Investigating the Role of FOXO1 in the differentiation of Mesenchymal Stem Cells towards Chondrocytes

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Abstract

Human mesenchymal stem cells (MSCs) have a profound potential in regenerative medicine. MSCs ability to differentiate into various tissue types *in vitro* provides a promising approach to tissue engineering and subsequently new clinical treatments. The ultimate and long-term goal of the research of which this thesis is a part of, is to generate a healthy hyaline cartilage that can be implanted in cartilage lesions. In this study the role of *FOXO1*, a gene significantly up-regulated in early chondrogenic differentiation of MSCs *in vitro,* was investigated. A three-dimensional scaffold aiming at mimicking the environment in the human body has been constructed for *in vitro* chondrogenic differentiation of MSCs. *FOXO1* has been knocked-down by FOXO1siRNA over the course of 6 days and the effects of the down-regulation on chondrogenic, osteogenic and other relevant genes were analyzed by RT-qPCR and nanostring technology. The results showed that *FOXO1* depletion in differentiating MSCs altered the expression of some chondrogenic related genes considerably; however the findings need to be validated further and in multiple donors, as donor variability constitutes an important factor to be considered in result interpretation and conclusion drawing. In addition, *FOXO1* has also been over-expressed in MSCs in 2D and the effects of up-regulation on pre-selected genes were analyzed by RT-qPCR. The results show that over-expression of *FOXO1* in MSCs has not had an impact on the studied genes.

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Abbreviations

ACI	Autologous chondrocyte implantation
BM- MNC	Bone marrow-mononuclear cells
BM- MSCs	Bone marrow- mesenchymal stem cells
BMP	Bone morphogenic protein
CFU-F	Colony forming unit-fibroblasts
IGF	Insulin growth factor
IGFBP	Insulin growth factor binding protein
IHH	Indian hedgehog
ESC	Embryonic stem cells
FBS	Fetal bovine serum
TGF-β	Transforming growth factor B
FGF	Fibroblast growth factor
FOXO1	Forkhead box O1 (small letters indicate the mouse version of the gene/protein)
GAPDH	Glyceraldehyd-3-phosphat dehyrogenase
HSC	Hematopoietic stem cells
MSC	Mesenchymal stromal cells

PLP	Platelet lysate plasma
PPAR-γ	Receptor peroxisome proliferator-activated receptor-γ
ROS	Reactive oxygen species
RT	Reverse transcription
SOX	SRY (sex determining region Y)-box

1. Introduction

1.1 Stem cells

A stem cell is a cell from the embryo, fetus, or adult that has, under certain conditions, the ability to reproduce itself for long periods or, in the case of adult stem cells, throughout the life of the organism. It can give rise to specialized cells that make up the tissues and organs of the body[1]. Thus stem cells are by definition also undifferentiated, and when they divide each daughter cell has a choice of; either remaining a stem cell (in order to maintain the population), or embark on a course that commits it to terminal differentiation. However, it should be noted that stem cells do not necessarily have to divide rapidly; in fact, stem cells usually divide at a relatively slow rate[2].

There are two main categories of stem cells depending on their potency/ability of differentiation: *pluripotent stem cells* and *multipotent stem cells*. In the following section both will be introduced briefly with examples, however mesenchymal stem cells (one type of adult multipotent stem cells) will be discussed in more details as this category is the core of this research.

Pluripotent stem cell

A single pluripotent stem cell has the ability to give rise to types of cells that develop from the three germ layers (mesoderm, endoderm, and ectoderm) from which all the cells of the body arise. The only known sources of human pluripotent stem cells are those isolated and cultured from early human embryos (embryonic stem cells)[3] and from fetal tissue that was destined to be part of the gonads (embryonic germ cells)[1]. Additionally induced pluripotent stem cells (iPSCs)[4] also falls under this category of pluripotent stem cells[5]. Embryonic stem cells (ESCs) are derived from a group of cells called the inner cell mass, which is part of the early (4- to 5-day) embryo called the blastocyst[3]. While embryonic germ cells are derived from fetal tissue, more specifically, they are isolated from the primordial germ cells of the gonadal ridge of the 5- to 10-week fetus. Later in development, the gonadal ridge develops into the testes or ovaries and the primordial germ cells give rise to eggs or sperm[1, 6] _ENREF_5, while embryonic stem cells and

embryonic germ cells are both pluripotent, they are not identical in their properties and characteristics[5]. The third type of the pluripotent stem cells (iPSCs) have been generated recently by reprogramming specialized adult cells by introducing genes important for maintaining the essential properties of embryonic stem cells (ESCs)[4]. Since the initial discovery, by Takahashi K and Yamanaka S. in 2006, researchers have rapidly improved the techniques to generate iPSCs, creating a powerful new way to "de-differentiate" cells whose developmental fates had been previously assumed to be determined.

Multipotent stem cell

Multipotent stem cells have the capability of developing multiple different and differentiated cells of the same germ layers. [1]An adult stem cell is a multipotent stem cell that is undifferentiated/unspecialized, which resides in a differentiated (specialized) tissue. It has the potential to renew itself, or specialize to yield any of the cell types of the tissue from which it originated[7]. Adult stem cells are capable of dividing and replenish themselves throughout the lifetime of the organism. This property is referred to as "self-renewal." Adult stem cells usually divide to generate progenitor or precursor cells, which then differentiate or develop into "mature" cell types that have characteristic shapes and specialized functions, e.g., muscle cell contraction or nerve cell signaling.[1] A progenitor or precursor cell occurs in fetal or adult tissues and is partially specialized[8]. Researchers[8-10] often distinguish precursor/progenitor cells from adult stem cells in the following way: when a stem cell divides, one of the two new cells is often a stem cell capable of replicating itself again. In contrast, when a progenitor/precursor cell divides, it gives rise to differentiated/specialized cells, neither of which is capable of replicating itself. Progenitor/precursor cells can replace cells that are damaged or dead, thus maintaining the integrity and functions of a tissue such as liver or brain. Progenitor/precursor cells give rise to related types of cells-lymphocytes such as T cells, B cells, and natural killer cells, for example—but in their normal state do not generate a wide variety of cell types.[1]
Sources of adult stem cells include the bone marrow, blood (transient state), the cornea and the retina of the eye, brain, skeletal muscle, dental pulp, liver, skin, the lining of the gastrointestinal tract, and pancreas[1]. The most abundant information about adult human stem cells comes from

studies of hematopoietic (blood-forming) stem cells isolated from the bone marrow and blood. These adult stem cells have been extensively studied and applied therapeutically for various diseases. Adult stem cells are rare and often difficult to identify, isolate, and purify. There are insufficient numbers of cells available for transplantation and adult stem cells do not replicate indefinitely in culture[1].

1.2 Mesenchymal stem cells/ Bone marrow stromal cells

In the bone marrow of many mammalian species at least two distinct stem cell populations exist: hematopoietic stem cell (HSCs) and a population of stem cells responsible for maintenance of the non-hematopoietic bone marrow elements called mesenchymal stem cells (MSCs also called bone marrow-BM- stromal cells)[1]. HSCs are responsible for forming all of the types of blood cells in the body, whereas MSCs are a mixed cell population that generates bone, cartilage, fat, fibrous connective tissue, and the reticular network that supports blood cell formation.[1, 11]. In addition, a third population of progenitor cells that differentiates into endothelial cells (giving rise to blood vessels), was recently isolated from circulating blood and identified as originating in bone marrow. [3]Thus, the bone marrow appears to contain three stem cell populations—HSCs, MSCs, and (possibly) endothelial progenitor cells.[1]

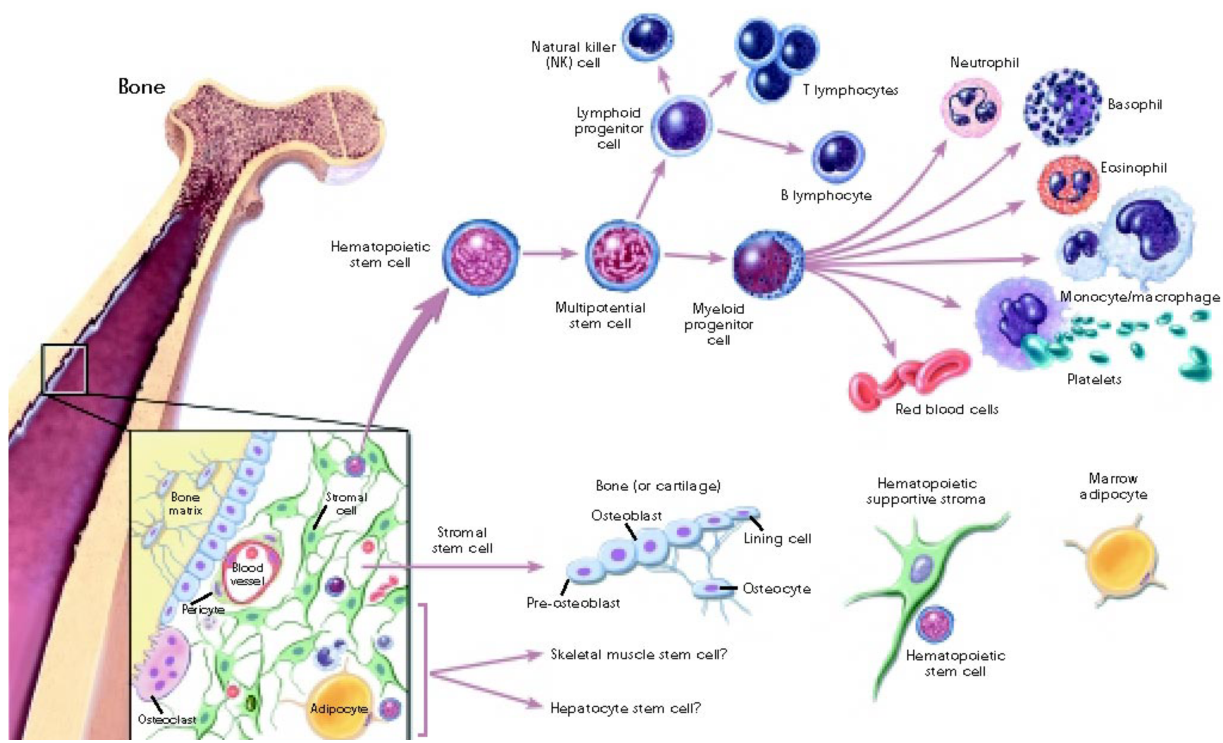


Figure 1-1. Hematopoietic and Stromal Stem Cell Differentiation. Hematopoietic stem cell form all the types of blood cells in the body, whereas stromal stem cells (MSCs) can form can bone, cartilage, fat, cells that support the formation of blood, and fibrous connective tissue. From a stem cell report by The National Institute of Health, 2001.[1]

MSCs have also long been recognized for playing an important role in the differentiation of mature blood cells from HSCs, by providing the physical environment in which HSCs differentiate[12, 13]. Like HSCs, MSCs arise from embryonic mesoderm during development, although no specific precursor or stem cell for stromal cells has been isolated and identified[1]. MSCs have many features that distinguish them from HSCs. The two cell types are easy to separate *in vitro*; when bone marrow is dissociated, and the mixture of cells it contains plated at low density, the MSCs adhere to the surface of the culture dish, while the HSCs do not[9]. Actually the ability of adherence to plastic is one of three criteria that define MSCs[14]. Methodology employed in the isolation and enrichment of human MSCs is strongly reliant on the ability of these cells to adhere to and subsequently proliferate on tissue culture plastic. Given specific *in vitro* conditions, MSCs form colonies from a single cell called the colony forming unit-F (CFU-F)[9] and unlike HSCs, which do not divide *in vitro* (or proliferate only to a limited extent), MSCs can proliferate for up to 35 population doublings *in vitro*[15]. They grow rapidly under the influence of mitogens such as platelet-derived growth factor (PDGF), epidermal growth factor

(EGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor-1 (IGF-1)[9, 16]. Thus although MSCs are rare in the bone marrow representing about 1 in 10 000 nucleated cells and though they are not immortal, they have the ability to expand many fold in culture while retaining their growth and multilineage potential[17]. In addition to bone marrow MSCs, MSCs-like cells have now been isolated from various other sites, including adipose tissue, amniotic fluid, periosteum, and fetal tissues, however some phenotypic heterogeneity has been shown[18, 19].

Despite the variation in reported phenotypes of mesenchymal subpopulations, it is widely accepted that a second criteria for the cultured cells, regardless of the methods employed in their isolation and culture, is the lack expression of prototypic hematopoietic antigens like CD45, CD34,CD11b and CD14. MSCs have also been reported to express SH2 (CD105), SH3/SH4 (CD73), CD29, CD44, CD90, CD71, CD106, CD166, STRO-1, GD2 and CD146[14, 20-25]. In addition adult human MSCs are reported to express intermediate levels of major histocompatibility complex (MHC) class I, but do not express human leukocyte antigen (HLA) class II antigens on the cell surface[26]. The third criterion to MSCs is their ability of differentiating into various tissues *in vitro*[14], such as adipocytes, bone, cartilage, muscle, bone marrow stroma, tendon/ligament, fat, dermis, and other connective tissues as diagrammed in figure 1-2 [11, 22]. Hence, these cells can potentially be used for the regeneration of these mesenchymal tissues through the principles and practices of tissue engineering[11]. MSCs have another profound capacity of secreting a broad spectrum of bioactive macromolecules that are both immunoregulatory and serve to structure regenerative microenvironments in fields of tissue injury. This capacity of MSCs to home to injured tissues or to participate in the injury response by providing a broad array of paracrine factors is referred to as their ‘‘trophic activity;’’ these capacities define and embody the concept of Regenerative Medicine[11].

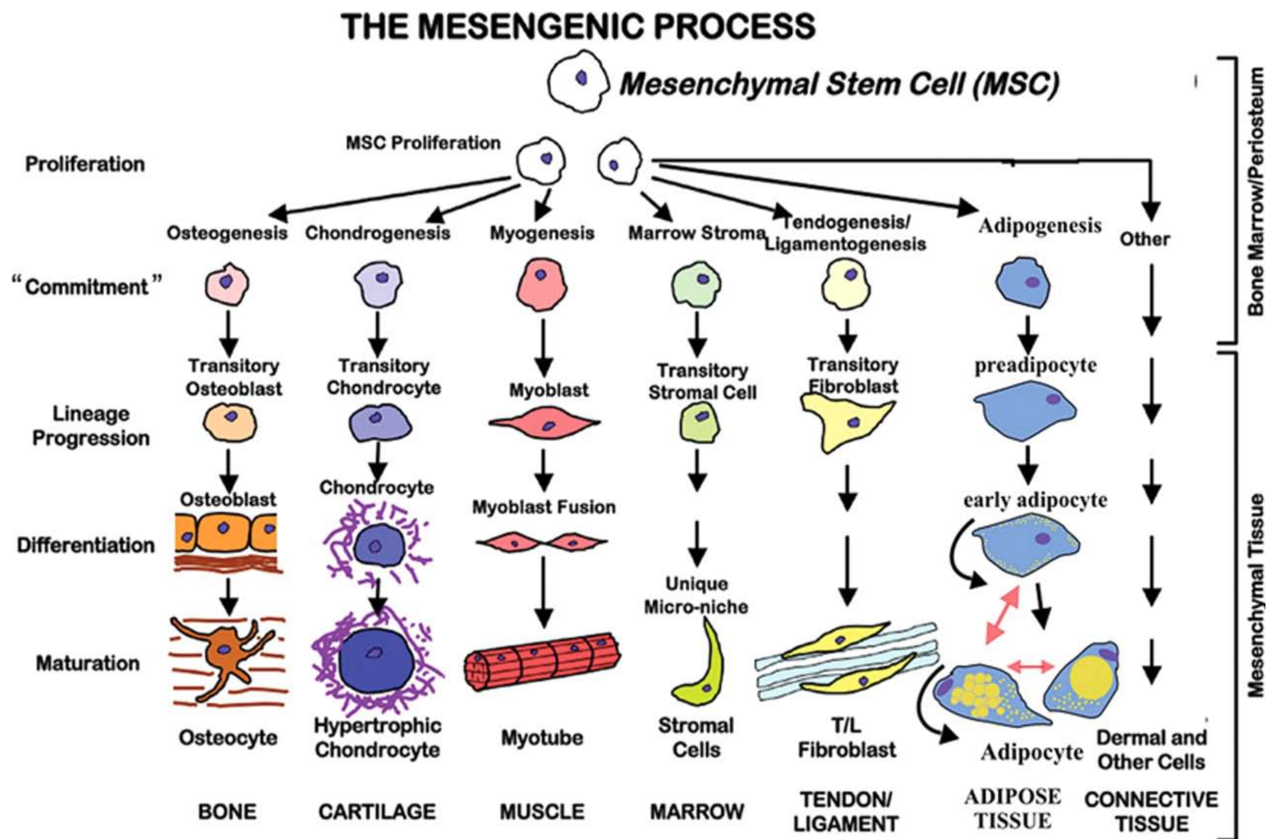


Figure1-2. The mesengenic process diagram. MSCs can differentiate into distinctive end-stage cell types, such as those that fabricate specific mesenchymal tissues including bone, cartilage, muscle, bone marrow stroma, tendon/ligament, fat, dermis, and other connective tissues. Figure taken from Nora G. Singer and Arnold I. Caplan et al 2011[27].

1.3 Chondrogenesis

Chondrogenesis is an essential process in vertebrates that can either lead to the formation of cartilage growth plate and ultimately endochondral ossification, or the formation of permanent cartilaginous tissues that provide the major structural support in the articular joints and respiratory and auditory tracts throughout life[28]. There are three distinct cartilaginous tissue types within the adult vertebrate skeleton: hyaline cartilage, fibrocartilage, and elastic cartilage. Each is defined by the unique molecular composition and organization of its extracellular matrix ECM.[28]

Hyaline cartilage is the most prevalent, and the only one to be discussed here. Within the hyaline cartilage tissue types, articular cartilage of the diarthrodial joint is both the most common and most studied[29, 30]. Diarthrodial joints are capable of extensive ranges of nearly frictionless movement and account for most of the skeletal articulations found within the vertebrate body. Inside the diarthrodial joint, hyaline cartilage is a smooth and resilient connective tissue that functions as both a weight-bearing and gliding surface [26].

1.3.1 Hyaline Cartilage Molecules

Molecules in the hyaline cartilage vary depending on the developmental stage of the cartilage. The following section introduces the most important and highly relevant (to this research) molecules that are found in the pre-cartilage and the cartilage stage.

Precartilage Matrix Molecules

After the migration of chondroprogenitor from cells to the location of their future skeletal element, and before noticeable chondrogenesis occurs, these undifferentiated mesenchymal cells produce a transient ECM that functions to temporally regulate both their aggregation and differentiation into chondrocytes *in vivo*[26]. This precartilage matrix is defined by a unique composition of molecules residing in the extracellular space (Figure 1-4), including collagen types I and IIA, hyaluronan, fibronectin, tenascin- C, neural-cadherin (N-cadherin), and neural cell adhesion molecule (N-CAM)[31-33]. Only the most relevant molecules will be discussed further. Collagen type II is the major collagen type found in both embryonic and adult cartilages [26]and is responsible for the tensile properties of cartilage tissue [34].

Hyaluronan (hyaluronic acid) is a widely distributed glycosaminoglycan (GAG) of high molecular weight that is found in most embryonic and adult tissues[26]. Hyaluronan is believed to perform dual roles in the precartilage matrix; both preventing intimate mesenchymal cell-cell interaction and facilitating cellular migration. Hyaluronan has also been shown to be important regulator of the transforming growth factor-beta TGF-β[35], bone morphogenic protein BMP[36], epidermal growth factor EGF[37], insulin like growth factor IGFI, and platelet-derived growth factor (PDGF) [38]signaling system and thus may function to regulate chondrogenesis via modulation of growth factor signaling in addition to its physical regulation of mesenchymal

condensation. Fibronectin [39]and Tenascin[40] are important glycoproteins that promote mesenchymal condensation *in vitro*

Hyaline Cartilage Matrix molecules

Hyaline cartilage ECM can be divided into three regions: the pericellular, territorial and interterritorial matrices (fig.1-5), each dually defined by specific location within the ECM and a unique composition (fig. 1-3) of ECM molecules[26]. Chondroblasts secrete two categories of major constituents that comprise the hyaline cartilage ECM: the collagens and proteoglycans. Collagen II is the predominant collagen type within the ECM, however collagen type IX, XI, and VI are also present. Collagen type II is able to contract with chondrocytes via binding to its cell surface receptor annexin V [41].

The two most abundant proteoglycans in the cartilage matrix are aggrecan and decorin. Aggrecan is a large proteoglycan consisting of core protein to which keratin sulfate and chondroitin sulfate GAG side-chains are attached.

The sulfated GAG side-chains that are attached to the aggrecan protein are highly negatively charged, and thus have the ability to attract osmotically active cations and their associated water, allowing cartilage to withstand compressive force [42]. Aggrecan mRNA begins to accumulate at the onset of cellular condensation and continues to be expressed throughout differentiation[43]. Aggrecan is found throughout hyaline cartilage ECM, but is concentrated in the pericellular and territorial regions (figure 1-5)

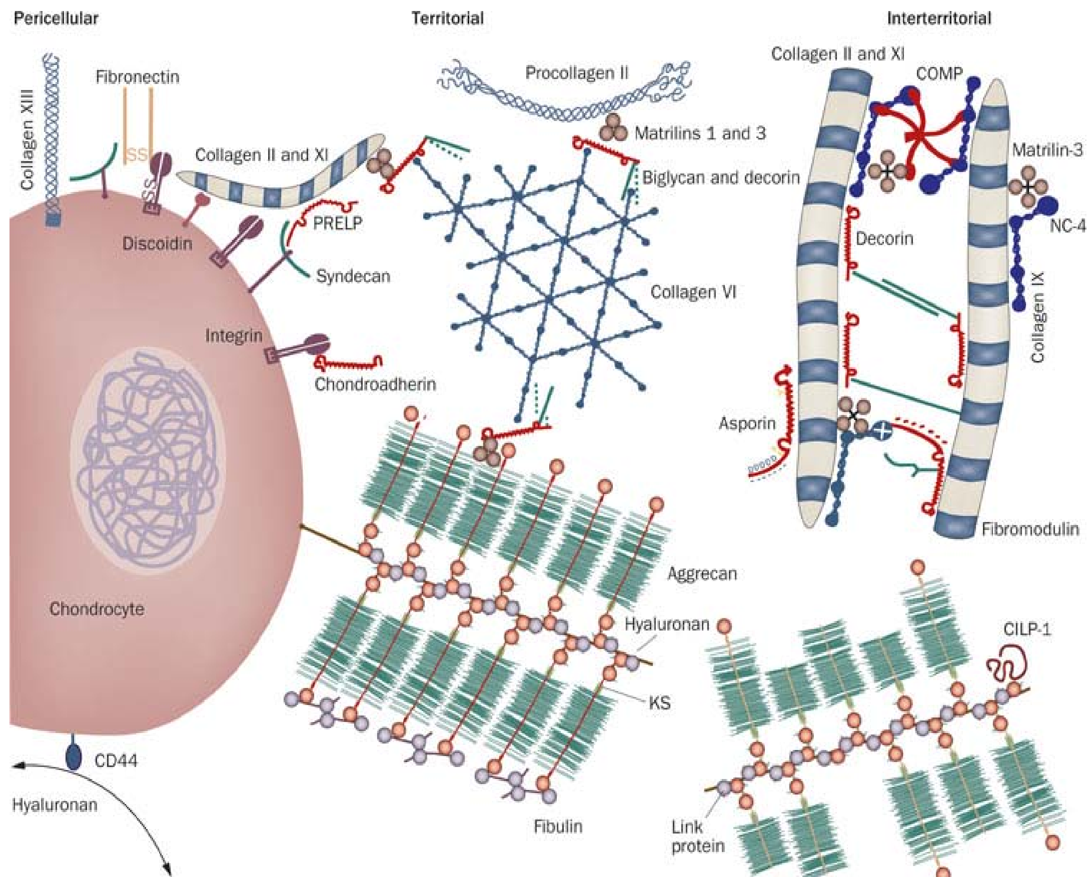


Figure 1-3. The cartilage matrix surrounding chondrocytes in healthy articular cartilage is arranged into zones defined by their distance from the cell. The pericellular matrix lies immediately around the cell and is the zone where molecules that interact with cell surface receptors are located; for example, hyaluronan binds the receptor CD44. Next to the pericellular matrix, slightly further from the cell, lies the territorial matrix. At largest distance from the cell is the interterritorial matrix. The types of collagens and the collagen-binding proteins that form the matrices are different in each zone. Taken from Dick Heinegård and Tore Saxne, 2011[44].

