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Oslo, May 2008

Biruk Luelseged

Abstract

The human *HMGA2* gene is located at chromosomal band 12q14-15. In normal cells it encodes for the HMGA2 protein (109 amino acids). HMGA2 is a transcription factor that binds to the AT rich sequences on DNA and thereby changing its conformation. The gene consists of five exons and between the third and the fourth exon, there is an intron with a size of 140Kb which may be broken in tumours. Such rearrangements have been identified in benign neoplasms and several types of malignant mesenchymal tumours. Mesenchymal stem cells (MSCs) are multipotent stem cells capable of differentiating into adipocytes among others.

In this study we have induced adipogenic differentiation in the immortalized MSC line iMSC#3b. We found that theses cell lines are capable of differentiating into adipocytic lineage but difficult to transfect, and changed to another MSC line, hMSCtert20. hMSC-Tert20-HMGA2 clone t4 cell line was used for overexpression of *HMGA2* during adipogenic differentiation and expression levels were measured by real time RT-PCR. It turned out that forced expression of exogeneous *HMGA2* led to further overexpression during differentiation in most of the measurements. The effect of overexpression of *HMGA2* was investigated on one of the markers of adipogenic differentiation Peroxisome proliferator-activated receptor γ (PPARγ). Also *PPARγ* expression was enhanced during differentiation by overexpressing *HMGA2*. It was also possible to see a slight increase in its expression in cells not induced for adipogenesis.

We have also attempted to confirm the HMGA2 and NF-$_k$B interaction. We used antibodies against HMGA2 and NF-$_k$B-p65 to to investigate the expected interaction by immunoprecipitation. No interaction could be detected in the samples (HMGA2 and NF-$_k$B) investigated, but this could be due to low sensitivity.

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1. Introduction

1.1. Cancer in general

Cancer development is viewed as the transformation of a normal cell into malignant cells. The transformation is accompanied by sequential mutations in tumour suppressor genes promoting oncogenes. The damage to the genome, resulting in sequential occurrence of mutations can be as a consequence of endogenous and exogenous processes for example DNA replication errors, chemical instability of DNA bases, interaction with exogenous agents (i.e. chemical or radiation). Cancer cells have different shapes compared with healthy cells as a consequence of which they don't behave and function properly.

Tumours are lesions formed due to abnormal growth of tissue. They are collection of cells that divide and grow uncontrollably. Tumours can be benign or malignant. Benign tumours are tumours that can grow very big and have the capacity to invade surrounding tissues. The progression of benign tumours to malignancy and metastasis is accompanied by the loss of growth factors that control cellular proliferation and diversity (Nicolson, 1991) at early stage and acquire the capacity to invade and colonise other tissues. In contrast, malignant tumours tend to divide uncontrollably and spread aggressively to the surrounding tissues thereby destroys the nearby normal tissues. Normal cells are destroyed by the metastatic potential of these tumours by which they send cells to other tissues transported by blood (circulator system) or the lymphatic system in the body. The original tumour cells (primary tumours) will form new tumours after being destined in other organs (secondary tumours) in the body.
There are different types of cancers. *Carcinomas* are cancers of the epithelial tissues. *Lymphoma* is a cancer in the lymph nodes and glands of the lymphatic system. *Leukaemia* sometimes referred as blood cancer, is a cancer of the bone marrow. *Myelomas* are cancers of plasma cells of the bone marrow. *Sarcomas* are malignant tumours in connective and supportive tissues.

1.1.1 Sarcoma

The word sarcoma is derived from Greek word *sarx* which means fleshy substance. Sarcoma is a heterogeneous group of malignant tumours which consist of a various group of mesenchymal neoplasms. They are relatively rare malignant tumours that comprise 1% of all human malignancies but about 10% of pediatric cancers. They arise in mesenchymal tissues and often display highly aggressive behavior towards early haematogenous metastasis (Riggi et al., 2007).
They can be grouped into two general categories, soft tissue sarcoma and primary bone sarcoma.
Soft tissue sarcomas, which represent fewer than 1% of malignancies, may arise in adipose tissue, smooth or striated muscle and cartilage, as well as other soft tissue (Skubitz & D'Adamo, 2007). Sarcomas which arise and resemble fat tissue are termed as liposarcoma. Several types of liposarcoma exist, including well-differentiated liposarcoma, myxoid liposarcoma, round-cell liposarcoma, dedifferentiated liposarcoma, and pleomorphic liposarcoma. The primary bone sarcoma arises from bone cells or tissues. The most common bone sarcoma is osteosarcoma.

Cytogenetic studies have shown that in human chromosome 12, the region q13-q15 is strongly implicated in development of malignant and benign solid tumours, suggesting the location of tumour associated genes. Malignant tumour types with recurrent aberrations in 12q13-q15 include myxoid liposarcoma, soft tissue clear cells (Schoenmakers et al., 1995), well differentiated liposarcoma and osteosarcoma. The HMGA2 proteins as well as other oncogenes like MDM2 are located at chromosomal locus 12q14-15 in man.

1.1.2 HMGA2

The high mobility group (HMG) proteins are among the largest and best characterized group of non-histone chromosomal proteins. The 'high mobility group' HMGA protein family consists of four members encoded by two different genes: HMGA1a, HMGA1b and HMGA1c, are translated by alternative splicing of one gene. And HMGA2 is encoded for by another gene (Cleynen & Van de Ven, 2008). All HMGA proteins contain about 100 amino acid residues and have many functions and are involved in various cellular processes, including gene regulation, cell cycle, and adipogenic differentiation. HMGA protein family plays also a role in the promotion of gene activation. They have important role in gene transcription regulation through both protein-protein and protein-DNA interaction (Friedmann et al., 1993).
These proteins are preferentially expressed during embryonic and fetal stages of development. In adults *HMGA2* is not expressed, and HMGA1 is expressed at very low level.
The *HMGA2* gene has 5 exons in which between the third exon and the fourth exon there is an intron with a size of approximately 140 kb. Upon transcription the mRNA contains the three exons that have three DNA binding domains.

Figure 1.1: Schematical view of the HMGA2 protein.

All members of the HMGA family are characterized by the presence of highly conserved DNA-binding domains called 'AT hooks' that bind to AT rich sequences on DNA HMGA2 proteins acts as architectural transcription factors by organizing the assembly of transcriptional complexes at the promoter regions of several genes (Fusco & Fedele, 2007), enhancing or suppressing transcription by binding to transcription factors or DNA (Sgarra et al., 2004). *HMGA2* is differentially expressed in a single or multiple cell lines (Ayoubi et al., 1999).

The *HMGA2* gene codes for an architectural transcription factor target of frequently rearranged by translocation in lipomas and other benign mesenchymal tumours mostly, uterine leiomyomas, pleomorphic adenomas, fibroadenomas (Noro et al., 2003). In sarcomas, the gene is also found to be rearranged, but in addition amplified and overexpressed (Berner *et al.*, 1997). In most cases, the rearrangements resulted in

the separation of the three HMGA2 DNA-binding sites and lose of the c-terminal domain (Geurts et al., 1997). This c-terminal domain might have a regulatory role since in the absence of it cells turnout to become tumorigenic. Binding of HMGA2 to targets within the minor groove affects the DNA conformation and facilitates binding of transcription factors (Wisniewski & Schwanbeck, 2000).

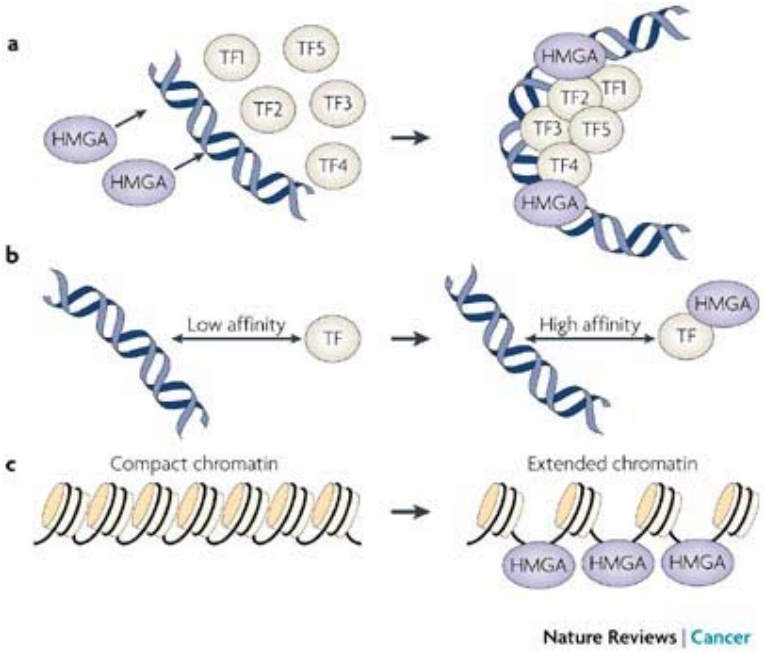

Figure 1.2 (A). HMGA interact with both DNA and transcription factors to form multiprotein complex. (B) HMGA proteins can affect gene transcription by protein-protein interaction. (C) HMGA proteins have the ability to form heterochromatin. From (Fusco & Fedele, 2007).

1.1.3 HMGA2 as transcriptional apparatus

MARs (matrix associated region) are segments in DNA that have AT-rich sequences and have high affinity to nuclear factors. HMGA2 proteins have been implicated as an important component of MARs. Some examples could be mentioned, HMGA2 directly regulate transcription of the *IMP2* gene by binding to an AT-rich site of DNA located in the first intron (Cleynen et al., 2007). HMGA proteins have been shown to bind PRDII element (positive regulatory domains) together with p50/p65 NF-$_k$B heterodimer (Thanos & Maniatis, 1992). Moreover HMGA2 was also implicated in activating transcription by forming association with p50/p65 NF-$_k$B heterodimer (Mantovani et al., 1998). The HMGA2 proteins amino acid 54-73 is critical for assisting binding of NF-$_k$B to the PRDII element (Noro et al., 2003). Moreover HMGA2 was implicated in heterochromatin formation (Narita *et al.*, 2006).

