcripts targeted	Per cent (%) compatibility with expected modulation
Osteoblast: 188 genes	MSCs: differentiation in HA scaffold (3D) for 28 days	34	89 (14%)	76
	MSCs: differentiation in monolayers (2D) for 3 days	17	53 (28%)	18
Chondrocytes: 261 genes	Chondrcytes: Embedded in hyaline cartilage, dedifferentiated in 3D for 28 days	52	136 (52%)	88
	MSCs: differentiation in micropellets (3D) for 3 days	54	142 (54%)	16

Figure 4B. Computation of compatibility score between osteoblast and chondrocyte transcriptomes and microRNA profiles. Gene transcript (mRNA) and microRNA networks were generated using osteoblast mRNA fingerprints from separate experiments (published in the literature) and microRNA-arrays from own experiments (see chapter text). Percentage of predicted mRNA-microRNA interactions in accordance with expected up-and down-regulation of gene expression in osteoblasts and chondrocytes were calculated. The higher the percentage, the better the differentiation process obtained, and (theoretically) the higher probability of success when using engineered osteochondral cells for tissue replacement.

11. Summary and future perspectives

This chapter summarizes the concept of single factor permutations in order to arrive at the optimal scheme for generating osteochondral cells for tissue replacement. To be considered is the use of trimmed osteoblast or chondrocyte transcriptomes (between 200 and 400 transcripts) obtained from clean cell populations residing within healthy bone and cartilage, along with a defined number of microRNA species (not more than 20-30) as markers and guidance for the use of a set of manipulations eventually leading to functional and stable cell phenotypes.

One scheme may consist of the following materials and factors: MSCs or ASCs exposed to a growth factor in a serum-free differentiating medium, mechano-stimulation (adapted to optimalize differentiation of osteoblasts or chondrocytes), preferably within scaffolds (designed to display a porosity gradient), transient adjustments of the levels of certain microRNA species (down-regulated in differentiating osteoblasts, up-regulated in differentiating chondrocytes).

If the disease necessitating tissue replacement can be handled/treated successfully, manipulations of the engineered cells to withstand phenotype alterations, may not be necessary. However, in the case of osteochondral replacement in joints being subject to inflammation, it may be necessary to protect the engineered cells from changing their function (e.g. stimulating osteoclastogenesis) or showing an accelerated development of senescence, by permanently modulating expression of selected genes or microRNAs.

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