1.3.2 Chondrogenesis *in vivo*

The process of chondrogenesis occurs in stages (fig.1-4), commencing with mesenchymal cell recruitment and migration, proliferation and condensation, regulated by mesenchymal-epithelial cell interaction[32, 45]..

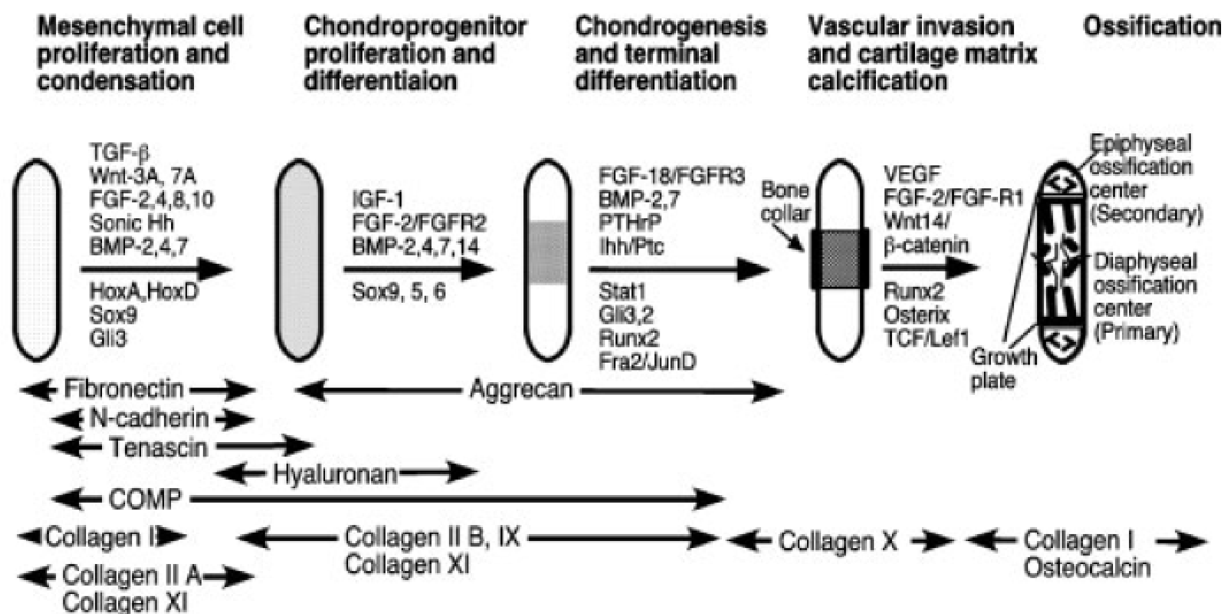


Figure 1-4. The different stages of chondrogenesis. The figure shows the temporal patterns of growth and differentiation factors above the arrows. The transcription factors are indicated below the arrows. The extracellular matrix proteins distinguishing the various stages are also indicated below. Taken from Mary B. Goldring et al. 2006 [46].

Transcriptions factors expressed by chondrogenic cells

The main transcription factors involved are the nuclear transcription factor (SOX) family and the transforming growth factor-β (TGF-β) superfamily and both families are also involved in the *in vitro* pathway. The transcription factor SOX9 is required for both precartilage condensation and overt differentiation of chondroprogenitor cells into chondroblasts. SOX9 is required for the

expression of type II collagen and other cartilage-specific matrix proteins, prior to matrix deposition in the cartilage anlagen[47]. Two other members of the SOX transcription factor family; L-SOX5 and SOX6 (not present in early condensation), and are co-expressed with SOX9 in all chondroprogenitor cells and function in cooperation with SOX9 to activate the col2al gene[48, 49]. During embryonic cartilage formation the expression of SOX9 precedes the appearance of either L-SOX5 or SOX6[50]. This is mimicked during the chondrogenic differentiation of adult human bone marrow-derived MSCs in high-density pellet culture[51]. L-SOX5 and SOX6 are required for the expression of COL9A1, aggrecan, link protein as well as COL2A1 during chondrocyte differentiation[52] (see also figure 1-4).

The aggregation of chondroprogenitor mesenchymal cells into cartilage condensation[53] is dependent upon signals initiated by cell-cell and cell-matrix interactions and changes in the cytoskeletal architecture. The initiation of condensation is associated with increased hyaluronase activity and the appearance of cell adhesion molecules; neural cadherin (N- cadherin) and neural adhesion molecule (N-CAM) as mentioned earlier. N-CAM is regulated by fibronectin which is synthesized in early chondrogenesis due to stimulation by TGF-β, one of the earliest signals in chondrogenic condensation and a major chondro-regulator[31, 32] (figure 1-4). The expression of the SOX proteins is dependent upon BMP signaling via BMPR1A and BMPRIB which are active in chondrocyte condensations, however not in the perichondrium[54]. BMPs set the stage for bone morphogenesis by initiating chondroprogenitor cell determination and differentiation, but also regulate the later stages of chondrocyte maturation and terminal differentiation to the hypertrophic phenotype[46]. *In vitro* and *in vivo* studies have shown that BMP signaling is required for both the formation of precartilaginous condensation and for the differentiation of precursors into chondrocytes[54, 55].

Chondrocyte differentiation is characterized by the deposition of cartilage matrix containing collagens II, IX, XI and aggrecan [46](figure 1-4). The balance of signaling by BMPs and FGFs determines the rate of proliferation throughout chondrogenesis, thereby adjusting the rate of differentiation[56].

Chondrocyte hypertrophy follows terminal differentiation in the process of endochondral ossification[57-59]. Hypertrophy is initiated when the cells in the central region of the anlage (figure1-5) begin to hypertrophy, increasing the cellular fluid volume by almost 20 fold[46]. Ihh

(required for endochondral bone formation[60] and synchronizes skeletal angiogenesis with the perichondrial maturation) is expressed in the prehypertrophic chondrocytes as they exit the proliferative phase, enter the hypertrophic phase, and begin to express the hypertrophic chondrocyte marker, type X collagen (Col10a1) and alkaline phosphatase. The runt-domain transcription factor, Runx2 (Core binding factor, Cbfa1, and Osf2) is also expressed in all condensations, including those that are destined to form bone. Runx2 serves as a positive regulatory factor in chondrocyte maturation to hypertrophic phenotype[61] is expressed in the adjacent perichondrium and in prehypertrophic chondrocytes[62, 63], overlapping with Ihh, Col10a1, and BMP-6[57, 64]. In summary, a complex interplay of positive and negative factors balance and regulate the rate and progression of chondrogenesis.

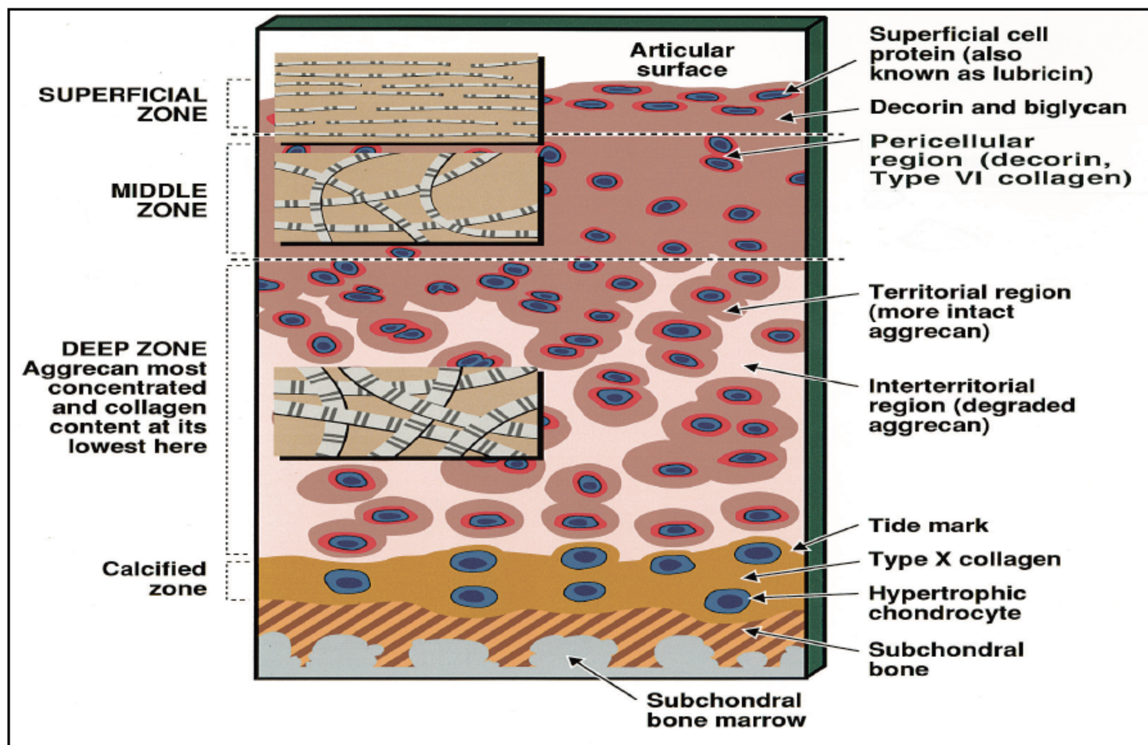


Figure 1-5. Diagrammatic representation of the general structure of human articular cartilage from an adult indicating the zones, regions and relationship with subchondral bone. The insets show the relative diameters and organization of collagen macrofibrils in the different zones. Some special features of molecular content and properties are also indicated. (Poole *et al.*, 2001)[65]

1.3.3 Chondrogenesis *in vitro*

<u>*In vitro Modeling of Hyaline Cartilage Formation*</u>

Most of the current knowledge and understanding regarding the regulation of normal *in vivo* developmental chondrogenesis has been delineated *in vitro* utilizing cell culture systems characterized by high cellular density - in particular, the micromass culture model system[26]. A micromass culture is a three-dimensional, high-density cell culture that is comprised of precursor cells characterized by the potential to differentiate into chondrocytes[66]. The high seeding density stimulates the precartilage cells to mimic the condensation and differentiation events that normally occur during embryonic hyaline cartilage formation *in vivo*[26]. Specifically, the chondrogenic progenitors first undergo condensation, giving rise to aggregates that subsequently differentiate into cartilage nodules, thus simulating the normal progression of *in vivo* chondrogenesis in which mesenchymal condensation precedes a noticeable differentiation (reviewed in DeLise et al., 2000b)[67]. Moreover, the sequence of gene expression during the chondrogenic differentiation process, characterized by the appearance of the transcription factor Sox9 before the accumulation of either collagen type II or aggrecan, is comparable between the *in vitro* micromass culture and *in vivo* situations[68]. This phenomenon is demonstrated as an indication of chondrogenesis *in vitro* in this study (fig. 3-2-B, C). Adding growth factors to micromass cultures allows cells differentiate towards chondrocytes, and therefore provides a convenient means for analyzing the roles of specific molecules in the chondrogenic differentiation process. Finally, the micromass culture model system has been employed to study normal developmental chondrogenesis in a variety of different chondroprogenitor cell types[66, 69, 70]. It is important to note that chondrogenesis can be initiated at low cell density and consequently, in the absence of precartilage condensation. However, the chondroprogenitor must be deceived into acting as though the condensation event has concluded. This can be accomplished by seeding chondroprecursor cells in scaffolds, as is commonly performed during the generation of cartilage constructs, so that ECM molecules accumulate in the pericellular environment in a way that normally occurs post- condensation *in vivo* (reviewed in Hall, 2005)[33]. In this study the scaffold/micromass environment used constitutes of alginate, as done in the groups' previous studies[71] and have been reported by others[72, 73].

Induction of chondrogenesis *in vitro* is similar to the *in vivo* commencement, in terms of both depending on members of the TGF- β Growth Factor Superfamily for induction[26]. Consequently TGF- β and BMPs (lies within the TGF-β superfamily) are required for inducing chondrogenesis *in vitro* as well. TGF-β1 may regulate chondrogenesis at the level of precartilage condensation as its application has been shown to upregulate fibronectin mRNA expression in micromass culture [74]mimicking the *in vivo* process- . TGF-β1, β2 and β3 are frequently employed to induce the transition from fibroblastic to chondrocytic phenotype in high density cultures of adult vertebrate MSCs (reviewed in Chen et al., 2006). TGF-β2, β3 seem to be more chondroinductive than TGF-β1 in high density pellets of adult human bone marrow-derived MSCs, as indicated by enhanced relative production of collagen type II protein, as well as increased mRNA transcripts levels for a variety of cartilage genes, including type II collagen, aggrecan, COMP, and decorin[75].
BMPs have been shown to induce the *in vitro* chondrogenic differentiation of C3H10T1/2 cells, mouse ESCs and human MSCs[76-78]. One of the earliest roles played by BMPs in the chondrogenic differentiation is to promote cell-cell interaction by upregulating expression of N-cadherin[26].
Other growth factors shown to regulate chondrogenesis *in vitro* are IGF, EGF and FGF. IGF1 has been implicated as a positive regulator of chondrogenesis in cell culture[26, 79], in contrast to EGF which has been implicated to be a negative regulator of chondrogenesis[26, 80]. FGF on the other hand can both be positive and a negative regulator *in vitro* depending on the cell type[81-83].

Another positive regulator of chondrogenesis *in vitro* is the glucocorticoid dexamethasone. A study by Derfoul *et al.* (2006)[84] showed that dexamethasone enhances the chondrogenic differentiation of an adult human multipotential mesenchymal cell line established from trabecular bone and further stimulates cartilage-specific gene expression during TGF-β3-enhanced mesenchymal cell line chondrogenesis.

1.4 FOXO1

FOXO1 also known as FOXO1a (and originally as FKHR)is a member of the FOXO family(the forkhead family proteins of the subclass O), which metabolic functions are relevant to glucose metabolism, tumor suppression, hematopoiesis, angiogenesis, cell cycle regulation, modulation of inflammation, apoptosis, and antioxidant defense[85-90].

The forkhead family of transcription factors is characterized by a 100 amino acid monomeric DNA binding domain (DBD) called the FOX domain[91, 92] which is able to up-and downregulate a variety of gens via binding of its consensus Forkhead Recognition element, or similar sequences including Insulin Response Element[92]. Other domains of the forkhead proteins, such as the DNA transactivation or DNA transrepression domain, are highly divergent amongst the forkhead family[93]. The functions of FOXO1 are dynamically regulated by a complex set of post-transcriptional modifications including phosphorylation[94-96], acetylation[97] and methylation and polyubiqiutilation (reviewed by Miranda S.C)[92]. These covalent modifications affect stability, subcellular localization, gene target specificity, and DNA-binding activity. One of the most known and important FOXO1regulators is the serine/threonine kinase Akt(PKB) working downstream of the phosphatidylinositol-3kinase (PI-3kinase). Akt inhibits FOXO1 activity by phosphorylating three sites (Thr-24, Ser-256, Ser -319- in humans)[94-96] (figure 1-6)[92]. The phosphorylation of FOXO1 leads to its cytoplasmic retention and inhibition of its transcriptional activity. Endogenous inhibitors of FOXOs are growth factors, such as insulin and insulin-like growth factors that signal the phosphorylation of FOXOs via the Akt kinase. Dephosphorylation localizes FOXO1 to the nucleus, where FOXO1 binds to the forkhead response element in the promoter of target genes and interacts with transcriptional coactivators, resulting in transcriptional regulation[98].

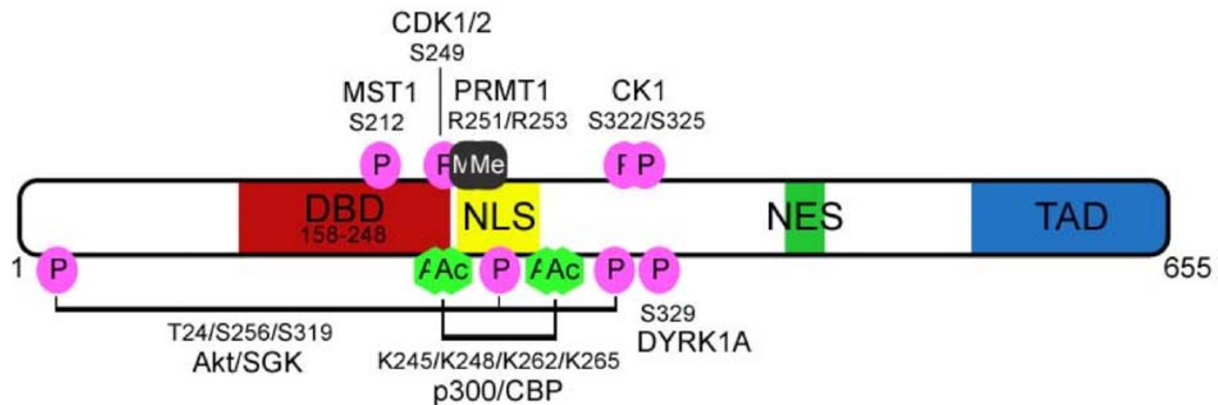


Figure 1-6. Phosphorylation and other modifications of FOXO1. Schematic figure showing confirmed sites of phosphorylation in FOXO1, and the kinases and acetyl transferases involved. Phosphorylation by MST increases FOXO1 activity, while Akt, and SGK are inhibitors. DBD = forkhead DNA binding domain, NLS = nuclear localisation sequence, NES = nuclear export signal, TAD = transactivation domain. (Miranda S.C. *et al*, 2009)[92]

However the function of FOXO1 can be enhanced by phosphorylation as well, at other phosphorylation sites, by activators such as the kinases JNK and MST1, which act in response to oxidative stress, leading to FOXO1 translocation into the nucleus. Once in the nucleus FOXOs define cell fate by affecting genes of various functions, such as transactiviating specific cyclins, cyclin-dependent kinase inhibitors, DNA repair, apoptosis.control genes, and antioxidant enzymes (as reviewed by Stavroula Kousteni, 2010)[99]

<u>FOXO1 *and oxidative stress*</u>

The cell responds to oxidative stress by the transcriptional activation of Foxos[100-102] (one of two major components that the cell uses to counteract the adverse effects of oxidative stress) and subsequent upregulation of the expression of three main Foxo targets: 1) The mitochondrial enzyme superoxide dismutase 2(SOD2) which convert hydroxyl radicals to H_2O_2. 2) The peroxidase catalse which converts H_2O_2 to H_2O. 3) GADD45, the growth-arrest and DNA-

damage inducible protein. Deletion of Foxo1 (in mice) from osteoblasts and osteoblasts progenitors decreases osteoblast numbers, bone formation rate, and bone volume[103, 104]. A Foxo1 knockout model was correlated with increased oxidative stress levels, as evidenced by elevation of both reactive oxygen species (ROS) and lipid peroxidation products. Oxidative stress was secondary due to a suppression of antioxidants mechanisms, as indicated by a decrease in the activity of SOD2 and the levels of glutathione (a protein that in its reduced form scavenges free radicals and detoxifies cells.

Foxo1 *and metabolism through the skeleton*

Foxo1 is a transcription factor that orchestrates the endocrine function of the skeleton in regulating energy metabolism[105-107]. The metabolic actions of osteoblast-expressed Foxo1 are likely mediated, at least in part, by osteocalcin. It has been shown in several studies that osteocalcin in its uncarboxylated state favors β-cell proliferation, insulin secretion, and sensitivity[106, 108-112]. Osteocalcin carboxylation is promoted by tyrosine phosphatase (the product of Esp- function as osteocalcin inhibitor), and thus inhibiting insulin signaling in osteoblasts[106, 107] (fig.1-7). On the other hand insulin signaling in osteoblasts promotes bone resorption in a Foxo1 dependent manner and as a result induces the acidification of the bone extracellular matrix. The acidic environment generated during osteoclastic bone resorption in turn promotes osteocalcin decarboxylation[107]. Foxo1 suppresses pancreatic β-cell proliferation and function (fig.1-7) and thus is a negative regulator of insulin sensitivity in β-cell, hepatocytes, and adipocytes[113, 114]. Collectively, these events compromise glucose metabolism and increase blood glucose levels.

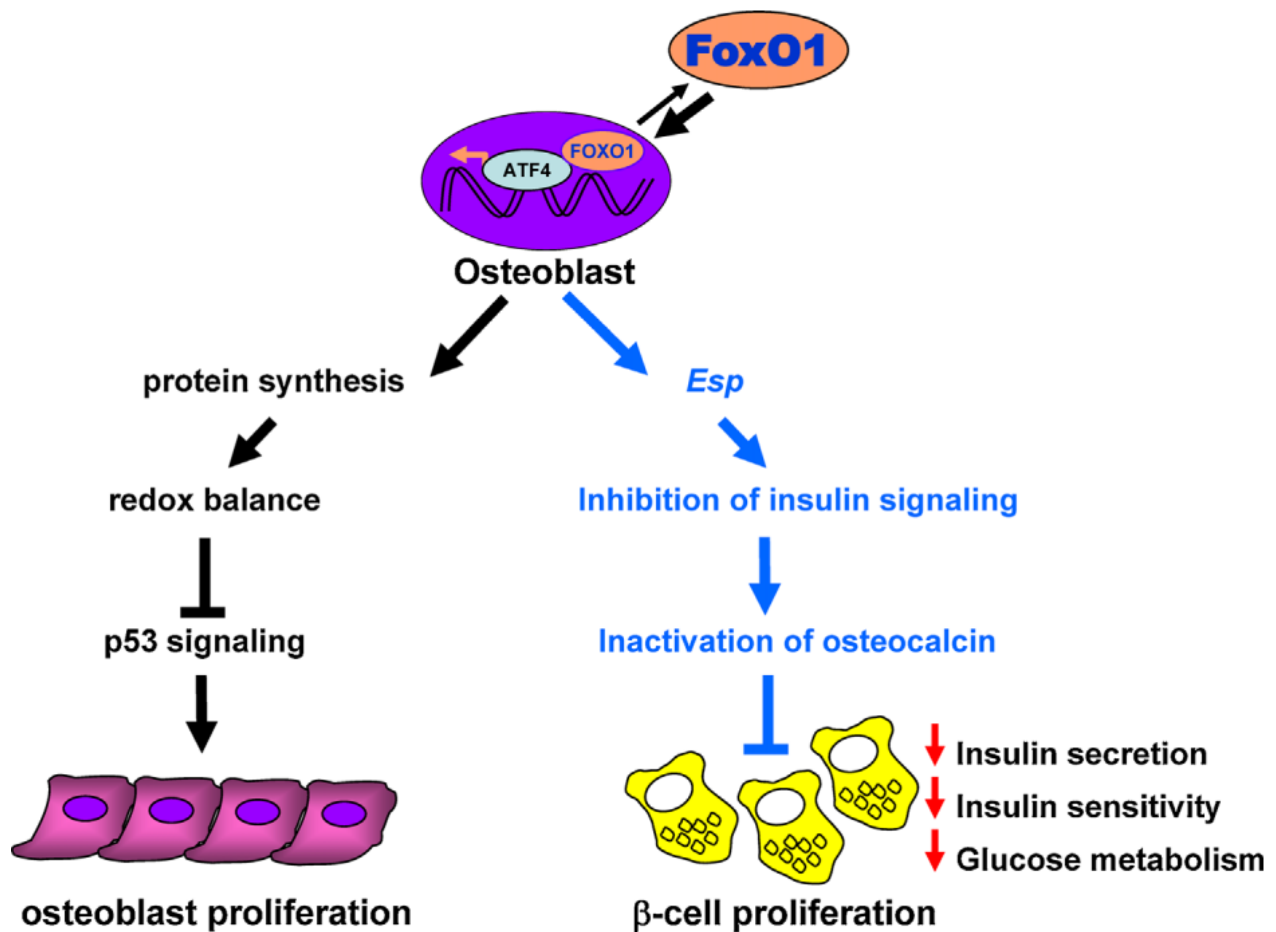


Figure1-7. Local and long distance actions of osteoblast-expressed Foxo1. Foxo1 shuttles between the nucleus and the cytoplasm. Physiological levels of stress promote translocation of Foxo1 in the nucleus, where it interacts with ATF4. This interaction promotes the transcriptional activity of Foxo1 and is required for protein synthesis. Normal protein synthesis allows Foxo1 to maintain redox balance by preventing the increase in ROS levels and thus by suppressing subsequent, ROS-induced activation of an antiproliferative p53 cascade. Repression of a p19ARF/p16/p53 pathway prevents cell cycle arrest in osteoblasts and maintains their normal proliferation and skeletal homeostasis. (From Kousteni, 2010)[99]

This opposing effect of oxidative stress and insulin on osteoblasts permits a dual mode of regulation. In one mode it preserves metabolic balance in conditions of increased oxidative stress. In early stages of aging, a modest increase in oxidative stress can increase FOXO1 activity in bone, thus maintaining osteoblast numbers and preserving their function as endocrine cells that

favor glucose availability. In another opposite context, in situation s of metabolic stress (starvation) reduced insulin levels would lead to an increase in FOXO1 activity in bone. In turn, this increase in Foxo1 activity in bone would raise blood glucose levels, providing a source of nutrients to the brain. Thus a dual mode of regulation provides a dual mode of rescue mechanism. Moreover along with the pancreas and liver, bone becomes another organ that determines energy supply under stress.[99]

Foxo1 a regulator of osteoblast differentiation and skeletogenesis:

Foxo1 interacts directly with the promoter of Runx2 and regulates its expression; therefore silencing Foxo1 decreases the expression of Runx2 and impairs bone formation[91]. It has been shown that silencing of Foxo1 blocks also the expression of other osteogenic markers such as alkaline phosphate, and osteocalcin and results in decreased culture calcification even in the presence of strong osteogenic stimulants. Conversely the expression of these markers increases significantly in response to Foxo1 overexpression. It has also been demonstrated that activation of Foxo1 prevents mesenchymal cells from differentiating into fat or muscle cells[115-117]. The role of FOXO1 in skeletogenesis is discussed further in chapter 4.2.