Besides the role of HMGA2 as architectural factors, they have also been shown to be involved in the process of adipogenesis. Previously done experiment in our group suggest that overexpression of *HMGA2* inhibits adipogenesis. Moreover *HMGA2* null mice turned out to have a pygmy phenotype with greatly reduced fat tissues indicating the role of HMGA2 in adipogenesis (Zhou *et al.*, 1995). HMGA2 modulation in vivo also reduces obesity (Anand & Chada, 2000).

1.2 Mesenchymal stem cells

Mesenchymal stem cells are adult stem cells that are derived from the mesoderm which are able to differentiat into multiple cell lineages and also serve as reservoirs for a variety of stem cells. They have been used as a model system due to their various applications. They can take up and keep introduced genes which are crucial for the delivery of important molecules to targeted sites (Clark & Keating, 1995). They can also be frozen and thawed as needed without any effect on the normal functioning of the cells. Moreover mesenchymal stem cells have therapeutic role (Horwitz et al., 1999), stem cell can be taken from small bone marrow cells, expanded in culture and can be returned back to the patient. They are also important tools in expressing receptors and growth factors (Majumdar et al., 1998).

1.2.1 Stem cells and pluripotency

A stem cell is an undifferentiated cell that produces daughter cells that are either stem cells by themselves by the process of self renewal or committed cells that undergo several differentiation steps to form a mature more committed cells (Serafini & Verfaillie, 2006). Stem cells can be classified as totipotent, pluripotent, multipotent, and unipotent.

Totipotent cells are speciallized cells capable of differentiating into all types of cells in an organism. They are created when a sperm cell fertilizes an egg. Days after fertilization, totipotent stem cell matures and specializes into pluripotent cells. Pluripotent cells have the ability to give rise to a variety of differentiated cell types found in embryonic germ layers (Figure 1.3). Development in mammals is characterized by proliferation of pluripotent stem cells followed by differentiation and spatial organization (Brickman & Burdon, 2002). Unipotent cells arise from multipotent cells and differentiate into a single cell type for instance skin cells. They can differentiate and proliferate repeatedly.

Multipotency refers to the ability of a cell to differentiate into multiple cell types of a single tissue. Human mesenchymal stem cells are derived from mesodermal germ layer during embryo development. They are thought to be multipotent cells, which are present in adult marrow, can replicate as undifferentiated cells and have the potential to differentiate to lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle, and marrow stroma (Pittenger et al., 1999).

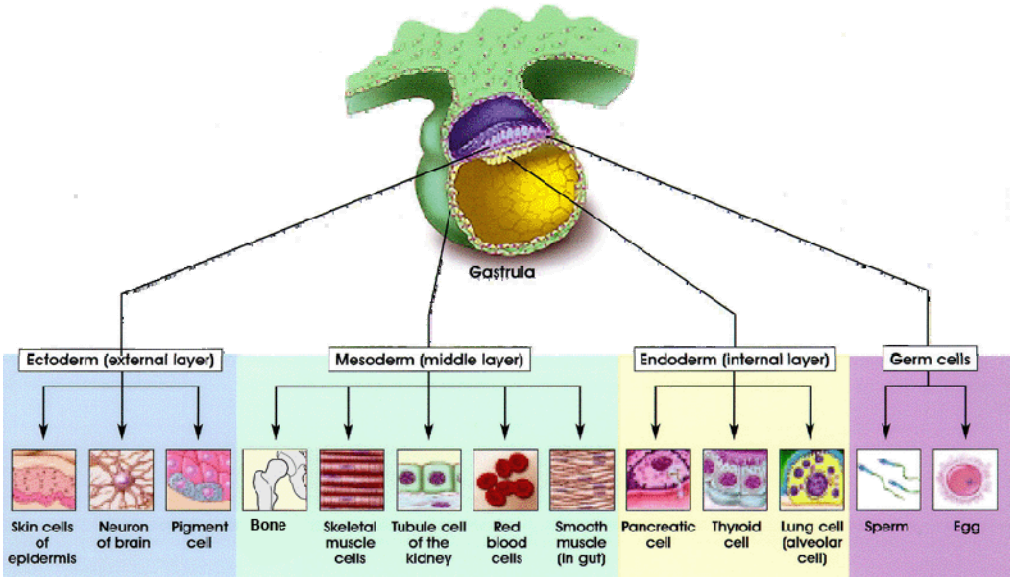

Figure 1.3 A schematic view showing a variety of differentiated cells found in embryonic germ layers. From J. Bottenstein.

The mesenchymal stem cells have the ability to self renew and differentiate that makes them preferable for clinical application in regenerative medicine (Mishra et al., 2009).

1.2.2 Adipocytic differentiation

Adipose tissue is connective tissue which serves as a site for storage of fats in the form of triglycerides. There are two types of adipose tissue, white adipose tissue and brown adipose tissue. White adipose tissue formation begins before birth and function as heat insulator and source of energy. Its expansion takes place rapidly after birth as a result of increased adipocyte size and number. Adipocytes are the main cellular components of white adipose tissue. Brown adipose tissue is present in small mammals and newborn infants in human. They function as a vital source of heat.
Several studies on multipotent clonal cell lines indicate that adipocytes are derived from mesenchymal stem cells (Pittenger *et al.*, 1999). Adipogenic differentiation involves the conversion of mesenchymal stem cell into mature adipocyte.

Initiation and progression in adipocytic differentiation involves multiple signals through signal transduction at the cell membrane and in the nucleus. During this process cells convert to a spherical shape and accumulate small lipid droplets. Later these droplets fuse together and give a rounded shape (Smas & Sul, 1995) appearance of cells similar to white adipose tissue. Transcriptional regulation in the process of adipogenesis requires the activity of several transcription factors that control growth arrest and the coordinated expression of adipocyte-specific genes.
Adipocytic differentiation is characterized by successive expression of numerous genes. This is shown by the expression of early, mid and late adipogenic markers (Smas & Sul, 1995). Genes that are involved in the process of adipogenic differentiation are crucial to study adipogenesis at a molecular level. Changes in gene

expression during adipocyte maturation is characterized by the use of preadipose cell lines (Gregoire et al., 1998).

1.2.3 Markers of adipocytic differentiation

The peroxisome proliferator-activated receptor (PPAR) is a nuclear hormone receptor. Structurally these proteins have 4 domains namely A/B (ligand independent activation domain), C (DNA binding domain), D (hinge domain), and E/F (ligand-binding domain – LBD) (Owen & Zelent, 2000). In vertebrates there are three PPAR isotypes; PPAR-alpha, -delta, and –gamma (Michalik & Wahli, 1999).

PPARγ binds to peroxisome proliferator response element by forming a heterodimer complex with retinoid X receptor RXR (Kliewer et al., 1992), and it is required for adipocyte development (Rosen et al., 1999). Expression of CCAAT-enhancer-binding protein ß (C/EBPß) is activated by transcription factors that are expressed at the early or late stages in the adipogenesis process. PPARγ is expressed early in the adipocytic differentiation process (Tontonoz et al., 1994) and is activated by C/EBPß expression. C/EBPα function cooperatively to establish terminal adipocyte differentiation (Shao & Lazar, 1997) as shown in Figure 1.3.

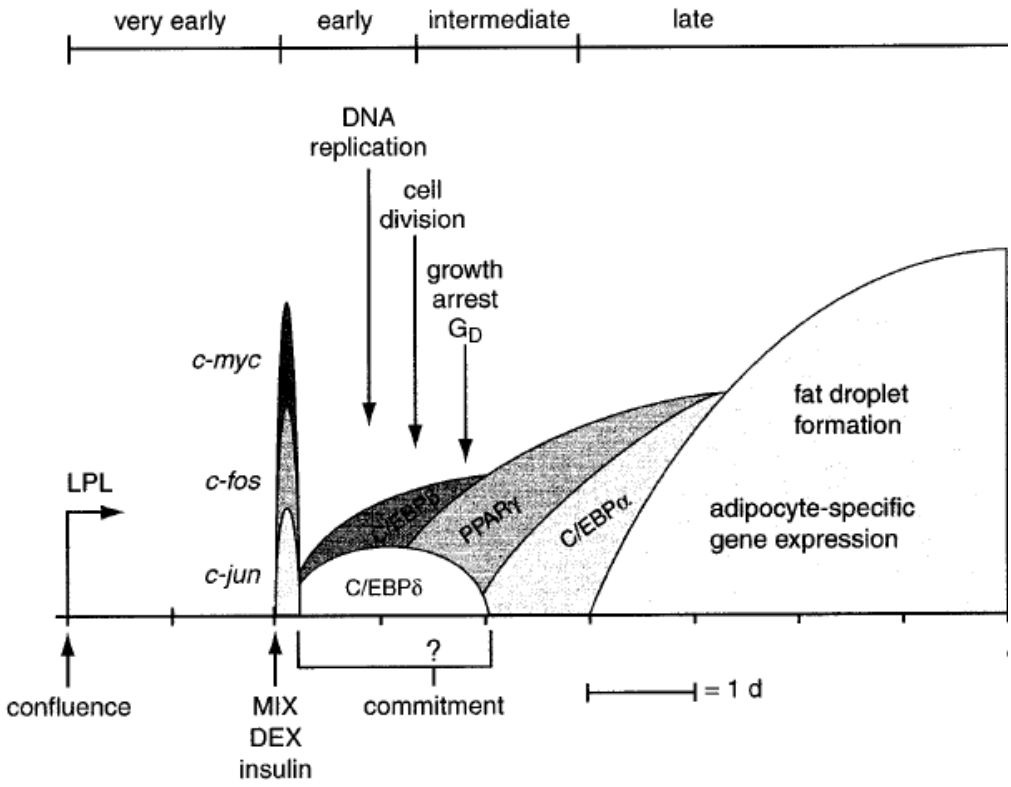

Figure 1.4 Progression of preadipocyte differentiation. The chronological expression of adipogenic markers at distinct stages of differentiation (very early, early, intermediate and late). LPL, lipoprotein lipase; C/EBP, CCAAT/enhancer binding protein; PPAR, Peroxisome proliferator-activated receptor; MIX, methylisobutylxanthin; DEX, dexamethasone. From (Ntambi & Young-Cheul, 2000).