1.5 articular cartilage injuries and treatment

Normal synovial joint function depends on the smooth, low friction gliding surface provided by articular cartilage[118]. Although at most only a few millimeters thick, articular cartilage has surprising resistance to compression and resilience. It has an exceptional ability to distribute loads, thereby minimizing peak stresses on subchondral bone. Articular cartilage has remarkable durability and is able to provide normal joint function for 80 years or more in many people [11]. Yet despite the durability of articular cartilage under normal joint loading, excessive joint loading can damage articular cartilage causing loss of joint motion, instability, deformity and pain.

Injuries to articular cartilage in the knee are fairly common[119, 120]. Traumatic cartilage injuries are often seen in young active people (athletes, or people exposed to accidents), while degenerative cartilage injuries are most common in elderly. The ultimate goal for cartilage repair is to regenerate a repair tissue that is capable of bearing load and fulfilling various other functions of normal cartilage with a perfect integration into surrounding tissues[121]. To date a treatment that

fulfills these requirements does not exist. However there are various methods of treatment today either cell based or marrow stimulatory based therapies. Within the marrow stimulatory approaches the mircrofracture[122] is the most frequently applied therapy, which involves the penetration of the subchondral bone in several places with 3-4mm distance apart leading to bleeding and formation of a fibrin clot that functions as repairing tissue. Within the cell based therapies the autologous chondrocyte implantation (ACI)[123] is the most commonly used approach today. ACI is a regenerative approach and the current therapeutic aspect of our research. The principle of the approach is to isolate healthy cartilage tissue from around the site of injury from patients, suffering from cartilage damage; followed by culturing the healthy cells (chondrocytes) in the lab. After achieving a sufficient number of cells, the cells will be injected back to the site of the injury (fig.1-8), and the healthy cells will then home to the site of injury and “heal the lesion”.

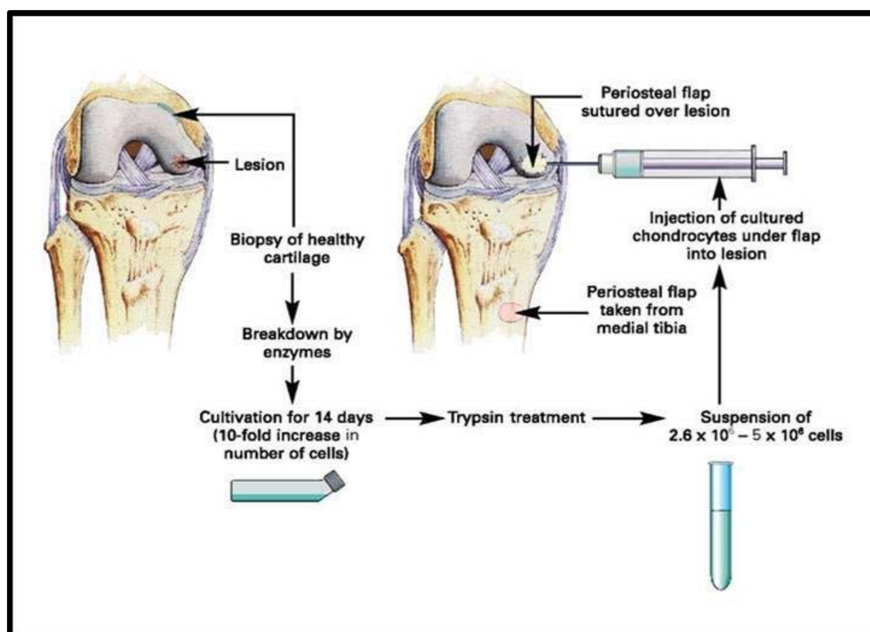


Figure 1-8. Articular cartilage biopsy from a patient's knee during arthroscopic surgery. The chondrocytes from the cartilage are isolated and grown in the laboratory. After a sufficient number of chondrocytes has been cultured, they are injected back into the defect of the knee, where a periosteal flap covers the defect. From Dr C. Benjamin Ma, 2012.[124]

Most of the patients respond well to the treatment[125, 126], however a some showed a relapse into pain and malfunction of the knees after a few years from the treatment (2-5 years), according to the trials that have been done so far. Theoretically this treatment is designed to be permanent, however as many studies that have been done so far are recent studies and the course of treatment has not been longer than 10 years, it is unclear and too early to assume that the treatment is truly permanent. Thus the challenge still remains to figure out why some patients do relapse. What are the factors involved in reversing the effect of the treatment? How can we regulate them? Which genes should be on and which should be off? How can we keep the tissue healthy and functional permanently? In order to answer these questions a thorough understanding of the basic biology of stem cells and chondrogenesis is required. Furthermore more molecular research is ought to be carried in order to identify more factors that are involved in the differentiation of MSCs towards chondrocytes, the factors that regulate terminal differentiation of chondrocytes, and which factors can be regulated to maintain this state of differentiation in order to prevent cartilage hypertrophy (refer to section 1.3.2). Highly importantly we need to acquire an understanding of the interplay of those factors that are/will be indentified. In this research group where this work was carried out, cartilage construction (tissue engineering) is the ultimate goal. The principle of the ongoing research is to direct MSCs towards cartilage differentiation by capturing the cells in a three dimensional scaffold/culture and providing the cells with specific stimulating factors (reviewed in section 1.3.2 and 1.3.3). In a previous study performed by Jackobsen *et. al* (unpublished data) that aimed for defining genes that were significantly altered at the commencement of chondrogenesis *in vitro,* it has been shown that *FOXO1* expression increases in the early days of differentiation of mesenchymal cells into chondrocytes in the duration of 1 week. *FOXO1* had its highest peak of expression on day 3(figure1-9-A). Additionally during this period collagen type II expression was increasing (Figure.1-9-B.) The same study has shown that *FOXO1* expression exhibits a pattern similar to that of *SOX9* (figur1-10). Thus the aim of this study was to investigate and identify the molecular mechanisms of which by *FOXO1* operate in chondrogenesis.

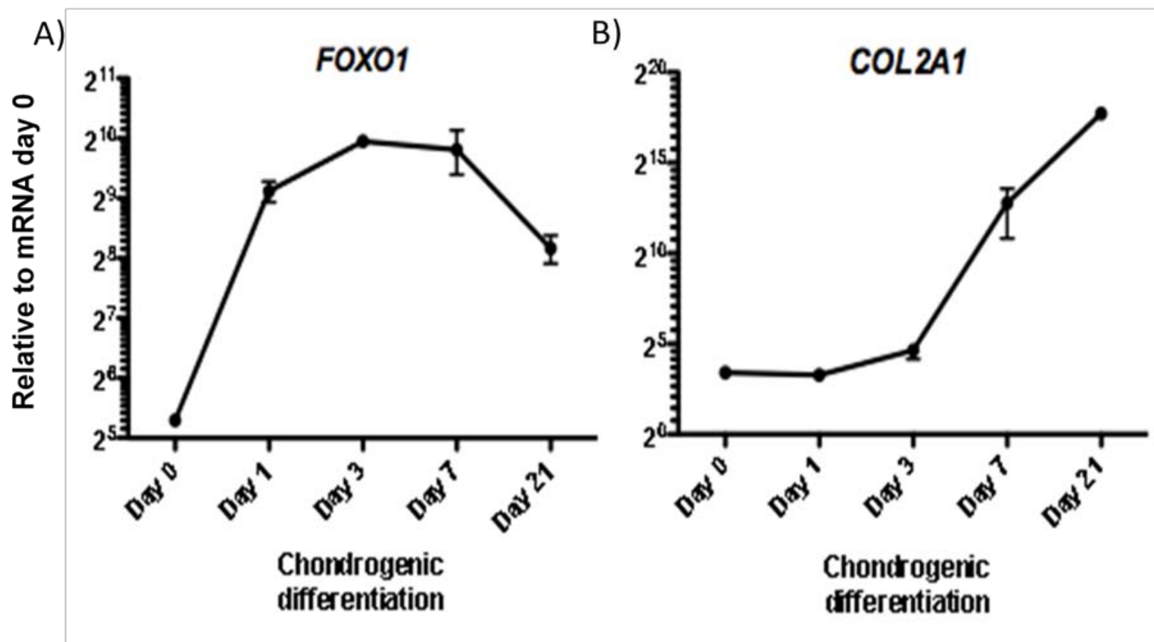


Figure 1-9.mRNA levels of *FOXO1* and *COL2A1*. Cells in both A) and B) were cultured in alginate and differentiated towards chondrogenesis by adding differentiation medium containing TGF-β1, BMP2, dexamethasone, ascorbic acid, sodium pyruvate, ITS, HSA, and glucose. Jakobsen *et. al* (unpublished data, 2011)

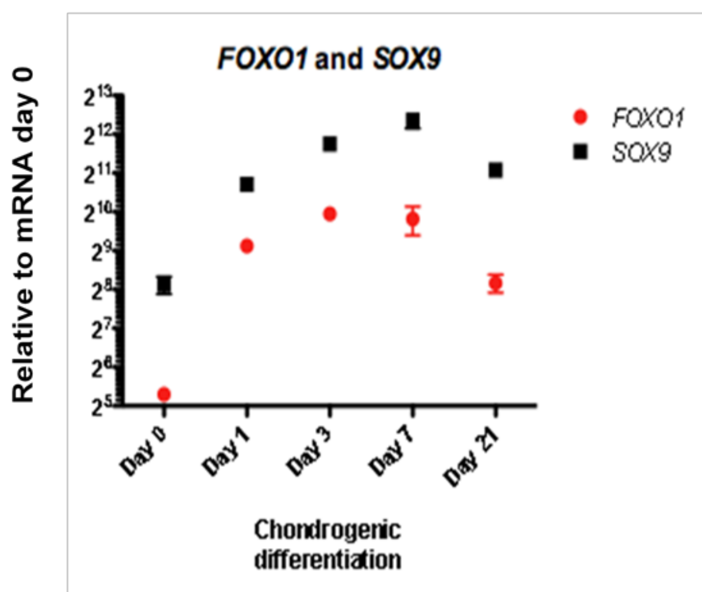


Figure 1-10. mRNA levels of *FOXO1* and *SOX9*. MSCs cultured in alginate and differentiated towards chondrogenesis with the same differentiated medium in figure 1-10. Jakobsen *et.al*(unpublished data, 2011)

1.6 Aims of study

Based on the unpublished results from Jakobsen *et al.* reviewed above (section 1.5), this study aimed for exploring the role of FOXO1 in the differentiation pathway of MSCs towards chondrogenesis. The investigation was designed as follows:

1) To characterize the effects of FOXO1 knockdown on MSCs differentiated towards chondrogenesis in a three dimensional scaffold, this implies the following:
    a. Constructing the alginate three-dimensional scaffold and differentiate the transfected MSCs captured in it towards chondrogenesis with the required stimulatory chondrogenic factors.
    b. Identifying an ultimate functional siRNA that knocks down FOXO1 with high efficiency and stays stable for the time required (optimally 6 days). When the most efficient FOXO1 siRNA is found, it is to be transfect into MSCs determined for differentiation.
    c. Analyzing the effects of *FOXO1* knock-down exhibited on target genes in differentiated cells with RT-qPCR and nanostring technology.

2) To study the effects of FOXO1 up-regulation on MSCs cultured in a two-dimensional system, which implies
    a. Identifying a suitable plasmid expressing the *FOXO1* gene and subsequently transfect MSCs with the plasmid to over-express *FOXO1*.
    b. Analyzing the effects of *FOXO1*over-expression on target genes by RT-qPCR.

Collectively evaluate the overall effects of *FOXO1* down regulation and upregulation on the selected genes in two different systems (2D and 3D).

2. Materials and Methods

2.1. Cells

Theoretical Background

In order to isolate MSCs, the marrow mononuclear cells (BM-MNC) are isolated from bone marrow from various young and healthy donors of both genders using gradient centrifugation where cells are separated according to their density (Arne Bøyum 1968)[127]. Bone marrow aspirate is diluted with a diluting medium, applied to the top of the centrifugation medium (Lymphoprep) and centrifuged at 800g for 20 min. This is the so-called gradient centrifugation where cells are separated according to their density. The lymphoprep has a density of 1.077 g/cm^3 [2] and cells with higher density (erythrocyte and granulocytes) than the Lymphoprep will go through the medium, while cells with a lower density such as the mononuclear cells (monocytes, lymphocytes and MSCs) will be retained at the medium interface above the lymphoprep[2] (see figure 2-1)

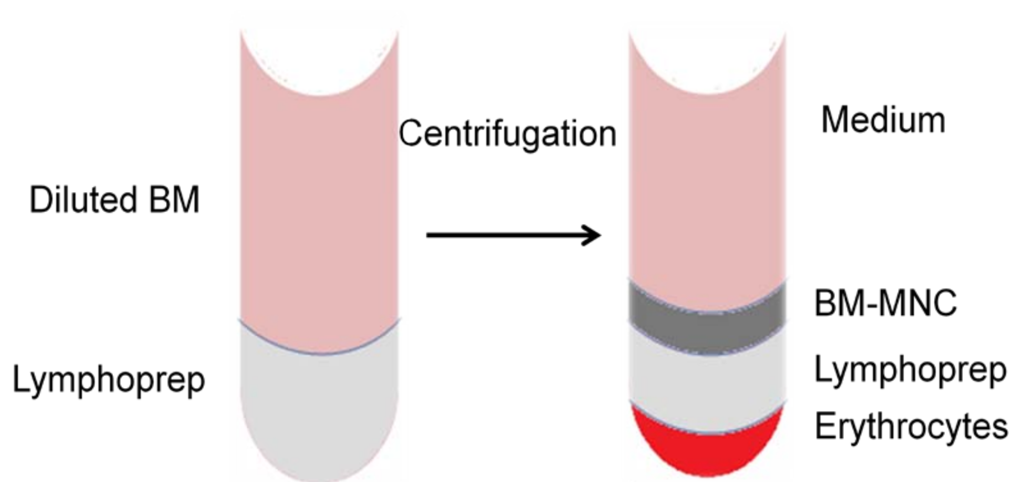


Figure 2-1.Density gradient centrifugation. The method allows for the separation of cells according to their density in distinct layers. Centrifugation is performed at 800 g for 20 min. The mononuclear cells are to be found in the grey-like phase.

Procedure: Isolation and culture of hBM-MSCs

BM-MSCs were isolated from human bone-marrow taken from the iliac crest of 3 donors according to previous publication (Herlofsen 2010). Briefly, 50 ml bone marrow was diluted in a diluting medium; DMEM/F12 containing 2.5µg/ml amphotericin B, 100units/ml penicillin and 100µg/ml streptomycin P/S (Sigma), and isolated by density gradient centrifugation using BM-DMEM:Lymphoprep at a ratio of 1:3. The mononuclear fraction was seeded out in T175 flasks (5 per donor) in DMEM/F12 containing 20% serum (PLP- Platelet Lysate Plasma made in the lab following Shallmoser *et al.*'s protocol[128]), 2.5µg/ml amphotericin B, 100units/ml penicillin and 100µg/ml streptomycin P/S and kept for 10 days with medium change every 3 days. After 10 days, cells were passaged using Trypsin-EDTA 1X(Sigma), counted and seeded out about $1x10^6$ cells/T175cm^2 flask with 35ml culture medium (DMEM/F12, 10% PLP, and P/S).

The culture established directly after isolation is referred to as P0 (passage 0) and cells are usually trypsinated/passaged every 3-4 days depending on their confluence. In this study cells from several donors were used at passage3-passage 6.

2.2 Chondrogenic differentiation

The protocol for chondrogenic differentiation (according to Herlofsen et. al 2011) consists of the embedding of cells into alginate discs, followed by culturing in a chondrogenic differentiation medium. To prepare alginate discs cells were trypsinated and pellets were resuspended in 4.6% D-mannitol (Sigma-Aldrich) and counted. Cells were spun down at 300g for 5 min, and pellet was resuspended in 1%LVG (low viscosity sodium alginate- from NovaMatrix). The LVG-pellet mixture was then mixed with 1% Sodium alginate gel from NovaMatrix and left to polymerize/gel for 15 min, followed by washing 3X with DMEM. The discs were cultured in differentiation medium (table 2-1) for various times before analyzed.

In this study before cells were embedded in alginate they were transfected with FOXO1siRNA and the negative control (scrambled siRNA), seeded in T75 flasks and let recover for a day. The following day some of the cells were harvested from the flask and snap-frozen to isolate RNA for RT-qPCR analysis. These cells are called monolayer, or Day 0 cells, as they have not been in

alginate. The rest of the cells were embedded in alginate discs at the density of $10x10^6$ cells/ml and harvested on Day1, Day 3, and Day 5 after being cultured in differentiation medium.

Table 2-1. Chondrogenic differentiation medium reagents.

Reagents	Concentration	Manufacturer
Sodium pyruvate	1mM	Gibco BRL
Ascorbic acid-2-phosphate	0.1 mM	Sigma
Dexamethasone	0.1µM	Sigma
ITS(Insulin, Transferin, Sodium selenite)	1%	Sigma
Glucose	4.5g/L	B.Braun
BMP2	500ng/ml	R&D Systems
TGF-β1	10ng/ml	R&Dsystems
HSA	40mg/ml	Octapharma

2.3. Transient transfection – Amaxa ™ Nucleofactor ™ Technology

Theoretical background

Nucleofection™ is a technology based on the momentary creation of small pores in cell membranes by applying an electrical pulse. The comprehensive way in which Nucleofector™ Programs and cell type-specific solutions are developed enables nucleic acid substrates delivery not only to the cytoplasm, but also through the nuclear membrane and into the nucleus. This allows for high transfection efficiencies up to 99% and makes the transfection success independent from any cell proliferation.6

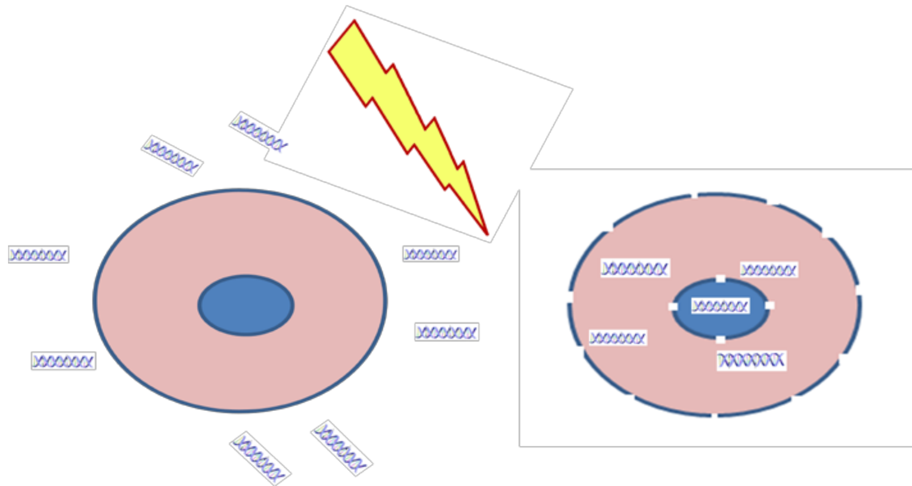


Figure 2-2. Nucleofection. The electric pulse creates pores in the cell membrane and the nucleus membrane allowing the short siRNAs to enter the cell from the surrounding medium. Once inside the cell their down-regulating effect commences.

Procedure

Transfection with siRNA and plasmid was performed using Amaxa™ Nucleofector™ kit for human chondrocytes (Lonza) according to the protocols from the manufacturer. Briefly cells were dissolved in a transfection buffer (Human chondrocyte Nucleofactor solution) at a concentration of $1x10^6$ cells/100µl buffer, together with either siRNA (1µM or 5µM- see fig.3-4-B) or plasmid (1µg/µl- see fig.3-10). The mixture was transferred to a cuvette and pulsed for 2 seconds. Cells were then carefully and quickly transferred to flasks with culture medium (DMEM/F12, 20% PLP and no antibiotics added) to allow cells to recover. Cells were harvested the following day.

2.4. siRNA technology

siRNA (small interfering RNA) is also known as short interfering RNA or silencing RNA[129-131], is a class of double-stranded RNA molecules, 20–25 nucleotides in length, that play a variety of roles in biology. Most notably, siRNA is involved in the RNA interference (RNAi) pathway, where it interferes with the expression of specific gene. RNA interference is an inherent regulatory mechanism in living cells that takes part in fine-tuning transcriptional regulation. siRNA is one central mediator of the RNAi system and the other is microRNA(miRNA). An enzyme complex (Dicer) is mediating cleavage of dsRNA. Dicer contains domains for dsRNA binding, RNA unwinding, and ribonuclease activity, and is associated with additional proteins to drive the cleavage of dsRNA in an ATP-dependent manner[132, 133]. The resulting siRNA as part of a multiprotein RNA-inducing silencing complex (RISC) is targeted to the complementary RNA species which is then cleaved[134]. After binding to this homologous mRNA, RISC cleaves it in the middle of the region bearing complementary base pairing to the guide strand. The guide is not cleaved and RISC can thus attach to and cleave multiple copies of target mRNA, leading to effective RNAi (Saurabh Singh et. al 2011)

miRNAs are endogenous, non-coding RNAi molecules about 22 nt long and are capable of negatively modulating posttranscriptional expression of genes by binding to their complementary sequence in the 3' untranslated (UTR) region of mRNAtargets. miRNA, like siRNA, need to be incorporated into a RISC to cause RNAi. Unlike siRNA, miRNAs are generated in the nucleus and transported to the cytoplasm as mature, hairpin structures. The precursor units of miRNA in the nucleus are the pri-miRNAs, which are several kilobases in length and are transcribed by RNA polymerase II (Saurabh Singh et. al 2011)

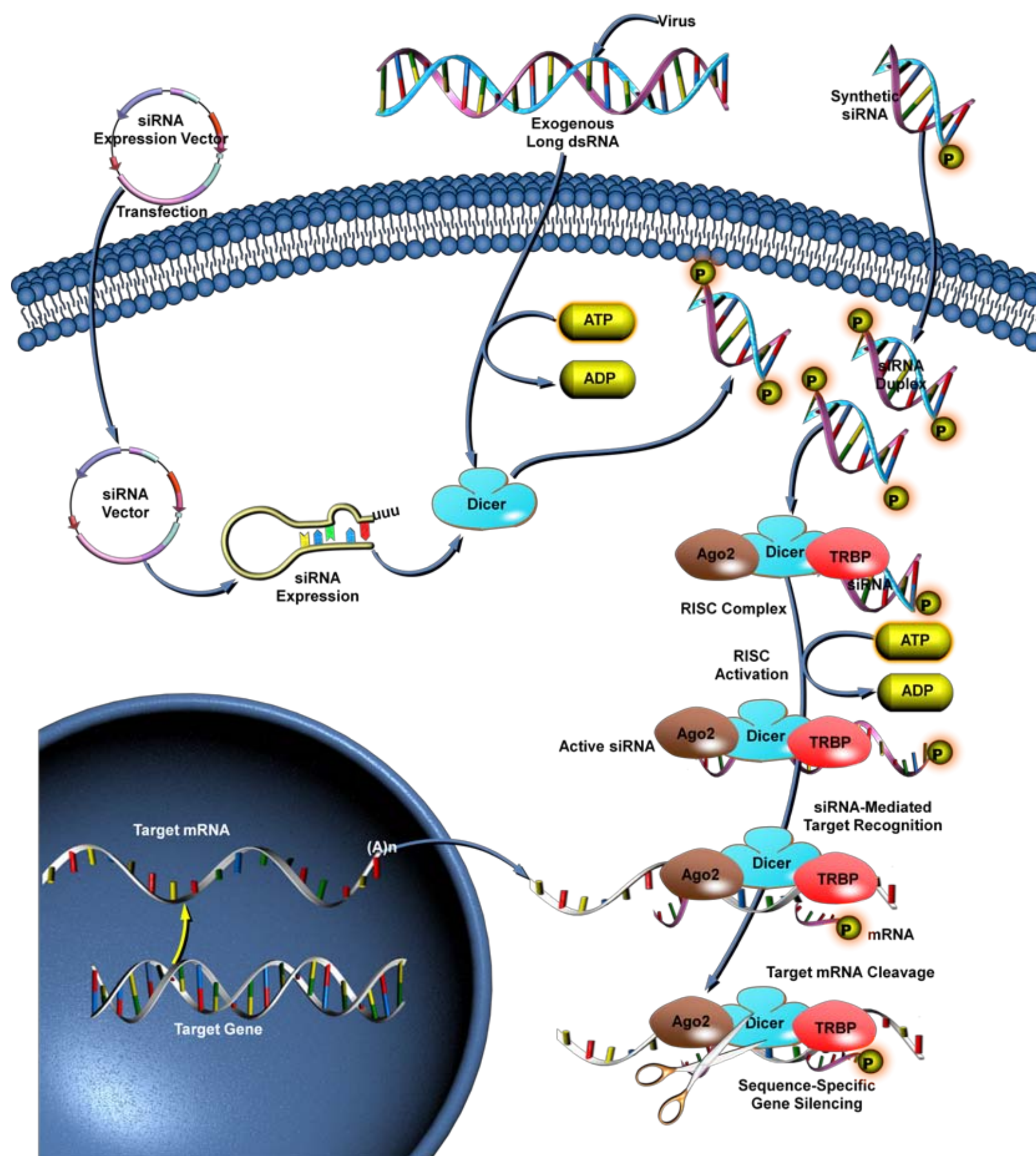


Figure 2-3. siRNAs as mediator of RNAi and as a tool for gene function analysis. dsRNA from various sources is cleaved by the Dicer multiprotein complex to generate short duplexes (siRNAs). Multiprotein complexes then are targeted to complementary RNA species to mediate gene silencing. siRNAs become part of a RNA-inducing silencing complex which mediates gene silencing by target RNA cleavage. This mechanism can be used to analyze gene function *in vitro* and *in vivo*. Short hairpins generated from vector systems in cells are converted to siRNAs (which can also be applied exogenously)[134]. Chemically synthesized siRNAs that are introduced into cells bypass the 'dicing' step and are incorporated into the RISC for targeted mRNA degradation [135]. Figure taken from QIAGEN Sample and Assay technologies.

It is essential to find an efficient siRNA sequence. Several different siRNAs should be tested in

the experimental system. An effective siRNA should be titrated; siRNAs are functional at surprisingly low concentrations and they should be used at the lowest effective level in order to minimize potential side effects since there is experimental evidence that the RISC complex is saturable.[134]

Table 2-2. List of FOXO1 siRNAs tested for their knockdown efficiency.

siRNA Name and description		Sequence targeted	Producer
1. Hs_ FOXO1A_6	Experimentally Verified	AACCAAGTAGCCTGTTATCAA	QIAGEN
2. Hs_FOXO1A_7	Experimentally verified	CCCGAGTTTAGTAACAGTGCA	
3. Hs_FOXO1_3	Not verified	AAGAGCTGCATCCATGGACAA	
4. Hs_FOXOA_1	Not verified	CTCGAACTAGCTCAAATGCTA	

2.5. Real-time qPCR

Theoretical background

Real- time qPCR is the most powerful tool for quantitative nucleic acids analysis. RT-qPCR allows the sensitive, specific and reproducible quantification of nucleic acids[136]. The PCR reaction generates copies of a DNA template exponentially; this results in a quantitative relationship between the amount of starting target sequence and the amount of PCR product accumulated at any particular cycle. The measurements of PCR products as they accumulate "in real time" allow quantification in the exponential phase of the reaction and therefore is a reliable detection and measurement of the products generated during each cycle, as they are directly proportional to the amount of template prior to the start of the PCR process. In contrast to traditional PCR where the accumulated PCR products are measured only at the end of the PCR cycle, making it only semi-quantitative and unreliable as reagents eventually are used up, presence of inhibitors in the sample and accumulation of inorganic phosphate eventually lead to

slow the polymerase reaction rate and PCR product no longer being doubled each cycle. Carrying out the process of real-time qPCR involves two pre-steps prior to the reaction itself: 1) RNA isolation. 2) Reverse transcription of RNA to cDNA (complementary DNA). Further the amplification process of the qPCR follows and finally data analysis of the results.

There are two types of quantitative real-time PCR: absolute and relative[137]. In this study only the former approach has been utilized, therefore relative quantification will discussed in more details. Absolute quantification or standard curve: determines the input copy number of the transcript of interest, usually by relating the PCR signal to a standard curve, and should be performed in situations where it is necessary to determine the absolute transcript copy number.

Relative quantification (RQ): determines the change in expression of a nucleic acid sequence (target) in a test sample relative to the same sequence in a calibrator sample. The calibrator sample can be an untreated control (an empty plasmid or a scrambled siRNA in this study), or a sample at time zero in a time-course study. RQ is commonly used to compare expression levels of wild-type with mutated alleles or the expression levels of a gene in different tissues. In this study relative quantification is used to compare the regular expression of a chosen gene and the down regulated version of the gene and subsequently the effects of this down regulation on some selected chondrogenic genes. RQ provides accurate comparison between the initial levels of template in each sample without requiring the exact copy number of the template. Another advantage of RQ is that the relative levels of templates in samples can be determined without the use of standard curves. RQ is also known as comparative threshold method ($2^{-\Delta\Delta Ct}$ method)[136]. The amount of target gene in the sample, normalized to an endogenous housekeeping gene and relative to the normalized calibrator, is then given by $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = \Delta Ct$ (sample)- ΔCt (calibrator), and ΔCt is the Ct of the target gene subtracted from the Ct of the housekeeping gene. In order for this calculation to be valid and to obtain reliable results, it is imperative that the amplification efficiencies of the housekeeping and target gene are approximately equal (above 90%). This can be established by looking at how ΔCt (of both sample and calibrator) varies with template dilution if the plot of complementary DNA (cDNA) dilution versus ΔCt close to zero, it implies that the efficiencies of the housekeeping and target gene are very similar. If a

housekeeping gene cannot be found whose amplification efficiency is similar to the target, the standard curve method is then preferable.

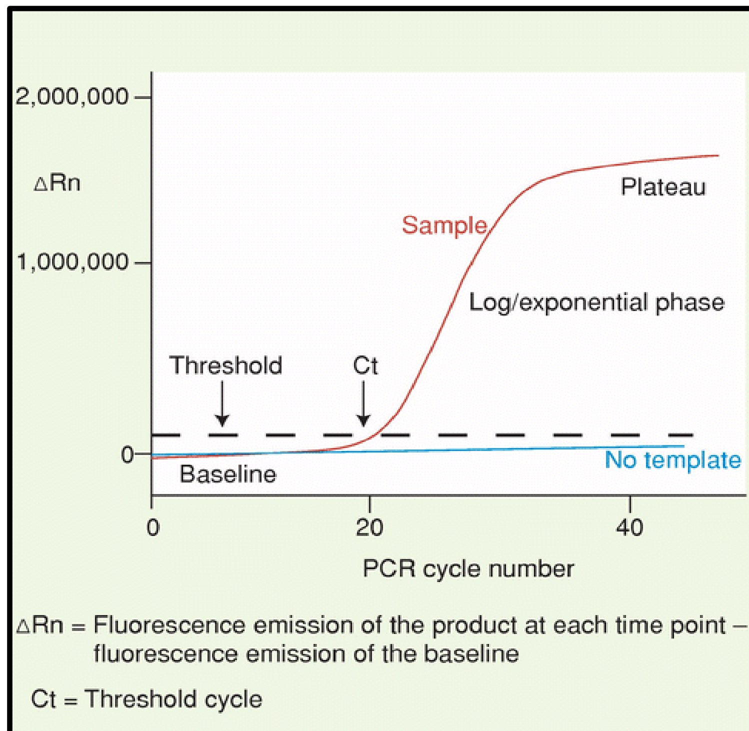


Figure 2-4. qPCR amplification plot. The computer software constructs amplification plots using the fluorescence emission data that are collected during the PCR amplification. The baseline is defined as the PCR cycles in which a fluorescence signal is accumulating but is beneath the limits of detection of the instrument. Threshold is an arbitrary threshold is chosen by the computers, based on the variability of the baseline. The threshold can also be adjusted manually. A fluorescent signal is detected above the threshold is considered a real signal that can be used to define the threshold cycle (Ct). Ct is defined as the fractional PCR cycle number at which the reporter fluorescence is greater than the minimal detection level. As reaction components become limiting, the rate of target amplification decreases until the PCR reaction is no longer generating template at an exponential rate (plateau phase) and there is little or no increase in PCR product. From Arya *et. al* 2005[136].

Normalization of RNA is necessary due to the specific errors that occur in qPCR, as a consequence of the minor differences in the starting amount of RNA, quality of RNA, or differences in efficiency of cDNA synthesis and PCR amplification. The most common genes

used for normalization are housekeeping genes such as β-actin, *GAPDH* (glyceraldehyd-3-phosphat dehydrogenase), glycolytic enzyme and ribosomal RNA (rRNA). In this study *GAPDH* was used for normalization. These genes should theoretically be expressed at a constant level among different tissues of an organism, at all stages of development, and their expression levels should also remain relatively constant in different experimental conditions. However none of these housekeeping genes are ideal. It has been shown that GAPDH expression levels are altered by glucose, insulin, heat shock and cellular proliferation (as reviewed by Arya *et. al* 2005). Alteration of *GAPDH* might be the reason for the results observed in fig-3-12.

<u>*Amplicon detection*</u>

There are two types of general chemistries that are used to detect PCR products on real-time instruments; 1) double stranded DNA-intercalating agents (DNA binding dyes). 2) Fluorescent probes. Only the last chemistry will be introduced further.

With fluorogenic probes, nonspecific amplification due to mispriming or primer-dimer artifact does not generate signal as specific hybridization between probe and template is necessary for fluorescence emission. Also, fluorogenic probes can be labeled with different and distinguishable reporter dyes, thus allowing the detection of amplicons that may have been produced by one or several primer pairs in a single PCR reaction – termed multiplex real-time PCR.[2]

In this study the second chemistry was chosen; hydroloslysis probes, and used TaqMan probes for detection. Therefore this approach will be discussed in more details.

In this process a forward and reverse primer and a probe are used. The efficiency of the assay is mainly dependent on 5´ to 3´ nuclease activity – the most commonly used enzyme is Taq-polymerase but any enzyme with 5´ nuclease activity can be used. The oligonucleotide probe has a covalently bonded fluorescent reporter dye and quencher dye at the 5´ and 3´ ends, respectively. When the probe is intact the proximity of the reporter and quencher dyes permits FRET, and fluorescence emission does not occur. During PCR amplification the probe anneals to the target and Taq-polymerase cleaves the probe, allowing an increase in fluorescence emission. The increase in fluorescence intensity is directly proportional to the amount of amplicon produced.[2]

The TaqMan chemistry is the most widely used real-time PCR assay.[2]

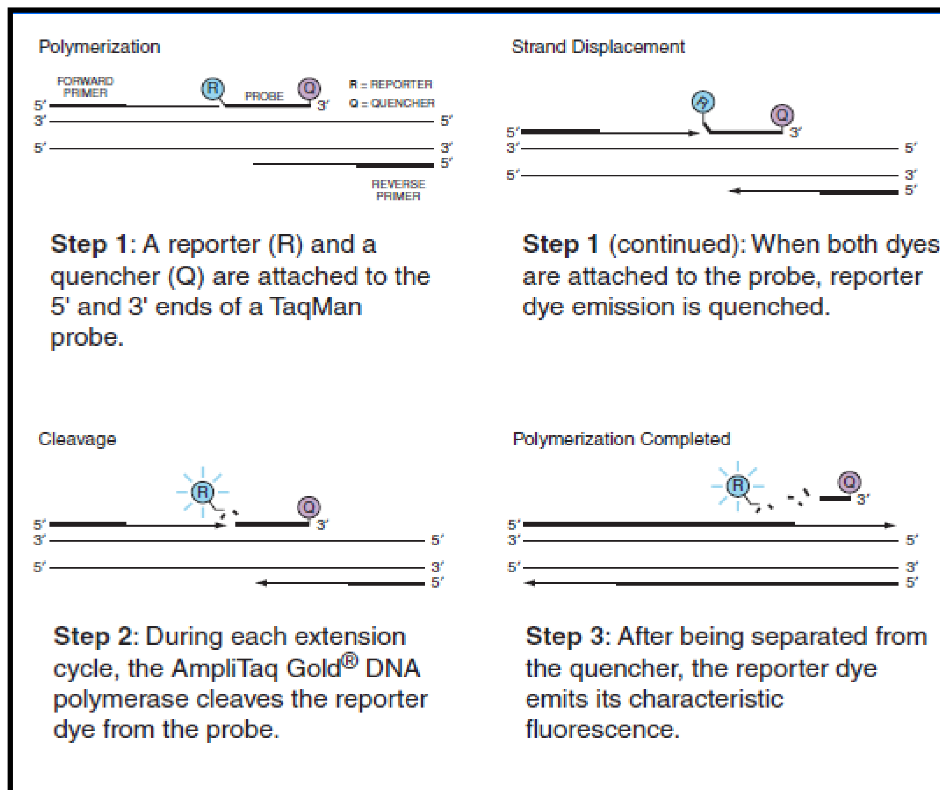


Figure 2-5. TaqMan assay. TaqMan reagent-based chemistry uses a fluorogenic probe to enable detection of a specific PCR product as it accumulates during PCR. From Applied Biosystems User's manual.

Procedure (including pre-preparations)

2.5.1 RNA isolation and RNA and cDNA synthesis

Total RNA was isolated from 0.5-1.0x10^6 cells using the RNAqueous®-Micro Kit (Ambion). The purified RNA was DNAase treated according to producer's protocol (Ambion) before subjected to cDNA synthesis. Reverse transcription was performed utilizing high capacity cDNA

reverse transcription kit (Applied Biosystems) according to the producer's protocol. Briefly 200 ng (RNA concentration was measured by Nanodrop® ND-1000 (Thermo Scientific)) in a 20 µl cDNA synthesis reaction mix containing dNTPs, random primers, buffer, multiscribe, and RNAase free water, was subjected to the following: step 1 at25°C for 10 min, step 2 at 37°C for 120 min and step 3 at 85°C for 5 sec. cDNA was always stored at -20°C, and RNA was always stored at -80°C.

2.5.2 RNA isolation from alginate discs

Alginate discs were harvested at different days and snap-frozen in liquid nitrogen. The frozen discs, while kept in liquid nitrogen, were pulverized using a pestle (1.5ml pestle from VWR) and the RNA was isolated using the RNeasy mini kit (QIAGEN). The purified RNA isolated was then DNase treated as described in 2.4.1.

2.5.3 Real time qPCR

Quantitative PCR analysis was carried out on the 7300 Real-Time RT PCR system (Applied Biosystems) using Taqman® Expression Assay Protocol (Applied biosystems). Briefly each sample was run in triplicate containing 26 µl RNase free water, 30µl PCR master mix, 2µl cDNA of the sample to be investigated, and 3 µl of the particular Taqman probe that targets the gene of interest. The assay ID for the probes used are listed in table 2.3. GAPDH is used as an internal control and calculation for relative expression were performed.

The following genes were investigated: *FOXO1, SOX9, COL1A1, COL10A1, COL2A1, RUNX2, BGLAP* and the endogenous control *GADPH.*

Table 2-3. Assay ID for targets used inn RT-qPCR

Target	Assay ID
FOXO1	Hs00231106_m1
SOX9	Hs00165814_m1
COL1A1	Hs00164004_m1
COL10A1	Hs00166657_m1
COL2A1	Hs00264051_m1
RUNX2	Hs00231692_m1
BGLAP	Hs015877813_g1
GADPH	Hs00231112_m1

2.6. *E.coli* transformation and DNA isolation

The expression plasmid (Myc-DDK-tagged ORF clone of *FOXO1*, fig. 2-6) containing myc-DDK-tagged FOXO1 was obtained from Origene. It was amplified by transformation into the E.coli strain DH5α according to general protocols (Sambrook, Molecular protocol, third edition, 2001). Briefly, 1 µg plasmid was mixed with 50 µl bacteria and subjected to heat shock using the following conditions: 10 minutes on ice, 2 minutes 42°C and 10 minutes on ice. 400 ml LB medium was added to the cells before incubation at 37°C for 15 minutes and plating out of 50 ml on an LB-agar plate containing 50µg/ml kanamycin. One bacterial colony was grown as pre-culture over day in 5ml LB/kanamycin and transferred to 100 ml LB/kanamycin for overnight growth, at 37°C with vigorous shaking. The bacteria were pelleted(centrifuged at 6000xg for 15 min at 4°C) and the DNA was purified using the alkaline lysis-based kit QIAfilter Plasmid Maxi

according to the producer's protocol (QIAGEN). The purified plasmid was run on a 1% agarose gel to assess the purity and integrity of the DNA.

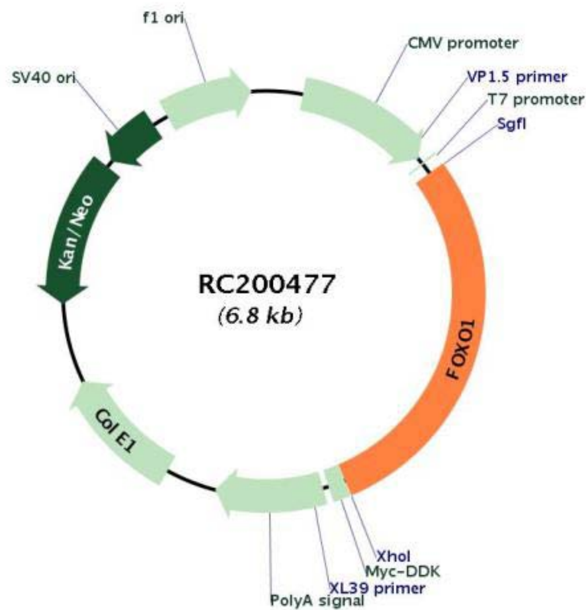


Figure 2-6. The plasmid pCMV6-Entry containing the ORF of the FOXO1 gene, various restriction sites, and the antibiotic kanamycin and Neomycin resistant gene. The plasmid has a Myc-DDK tag. Taken From QIAGEN.

2.6. Western blot

Theoretical Background

Western blot is an analytical technique where proteins, after being separated by electrophoresis, are transferred to a membrane and then detected.

The procedure includes six steps[138]; 1) preparation of the protein (antigen) sample. 2) Separation of proteins by electrophoresis. 3) Transfer of the separated polypeptides to a membrane. 4) Blocking by usually 5% milk or 3% BSA, to minimize the non-specific binding. 5) Addition/incubation with antibodies. 6) Detection of protein using ECL or AP.

Procedure

Western blot was performed according to general procedures (Abcam). Protein lysates were extracted using either 1X Laemmli (Bio-Rad) or 1X RIPA buffer (prepared according to Abcam's protocol), were separated on a 10% SDS-polyacrylamide gel (composition of gel see table2-4), transferred to a PVDF(polyvinylidene difluoride) membrane and ran in 1X transfer buffer at 300 mA for 2hr. The membrane was blocked for non-specific binding with 5% dry non-fat milk in TBS-Tween (0.1% tween 20) for 1 hr at RT. Membranes (fig.3-10) were washed with 1X TBS-T (washing buffer 1, see table 2-5) on shaker for 15 min three times, while membrane in fig3-4 was washed four times for 10 min each with first TBS, with1X TBS-T (washing buffer 2) twice for 10 min, and finally again with TBS for 10min and subjected to secondary antibodies (either biotinylated followed by incubation with streptavidin-HRP or HRP-conjugated) for 2 hours at RT. After washing (same way as after the primary antibody), proteins were detected using ECL (Enhanced ChemoLuminesence, Thermo scientific) on the Carestream Molecular Imaging.

Table 2-4. Composition of the gel.

10 % Tris –glycin-SDS-Polyacrylamide gel	5% Stacking gel
4.8 ml ddH2O	1.82 ml ddH2O
2.5 ml 40% acrylamide mix	0.312 ml 40% acrylamide mix
2.5 ml 1.5M Tris (pH 8.8)	0.31 ml 1.0M Tris-base (pH 8.8)
0.1 ml 10% SDS	25µl 10% SDS
0.1 ml 10% Ammonium persulfate	25µl 10% Ammonium persulfate
4µl TEMED	2.5µl TEMED

Table 2-5. Description of antibodies tested.

Antibody name	Antibody type	Producer	Concentration
Anti-FOXO1A, clone 4C8	Mouse Monoclonal	Millipore	1/1000 in milk
Anti-FOXO1A-ChIP Grade(ab39670)	Rabbit polyclonal antibody	abcam	1/1000 in milk
Anti-c-Myc antibody [9E10]-ChIP Grade. (ab32)	Mouse monoclonal antibody	abcam	1/1000 in milk

Table 2-6 Western blot reagents. The reagents are standard reagents, prepared according to abcam's protocol.

2X Laemmli buffer (stock)	2X Ripa buffer (stock)	10X 5L Running buffer	10X 5LTransfer buffer with 20% methanol	1L 10X Washing buffers (TBS-T)
Purchased as 2X from Bio-Rad pH 6.8	150 mM NaCl 1% NP-40 0.5% Sodium deoxylate 0.1% SDS 50mM Tris pH 8.0 ddH_2O pH 8.3	150g Tris-base 720G glycine 50g SDS ddH2O to 5L pH 8.3	189.5g Tris-base 900g glycine pH 8.3	**1.** 10X (TBS-T) 30g tris-base 80 g NaCl 0.1% Tween 20 pH 7.4-7.6 + $ddH_2O \rightarrow$ 1X(TBS-T) **2.** 1X TBS-T 0.05% tween 20 0.05% NP-40

2.7. Nanostring

Theoretical Background

The Nanostring nCounter system is a digital mRNA profiling system that has a sensitivity higher than that of microarrays and about equal to that of TaqMan RT-qPCR[139]. This method requires only a small amount of total RNA (100ng), and does not require cDNA synthesis or enzymatic reaction. The method involves mixing total RNA with pairs of capture and reporter probes tailored to each mRNA, hybridizing, washing away excess probes, immobilizing probe-bound mRNAs on a surface and scanning color-coded bar tags on the reporter probes. Refer to figure 2-8 for an overview of the method. This solution-phase hybridization scheme is expected to minimize background signal and improve detection of low-abundance mRNAs providing higher sensitivity- at or below a single mRNA molecule per cell compared with microarrays.