1.3 Aims of the study

The aim of this study was to investigate the role of HMGA2 in adipogenic differentiation by overexpressing it in a mesenchymal model system. The project had the following sub-aims:

1. Construction of plasmids for regulatable expression of *HMGA2* using the Gateway system.

2. Transfection of these into iMSC#3b.

3. Investigate the effect of *HMGA2* induction on markers of adipogenic differentiation.

4. An attempt to confirm protein association between HMGA2 and NF-kb transcription factors, as was suggested by a previous study in the group.

2. Materials and methods

2.1 Immunoprecipitation

Preparation of lysates was done without denaturing the cells. hMSC-Tert20 cells which constitutively express HMGA2 fused with EGFP were cultured in DMEM and maintained until 70% confluency. The cells were washed with ice cold PBS afterwards. After draining the PBS, ice cold lyses buffer; RIPA (50mM Tris HCl pH 8, 150mM Nacl, 1% NP-40, and 0.5% sodium deoxycholate) was added. Adherent cells were scraped off the dish. Constant agitation for 30 minutes at 4°C and centrifugation in a micro centrifuge at 4°C was proceeded. Goat polyclonal HMGA2 (Santa Cruz: sc-23684) antibody was diluted in 1:50 and that of rabbit polyclonal antibody NF-$_k$B (NF-$_k$B subunit p65 (A): sc-109**)** was diluted in a 1:100 ratio for the protein-protein interaction assay. The cell lysates were incubated with the antibody at 4°C for 1 hour. nprotein A sepharose^{TM} was added to the samples and then incubated at 4°C with rotation for 4 hours. Samples were then centrifuged, supernatant was removed and beads were washed with lysis buffer (each time centrifuging at 4°C and removing supernatant).

2.2 Western blot analysis

hMSC-Tert20 cells constitutively expressing the HMGA2-EGFP fusion protein were trypsinized, collected and were spun down once at 1000 rpm for 5 minutes. Supernatant was removed and washed with PBS for once more spinning. Protein was extracted by using lysis buffer 5× solution buffer [150mM Nacl, 50mMTris-Hcl, 0.1%NP-40, 1mM EDTA, protease inhibitors (all from SIGMA)]. SDS page gel was run prior to blotting.

The set up for blotting was as follows respectively, [blot pad, 7×10 cm pieces of whatman paper, Nylon membrane (MILLIPORE), gel, whatman paper and the blotpad, all soaked with 10×BSN buffer (Tris;58mM, Glycin;29mM) except for the gel]. The blotting was done overnight at 4^{o}C and at 30V. The filters were blocked by blocking solution [5% dried milk and low salt TBST; Tris p^{H}=7.5 (MERCK), 150mM Nacl, 0.1% Tween 20 (MERCK)]. The filter used for HMGA2 (sc-23684) detection was incubated overnight with 1°antibody to blocking solution in a (1:1000) dilution ratio for HMGA2 in a (1:5000) blocking solution for α-tubulin. The 2° antibody used for the final detection of NF-$_k$B interaction with HMGA2 was incubated in a (1:1000) with the blocking solution.
Both filters were washed with TBST; to remove the salt in the filter. The filters were incubated for 1 hr with 2° antibody of α-tubulin (Calbiochem) from mouse and that of HMGA2 from Goat in a dilution ratio of 1:5000 in a blocking solution. After the filters were washed 3 times with TBST for 10 minutes each, immunodetection was done by using chemiluminescence (ECL) and was recorded by film.

2.3 Gel electrophoresis

Determination of the size of DNA was done on [1% agarose gel (0.5g) and 50µl 1×TAE buffer, 3µl ethidium bromide]. Gene Ruler^{TM} 100bp DNA ladder (Fermentas) was used for comparison. The gel was run at 80V for 30 minutes. For western blot

analysis, the gel used is NUPAGE®- Gel- 4-12% BT 1.0 (Invitrogen), MES buffer. The gel was run at 200V for 35 minutes.

2.4 DNA isolation and purification

By using the fast plasmid™ mini kit (Invitrogen), plasmid DNA was isolated according to manufacturere instructions. From PCR reactions involving advantage 2 polymerase, DNA was isolated and purified by using the wizard® sv gel and PCR clean-up system protocol.

2.5 PCR

Standard PCR reaction was used with DNA polymerase or Advantage 2 polymerase (Clontech). In all the PCR reactions, primers were always diluted to a final concentration of 2μM. For reactions with advantage 2 polymerase, the denaturation temperature was at 95°C for 1 minute. Both the annealing and extension step was performed at 68°C with 30 number of cycles. In all the reactions not involving Advantage 2 polymerase , a single cycle of denaturation at 94°C and 30 cycles of annealing and elongation at 55°C and 72°C respectively were performed.

2.6 Gateway cloning

The Gateway® cloning system (Invitrogen life technology) was used to clone the coding region *HMGA2*. This cloning system is based on site specific recombination of bacteriophage λ. The donor vector is used to clone *att*-B flanked PCR products (genes of interest) to generate entry clones. The destination vector on the other hand recombines with the entry clone in an LR reaction to generate an expression clone.
The coding region *HMGA2* was amplified by PCR with primers containing two *att* sequences, *attB1* and *attB2*.
*att*B1 Forward primer:
5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAGCGCACGCGGTGAG3'
*att*B2 Reverse primer :
5'GGGGACCACTTTGTACAAGAAAGCTGGGTACTAGTCCTCTTCGGCAGA3'
In the BP reaction, the *att*B flanked PCR product was recombined into donor vector / pDONR221. In the LR reaction, the entry clone pDONR221-HMGA2 was recombined with the destination vector (pCDNA5/FRT/TO/GW) according to the manufacturer's instructions.

2.7 Restriction digests and transformations

All restriction digestion reactions were set up using enzymes from (NEB) according to manufacturer's instruction. The entry clone was transformed into competent DH5α cells, by using general guide lines from the manufacturers. The vector pcDNA5/FRT7TO/GW-HMGA2 was transformed into DH5α cells, placed on ice for 30 minutes after which it was taken and immersed in water bath for 30 seconds at 42^{o}C. It was incubated on ice for 2 minutes and 250 μl SOC medium was added afterwards. The cells were then placed at 220 rpm shaker, 37^{o}C for 1 hour. The transformed cells were plated on kanamycin containing plates. Then the plates were incubated overnight at 37^{o}C.

2.8 Sequencing

The vectors were verified by sequencing which was performed at the institute for molecular biosciences (IMBV) sequencing facility. 250 ng/ µl of plasmid and 5 µM of the primer was used for sequencing of the plasmid.
M13 (-20) Forward primer 5'-GTA AAA CGA CGG CCA GT-3'
M13 Reverse primer 5'-AAC AGC TAT GAC CAT G-3'

2.9 Cell culture

For this study, 4 different cell lines were used:

1. iMSC#3b

2. hMSC-Tert20; and from this cell line two sublines have been created:

2.1 hMSC-Tert20-EGFP-HMGA2 – which constitutively express *HMGA2* fused to EGFP in the N-terminal
2.2 hMSC-Tert20-HMGA2 clone t4 – which express *HMGA2* under an inducible promoter

Adipogenic differentiation assay was done on all cell lines except for hMSC-Tert20-EGFP-HMGA2. Except for iMSC#3b, all the other cell lines were cultured in DMEM (Biowhittaker) medium supplemented with fetal bovine serum, penicillin, hepes and glutamine. The cells were kept at 37°C incubator with 5% CO_2 (Steri-cycle CO_2 incubator). Subconfluent cells were splitted in 1:20 ratio. iMSC#3b cells were cultured in standard medium [α-MEM (GIBCO) supplemented with heparin, fetal bovin serum, penstrep]. At 70% of confluency, 2×10^5 cells were resuspended in 6 well plates for differentiation analysis at different time frames (T=0,8,24,48,72 hours). When the cells were subconfluent, hMSC-Tert20-HMGA2 clone t4 cells were induced with doxycycline for overexpression of *HMGA2* 12 hours prior to addition of the differentiation mix (dexamethason, insulin, IBMX) into the cells. For differentiation assay, hMSC-Tert20-HMGA2 clone t4 cells were categorized into four groups and treated differently as follows; (cells with doxycycline only, cells with both differentiation mix and doxycycline, cells with differentiation mix only and cells without doxycycline and differentiation mix). iMSC#3b cell differentiation was induced by adding differentiation mix to the cells suspended in α-MEM. The cells were allowed to stay for 21 days before analyzing the result by taking a picture of the cells with camera (Axiovision, ZEISS). The medium was changed twice per week for all the cell lines.

2.10 Human telomerase reverse transcriptase (*hTERT*)

Telomeres are repetitive sequences which have TG and CA rich sequences in each complimentary DNA strands. They shorten in each cell division leading to inhibition of cell growth and proliferation (Vaziri & Benchimol, 1999). Telomerase, a ribonucleoprotein (Greider & Blackburn, 1987) is involved in immortalizing cells by adding telomeric sequences to the 3' end of DNA strands thereby extending the telomere length allowing DNA replication and keeping the end of the chromosome from attaching with each other.
Human telomerase has three components; human telomerase RNA component, human telomerase-associated protein 1 (hTEP1) and human telomerase reverse transcriptase

(hTERT) (Poremba et al., 2000). Transduced hMSC-TERTs were shown to undergo more than 260 population doublings (Simonsen *et al.*, 2002).

2.11The TET ON system

The Tet on system is based on the repressor gene (*tetR*) of the Tn10 tetracycline resistance operon. This binds to operator sequences (*tetO*) of the Tet operon in a tetracycline-dependent manner. This system was used for the regulated expression of our gene of interest *HMGA2* for functional analysis. In the presence of doxycycline, the *tetR* gene will unbound the Tet operator sequences located downstream of the CMV promoter region and induces the expression of *HMGA2*. In the absence of doxycycline the *tetR* remains bound to the *tetO* sequences thereby inhibiting expression of the gene of interest.

2.12 Oil Red O staining

Cells were fixed with ice cold 70% ethanol and were incubated at 4°C for 1 hour. Soon after the incubation period, the cells were stained with Oil Red O solution (0.5%) for 30 minutes. Pictures were taken with camera after aspiration and wash with water.

2.13 RNA isolation and cDNA synthesis

RNA was isolated by using TRIzol®reagent (Invitrogen) according to manufacturer's instructions.
Using the HIGH CAPACITY RNA-TO-cDNA master mix kit (Appplied Biosytems), 100ng RNA template was reversibly transcribed by using a thermal cycler (GeneAMP PCR system 9700, Applied Biosystems). 25°C denaturation step for 5 minute and further annealing and elongation steps were performed at 42°C and 85°C for 30 and 5 minutes respectively.

2.14 Quantitative real time PCR

The gene expression was measured by using an ABI 7500 Real time PCR system (Applied biosystems). The assays used are PPARγ (Hs 00234592-m1), HMGA2 (Hs 00171569-m1), and TBP (Hs 99999910-m1) as an endogenous control. In 96 well reaction plate, two identical replicates were included for every cDNA. The default on the thermal cycling conditions comprised of enzyme activation step at 95°C for 10 minutes. Moreover 40 cycles of denaturation and annealing/extension steps were done for 15 seconds and 1 minute at 95°C and 60°C respectively. The fluorescence emitted by the reporter dye found on the Taqman probe was detected in each cycle of PCR reaction. The C_T value obtained which refers to the number of cycles when the fluorescence emitted by the probe passes a threshold level set by the user, with in the growth region of an amplification curve, was used to normalize the samples with the endogenous control; TBP. Further analysis of the data was done on Office Excel 2003 (Microsoft).

3. Results

3.1 Differentiation of immortalized bone marrow stromal cells

The iMSC #3b cell line is a non-tumorigenic and stably transduced with vector containing the *hTERT* gene (Noordhuis et al, in prep). Maintenance of these cells was done in αMEM (changed twice a week) with or without the differentiation mix. Quantification of adipogenic differentiation analysis after 21 days treatment with the differentiation mix shows that the immortalized bone marrow stromal cells are capable of adipogenic differentiation (red stained fat droplets) as can be seen in Figure 3.1.

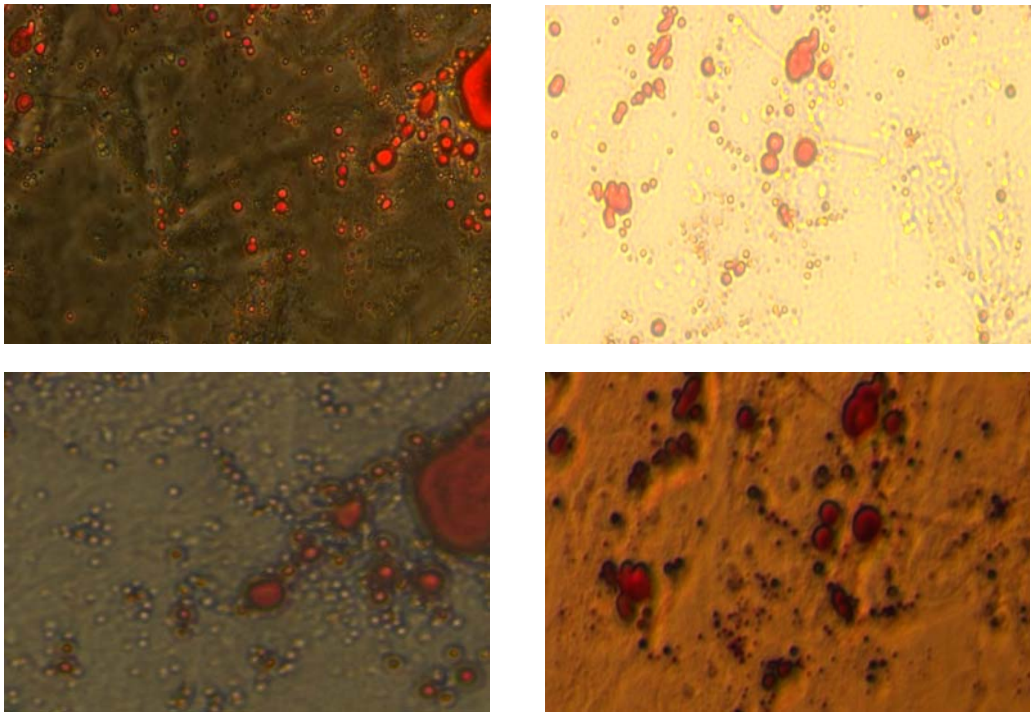

Figure 3.1 Differentiaiton of iMSC#3b cells: Analysis for adipocytic differentiation (red stained fat droplets due to the higher solubility of Oil Red O in fat cells) after 21 days of differentiation.

Unfortunately, the iMSC#3b cells turned out to be difficult to transfect. For further analysis we used an already existing cell line which has been transfected previously by others in our group with either *HMGA2* expressed from an inducible promoter or Hmga2 tagged with EGFP under a constitutive promoter (see Materials and Methods section).

3.2 Cloning of HMGA2 *by Gateway cloning technique.*

Previous findings in the Myklebost group have shown that overexpression of stably transfected *HMGA2* during adipogenesis of mesenchymal stem cells inhibits attempts to differentiation. For regulated expression analysis in mesenchymal model system,

HMGA2 was cloned by introducing the *att*B flanked PCR product (A) in Figure 3.2 into the pDONR^{TM}221 vector by using Gateway technology. In order to check whether there is an insert or not, a double digestion of the entry clone was done by PvuII. The size of the entry clone was verified by comparing the isolated plasmid DNA with a standard ladder. The destination vector pcDNA5/FRT/To/GW containing the Tet on promoter system was recombined in the LR recombination reaction with pDONR^{TM}221-hmga2 (entry clone) to form the expression clone.

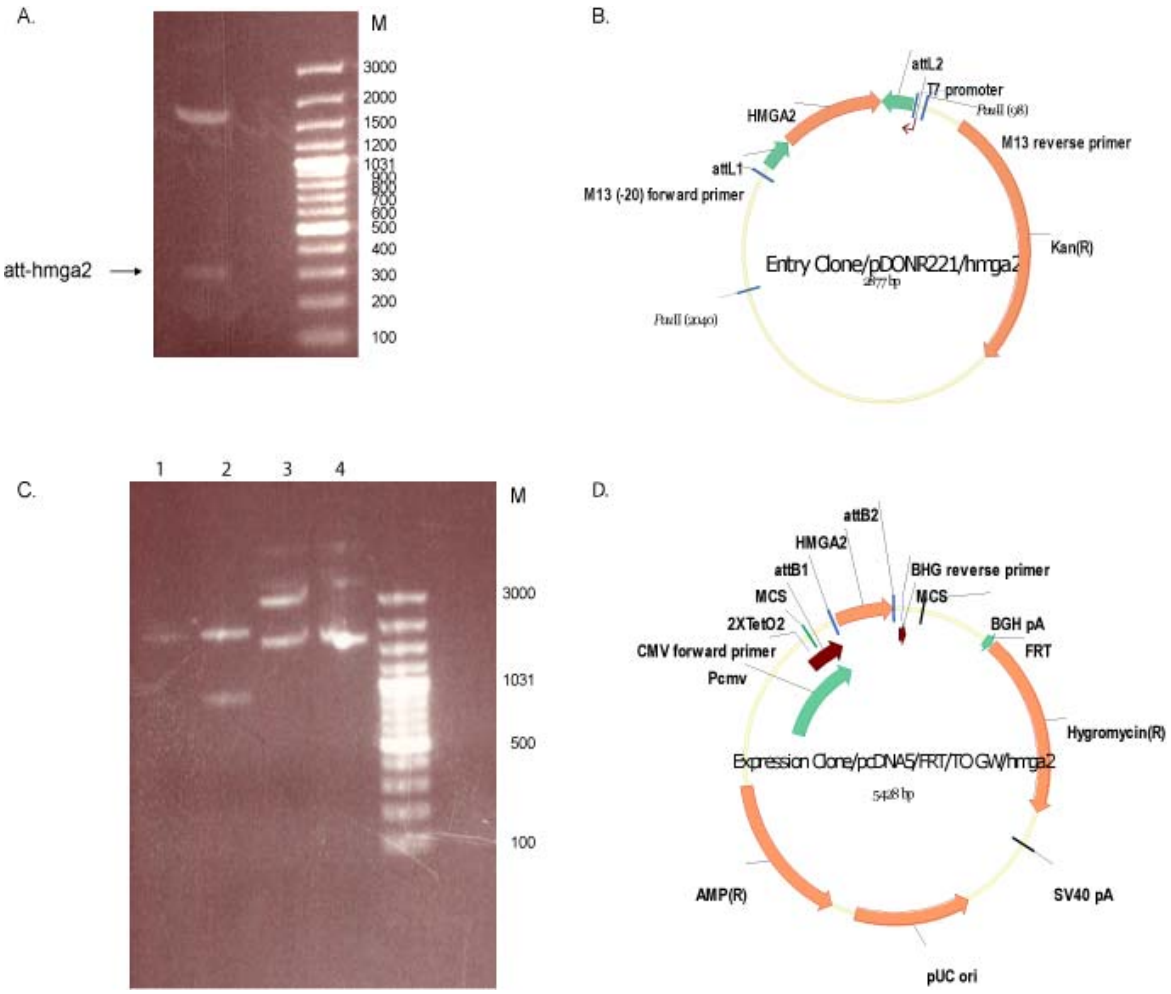

Figure 3.2 (A) Left panel, lower fragment *att*B flanked PCR product, upper fragment is a byproduct (vector). Right panel, GeneRuler^{TM} 100bp DNA ladder plus. (B) pDONR^{TM}221-HMGA2 entry clone map after BP reaction by Gateway® cloning system. It shows the forward and reverse primers used to amplify a PCR product and the restriction enzyme cutting site for PvuII. attL1 attL2 are attachment sites in the donor vector used for site specific recombination reaction. (C) Restriction cut of the entry clone with PvuII. Lanes 1 and 2 show pDONR^{TM}221-HMGA2 that was cut with PvuII. The upper and lower band indicates the larger and smaller cut fragments respectively. Lanes 3 and 4 represent an entry clone which was not cut with PvuII. The lower band shows the size of the whole vector while the upper band is a byproduct. GeneRuler^{TM} 100bp DNA ladder plus was used for the size determination. (D) pcDNA5/FRT/To/GW-HMGA2 expression clone. A destination vector containing a CMV promoter that is under control of the *tet* repressor gene in a tet on system.