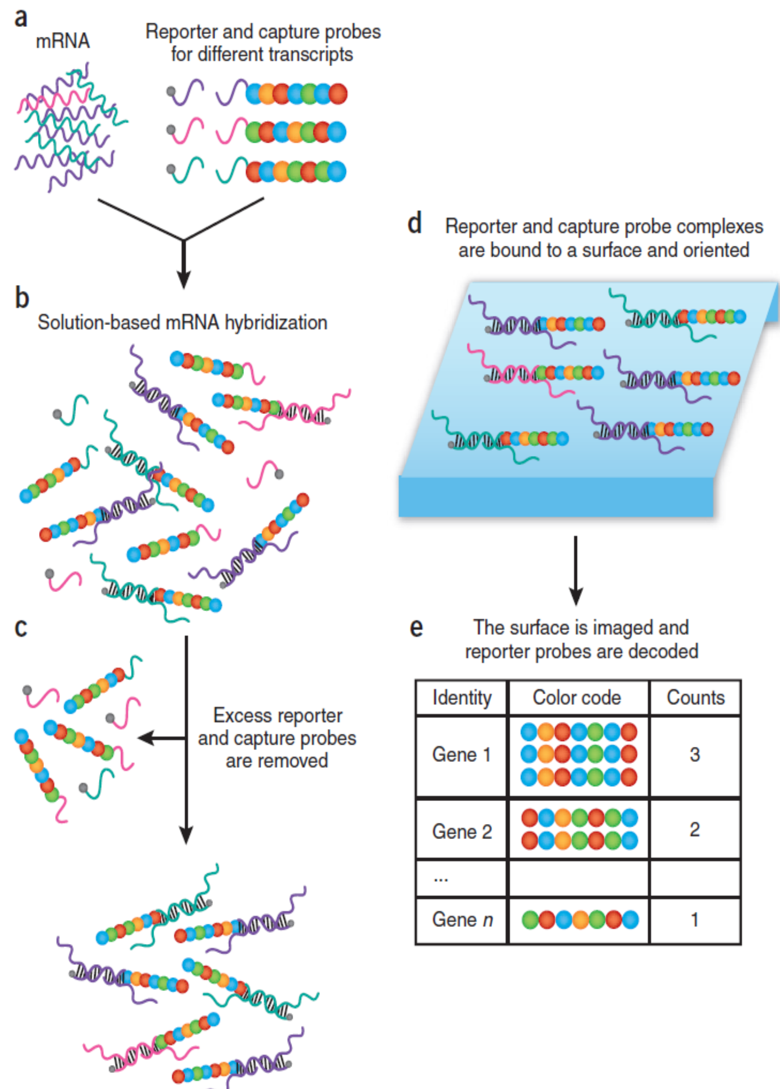


Figure 2-7. Overview of the digital mRNA profiling technology. (a)Total RNA is mixed directly with nCounter reporter and capture probes. (b-d) After hybridization (b) excess reporters and capture probes are removed (c) and purified ternary complexes are bound to the imaging surface, elongated and mobilized (d). (e) Reporter probes, representing individual copies of mRNA, are tabulated for each gene. In this study 370 genes are multiplexed in a single reaction. Taken from Paolo Fortina *et. al* 2008[139]

Data analysis

After receiving the data and prior to data interpretation, slight differences in hybridization, purification, binding efficiency and other experimental variables must be normalized. To accomplish this, it is recommended by Nanostring, to utilize the internal positive controls that are present in each CodeSet. Since these targets are independent of the sample, normalizing these controls will help to eliminate variability unrelated to the sample.

The positive spike in RNA hybridization controls for each lane may be used to estimate the overall efficiency of hybridization and recovery for each lane. This has been done by calculating the geometric mean of all positive controls (6 positive controls one for each RNA sample). Then the average of the geometric mean was calculated and divided by each geometric mean separately in order to calculate a lane specific scaling factor. Further all positive, negative controls and gene counts were multiplied by the lane-specific scaling factor. For further details on calculations refer to the nCounter Expression Data Analysis Guide.

A second normalization was also performed; it is the so-called reference/housekeeping gene normalization. This normalization was performed in order to adjust counts of all probes relative to a probe that is not expected to vary between samples or replicates. In this analysis 5 reference genes have been used for normalization. This has been done as follows; the geometric mean and its average for each lane of the reference gene were calculated, the product was then divided by the geometric mean in each lane to get a lane-specific normalization factor. Finally all gene counts in a lane were multiplied by its lane normalization factor.

Assessing background

Accurate estimation of probe background is essential for interpreting expression data. The negative controls have been used to estimate background values in this experiment. A background threshold was established and values below this threshold were considered background (insignificant changes) and thus not taken in results for interpretation. The background threshold is determined by calculating the average and the standard deviation of all negative control counts for each lane. Then the standard deviation was multiplied by 2 before adding to the average, the result is the background threshold. For further details refer to the nCounter Expression Data Analysis Guide.

3. Results

3.1 Validation of *in vitro* chondrogenesis in alginate

In order to investigate the role of *FOXO1* in chondrogenesis, the process of chondrogenesis was mimicked *in vitro* by utilizing a three-dimensional alginate scaffold where MSCs were captured at a specific cell density and stimulated towards chondrogenesis by a mixture of differentiating factors[71]. MSCs exhibit a different morphology at differentiation commencement.

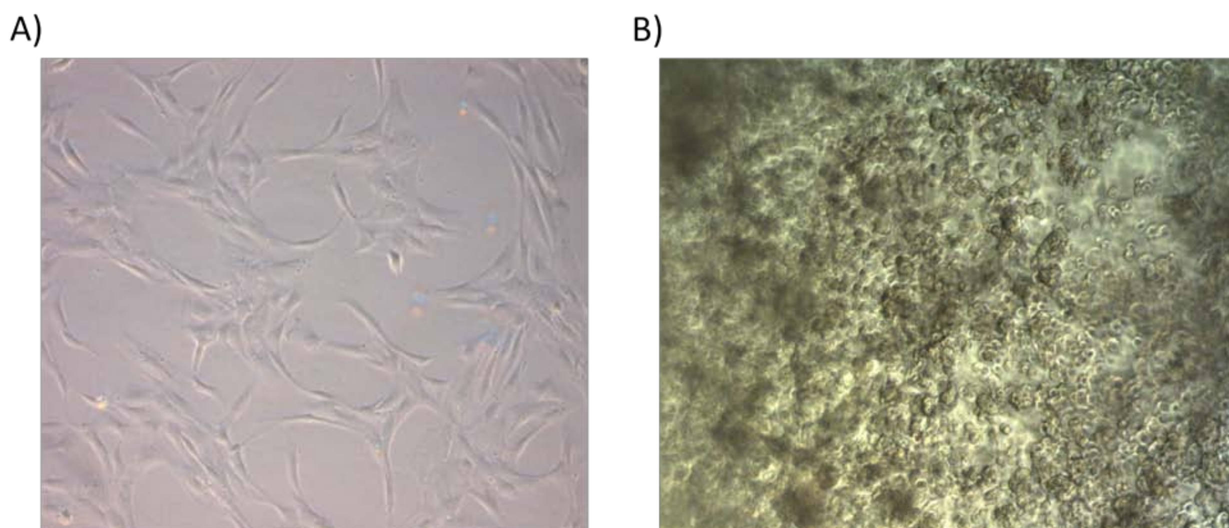


Figure 3-1. Morphological changes associated with chondrogenesis *in vitro*. Light microscopy pictures of MSCs in 2D and 3D. A) MSCs in 2D; cultured in culture medium in T175cm^2 flasks. B) MSCs in 3D are cultured in an alginate scaffold with a cell density of 10x10^{6}cells/ml, provided with chondrogenic differentiation medium (refer to table 2-1).

MSCs grown in 2D are adherent to the plastic (a criteria that defines MSCs *in vitro*, section 1.2), clustered together and keep their characteristic filament-like shape. In contrast MSCs in 3D are round with rough edges and tend to cluster in aggregates when differentiated towards chondrogenesis. It has been shown that MSCs embedded in alginate and cultured in differentiation medium display features associated with chondrogenesis; like the deposition of

hyaline ECM molecules along the intercellular space, following an up-regulation of well-known chondrogenesis signature genes(Herlofsen et al. 2011)[71], like *COL2A1*, *SOX9* and *ACAN*. To further verify that chondrogenesis occurred *in vitro* in transfected MSCs, transcriptional activity of some signature chondrogenic genes and other relevant genes was investigated; collagen type II, and *SOX9* (both are major chondrogenic differentiation markers, reviewed in figure 1-4), collagen type I, a fibrocartilage and bone marker, required at the onset of chondrogenesis *in vivo*, but undesirable during differentiation both *in vivo* and *in vitro* and collagen type X which is a chondrogenic hypertrophic marker, also undesirable during differentiation.

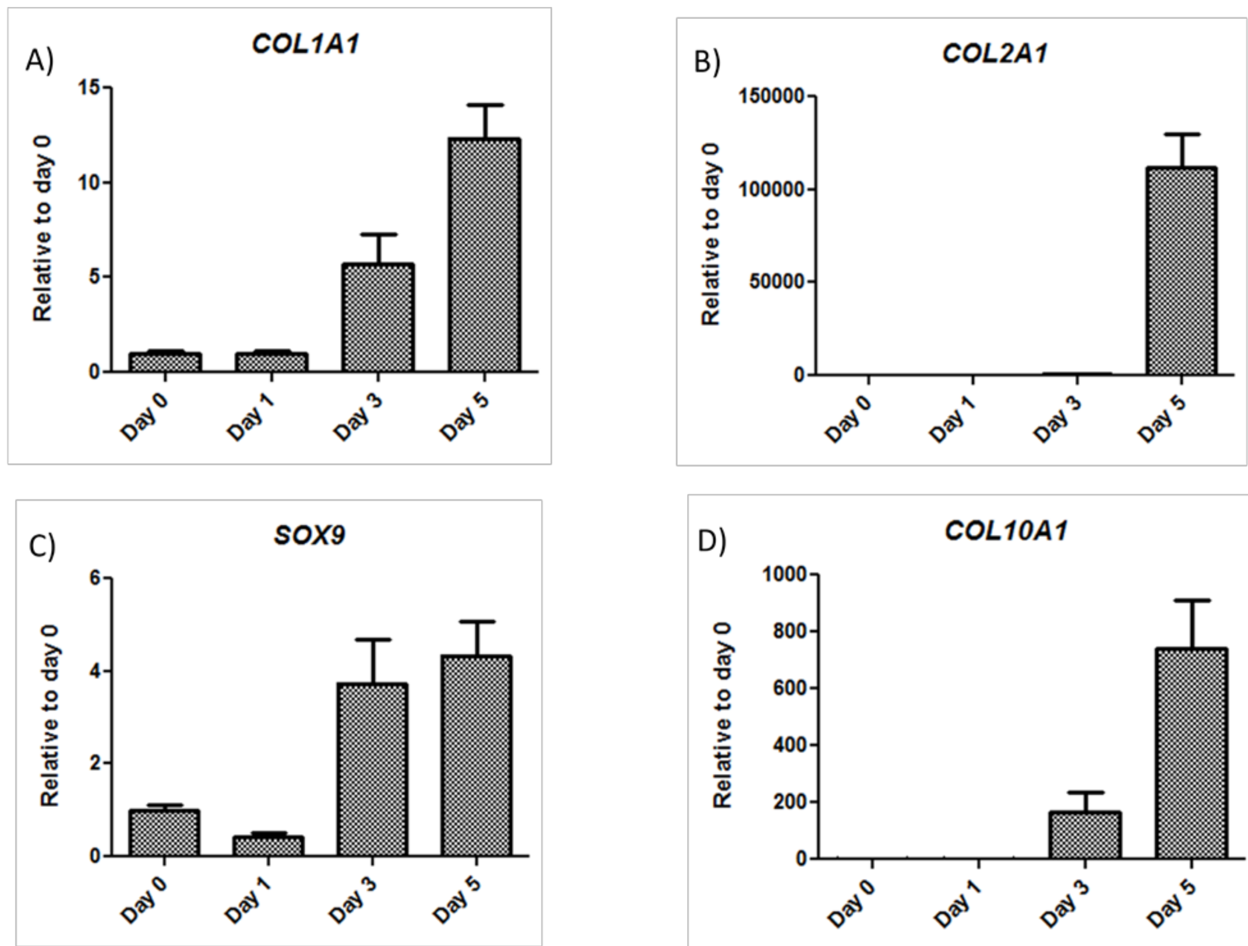


Figure 3-2. RT-qPCR analyzing mRNA levels of relevant genes in MSCs (from donor 1)transfected with scrambled siRNA and embedded in alginate , followed by chondrogenic stimulation over 5days. Day 0 indicate levels of mRNA in MSCs before the cells were differentiated.. A) *COL1A1* mRNA levels. B)

COL2A1 mRNA levels. C) *SOX9* mRNA levels. D) *COL10A1* mRNA levels. Error bars indicate 95% confidence interval for triplicates.

As shown in fig. 3-2-A, *COL1A1* mRNA levels increase as chondrogenesis proceeds, from day 3 and onwards, and at day 5 it has reached an approximate 12 times upregulation compared to day 0. *COL2A1* levels go from barely detected at day 0 and day 1 to very low on day 3, but on day 5 a dramatic increase in the mRNA is observed (fig-3-2-B) resembling what has been observed in fig.1-10-B (Jakobsen, unpublished data). *COL2A1* is dependent on SOX9 to be transcribed and its expression is therefore observed after SOX9 expression[47, 49] (fig.1-11 vs. 1-10-B). The transcription pattern of *COL1A1* is also in accordance with previous data from the group Jakobsen (unpublished data) and Herlofsen *et. al* (2011). *SOX9* levels increases as chondrogenesis proceeds as expected (fig.3-2-C), resembling what has been observed earlier (fig.1-11) in Jakobsen's unpublished data. *COL10A1* (fig. 3-2-D) is first detected on day 3 with a dramatic 200 fold increased levels and increases further 4 fold to day 5, the *COL10A1* mRNA levels increases with 4 fold. All in all the results show that cells that have been subjected to transfection with scrambled siRNA are able undergo chondrogenesis in vitro and resemble the wild type MSCs as observed before in Jakobsen *et.al* unpublished data (2011) and Herlofsen (2011). The cells transfected with scrambled siRNA could be considered a control in the experiments, since they behaved like the wild-type cells.

3.2 Knock-down of *FOXO1*

To study the role of FOXO1 in *in vitro* chondrogenesis, an efficient FOXO1siRNA that could be used in knock-down experiments needed to be identified. For this purpose four siRNAs were tested for their efficiency to down-regulate *FOXO1*in MSCs, singularly or mixed, and analyzed by RT-qPCR. The results show that the four siRNA vary dramatically in their ability to affect *FOXO1* mRNA levels (fig.3-4-A). Whereas FOXO1siRNA_ 1 was very efficient and displayed a knock-down of FOXO1 of 86% 1 day after transfection, FOXO1siRNA_2 and FOXO1siRNA_4 lead to approximately 2 fold down-regulation, whereas FOXO1siRNA_3 had no effect. When all four siRNAs were mixed, the knock-down is similar to that of FOXO1siRNA 1 alone, suggesting

that the observed effect is due to this particular siRNA. Two different concentrations (1µM and 5µM) of the FOXO1siRNA were compared (fig.3-3-B). The lower concentration of FOXO1siRNA is as efficient as the higher, and this was chosen for further experiments to minimize the probability of off-target side effects.

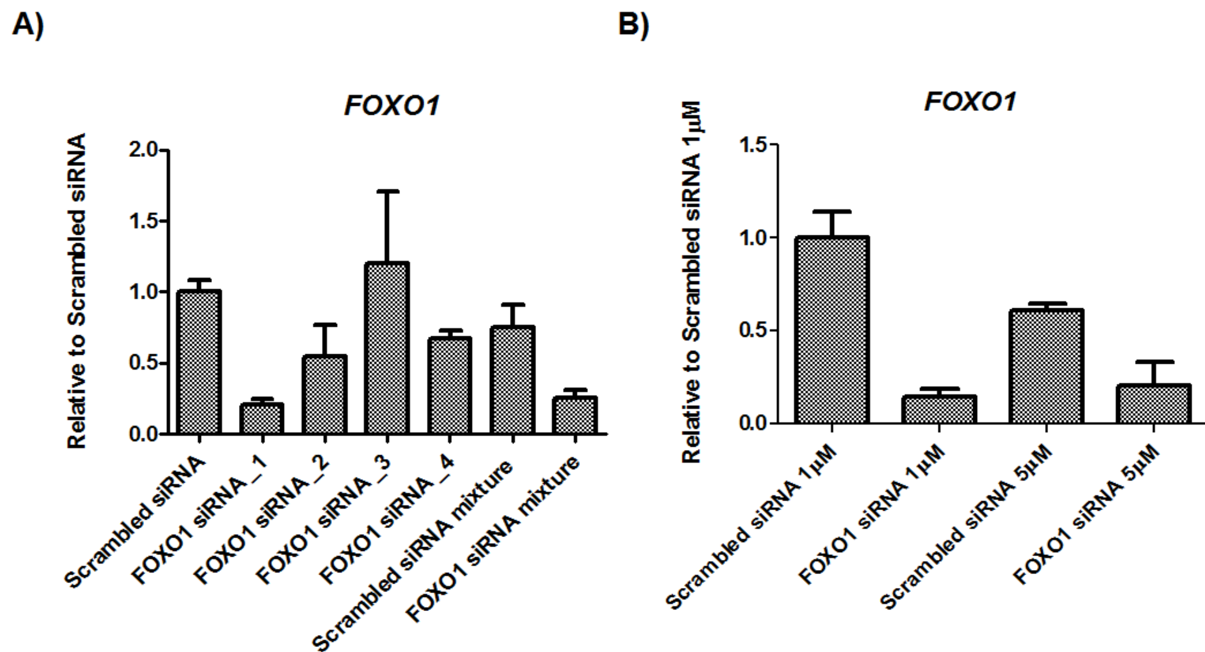


Figure 3-3. Identifying the most efficient FOXO1siRNA. A) siRNA efficiency test. Four different siRNAs; FOXO1siRNA _1, FOXO1siRNA_2 ,FOXO1siRNA_ 3 and FOXO1siRNA_ 4 (see table 2-1) have been tested for their efficiency to down-regulate the FOXO1 mRNA separately or mixed(FOXO1siRNA mixture) by transfection with Amaxa into MSCs and analyzed after 1 day after transfection. Scrambled siRNA is the negative control for each one of the siRNAs separately (1µM), Scrambled siRNA mixture (5µM) is the negative control for the FOXO1siRNA mixture of the four siRNAs. B) siRNA titration. Cells were transfected with FOXO1siRNA_1 at two different concentrations 1µM and 5 µM.

The results show that FOXO1siRNA_1 was very efficient at knocking down the mRNA levels of *FOXO1* after 1 day. However as the knowledge of how affects the protein level is limited since it is dependent on the degradation rate of FOXO1, the effects of the knock-down at protein level ought to be assessed. The knock down of *FOXO1* was therefore validated by western blot. MSCs were transfected with FOXO1siRNA or scrambled siRNA and analyzed.

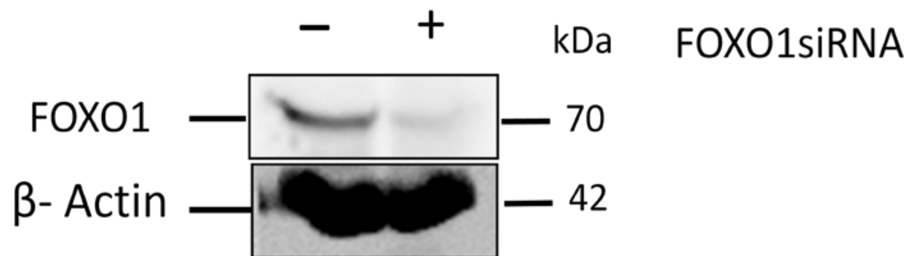


Figure 3-4. FOXO1 protein levels in MSCs transfected with FOXO1siRNA and scrambled siRNA. The left lane represents FOXO1 protein levels in control cells transfected with scrambled siRNA, while the right lane represents protein levels in cells transfected with FOXO1siRNA. Proteins were extracted and run on an SDS-PAGE gel. β-actin was used as an internal control.

Figure 3-4 show that the FOXO1siRNA_1 led to a dramatic decrease in protein levels over the course of 2 days, since FOXO1 is hardly detectable (right lane), concomitant with the reduction of FOXO1on mRNA levels (fig. 3-4). These results show that FOXO1siRNA_1 can be used to knock-down and reduce *FOXO1*, both at the mRNA and protein level.

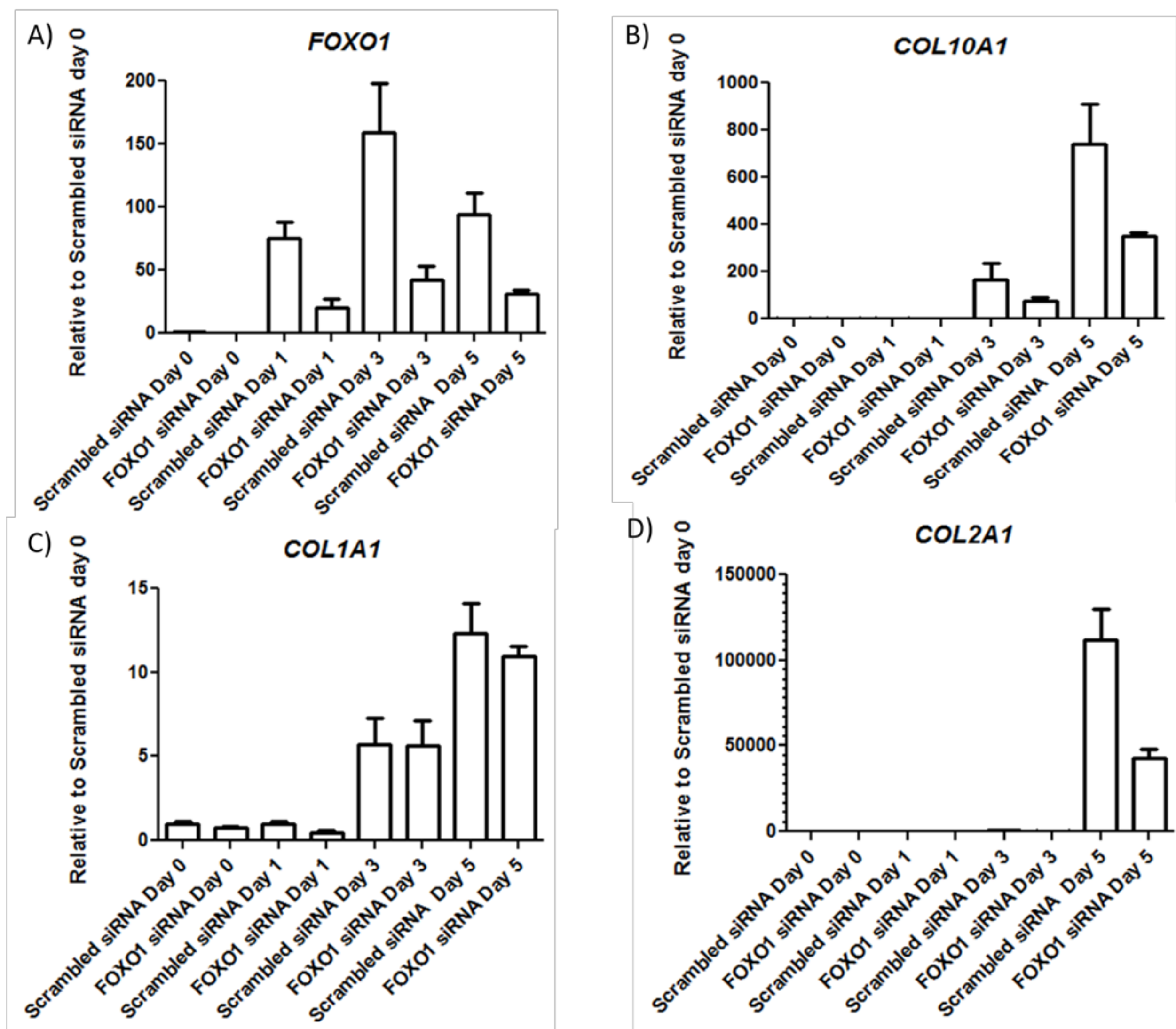
3.3 The effect of FOXO1siRNA on selected genes relevant for *in vitro* chondrogenesis using RT-qPCR

To investigate the FOXO1siRNA effect on a group of selected chondrogenic signature genes (*COL2A1*, *SOX9* and *ACAN*), a hypertrophic marker (*COL10A1*) and some osteogenic markers (*COL1A1*, *BGLAP*, and *RUNX2*) on MSCs (from 3 donors) that were embedded in alginate for chondrogenic differentiation over a time course of 5 days. mRNA levels of the selected genes were analyzed by RT-qPCR.

siRNAs have the reputation of exhibiting extremely short effects therefore after confirming the profound effect of FOXO1siRNA on the mRNA and the protein levels after one day, I next investigated the duration of this effect over a 6 days- time course. For this experiment MSCs from three bone marrow donors were transfected with FOXO1siRNA and the control siRNA and were analyzed over a time period. An aliquot of cells was harvested and analyzed by RT-qPCR at day 0 (before embedding in alginate), the rest was embedded in alginate, differentiated and analyzed by RT-qPCR on day 1, day 3, and day 5. Thus RT-qPCR analysis were carried out on all donors for all days to investigate two issues; the persistence of the FOXO1siRNA and the effects of FOXO1 down regulation on the chondrogenic relevant genes.