3.3 Overexpression of Hmga2 during differentiation into the adipocytic lineage

Hmga2 overexpression has been shown to inhibit adipogenic differentiation in our group (Henriksen et al. in prep). But how it inhibits in the adipogenic differentiation lineage is not known. In this study we have tried to see the effect of overexpression of *Hmga*2 on one of the adipogenic marker, *PPARγ*. These studies were done with the hMSC-Tert20-HMGA2 clone t4 cell line. The differentiation experiment was performed twice. Several real time PCR measurements were undergone however some measurements including those that deviate from results presented here are not shown. Previous experiment done in our group (Henriksen et al. in prep) shows that *Hmga2* was overexpressed after induction with doxycycline for 12 hours as can be shown in Figure 3.3. Two transfected cell lines were induced with doxycycline; overexpression of *Hmga2* in hMSC-Tert20-HMGA2 clone t4 cells was higher than the hMSC-Tert20-HMGA2 clone t2 cells overexpression. *HMGA2* was overexpressed and was more than twice higher than before induction time (t4 0). The hMSC-Tert20-HMGA2 clone t4 cell line having the largest induction was chosen for further analysis in gene expression assay. The cells were grown and maintained in 6 well plates with and without the differentiation mix. The expression level of *HMGA2* was monitored with quantitative real time PCR.

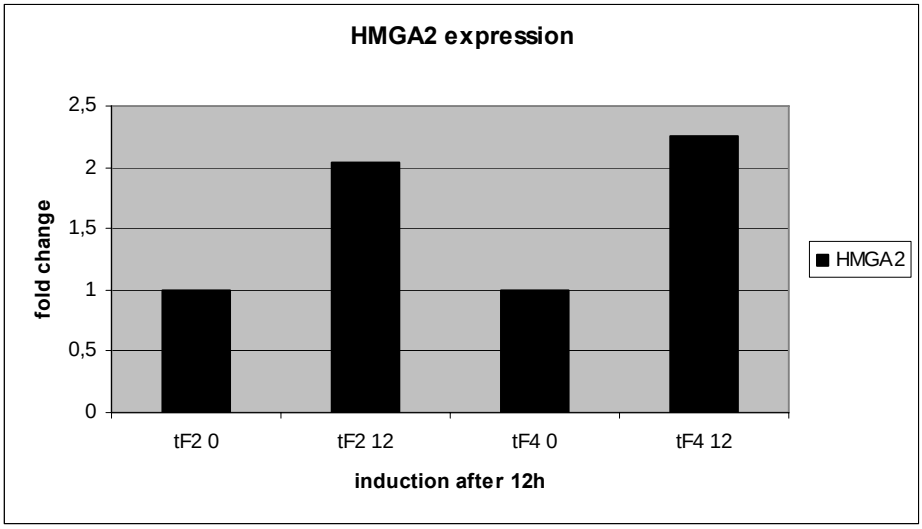

Figure 3.3 The expression level of *HMGA2* in two *HMGA2* transfected clones of hMSC-Tert20, t2 and t4. The expression of *HMGA2* increases after 12 hours of induction period. This expression assay includes both the endogenous and the transgene expression level.

Peroxisome proliferator activated receptor γ (PPARγ) is a nuclear receptor protein that functions as a regulator of gene expression. Transcription of the *PPARγ* gene commences only during adipogenic differentiation. Due to the ability of this gene to be turned on only during adipogenic differentiation, the gene is termed as an adipogenic marker.

3.3.1 Quantitative real time PCR assay

Expression of genes was analyzed through quantitative real time PCR technique. The analysis was done to see the effect of overexpression of *HMGA2*. After the cells were grown and maintained, RNA isolation followed by cDNA synthesis was done as referred in section 2.13 after which the real time PCR run took place. Because all results are relative to the amount of RNA added, the house-keeping gene TBP was used to normalize expression levels between samples. Figure 3.4 illustrates one real-time experiment and shows that expression of *HMGA2* is higher in cells with the adipocytic differentiation mix than in cells without the mix. And this overexpression increases as the differentiation period increases. The result shows a surprising increase in the level of *HMGA2* upon induction of differentiation, which is not observed with induction by doxycycline only.

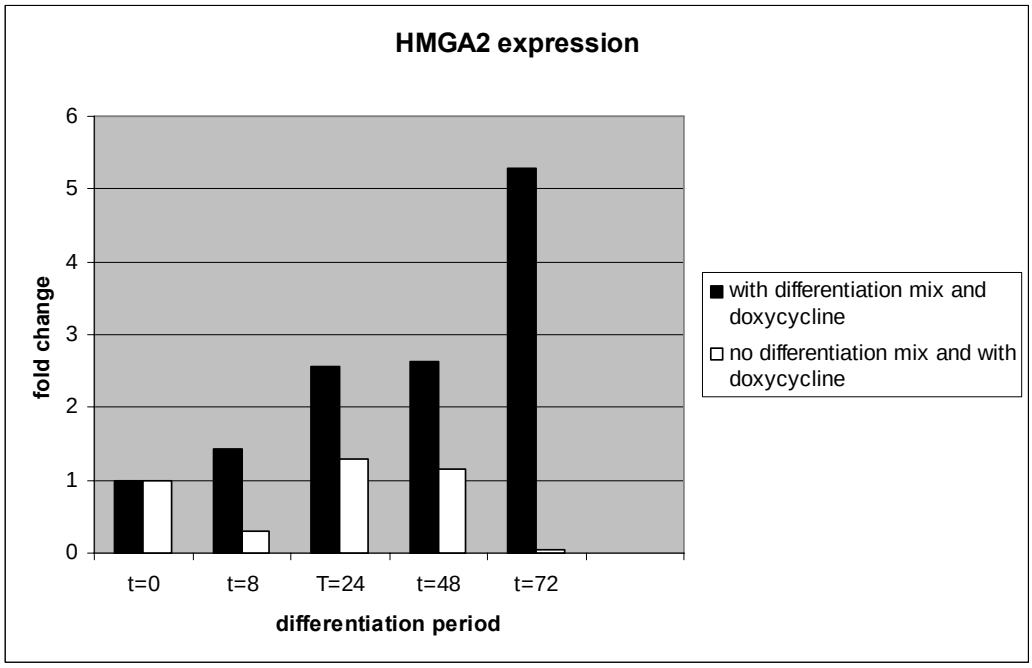

Figure 3.4 A diagram showing fold change in the expression level of *HMGA2*. The real time PCR products were normalized with an endogenous control, TBP. Black: Induced cells with adipocytic differentiation cocktail. Fold change is relative to T=0. NB doxycycline was added 12 hours prior to differentiation. White: Induced cells without the differentiation cocktail.

Similarly PPARγ expression was measured in the same experiment by quantitative real time PCR. In figure 3.5, it can be seen that its expression increases as the differentiation time proceeds when it is treated with and without the differentiation mix, although PPARγ expression is higher in cells with the adipogenic differentiation mix.

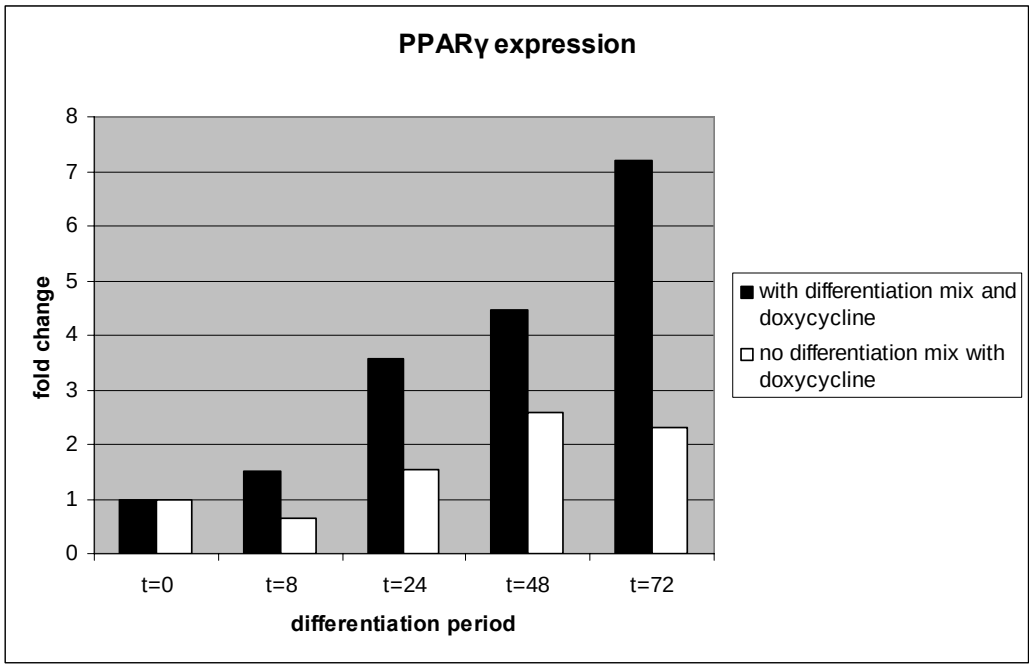

Figure 3.5 Quantification of PPARγ mRNA level by real time RT-PCR. The real time PCR products were normalized with an endogenous control, TBP. Expression of PPARγ in induced hMSC-Tert20-*HMGA2* clone t4 cells treated with differentiation mix (black) and without (white) for the time periods shown in Hours.