Surprisingly, *FOXO1* down regulation has persisted until day 5(actually day 6 after transfection) to satisfactory levels, indicating a satisfactory stability of the siRNA.
In donor 1(fig.3-5-A) FOXO1siRNA exhibits about 87% down regulation of *FOXO1* on day 0(not well demonstrated in the figure as the start mRNA levels are very low compared to day3 where it is on its highest expression). On day 1 the FOXO1siRNA exhibits a 73% down regulation on the mRNA, furthermore on day 3 when the mRNA reaches its highest peak the FOXO1siRNA down-regulatory efficiency does not decline and it exhibits about 74% downregulation. Finally on day 5 the FOXO1siRNA efficiency declines to 67% downregulation of mRNA compared to the control levels. Regarding donor 2(fig.3-6-A) FOXO1siRNA exhibits an 86% downregulation on *FOXO1* on day 0, a 84% down regulation on day 1, and on day 3 though mRNA is on its highest peak here as well the siRNA exhibits a down regulation of 87%. On day 5 the FOXO1siRNA down-regulates *FOXO1* by 63% efficiency declines presumably due to either low mRNA levels, or siRNA degradation. Donor 3(fig.3-7-A) shows an 80% downregulation of *FOXO1* on day 0, a 65% downregulation on day1 and day 3, and finally a 63%

down regulation on day 5. This donor differs from the two others by exhibiting its highest peak of *FOXO1* levels on day 5 in contrast to donor 1, donor 2 and previous data showed in fig. 1-9-A whereas the highest peak was observed around day 3. Donor 3 also differs in displaying the lowest downregulation of *FOXO1*on all days. The results suggest that there may be some donor variation in the effects of FOXO1siRNA, but collectively these observations demonstrate that the system works well and the effects of the knock-down persists over the time period of the experiment of which the *FOXO1* effects on chondrogenesis has been assessed.



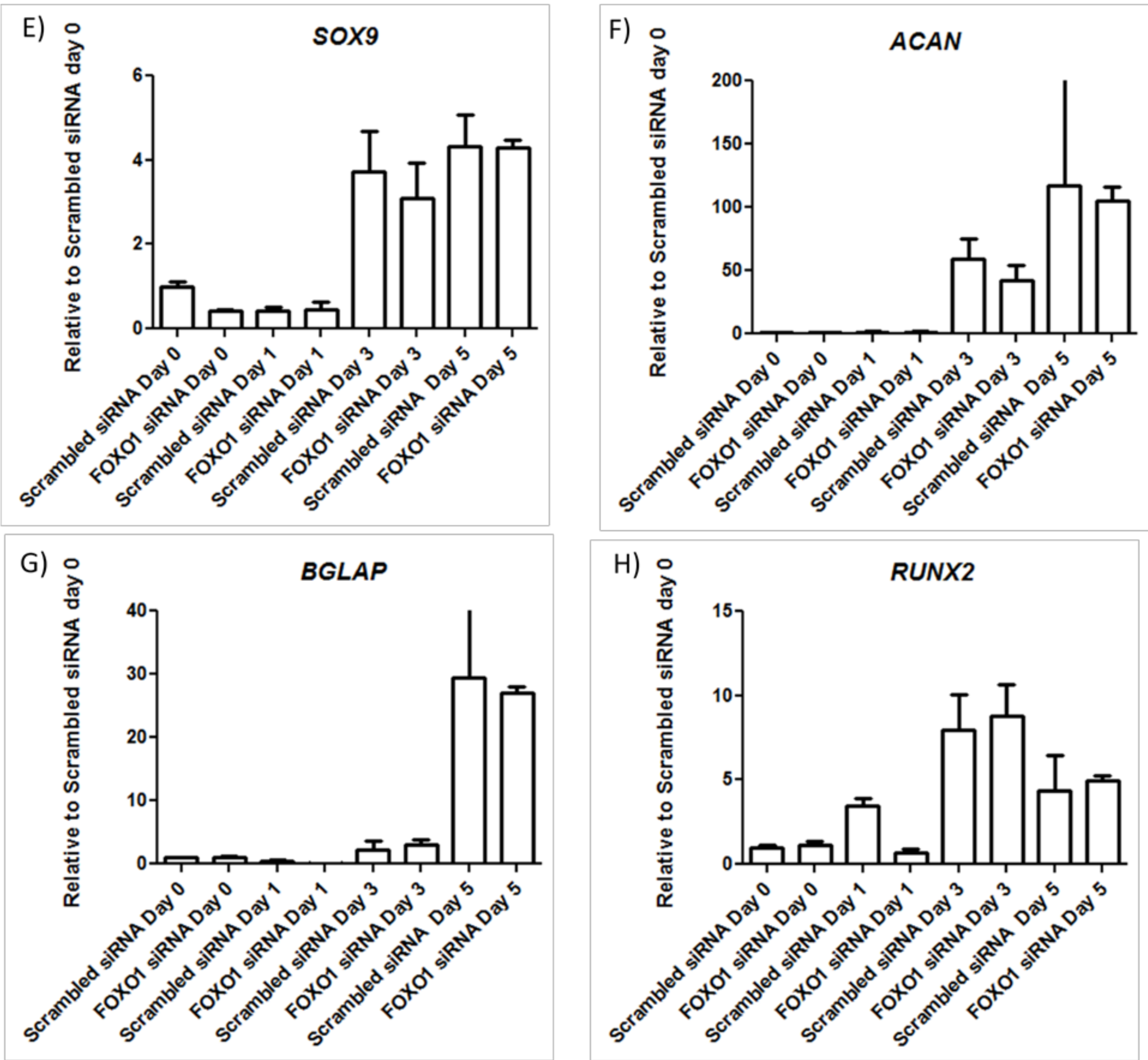
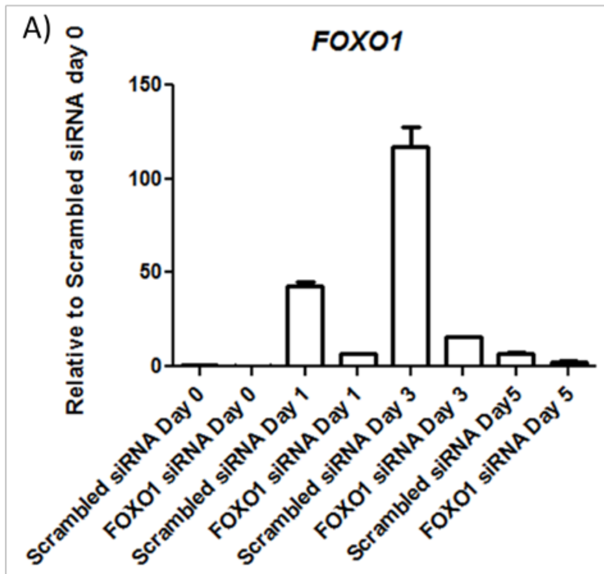
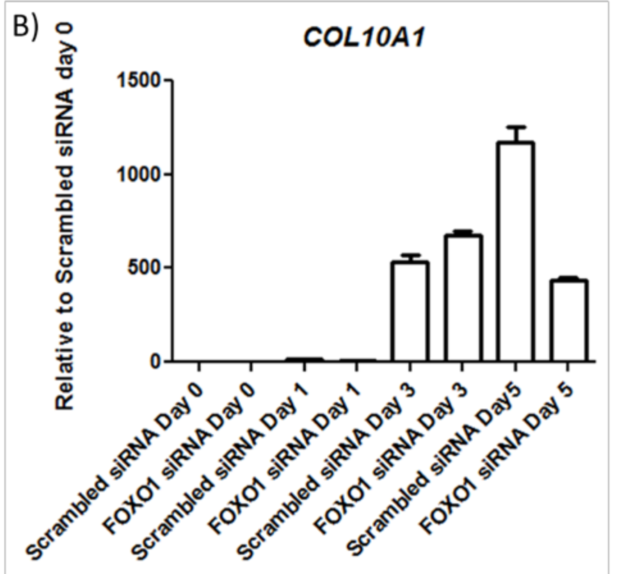


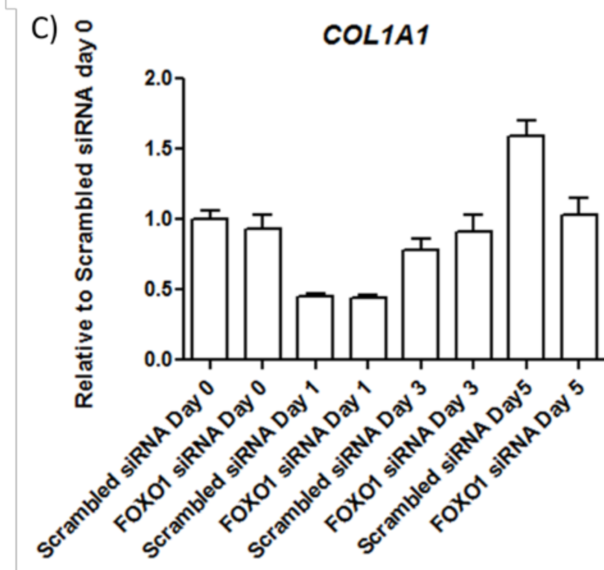
Figure 3-5. mRNA levels of chondrogenic and osteogenic markers in donor 1. Error bars indicate 95% confidence interval for triplicates. Day 0 data represent mRNA levels from cells cultured in 2D, day 1, 3, and 5 represent mRNA levels from cells cultured in 3D. Cells transfected with scrambled siRNA resemble the wild type mRNA levels of the respective gene. FOXO1siRNA resemble the mRNA levels of respective genes in cells where *FOXO1* is down regulated.

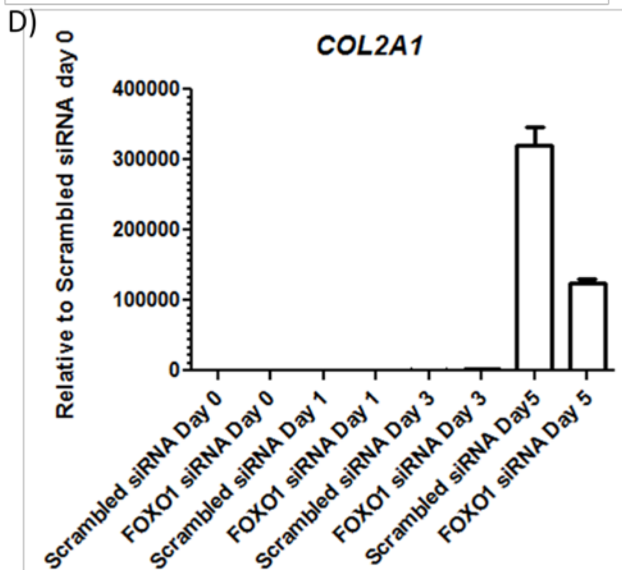
Looking at the hypertrophic marker *COL10A1* a 56% downregulation is observed in the *FOXO1* knock-down cells compared to the control on day 3 (fig. 3-5-B). A similar reduction in *COL10A1* levels is observed in the *FOXO1* knock-down cells on day 5, compared to the control cells. The chondrogenic marker *COL2A1*was expressed at very low levels until day 3 where it starts elevating, and a 68% downregulation of *COL2A1* is observed as an effect of *FOXO1* downregulation compared to control cells. The *COL2A1* levels increase dramatically in control cells on day 5 and *FOXO1* knock-down consistently lead to lead to a 62% down regulation of *COL2A1* on day 5 compared to the control. SOX9 is another chondrogenic signature marker and responsible for the transcriptional activation of *COL2A1*. *SOX9* (fig.3-5-E) is down regulated by 60% on day 0, however after cells were embedded in alginate no further significant downregulation is observed on the other days. SOX9 levels increase considerably from day 0 to day 5 as chondrogenesis proceeds. *ACAN* (aggrecan) is the third chondrogenic marker investigated. From being barely detected on day 0 and day 1 the levels of *ACAN* increases significantly on day 3, and even more on day 5. *FOXO1* down-regulation did not affect *ACAN* levels significantly and *ACAN* was therefore not investigated further in the following donors. Three osteogenic markers were also investigated; *COL1A1, BGLAP* (osteocalcin), and *RUNX2* (runt-related transcription factor). *COL1A1* levels (fig.3-5-C) increases from day 0 to day 5 as chondrogenesis proceeds. Collectively it does not seem like *COL1A1* is affected by *FOXO1* downregulation, though a reduction *COL1A1* to 50% compared to control on day 1 is observed. The levels of *BGLAP* (fig.3-5-G) also increases from day 0 to day 5 as chondrogenesis proceeds and the levels are similar in control cells and *FOXO1* knock-down cells, *BGLAP* was therefore not investigated any further. *RUNX2* (fig.3-5-H) increases from day 0 to day 3 and goes down again (2 fold reduction) from day 3 to day 5. *RUNX2* exhibits an 80% downregulation on day 1 as an effect of *FOXO1* down regulation; however the levels on day 3 and day 5 are equal in FOXO1 knockdown cells and control cells. Due to the inconsistency in the results of *RUNX2* throughout the experiment it has also been omitted for further investigation in the other donors.

Overall donor 2(fig.3-6) exhibits a similar pattern as donor 1 with regards to FOXO1 knock-down, COL10A1 knockdown on day 5 (though the overall levels of COL10A1 in donor 2 are higher), and the highly elevated levels of COL2A1 on day 5 on control cells (indicating good differentiation). Additionally both donors exhibit a similar down-regulation efficiency on *COL2A1* on day 5 in the *FOXO1* down-regulated cells. Similar to donor1 *SOX9* levels (fig.3-6-E) collectively increase as chondrogenesis proceeds, though an unexpected drop in the mRNA levels is observed from day 1 to day 3, but they increase again in day 5. Otherwise there are few insignificant changes in *SOX9* levels between the control and the cells where *FOXO1* is down regulated. *COL1A1* (fig.3-6-C) the only osteogenic marker investigated in donor 2 resembles *COL1A1* pattern in donor 1(fig.3-5-C) and no significant differences in mRNA levels between the control and the FOXO1siRNA transfected cells, were observed though the levels of *COL1A1* were 36% lower in *FOXO1* knockdown cells than in control cells on day 5.









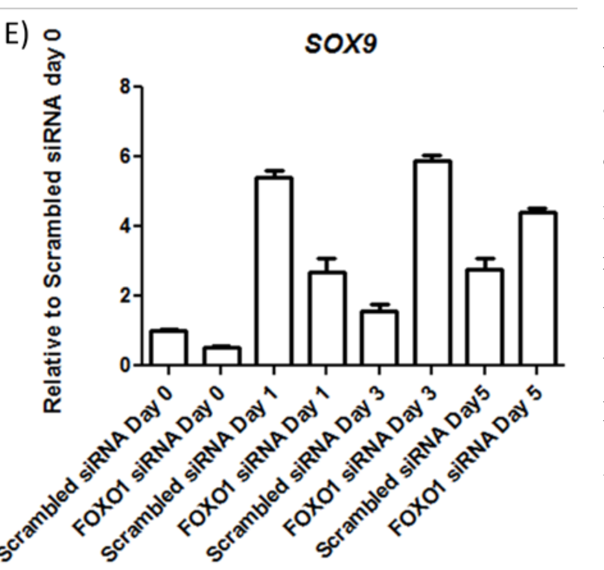


Figure 3-6. mRNA levels of chondrogenic and osteogenic genes in donor 2.Error bars indicate 95% confidence interval for triplicates. Day 0 data represent mRNA levels from cells cultured in 2D, day 1, 3, and 5 represent mRNA levels from cells cultured in 3D. Cells transfected with scrambled siRNA resemble the wild type mRNA levels of the respective gene. FOXO1siRNA resemble the mRNA levels of respective genes in cells where *FOXO1* is down regulated.

Taken together, the results show that the first and the second donor (fig. 3-5, 6) differentiated successfully as judged by elevated levels of the chondrogenic signature markers *COL2A1*, *COL10A1*, and *SOX9* , which are similar to the levels we previously have observed in this system (Herlofsen 2011). Importantly, the FOXO1siRNA levels has worked efficiently and maintained FOXO1 levels to at the most 65% in average after 6 days. Some donor variations were observed with regard to the efficiency with which the FOXO1siRNA worked over days but rather similar on day 5; donor1 displays a knockdown of FOXO1by ~ 3 fold, donor 2 displays a ~ 2.7 fold knockdown of FOXO1, while the third donor shows a ~2.6 fold on day 5 compared to scrambled siRNA. Although the *FOXO1* levels in donor 3 were low as an effect of FOXO1 knockdown (figure 3-7-A), the donor showed a contradicting result compared to the other two donors and to the general effects observed when differentiating MSCs, and the level of *FOXO1* appeared to be delayed compared to donor 1 and donor 2 (fig. 3-5,6 and 7-A). This suggested that the chondrogenic pathway was delayed in these cells, and therefore this donor was excluded from the following experiments and in result interpretation.

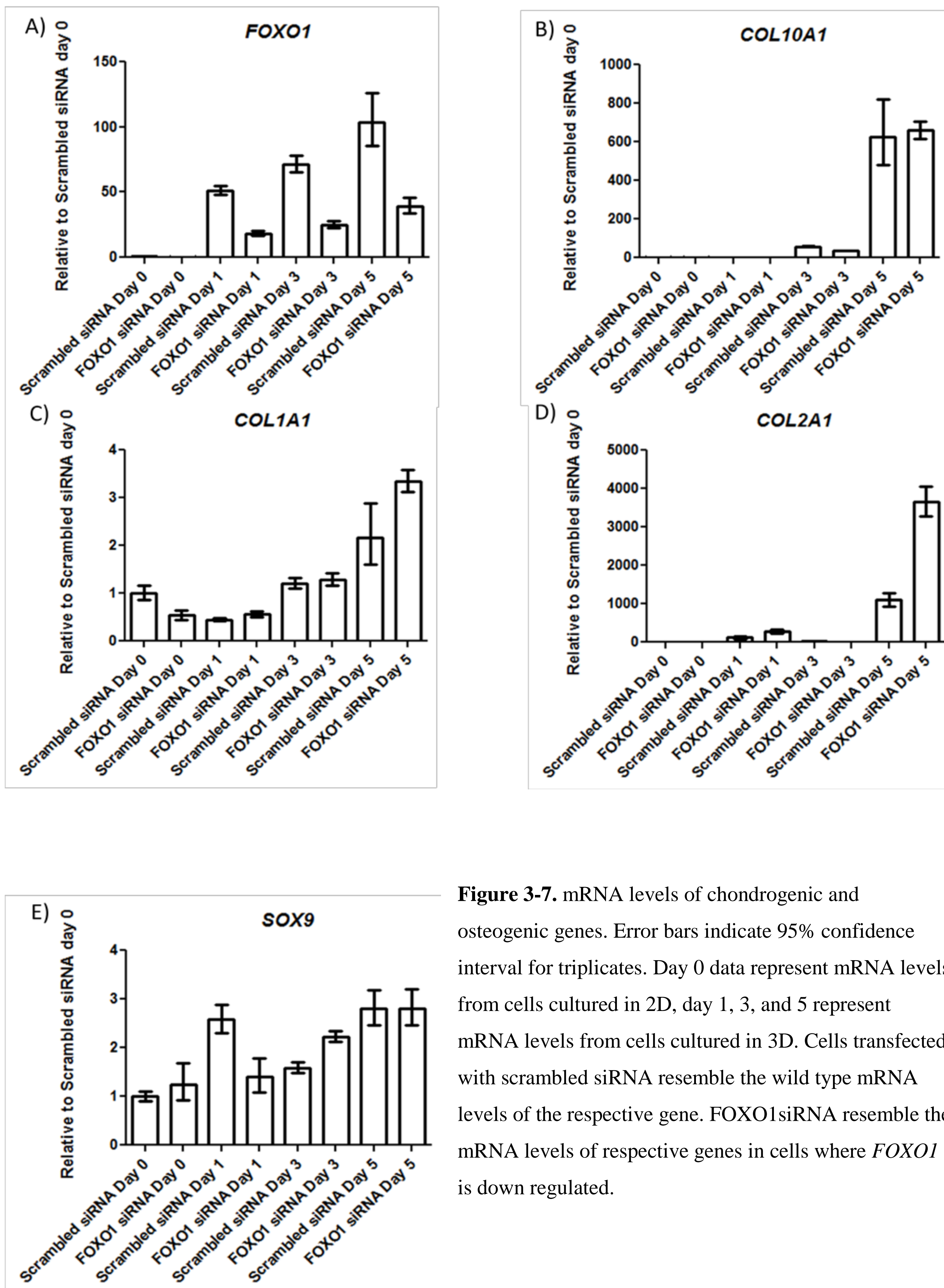


Figure 3-7. mRNA levels of chondrogenic and osteogenic genes. Error bars indicate 95% confidence interval for triplicates. Day 0 data represent mRNA levels from cells cultured in 2D, day 1, 3, and 5 represent mRNA levels from cells cultured in 3D. Cells transfected with scrambled siRNA resemble the wild type mRNA levels of the respective gene. FOXO1siRNA resemble the mRNA levels of respective genes in cells where *FOXO1* is down regulated.

3.4 The effect of FOXO1siRNA on a pre-selected assembly of genes using nanostring technology

As cells have undergone a successful differentiation (at least in 2 of 3 donors), and we identified some interesting effects of *FOXO1* knockdown on particular chondrogenic genes, I further aimed to investigate the effect of *FOXO1* down regulation on a broader spectrum of genes that could potentially be altered other than the selected chondrogenic and osteogenic markers. For this purpose a nanostring array was assessed. Due to technical issues with donor1, only results from donor 2 will be presented and discussed. The nanostring array screened 370 pre-selected genes related to chondrogenesis, adipogenesis, osteogenesis and further relevant genes that from previous studies (Herlofsen et. al 2011) have been shown to be affected in *in vitro* chondrogenesis. We analyzed mRNA levels for these genes after 5 days of differentiation and compared the levels of the control cells (scrambled siRNA) with FOXO1siRNA. The following criteria were used for identification of significant differences; the fold change minimum requirement was determined to be 1.5 and the average of 8 negative controls was used as a threshold established to eliminate background (very low mRNA levels) and thus unreliable fold changes. Refer to section 2.8 for further details. Highly interesting genes are highlighted in bold. Negative fold change indicates downregulation, and positive fold change indicates upregulation achieved by the calculated ratio between the FOXO1siRNA values on day 5 by the control scrambled siRNA.

Table 3.1.1. Genes down-regulated in donor 2 due to *FOXO1* down regulation on day 5 after differentiation.

	FOXO1siRNA	Scrambled siRNA	Fold change
FOXA2	UT	BAT	-9.2
COL9A1	**59,0**	**236,2**	**-4,0**
BAPX1	UT	BAT	-3,6
FZD9	UT	BAT	-2,5
ITGA3	17,8	40,7	-2,3
IHH	**181,2**	**391,0**	**-2,2**
PANX3	**985,7**	**2 080,0**	**-2,1**
COL10A1	**6 938,2**	**14 617,2**	**-2,1**
CDKN1C	**212,8**	**441,6**	**-2,1**
SLC13A5	135,9	276,9	-2,0
CHAD	**330,8**	**667,8**	**-2,0**
IRF6	32,9	66,5	-2,0
COL2A1_isoform2	**2 004,3**	**4 030,9**	**-2,0**
CA12	582,1	1 164,0	-2,0
IL20RB	BAT	40,7	-2,0
COL4A2	15,1	29,8	-2,0
MMP3	19,2	36,7	-1,9
FOXO1	57,7	110,2	-1,9
COL2A1_common	18 175,9	33 641,5	-1,9
FGF2	**38,4**	**70,5**	**-1,8**
BMP4	**30,2**	**54,6**	**-1,8**
PDPN	122,2	219,3	-1,8
CD24	**59,0**	**105,2**	**-1,8**
UNQ830	BAT	BAT	-1,8
CDKN2B	32,9	56,6	-1,7
MATN3	2 417,5	4 148,0	-1,7
COL12A1	**3 384,0**	**5 773,5**	**-1,7**

FGFBP2	200,4	320,5	-1,6
ALPL	1 662,5	2 622,8	-1,6
HAS2	79,6	125,0	-1,6
PTHR1	538,1	831,6	-1,5
PTCH1	60,4	93,3	-1,5
TRPV4	226,5	349,3	-1,5
ADAMTSL4	BAT	BAT	-1,5
COL2A1_isoform1	**917,0**	**1 394,2**	**-1,5**
TUBB2A	676,8	1 022,1	-1,5
CDH15	70,0	105,2	-1,5

Table 3.1.2. Genes up-regulated in donor 2 due to FOXO1 down regulation on day 5 after differentiation.

	FOXO1siRNA	Scrambled siRNA	Fold change
SERPINA3	269,1	172,7	1,6
PBX1	BAT	UT	1,6
CDKN2D	26,1	UT	1,6
FZD4	24,7	UT	1,7
MMP28	60,4	35,7	1,7
SPARCL1	60,4	34,7	1,7
IGFBP2	**31,6**	**17,9**	**1,8**
IGFBP5	**7 624,6**	**4 284,0**	**1,8**
IGFBP3	**245,7**	**137,9**	**1,8**
MMP8	22,0	UT	1,8
ANG	31,6	UT	1,9
FOXC1	35,7	UT	1,9
ADAMTS1	83,7	42,7	2,0

MAF	37,1	UT	2,0
TNFSF11	190,8	94,3	2,0
APOD	1 400,3	683,7	2,0
TMEM100	76,9	36,7	2,1
HEY1	**182,6**	**83,4**	**2,2**
CA9	177,1	70,5	2,5
BST1	24,7	UT	4,2

The table shows mRNA copy numbers of the respective gene in control cells (scrambled siRNA transfected cells) and in *FOXO1* down-regulated cells (FOXO1siRNA transfected cells) together with their respective fold change. Negative fold change represents genes that have been down regulated as a consequence to *FOXO1*down regulation, while positive fold changes represent genes that have been up regulated due to *FOXO1*down regulation. *COL2A1* common in table 3.1.1 represents the two isoforms of *COL2A1* seen in the same table. UT stands for values that are under the estimated Threshold, and BAT stands for values that are considered Barely Above Threshold. Values under threshold displayed in this table were not excluded as they do show a progress to above the threshold. However values that are barely or slightly above threshold are less reliable and must be interpreted with caution.

Selected chondrogenic-related genes exhibiting down-regulation

COL9A1 is the most affected gene in the table (table 3.1.1), experiencing a 4 fold change reduction in mRNA levels due to *FOXO1* knockdown. COL9A1 is a minor component of hyaline cartilage and is usually found in tissues containing COL2A1 (fig.1-4). Interestingly *FOXO1* knock-down lead to a reduction in the mRNA levels of all collagens displayed in the table, approximately 2.5 fold in average. *COL2A1* common is ~2 fold down-regulated, consistent with the results from the RT-qPCR fig.3-6-D (both data are from donor2). *COL10A1* is another collagen that exhibited a reduction in mRNA levels, which also is consistent with the RT-qPCR results (fig. 3-6.B). COL10A1 is a hypertrophic marker and is 2.1 fold down-regulated.

COL12A1 is down-regulated by 1.7 fold (table 3.1.1). COL12A1 is usually associated with COL1A1, and this association is thought to modify the interactions between collagen I fibrils and the surrounding matrix. Another collagen, COL4A2, is a major structural component of basement membranes. The C-terminal portion of the protein, known as canstatin, is an inhibitor of angiogenesis and tumor growth. *COL4A2* shows a down regulation by 1.9 fold.

Another interesting factor involved in chondrogenesis is IHH, required for endochondral bone formation[60] and synchronizes skeletal angiogenesis with the perichondrial maturation is expressed in the prehypertrophic chondrocytes as they exit the proliferative phase (section 1.3.2). IHH is 2.2 fold down-regulated as an affect of *FOXO1* knockdown, as shown in table 3.1.1. The cyclin-dependent kinase inhibitor 1C (CDKN1C), also known as P57^{Kip2}, is another marker that is involved in chondrogenic hypertrophy[140] have been showed to be required for the expression of *COL10A1*. The authors also suggest that it might be required for expression of other genes that facilitate the ossification of chondrocytes. It has also been demonstrated that p57^{kip2} mutant mice have short limbs, a defect attributable to abnormal endochondral ossification caused by delayed cell cycle exit during chondrocyte differentiation[141]. These results are in accordance with Zhang *et al.*'s findings[140]. *CDKN1C* exhibited 2.1 fold downregulation as an affect of *FOXO1* knockdown. FOXA2 is the third gene in table3.1.1 that is involved in chondrogenic hypertrophy. FOXA2 has recently been shown to be is a positive regulator[142] of *COL10A1*. However the considerably low mRNA levels of *FOXA2* make it difficult to estimate a true fold change as its mRNA goes from being above threshold to under threshold, though it suggests that it was down regulated as an effect of *FOXO1*-down regulation.

Further several chondrogenesis promoting genes were observed to experience a reduction in their mRNA levels owing to *FOXO1* knockdown. Pannexin 3 (PANX3) is a novel member of the gap junction pannexin family. PANX3 is expressed in cartilage and regulates chondrocyte proliferation and differentiation by regulating the intracellular ATP/cAMP levels[143]. *PANX3* was 2 fold down regulated. FGF2 has been shown to enhance mitogenic and chondrogenic potential[144] of MSCs. Handorf et al. (2011) have showed that FGF-2 pretreatment actually primed hMSCs to undergo enhanced chondrogenesis by increasing basal Sox9 protein levels. *FGF-2* experienced

1.8 fold down-regulation. BMP4 is another factor that induce chondrogenesis[145], that has also showed a1.8 fold down regulation as an effect of *FOXO1* knockdown.

CHAD codes for chondroadherin which is a cartilage matrix protein thought to mediate adhesion of isolated chondrocytes. Chondroadherin is a cell binding, leucine-rich repeat found in the territorial matrix of articular cartilage and was shown to bind to two sites on collagen type II[146]. *CHAD* is 2 fold down regulated.

Finally, 1.8 fold down regulation of *CD24* is observed. CD24 is a member of a large family of ATP-binding cassette genes encoding a family of transport proteins. *CD24* has been shown to exhibit a large increase in expression in the condensation phase of chondrogenesis[51] .*CD24* has also been shown to increase in Jakobsen's unpublished data.

<u>Selected chondrogenic-related genes exhibiting down-regulation</u>

HEY1 a Notch signaling component, is up-regulated 2.2 fold due to FOXO1 knockdown. The Notch pathway is known for its implication in repression of MSCs chondrogenic differentiation. It has been shown that overexpression of NICD (notch intracellular domain) and HEY-1 has lead to *COL2A1* repression[147] (more about this in the discussion section).

APOD- apolipoprotein increases 2-fold upon *FOXO1* knockdown. APOD is a high density lipoprotein which function in chondrogenesis is unknown, however it has earlier been observed that its expression increases during the course of chondrogenesis *in vitro,* and was mainly due to dexamethasone (in the differentiation medium) (Jakobsen *et. al* unpublished data).

Several insulin-like growth factor binding protein genes have displayed an upregulation as an effect of *FOXO1* knockdown. *IGFBP3* for instance exhibited 1.8 fold increase in expression. IGFBP3 exhibits an intrinsic bioactivity that is independent of IGF binding, it has been reported that IGFBP-3 has an IGF-independent antiproliferative effect in undifferentiated and early

differentiated chondrocytes but not in terminally differentiated chondrocytes[148]. *IGFBP-2* and *IGFBP-5* exhibited 1.8 fold upregulation and have been shown to be expressed in osteoblasts[149]. However IGFBP-5 was shown to be detected in mesenchymal condensation, whereas IGFBP-2 was not.

Collectively it appears that some chondrogenesis promoting genes (*PANX3, BMP4, FGF2*, and *CD24*), chondrogenic markers (*COL2A1, COL9A1*), as well as chondrogenic hypertrophy markers/related genes (*COL10A1, IHH, CDKN1C, and FOXA2*) are experiencing a reduction in mRNA levels due to *FOXO1* knockdown. On the other hand genes affecting chondrogenesis in a negative manner (*HEY-1* and *IGFBP-3*) seem to be up-regulated as an effect of *FOXO1* knockdown.

3.5 Overexpression of *FOXO1*

One angle of studying the role of chondrogenesis *in vitro* was to perform knockdown experiments and remove the elevated *FOXO1* levels as the MSCs differentiating towards the chondrogenic lineage. The inverted experiment was to look at the effects of over-expression of *FOXO1* on MSCs. *FOXO1* was overexpressed by using a Myc-DDK tagged ORF clone containing vector (pCMV6) containing the entire open reading frame (ORF) of *FOXO1*. The plasmid was first amplified, purified and analyzed on an agarose gel.

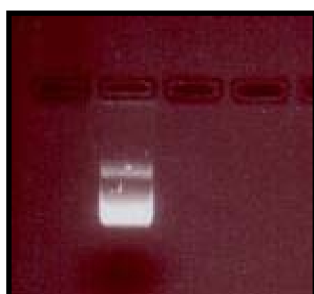


Figure 3-8. Gel electrophoresis of pCMV6 plasmid. Supercoiled and open circular DNA on a1% agarose gel.

After amplification and purification of the *FOXO1* containing, plasmid was transfected into MSCs to resemble the elevated levels of *FOXO1* exhibited on differentiating MSCs.

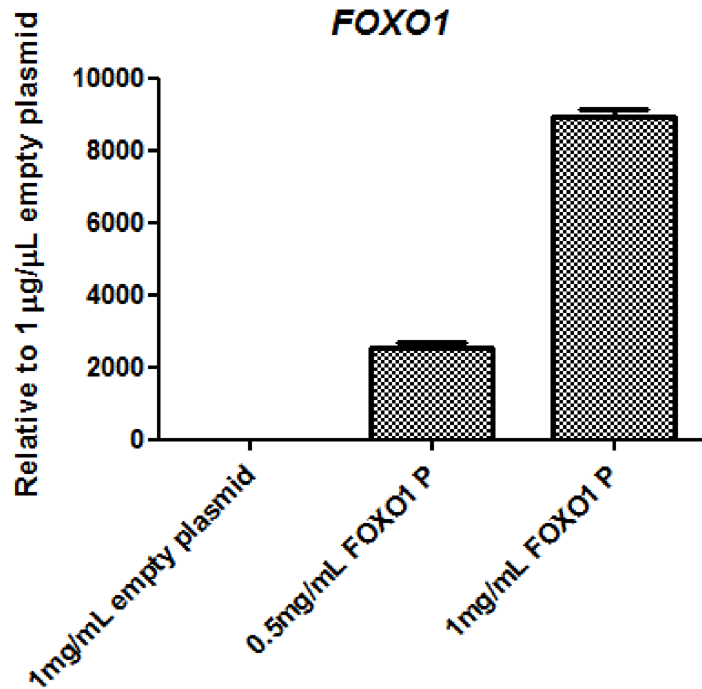


Figure 3-9. Titration of plasmid concentration. Cells were transfected with 0.5µg/µL and 1µg/µL FOXO1 plasmid. The negative control is an empty plasmid (PUC18-1µg/µl).

Transfecting cells with the plasmid has up-regulated *FOXO1* drastically. By transfecting 0.5 µg/µl FOXO1plasmid transcription levels increased profoundly with a 2000 fold, increasing the plasmid concentration accordingly increased transcription rate and about a 7000 fold increase in mRNA levels compared to the control (empty plasmid- where very low levels of *FOXO1* were detected) has taken place.

Further the over-expression of FOXO1 needed to be validated on protein levels by Western blot. In this experiment three antibodies were tested, together with two different extraction buffers.

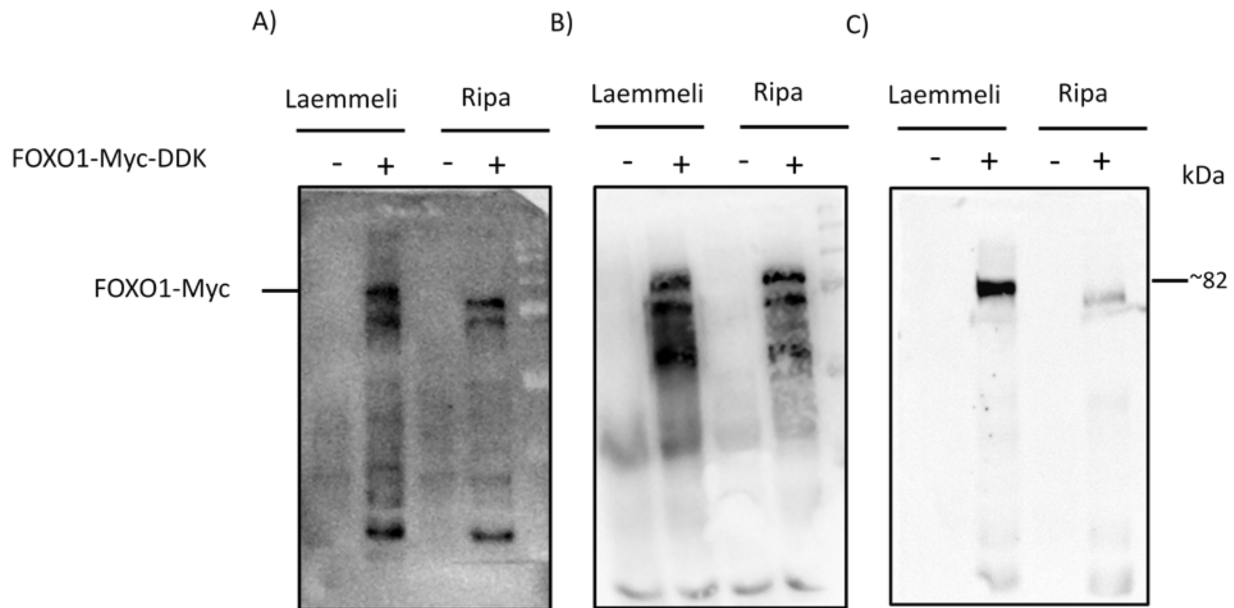
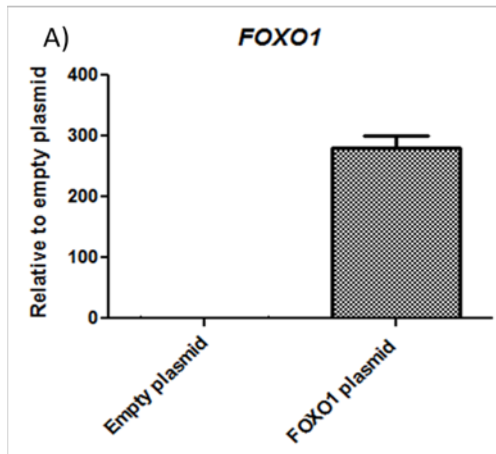


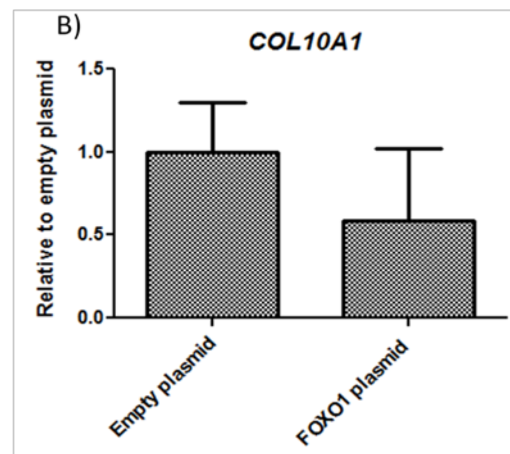
Figure 3-10. Over-expression of FOXO1 by Myc-DDK tagged ORF clone of FOXO1. For all three figures the first two lanes on the left are cells only treated with Laemmli buffer for protein extraction, while the following two lanes to the right are first treated with RIPA, then with Laemmli buffer. The visible FOXO1 bands represent the over-expressed exogenous FOXO1protein. Where there are no visible bands, the cells were treated with empty plasmid. Endogenous protein levels were not detected. The membrane was incubated with monoclonal anti-FOXO1 antibody (A), with the polyclonal anti-FOXO1 antibody (B) and with monoclonal anti-myc antibody (C).

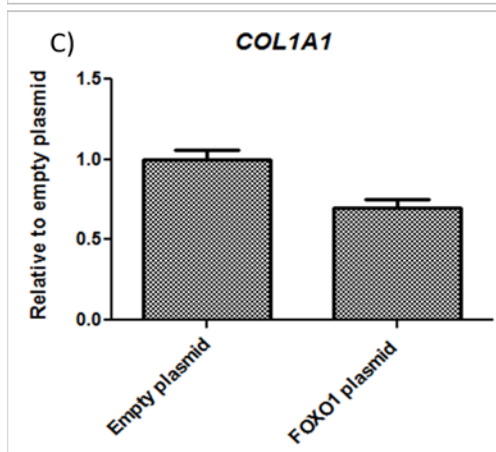
The extraction buffers, and antibody concentrations as well as blocking and washing conditions had to be optimized to obtain a successful western blotting result. The monoclonal anti-myc antibody (3-10-C) displayed almost no background in contrast to the mono- and polyclonal anti-FOXO1 antibodies (3-10-A, B). Using laemmli as the sole extraction buffer showed no significant difference compared to when RIPA was used as shown in fig 3-10-A, B, while in 3-

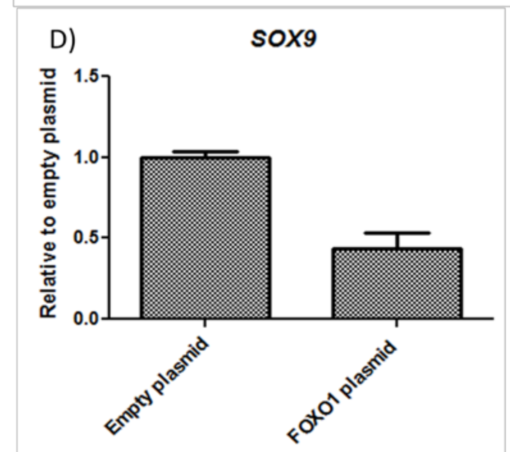
11-B there is considerable difference between than bands observed in the figure depending on the extraction method.

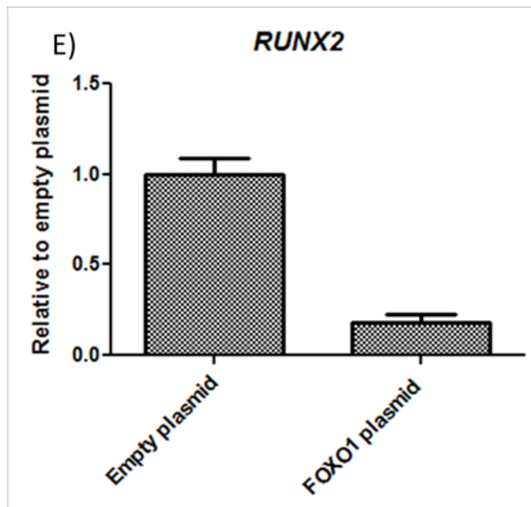
Further the over-expression of FOXO1 was carried out in MSCs from two donors and RT-qPCR analysis was assessed to study the effect of over-expression on selected chondrogenic and osteogenic genes.











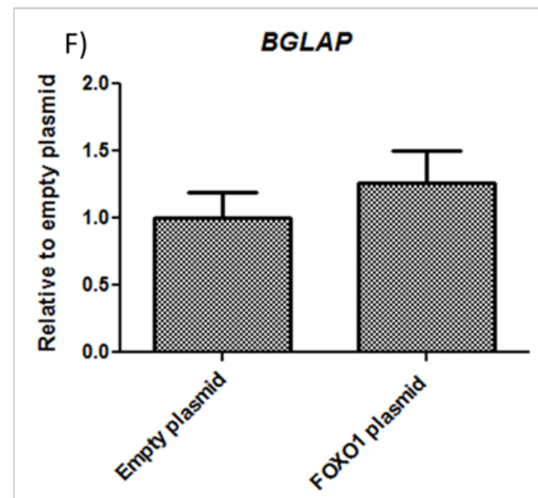


Figure 3-11. mRNA levels of chondrogenic and osteogenic genes in donor 4. Error bars indicate 95% confidence interval for triplicates. Empty plasmid (pUC18) is a control resembling the wild type mRNA levels of respective genes and FOXO1plasmid represents mRNA levels of the respective genes where *FOXO1* is up-regulated.

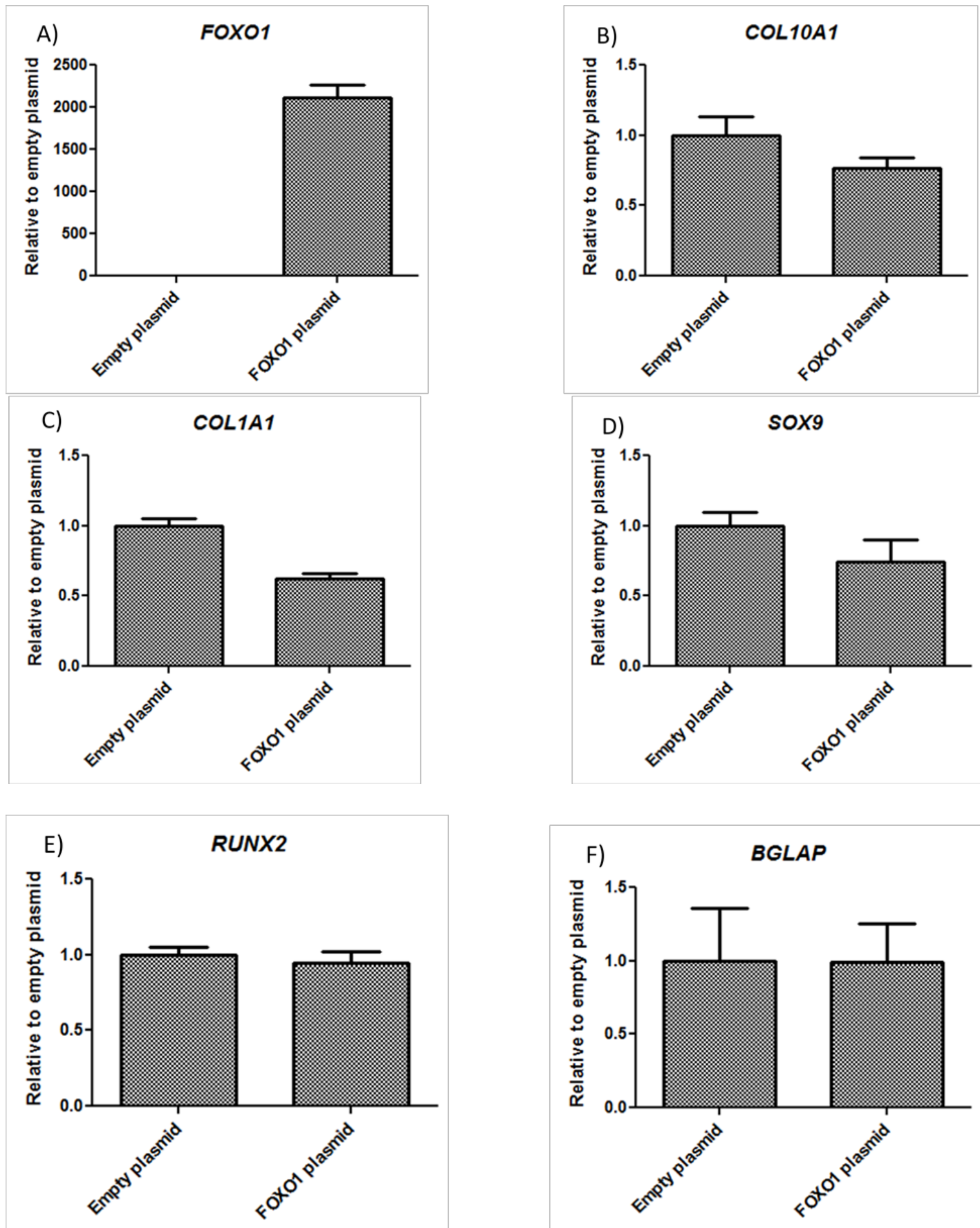


Figure 3-12. mRNA levels of chondrogenic and osteogenic genes in donor 5. Error bars indicate 95% confidence interval for triplicates. Empty plasmid (pUC18) is a control resembling the wild type mRNA levels of the respective genes and FOXO1plasmid resemble mRNA levels of respective genes where FOXO1 is up-regulated.

Both donors exhibited a profound expression of FOXO1; donor 4 with 300 fold up-regulation compared to the empty plasmid control (fig.3-11-A), while donor 5 expressed 2000 fold higher levels of FOXO1 than control cells (fig.3-12-A). Collectively speaking this profound upregulation of FOXO1 has not lead to a significant alteration in the transcription of any of these genes tested, as no consistency is observed. Surprisingly Runx2 is 82% down regulated in donor 4 (fig.3-11-E) while it was not altered at all in donor 5 (fig.3-12-E). BGLAP in donor 4 displayed a 21% upregulation compared to control, while in donor 5 it was not altered (fig.3-11, 12-F). However donor 4 showed an alteration in the internal control *GAPDH*, and therefore the results are difficult to interpret. Presumably there is no significant alteration of these genes in response to the over-expression of *FOXO1* in MSCs in 2D.