Additional samples were included in a second experiment to validate the results and see what effect of the doxycycline induction had, by including control samples without doxycycline. Cells that were induced with doxycycline and differentiated were included. Moreover, as a negative control cells without any treatments were also included in the gene expression assay. This differentiation measurement i.e. including all four samples was done twice and the RNA level of *HMGA2* expression was analyzed through real time PCR. The expression of *HMGA2* in experiment 2 (Figure 3.6) was similar to experiment 1, in that the level of *HMGA2* mRNA increased during induction of differentiation, but only when the transgene was induced. The result shows that overexpression of the transgene leads to further induction of *HMGA2* during adipogenic differentiation. It can also be seen that there is a variation in the expression of the endogenous *HMGA2* during the adipogenic differentiation induction and even in the control samples, probably due to technical errors.

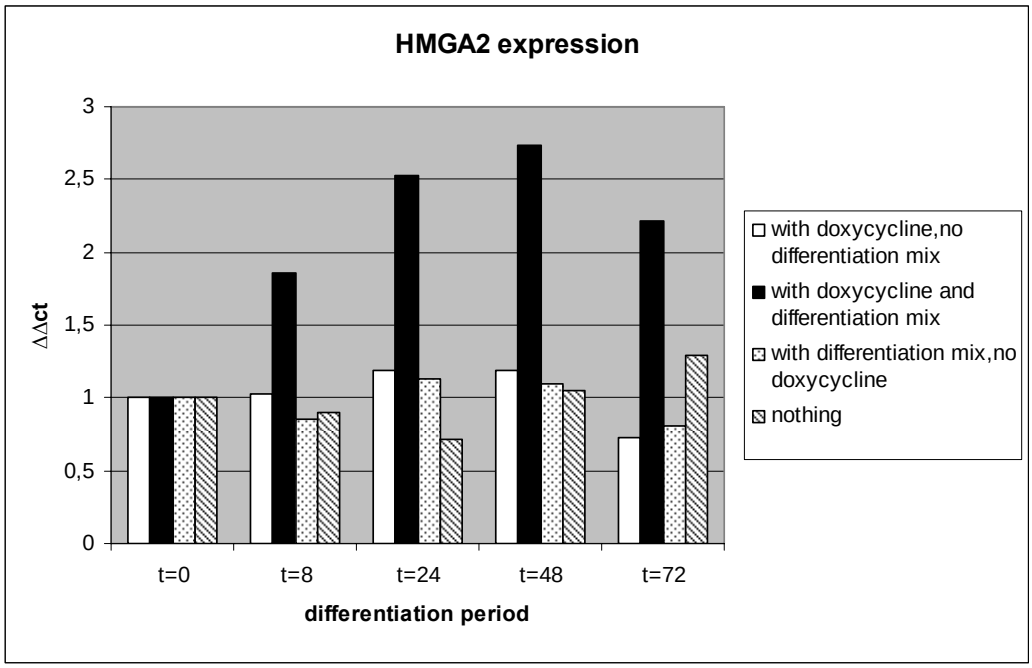

Figure 3.6 Analysis of *HMGA2* expressions in adipogenic differentiation. The real time PCR products were normalized with an endogenous control, TBP. Cells were treated as indicated in right panel on the Figure. White: real time PCR product from cells treated with doxycycline but not with differentiation mix. Black: cells with both doxycycline and differentiation mix. Dot: cells treated with differentiation mix but not with doxycycline. Grid: cells without any treatment. Cells were treated with doxycycline 12 hours prior to differentiation. NB. All are compared with their own t=0.

The induced level of *HMGA2* expression is more than twice higher than the endogenous level (without doxycycline treatment) during adipogenesis. However there is a slight increase in expression of *HMGA2* in cells which are not induced in third and fourth lanes at t=24, 48, 72 hours of differentiation periods.

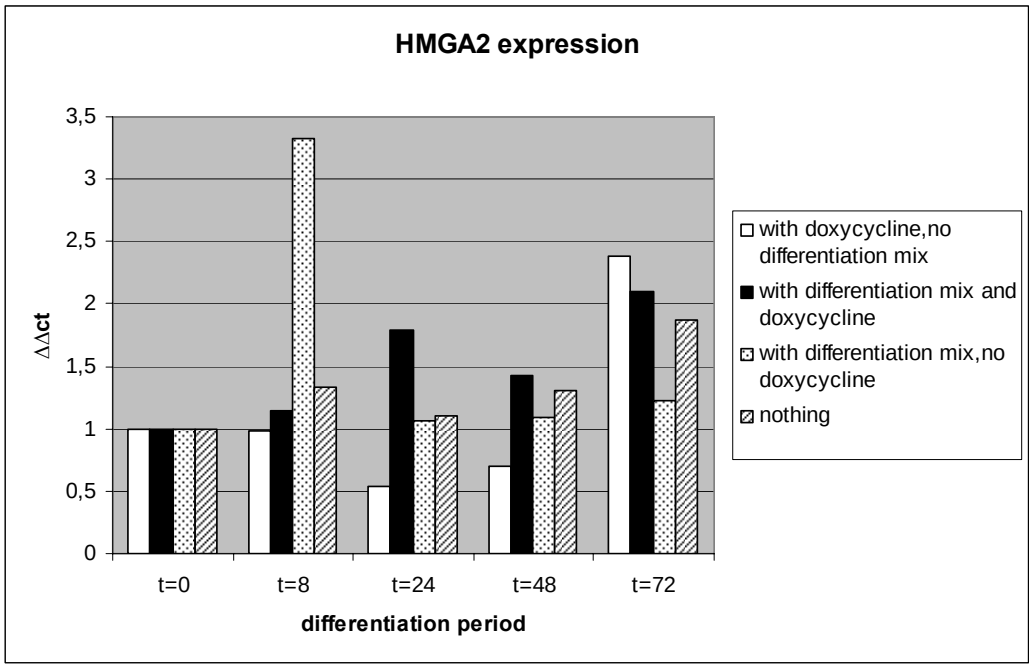

Figure 3.7 Analysis of *HMGA2* expression during adipogenesis. The real time PCR products were normalized with an endogenous control, TBP. White and Black show the level of overexpressed *HMGA2* with or without the differentiation mix; Dot and Grid represent the endogenous expression of *HMGA2* with and without the differentiation mix consecutively.

Figure 3.7 shows a third measurement done for the investigation of *HMGA2* expression during adipogenesis. Although there is considerable variation in the results, it seems that *HMGA2* is induced by transgene induction during differentiation. But the level of *HMGA2* expression during adipocytic differentiation is higher also without induction of the transgene. There is also overexpression of *HMGA2* in doxycycline induced cells but without adipogenic differentiation mix.

PPARγ expression was included for the investigation of how overexpression of *HMGA2* affects adipogenic differentiation. An increased level of PPARγ was observed in the cells that undergo adipogenic differentiation in a similar way as in experiment 1, and the increase was much higher when the transgene was induced. There was however considerable variation in the measurements in general. From the combination of the two results it can be said that *HMGA2* overexpression led to an increase in the induction of PPARγ expression during the early phase of adipogenic PPARγ differentiation.

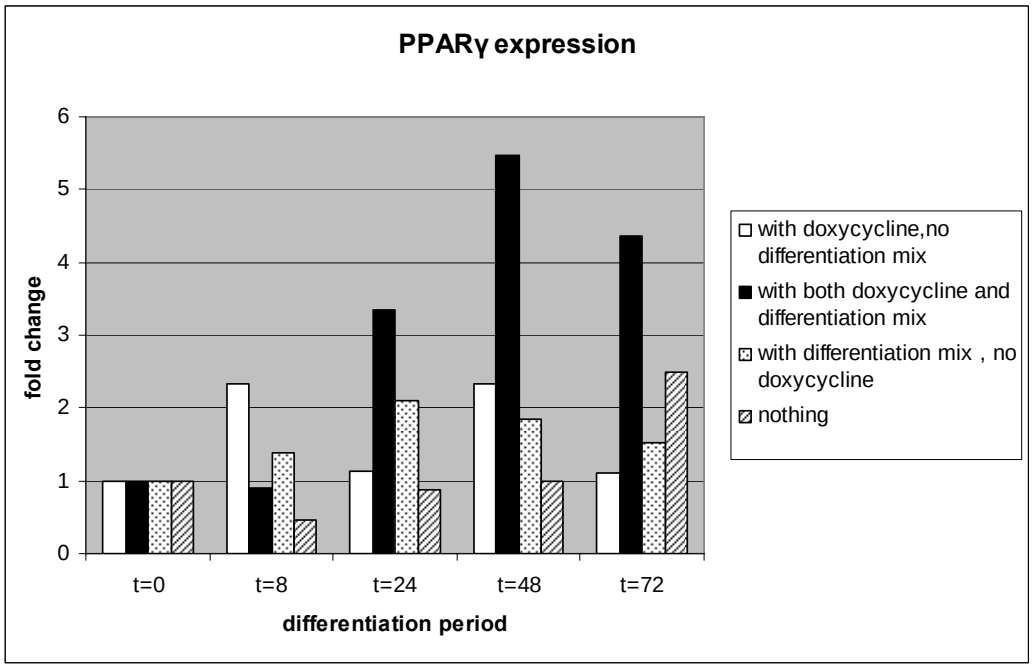

Figure 3.8 PPARγ expressions during adipogenic differentiation with and without induction of the *HMGA2* transgene. The real time PCR products were normalized with an endogenous control, TBP.

Pictures were taken from the control samples after 21 days of differentiation period. From the result it is possible to see the adipogenic differentiation of the samples just by looking at the red stained fat droplets. Although the levels of Oil red O should be quantitated for more precise conclusions, it can be concluded that adipogenic differentiation happened in all samples, both with and without induction either of the transgene or with the adipogenic mix.

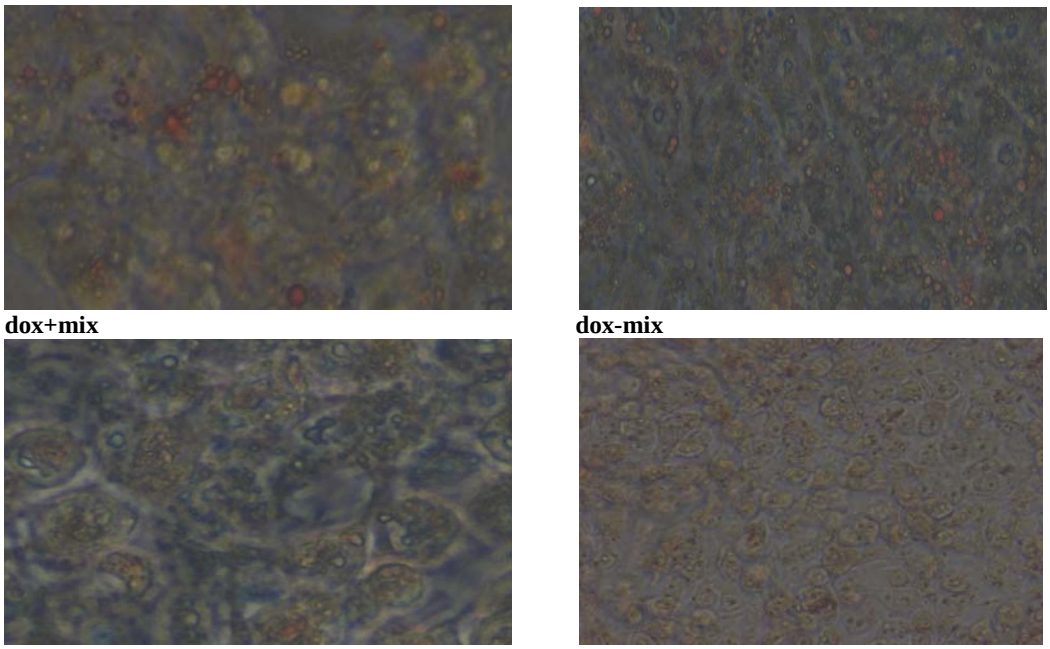

Figure 3.9 Adipogenic differentiation of hMSC-Tert20-HMGA2 clone t4 cells with Oil Red O stain. (A) Cells treated with differentiation mix and doxycycline. (B) Cells treated with doxycycline but not with differentiation mix. (C) Cells without treatment with both doxycycline and differentiation mix. (D) Cells treated with the differentiation mix.

3.4 Investigation of putative interactions between HMGA2 and NF-κB

Previously done experiments in our group (Henriksen et al. in prep) showed that HMGA2 interferes with NF-$_k$B pathway components. We wanted to investigate if protein-protein interactions could be detected that could explain this effect. HMGA2 was immunoprecipitated under non-denaturing conditions from cell lines that contain the transgene HMGA2 fused with EGFP and also from cells that express only the endogenous HMGA2. Because of this, we could expect to see two HMGA2 protein bands on the gel, one endogenous at 17kDa, and one fusion protein, at 50kDa. Similarly NF-$_k$B was immunoprecipitated as described in Section 2.1. Possible coprecipitation of the two proteins was investigated by detection of HMGA2 by western blot analysis. We could not show the interaction between HMGA2 and NF-$_k$B as can be seen from Figure 3.9.

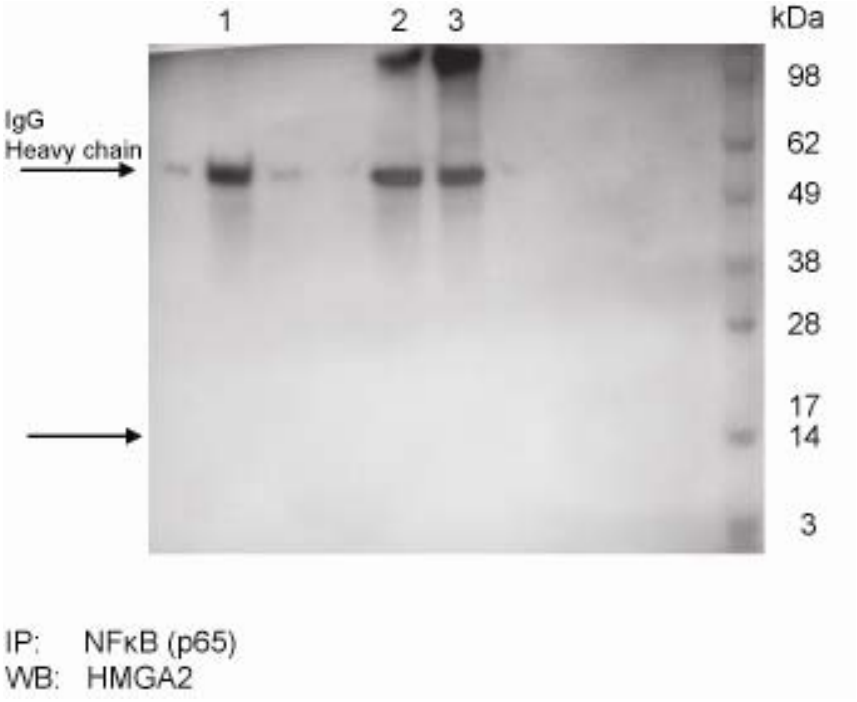

Figure 3.10 hMSC-Tert20-HMGA2-EGFP cell lysate was incubated with anti NF-$_k$B subunit p65 and immunoprecipitated with protein A sepharose™ beads. Possible interaction with HMGA2 was analysed by western blotting with goat polyclonal antibody against HMGA2. Lane 1, NF-$_k$B sample Lane 2, sample containing the fusion protein. Lane 3, sample containing endogenous HMGA2 only. Expected bands on lane 2 were at about 17kDa and 50kDa since the sample has both endogenous and exogenous HMGA2. Top arrow shows IgG heavy chain, and the lower arrow shows the expected position of endogenous HMGA2. Note that bands at 55kDa in all the lanes are IgG heavy chain. Size was compared by using the apparent molecular weights of the proteins in NuPage® MES buffer system.

As can be shown from the figure the endogenous HMGA2 could not be detected.

4. Discussion

4.1 Choice of cell line

We wanted to investigate how the HMGA2 protein influences the differentiation of mesenchymal cells towards adipocytes, and to generate a model system where we can investigate which genes and proteins are involved. In a previous study by our group, it was shown that stable transfectants expressing *HMGA2* under constitutive promoter were unable to efficiently differentiate to adipocytes. In the current study, using inducible expression, it should be possible to analyze this more precisely, as one could study the endogenous and induced expression in the same cell line. Furthermore, the intention was to do the study in a more normal cell line generated in our lab, the iMSC#3b cells. These have a normal karyotype and do not induce tumours in immunodeficient mice, as opposed to the hMSC-Tert20 cells. However, using a number of transfection techniques, we were unable to efficiently transfect these cells and due to time-constraints we chose to study a set of hMSC-Tert20 lines. These cells were used for subsequent analysis.

4.2 Overexpression of HMGA2 during adipogenesis

Preceding experiments performed in the group indicate that induction of these hMSC-Tert20-HMGA2 clone t4 cells results in double fold expression of *HMGA2* that is at a higher level when compared to the other hMSC-Tert20-HMGA2 clone t2 cells. Further expression analysis was done on t4 clones. These cells were induced with doxycycline 12 hours prior to addition of the adipogenic differentiation mix so that the transgene *HMGA2* was overexpressed during adipogenic differentiation. The process of adipogenic differentiation of cells requires a cascade of different gene expressions (Fajas et al., 1998). The important role PPARγ plays in this scenario has been noted. It has been referred as the marker gene for adipogenic differentiation though there are other transcription factors triggering its expression. Despite the transcriptional control of adipocyte - cell differentiation by many factors, PPARγ is thought to be sufficient and necessary for adipogenic differentiation (Tontonoz & Spiegelman, 2008). Initially, PPARγ expression was validated by real time RT-PCR, and indeed it is expressed during adipogenic differentiation. Then additional samples were included to the experiment in order to investigate whether the result is due to endogenous or exogenous expression of *HMGA2*. Overexpression of the transgene *HMGA2* did not block adipogenic differentiation in these experiments, as was observed previously in other hMSC-Tert20 clones.

The result shows that the gene is highly expressed which means that the overexpression of *HMGA2* did not inhibit but rather incease PPARγ expression and in turn would not be expected to have a negative impact on adipogenic differentiation. This may be explained by how HMGA2 influences the functioning of other transcription factors. The influence is either by binding to a transcription factor then favoring/inhibiting the expression of the downstream genes or by binding to the AT-rich sequences on the DNA likewise inhibiting or activating genes downstream in response to the conformational change of DNA. Without overexpressing the transgene *HMGA2*, PPARγ expression is less, which actually means that HMGA2 stimulates PPARγ expression. It is known that if cells do not undergo adipogenic differentiation,

then it means that directly or indirectly some of the genes regulating the transcriptional apparatus for adipogenesis are not expressed. In our result though there is slight increase in the expression of PPARγ in non-induced cells for adipogenesis, which could be explained by the spontaneous differentiation (see Figure 3.9).

My results show that HMGA2 mRNA was expressed at a more or less constant level in the control cells (with doxycycline, no differentiation mix). There was however considerable variation of the mRNA levels measured also in the controls, probably due to technical inaccuracy and the real time analysis had to be repeated several times. By looking at the cell cultures stained with the lipid-soluble dye oil Red O (Figure 3.9), no clear difference in level of differentiation could be seen, so the differentiation process may not have been completely successful. This could be caused by the cells being grown at the wrong density and other technical problems, although a strict protocol was followed. There is always some level of differentiation of these cells even without induction treatment. Since there is variability in differentiation within the hMSC-Tert20 cultures, it might be possible that the clone t4 cells don't differentiate efficiently.

When the cells were treated with the medium inducing adipogenesis, *HMGA2* appeared to be overexpressed further in two experiments. The real time assay does not distinguish between endogeneous and transgene expression, but if done again, assays could be used that could detect the endogeneous and exogeneous transcripts separately because the 3'UTRs are different. This would help in understanding how I got these results. Apparent induction of *HMGA2* during differentiation only in the presence of the exogenous protein was a surprising finding. Several conditions could contribute to explain these results.