4. Discussion

4.1 Chondrogenesis in self-gelling alginate

Chondrogenesis *in vitro* in this study has been carried out in a disc-shaped self-gelling alginate which functioned as a three dimensional scaffold for MSCs, aimed at mimicking the three dimensional environment in the human body. The alginate is prepared at a certain density to allow fusion of growth factors and other stimulating factors from the differentiation medium surrounding the gel and into the cells embedded in the gel. The gel also allows for diffusion of O_2 and CO_2. Successful chondrogenesis in this system has been reported earlier by the group (Herlofsen *et. al*.2011). The ultimate long-term goal of this setup is to produce functional hyaline autologous cartilage tissue which may be implanted in cartilage lesions. When the ECM produced by the differentiating MSCs has the right composition and is sufficiently strong, the alginate maybe removed by chelating agents or enzymatic digestion (Herlofsen *et. al* 2011). The cells are embedded in the gel at certain density, making sure it allows for cell-cell communication. The major chondrogenic regulatory transcription factor is SOX9, which is required for, amongst others collagen type II expression. Collagen type II is the most important chondrogenic marker and most predominant in cartilage giving cartilage its unique tensile property. The levels of *SOX9* and collagen type II both increased in our *in vitro* chondrogenesis system; figure 3-2 confirms the commitment of MSCs towards the chondrogenic pathway evident by the elevated levels of those markers as chondrogenesis proceeds. However an elevated levels of the undesired hypertrophic marker *COL10A1* and the osteogenic *COL1A1* were also observed significantly increasing from day 3 and onwards, concomitant with earlier observations (Jakobsen *et al.* unpublished data). There is a need to identify the molecular mechanisms that govern the expression of these various markers, and ultimately find a way to differentiate the MSCs towards chondrocytes without the expression of unwanted proteins like COL1A1 and COL10A1. We therefore hoped that by impairing the elevated levels of *FOXO1* in early chondrogenesis we could gain an insight into some of these molecular mechanisms involved.

4.2 FOXO1

FOXO1 has been shown to be a positive regulator of skeletogenesis by regulating the osteogenic markers Runx2 and ALP (Teixeira et. al 2009) also reviewed in chapter (1.4) . FOXO1 has also been shown to be a negative regulator of adipogenesis, by interacting with PPARγ (adipogenesis regulator) in adipose cells and negatively regulated its transcriptional activity[150]. FOXO1 can also bind to the PPARγ promoter region and suppress PPARγ expression[116]

The authors, Teixeira *et. al* thus hypothesized that inhibition of adipogenesis or myogenesis by Foxo1 concurrently signals mesenchymal cells toward osteogenesis (hypothesis supported by unchanged expression of collagen type II over the course of 1week) and showed that silencing Foxo1 significantly disturbs skeletogenesis *in vivo* and *ex vivo* and prevents expression of osteoblast markers and subsequent matrix calcification . However in our system with the cell density used collagen type II elevated dramatically from day 3 to day 21 as shown in Jakobsen's unpublished data, fig. 1-9-B and in this study a drastic increase has been observed from day 0 to day 5 (fig.3-2-B). Also the chondrogenic key regulatory factor SOX9 has during this period increased significantly as shown in fig. 1-10. *FOXO1* increases early during this course of chondrogenic pathway as shown in fig. 1-9-A and 1-10, therefore taken all above into consideration I hypothesized that early in differentiation under the conditions applied *in vitro* in our system, that FOXO1 is involved in chondrogenesis and therefore need to investigate the mechanisms by which it operates. One can wonder if FOXO1 is performing a similar regulatory effect in chondrogenesis as to what has been hypothesized by Teixiera *et al.* in the osteogenic differentiation pathway, where FOXO1 by its inhibitory effect on PPARγ consequently signal osteogenesis. Therefore maybe in this study FOXO1 is rather signaling chondrogenesis in this system evident by the elevated levels of the chondrogenic signature genes *SOX9* and *COL2A1*. Maybe FOXO1 is performing a dual role of regulation depending on the system, growth and differentiation factors applied. When cells were embedded in 3D, they were supplied by the differentiation medium (table2-1) that includes many growth factors that induces the expression of many chondrogenic genes as reviewed in section 2.5.1 and 2.5.2. Insulin, one of the factors in the differentiation medium has been shown to inhibit FOXO1 and vice versa (refer to chapter 2.6). However in this study, in the system of alginate *FOXO1* has not been shown a reduction on

the mRNA level, though this certainly does not exclude the probability of inhibition of FOXO1 protein by phosphorylation. If assuming FOXO1 is truly involved in early chondrogenesis and its up –regulation is in fact vital for this process, then there must be other molecular mechanisms and factors involved that can abolish the inhibitory effect of insulin on FOXO1, or else differentiation will be inhibited due to insulin.

4.3 *FOXO1* knock-down

Two strategies were performed to study the role of FOXO1 in chondrogenesis; *FOXO1* knock down and *FOXO1* up-regulation. In order to study the effects of *FOXO1* knockdown on differentiating cells an efficient siRNA with a persistent down-regulatory effect on *FOXO1* needed to be identified. Figure 3-3-A displays the siRNAs tested (table2-3), and FOXO1siRNA_1 exhibited the most profound downregulation (86%) compared to the control. Importantly, the effect of FOXO1siRNA lasted for at least 6 days after transfection, even though the cells exhibit a dramatic increase in *FOXO1* mRNA levels upon differentiation. Off-target effects are the gene perturbations caused by unintended interactions between the RNAi molecules and cellular components (Saurabh Singh *et al*. 2011). Broadly speaking, off-target effects can be specific or non-specific. The former are caused by the limited degree of siRNA or shRNA complementarity to non-targeted mRNAs. The latter, which causes immune and toxicity related response, are due to the RNAi construct itself or due to the delivery vehicle transporting the siRNA (Saurabh Singh *et al.* 2011) and according to previous studies of the group (Karlsen *et. al* unpublished data 2012) it has been shown that lipoplex mediated transfection induced an immune response in MSC. Therefore lipoplex mediated transfection as a method of siRNA delivery was excluded in this study, Amaxa electroporation was used instead. However regarding the specific off-target effects a new simple strategy known as "pooling" has been carried out. Pooling implies mixing of highly functional designed siRNAs targeting the same gene. Studies show that strong on-target gene knockdown can be achieved with minimal off-target effects if a pool consisting of highly functional multiple siRNA is substituted for individual duplexes. This finding is in contrast to speculation that mixtures of siRNAs can compound off-target effects (reviewed by thermo Fisher Scientific, 2008).

The impact of FOXO1siRNA_1 (1µM with a 86% down-regulatory efficiency) on FOXO1 needed to be further confirmed by western blot (figure 3-5) and the results show a profound impact on the protein level, indicated by the dramatic decrease in band size. FOXO1siRNA_1 is a potent siRNA exhibiting down-regulatory effects on both mRNA and protein levels of the gene and successful down-regulation of *FOXO1* on both mRNA and protein levels is a prerequisite for the performance of the following experiments where it is the particular effects exhibited by selected genes as a consequence of this down-regulation of *FOXO1* is the core of the study.

4.4 FOXO1down regulation in three donors (RT-qPCR)

FOXO1siRNA was transfected into MSCs before they were embedded in alginate, and RT-qPCR analysis was run to confirm *FOXO1* down regulation and to investigate the transcriptional alteration of certain genes due to this downregulation. FOXO1siRNA showed persistence (though with variations) in its ability to downregulate *FOXO1* during the entire duration of the experiment (6 days) as illustrated in figures 3-5-A, 3-6-A and 3-7 A (see chapter 3.3). The somewhat surprising persistence of the knock-down is maybe due to the fact that when cells are differentiating they are not dividing anymore and thus siRNA is not halved for each mitotic division. However the variation in the knock down efficiency between donors, present a potential problem for the performance of the experiments, since it can sabotage the interpretation of the results. This variability can be donor dependant, as different donors have different levels of *FOXO1* before and during down regulation. As seen in chapter 3.3 donor 1 and donor 2 exhibit a rather similar efficiency in *FOXO1* downregulation pattern, though donor 2 has clearly displayed the most potent siRNA down regulation over time. Donor 3 in contrast has displayed the poorest down regulation of *FOXO1*.

In the first donor (figure 3-5-B, D) *FOXO1* down regulation (67% down-regulation) has lead to a significant and subsequent downregulation of *COL10A1* (54%) and *COL2A1* (62%) on day 5. Donor 2 (figure 3-6.B, D) a 63% downregulation of FOXO1 on day 5 has lead to similar down-regulatory effects on the same genes; *COL10A1* (63%) and *COL2A1* (61%) respectively. Additionally donor 2 also indicated a down-regulation (36%- though not considered a profound down-regulation) of the *COL1A1*on day 5 compared to control, while donor 1 exhibited 12%

down regulation compared to control on the same day (figure 3-6-C and 3-5-C). However common for both donor 1 and 2 is the down-regulation of *SOX9* on day 0 as a result of *FOXO1* down regulation, donor 1 displayed 59% down regulation of SOX9, while donor 2 displayed a 46% down regulation of the same gene. Nevertheless this downregulation was not observed to be maintained after cells were embedded in alginate (day 1-day5). It is possible that FOXO1down regulation exhibits a negative impact on SOX9 transcription, as 2 of 3 donors showed this down regulation of SOX9, and that BMP2 and TGFβ in the differentiation medium alter *SOX9* expression and over-rules the effect of FOXO1, though this remains speculations at this point and would need further testing of more donors and conditions.

No effects were observed for the expression of the genes *ACAN*, *BGLAP*, and *RUNX2* in the first donor as a consequence of FOXO1 downregulation (figure 3-5) and these genes were therefore not investigated in the other donors.

Donor 1 and 2 showed a good progression in their chondrogenic differentiation pathway as evident by the profound increase in *COL2A1* levels (about 100000 fold- in donor 1 fig.3-5-D) on day 5, compared to day 0 and 1 where it was barely detected (thus not shown in the figure). Donor 2 showed even higher levels of *COL2A1* on day 5 (about 300000 fold compared to start levels fig.3-6-D). Donor 3 (figure 3-7) on the other hand displayed a rather poor chondrogenic differentiation, though *COL2A1* was detected on day 1 however it dropped again on day 3 and on day 5 the extreme increase in *COL2A1* levels observed in donor 1 and 2 was not observed here on the control. Peculiarly the *FOXO1* down-regulated sample exhibited higher *COL2A1* levels (70%) than the control that resembles the wild type, however though clearly increased *COL2A1* levels on donor 3 on day 5, it was only a 3000 fold upregulation compared to day 0.

In summary donor 1 and 2 have shown a successful chondrogenic differentiation and efficient and persistent *FOXO1* knock down over the course of the experiment. Both donors exhibited a significant reduction in *COL2A1* and *COL10A1* levels on day 5 in response to *FOXO1* down-regulation.

The profound donor variability in the potential to differentiate has been observed earlier and is an expected feature of primary cells; sometimes we observe that there is a delay in the differentiation of some donors, as maybe the case for donor 3. What this difference might be due to is a matter of speculation; metabolism rate varies among people and it is therefore possible that FOXO1 levels are affected by the metabolism rate of our donors (refer to chapter 1.4). Furthermore, oxidative stress, aging, and genetic factors that influence insulin levels will also affect FOXO1 levels.

4.5 FOXO1 knockdown- Nanostring

Since donor 1 and donor 2 have been demonstrated to undergo a successful chondrogenesis as determined by the levels of *SOX9, COL2A1* and *COL10A1* in the control cells, the next step was to investigate the impact of *FOXO1* downregulation on a larger selection of genes. Nanostring read out provides a much broader spectrum of genes involved, and represent genes involved in chondrogenesis, osteogenesis, adipogenesis, angiogenesis, apoptosis or cell among others. Donor 2 has been selected for this analysis due to technical issues with donor 1, however donor 2 is in fact the donor that showed the most successful differentiation within the time course of our study, in addition to exhibiting the highest level of knock-down on *FOXO1*. 370 genes were analyzed by nanostring and the readout data has been processed according the nCounter expression data analysis guide (chapter 2.7). 57 of the 370 genes were reviewed in table 3.1.1 and 3.1.2, those genes fulfill two requirements; overcome the background threshold and the minimum fold change (1.5) (refer to 2.8 or 3.4 for further details).

Surprisingly, the data shows that *FOXO1*down regulation appears to affect all collagens, shown in the table, in a negative manner. The highest down-regulation is observed with *COL9A1*with 4.0 fold, followed by *COL10A1* with 2.1, *COL4A2* 2.0 fold, *COL2A1* common 1.9 fold (the average of the two isoforms) and finally *COL12A1* exhibiting the lowest downregulation 1.7 fold. *COL1A1* is not reviewed in the table as it was automatically omitted due to not being able to fulfill the requirements of fold change; this coincides with RT-qPCR data (fig.3-7-C) where no valuable change has been observed. This common *FOXO1*downregulation effect exhibited on the collagens observed, could despite their various functions and engagement in different processes,

could be due to common structural similarity that allows for a common regulation in the decrease of *FOXO1* in the system. Whether that is the case, is yet to be investigated. However the probability of the coincidence of that *FOXO1* limitation exhibits various effects on some regulatory factors of the collagen leading to a common downregulation of the collagens is also present. Defining the mechanisms by which FOXO1 regulate collagens, and defining other cofactors could help us manipulate the expression of collagens in a more delibrate matter. Since different collagens are involved in different stages in chondrogenesis, some of them are desirable to be present and on at all times (*COL2A1*), while others are required only on the onset of chondrogenesis (*COL1A1*), and others are not desired at all (*COL10A1*).

Interestingly, the data also shows a common down-regulation of chondrogenic hypertrophic related genes; *IHH* was down-regulated by 2.2 fold, *CDKNIC* by 2.1 fold and finally *FOXA2* that clearly showed downregulation to under threshold (cannot determine a true fold change as the down regulated value is lower than threshold).IHH is expressed in the prehypertrophic chondrocytes as they exit the proliferative phase, enter the hypertrophic phase, and begin to express the hypertrophic chondrocyte marker, type X collagen (Col10a1) and alkaline phosphatase(reviewed in section 3.2.1). IHH is required for endochondral bone formation. CDKN1C has been shown to be required for the expression of the hypertrophic marker COL10A1, and has been suggested to facilitate ossification. Finally FOXA2 was recently shown to be involved in chondrogenic hypertrophy (Ionescu *et al.* 2012). The authors demonstrate that FoxA factors (in mice) are induced during chondrogenesis and bind to conserved binding sites in the collagen X (*COL10A1*) enhancer (another gene that is also down regulated as a consequence of *FOXO1* down regulation, see table 3.1) and can promote the expression of a collagen X-luciferase reporter in both chondrocytes and fibroblasts (Ionescu *et al.* 2012). This is consistent with what occurs in our system and could offer an explanation of how COL10A1 is down-regulated. It is tempting to speculate that the downregulation of *FOXO1* leads to a down regulation of *FOXA2* and *CDKN1C* which again lead to downregulation of *COL10A1*, as FOXA2 and CDKN1C presence is crucial for the expression of collagen X. In summary three important hypertrophic relevant genes appear to be effected in a negative manner by *FOXO1* depletion.

Further several positive regulatory factors of chondrogenesis were observed to experience a reduction in their mRNA levels owing *FOXO1* knockdown; *PANX3* was down-regulated by 2.1, *FGF2* and *BMP4* by 1.8 fold. PANX3 is expressed in cartilage and regulates chondrocyte proliferation and differentiation by regulating the intracellular ATP/cAMP levels (Iwamoto et. al 2010). FGF2 has been shown to enhance mitogenic and chondrogenic potential of MSCs. Andrew M. Handorf et al. (2011) have showed that FGF-2 pretreatment actually primed hMSCs to undergo enhanced chondrogenesis by increasing basal Sox9 protein levels. BMP4 is another factor that has been shown to induce chondrogenesis Semba and Nonaka *et al.* (2000). Depletion of such chondrogenesis promoting factors might provide an indirect explanation of why *COL2A1*and *COL9A1* experienced down-regulation.

Finally a matrix protein (CHAD) and transport protein (CD24) expression have also experienced a down regulation as a response to FOXO1 downregulation. *CHAD* experienced a 2.0 fold down regulation while *CD24* was down-regulated by 1.8 fold. *CHAD* was shown to be associated with and binding to two sites on collagen type II. Both chondroadherin and collagen interact with chondrocytes, partly via the same receptor, but give rise to different cellular responses. By also interacting with each other, a complex system is created which may be of functional importance for the communication between the cells and its surrounding matrix and/or in the regulation of collagen fibril assembly (Bengt Månsson *et. al* 2001). Maybe the association between those two allow for a reciprocal regulation, in other words it is possible that a down regulation of *CHAD* can lead to a downregulation of collagen type II and vice versa. Both molecules' mRNA is down-regulated in this system (table 3.1.1). *CD24* has been shown to exhibit a large increase in expression in the chondensation phase of chondrogenesis (Ichiro Sekiya et al 2002). *CD24* has also been shown to increase in Jakobsn's unpublished data. Maybe *CD24* is one of the factors that are required early in chondrogenesis, and its down regulation might lead to a delay of differentiation.

In summary various genes of various regulatory functions along the chondrogenic lineage have exhibited a down-regulation due to *FOXO1* down-regulation. Among them we observed both

chondrogenic promoting factors such as *Panx3, FGF2* and *BMP4*, and "perhaps" accordingly chondrogenic markers such as *COL2A1* and *COL9A1* to be depleted. Also chondrogenic hypertrophy promoting factors; *IHH, CDKN1C*, and *FOXA2* have exhibited down-regulation; their down regulation might provide an explanation of the *COL10A1* depletion. Finally a matrix protein coding gene, *CHAD*, associated with collagen type II and a transport protein, and *CD24,* expressed in chondrogenic condensation have both showed down-regulation.

Of the genes that have exhibited an up-regulation in expression due to *FOXO1* down-regulation, we interestingly find HEY1. HEY1 is a Notch signaling component that showed a 2.2 fold up-regulation. The Notch pathway is known for its implication in repression of MSCs chondrogenic differentiation. *COL2A1* gene expression was repressed following overexpression of Notch intracellular domain (NICD) (2-fold) and HES-1(another Notch signaling component) (3-fold) and was markedly repressed by overexpression of HEY-1 (80-fold) (Shawn et.al 2008). The authors also show that HEY1 repressed *ACAN* (aggrecan by 10 fold), and that HES-1 and HEY1 bind to *SOX9* binding site in *COL2A1* enhancer and prevent SOX9 from binding and thus transcriptional activation of *COL2A1*resulting in chondrogenic differentiation repression. This makes perfect sense and could be the explanation of how *COL2A1* is down-regulated. We do observe an upregulation of HEY1 (2.2 FOLD) and a down-regulation of *COL2A1* (1.9 fold). One can speculate if *FOXO1* decrease could directly lead to an upregulation of the Notch signaling proteins which again impair chondrogenesis by down-regulating *COL2A1*.

In summary, though the findings are premature and need further verifications, they give rise to interesting speculations when chondrogenic promoting genes and markers are down-regulated as an effect of *FOXO1* down-regulation, it is natural to expect that factors that affect chondrogenesis negatively are expected to exhibit an inverse response, which is the case in this study. Chondrogenic promoting factors; *Panx3, FGF2* and *BMP4* and the major and most important chondrogenic marker *COL2A1* are all down-regulated due to *FOXO1* down regulation. On the other hand chondrogenic repressing factor such as *HEY1* and the negatively regulator factor of chondrogenesis, *IGFBP-3* are both up-regulated. However which reaction occurs first as a consequence to *FOXO1* down-regulation is yet to be investigated as the interplay between

factors, positive and negative feedback loops involved form an incredible complex picture that we are, to date, ignorant off.

4.6 Over-expression of FOXO1

To see the effects on MSCs of an up-regulation of *FOXO1* a Myc-DDK-tagged FOXO1plasmid was transfected. Transfection with 0.5µg/µl of the plasmid resulted in a 2500 fold upregulation of *FOXO1* compared to endogenous levels (empty plasmid), and transfection with 1µg/µl resulted in an 8900 fold up-regulation, while up-regulated *FOXO1* due to chondrogenic differentiation is only about a 150 fold compared to endogenous levels. *FOXO1* has clearly been expressed to a higher level than the elevated levels observed in chondrogenesis. Two donors were used in this experiment to study the effect of *FOXO1* up-regulation on certain chondrogenic genes (*COL10A1, SOX9, and COL2A1*) and osteogenic genes *(COL1A1, RUNX2,* and *BGLAP*). Quite large donor variability has been observed in the donors' ability to over-express *FOXO1*, while donor 4 exhibited a 300 fold up-regulation of the gene, donor 5 exhibited a 2000 fold. The majority of tested genes exhibited no significant change in gene expression, the exception being *SOX9* (56% down-regulations, and RUNX2 (82% down-regulation) in donor 4 (fig3-11-D, E). This is inconsistent with previous data from FOXO1 knockdown experiments as well as with literature. *SOX9* has showed a down regulation due to *FOXO1* down-regulation in 2D in donor 1 and donor 2 (fig.3-6,7-E) in MSCs though the effect is abolished upon differentiation, possibly due to TGFβ and BMB2 as discussed previously in chapter 4.4, which contradict with the results in this study. It may be because FOXO1 is not in the same environment in 2D as in the 3D alginate; the MSCs in alginate have embarked on a differentiation route towards chondrocytes, they stop dividing and they have access to a different set of differentiation factors, other transcription factors and co-factors than in 2D that may affect the function and the pathways through which it works the two systems. Furthermore the over-expression of FOXO1 has altered the GAPDH expression, which has been used as an internal control in the RT-qPCR analysis. It has been shown that GAPDH expression levels can be altered by glucose, insulin, heat shock and cellular proliferation (Arya et al. 2005) see also chapter 2.5. The profound over-expression of *FOXO1* has probably altered insulin expression as reviewed in chapter1.4, and alteration in

insulin might have resulted in the alteration observed in *GAPDH* transcription. In this case samples need to be normalized to equal RNA concentration loaded.

5. Future approaches

As the nanostring analysis has been carried out on only one donor, results are unreliable statistically and it is premature to conclude from these experiments. Due to well-known donor variability in chondrogenic differentiation potential donor, the experiments should be repeated in at least three donors for firm conclusions to be drawn. The findings can further be verified by RT-qPCR using statistically valid methods. Another weakness with this setup is the limited time points included in the experiments. Having only looked at day 5, there is a potential risk that we pass over/over look small variations in the speed with which the differentiation process, thus the transcription, is developing. Ultimately should these analysis been carried out for various days in the course of differentiation and thus provide a differentiation curve, that would provide the necessary information about the effect of *FOXO1* knockdown throughout the process

To conclude that our observations are in fact due to *FOXO1* knockdown and not due to some off-targets effects, a rescue experiment further needs to be performed (Hoi Tang Ma *et.al* 2010). This could be done by co-transfection of FOXO1siRNA and myc-DDK FOXO1 into MSCs before chondrogenic differentiation in vitro. The reversion of the observed effects would confirm that they are FOXO1 specific. This control experiment has unfortunately not been performed due to limited time-frame. Since even 20% of remaining protein could perform most, if not all, of its function, a good approach for future experiments would be to perform a knockout of *FOXO1* using TALENs. Since FOXO1 is a transcription factor binding to Forkhead Recognition Element or similar sequences in promoters of the genes it regulates, it would be interesting to analyze the genes that are up- and down-regulated to get an idea whether the effects are directly due to FOXO1 and perform ChIP to confirm this.

Finally the experiment should be validated on the protein level, this would include developing a procedure for extracting proteins from alginate discs, since this does not exist to date and perform immune florescence (IF) on differentiating MSCs to confirm the expression and changes (existence or absence) of the protein.

6. Conclusion

The RT-qPCR analysis showed that *FOXO1* knockdown has led to a down-regulation of *COL10A1* and *COL2A1* in 2 of 3 donors. The nanostring analysis of one donor showed that *FOXO1* knock-down has lead to down-regulation of chondrogenic promoting genes such as *PANX3, FGF2*, and *BMP4*, chondrogenic hypertrophic genes such as *IHH, CDKN1C*, and *COL10A1* and the major chondrogenic marker *COL2A1*, additionally *FOXO1* knockdown has also led to an upregulation of negative regulators of chondrogenesis such as *HEY1* and *IGFBP-3*. Further, RT-qPCR analysis showed that the up-regulation of *FOXO1* has not altered gene expression of the genes tested and we can therefore conclude that *FOXO1* up-regulation in MSCs cultured in 2D has no impact. The nanostring results could imply a role for *FOXO1* in promoting chondrogenesis, through unknown mechanism, resulting in positively regulating chondrogenic promoting genes and negatively regulating chondrogenic impairing genes.

All in all these, while these findings are promising and suggestive of a function of *FOXO1* in chondrogenesis *in vitro*, it is premature to make any conclusions and further validations need to be performed.

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