The differentiation mix could affect the CMV promoter directly, or affect the doxycycline treatment e.g. stimulating the induction by affecting transport of doxycycline into the cell. The level of *HMGA2* expression is regulated by the let-7 microRNA (Lee & Dutta, 2007), and one could also think that the transgene *HMGA2* could repress the level of let-7 in the cells, and so lead to increased level of the endogeneous transcript. The transgene lacks the 3' UTR, containing the target sites for let-7, and is thus not sensitive to this effect. The doxycycline treatment itself, could affect the level of TBP, used as endogenous control. Expression analysis of a gene through real time RT-PCR depends on the stability of the reference gene to which it was normalized to, in our case TBP. Although TBP is regarded as a house-keeping gene, and supposed to have a more or less constant expression level, one could check this by using another house-keeping gene as control. The endogenous control should not vary with regard to different cell types. TBP was shown to be one of the least stable house keeping genes investigated for their stability (Ishii *et al.*, 2006). A change in the control gene could also explain the similar increase in PPARγ expression.

In addition to the pipetting inaccuracy, high quality RNA is critical in undergoing real time assays. The quality refers to the purity and stability of the RNA yield which depend on important steps involved in the RNA isolation.

4.3 Investigation of interaction between HMGA2 and NF-κB in hMSC-Tert20

Nuclear factor-$_k$B (NF-$_k$B) is a nuclear transcription factor which controls the expression of oncogenes, cytokines, growth factors and they have a role in development of many tissues. It regulates the expression of many components of the immune system (Li & Verma, 2002). The NF-$_k$B family is composed of, p50/p105, p52/p100, p65, c-Rel, and RelB (Ghosh, 1999). Each have Rel domain in the N terminal that are involved in DNA binding and dimerization. They are inactive unless stimulated by extracelllar inducers. The pathway in the translocation of NF-$_k$B dimers into the nucleus requires the phosphorylation and subsequent ubiquitination of the I$_k$B proteins which are found bound to NF-kB in the cytoplasm. Activation and regulation of NF-$_k$B are tightly controlled by I$_k$B proteins and are translocated to the nucleus where it regulates gene transcription (Verma et al., 1995). Once NF-$_k$B reaches DNA, transcriptional control commences through either by protein-protein or protein-DNA interaction. One example that could be mentioned here is its interaction with another nuclear transcription factor family, HMGA. From the literature HMGA proteins facilitate the recruitment of other transcription factors like NF-$_k$B in the assembly of enhanseosomes in INF-ß promoter through protein-DNA interaction. HMGA2 is also able to enhance the transcriptional activity of NF-$_k$B by binding to AT rich DNA repeats adjacent to NF-$_k$B consensus site (Cleynen et al., 2007). HMGA2 is also shown to interact with a subunit of NF-$_k$B, p50(Noro *et al.*, 2003).

In this study of HMGA2 - NF-$_k$B interaction, we were not able to see interaction between theses proteins which is not in agreement with the literature that used the same method employed here.

Activation or repression of a transcription factor is not only and directly determined by protein-protein interaction rather it could also be by binding to specific site in DNA. HMGA2 is able to enhance the transcriptional activity of NF-$_k$B by binding to AT rich DNA repeats adjacent to NF-$_k$B consensus site (Cleynen et al., 2007). Although we do not know the state of these promoters in our cells, we had expected to find an association. Since a positive control was not included that could infer that indeed the immunoprecipitation of NF-$_k$B was successful, it is impossible to conclude that there is no interaction. The antibody used might have low affinity for the protein during the immunoprecipitating process. Antibody or bead incubation period is critical step in assuring the binding of the beads or the protein to the protein. Moreover we did this assay by using p65 antibody but since NF-$_k$B has multiple subunits, we should have tried other antibodies. In our case the incubation of nprotein A sepharose^{TM} beads was done only for 4 hours with the NF-$_k$B subunit p65 antibody protein complexes. Then analysis for the protein-protein with HMGA2 was done. So probably the incubation period should have been increased for several hours and checked. Also precise conditions, clean equipments, and freshly made buffers usage is critical in assessing protein- protein interaction assay, as the complexes are unstable.

5. Conclusion

From the results it can be concluded more or less that forced expression of the transgene *HMGA2* to further induction of *HMGA2* mRNA during adipogenic differentiation and its overexpression is associated with an increase in PPARγ expression. However, we couldn't conclude on whether this had an effect on adipogenic differentiation as detected by lipid accumulation. No interaction between HMGA2 and NF-$_k$B could be detected. New experiments could be done to validate those results by including new expression assays that can help distinguish the endogenous and exogenous expression of HMGA2 and whether changes in the level of the house-keeping gene used for normalization could have led to apparent gene expression changes. One expression assay that could detect only the endogenous HMGA2 and another that detects the transgene and endogenous mRNA can be used to validate such experiment. Moreover it is important to do the gene expression assay with other house-keeping genes.

References

Anand A and Chada K. (2000).In vivo modulation of Hmgic reduces obesity *Nat Genet*, **24,** 377-380.

Ayoubi TA, Jansen E, Meulemans SM and Van de Ven WJ. (1999).Regulation of HMGIC expression: an architectural transcription factor involved in growth control and development *Oncogene*, **18,** 5076-5087.

Berner JM, Meza-Zepeda LA, Kools PF, Forus A, Schoenmakers EF, Van de Ven WJ, et al. (1997).HMGIC, the gene for an architectural transcription factor, is amplified and rearranged in a subset of human sarcomas *Oncogene*, **14,** 2935-2941.

Brickman JM and Burdon TG. (2002).Pluripotency and tumorigenicity *Nat Genet*, **32,** 557-558.

Clark BR and Keating A. (1995).Biology of bone marrow stroma *Ann N Y Acad Sci*, **770,** 70-78.

Cleynen I, Brants JR, Peeters K, Deckers R, Debiec-Rychter M, Sciot R, et al. (2007).HMGA2 regulates transcription of the Imp2 gene via an intronic regulatory element in cooperation with nuclear factor-kappaB *Mol Cancer Res*, **5,** 363-372.

Cleynen I and Van de Ven WJ. (2008).The HMGA proteins: a myriad of functions (Review) *Int J Oncol*, **32,** 289-305.

Fajas L, Fruchart JC and Auwerx J. (1998).Transcriptional control of adipogenesis *Curr Opin Cell Biol*, **10,** 165-173.

Friedmann M, Holth LT, Zoghbi HY and Reeves R. (1993).Organization, inducible-expression and chromosome localization of the human HMG-I(Y) nonhistone protein gene *Nucleic Acids Res*, **21,** 4259-4267.

Fusco A and Fedele M. (2007).Roles of HMGA proteins in cancer *Nat Rev Cancer*, **7,** 899-910.

Geurts JM, Schoenmakers EF and Van de Ven WJ. (1997).Molecular characterization of a complex chromosomal rearrangement in a pleomorphic salivary gland adenoma involving the 3'-UTR of HMGIC *Cancer Genet Cytogenet*, **95,** 198-205.

Ghosh S. (1999).Regulation of inducible gene expression by the transcription factor NF-kappaB *Immunol Res*, **19,** 183-189.

Gregoire FM, Smas CM and Sul HS. (1998).Understanding adipocyte differentiation *Physiol Rev*, **78,** 783-809.

Greider CW and Blackburn EH. (1987).The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity *Cell*, **51,** 887-898.

Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, et al. (1999).Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta *Nat Med*, **5,** 309-313.

Ishii T, Wallace AM, Zhang X, Gosselink J, Abboud RT, English JC, et al. (2006).Stability of housekeeping genes in alveolar macrophages from COPD patients *Eur Respir J*, **27,** 300-306.

Kliewer SA, Umesono K, Mangelsdorf DJ and Evans RM. (1992).Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling *Nature*, **355,** 446-449.

Lee YS and Dutta A. (2007).The tumor suppressor microRNA let-7 represses the HMGA2 oncogene *Genes Dev*, **21,** 1025-1030.

Li Q and Verma IM. (2002).NF-kappaB regulation in the immune system *Nat Rev Immunol*, **2,** 725-734.

Majumdar MK, Thiede MA, Mosca JD, Moorman M and Gerson SL. (1998).Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells *J Cell Physiol*, **176,** 57-66.

Mantovani F, Covaceuszach S, Rustighi A, Sgarra R, Heath C, Goodwin GH, et al. (1998).NF-kappaB mediated transcriptional activation is enhanced by the architectural factor HMGI-C *Nucleic Acids Res*, **26,** 1433-1439.

Michalik L and Wahli W. (1999).Peroxisome proliferator-activated receptors: three isotypes for a multitude of functions *Curr Opin Biotechnol*, **10,** 564-570.

Mishra PJ, Mishra PJ, Glod JW and Banerjee D. (2009).Mesenchymal stem cells: flip side of the coin *Cancer Res*, **69,** 1255-1258.

Narita M, Narita M, Krizhanovsky V, Nunez S, Chicas A, Hearn SA, et al. (2006).A novel role for high-mobility group a proteins in cellular senescence and heterochromatin formation *Cell*, **126,** 503-514.

Nicolson GL. (1991).Molecular mechanisms of cancer metastasis: tumor and host properties and the role of oncogenes and suppressor genes *Curr Opin Oncol*, **3,** 75-92.

Noro B, Licheri B, Sgarra R, Rustighi A, Tessari MA, Chau KY, et al. (2003).Molecular dissection of the architectural transcription factor HMGA2 *Biochemistry*, **42,** 4569-4577.

Ntambi JM and Young-Cheul K. (2000).Adipocyte differentiation and gene expression *J Nutr*, **130,** 3122S-3126S.

Owen GI and Zelent A. (2000).Origins and evolutionary diversification of the nuclear receptor superfamily *Cell Mol Life Sci*, **57,** 809-827.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. (1999).Multilineage potential of adult human mesenchymal stem cells *Science*, **284,** 143-147.

Poremba C, Scheel C, Hero B, Christiansen H, Schaefer KL, Nakayama J, et al. (2000).Telomerase activity and telomerase subunits gene expression patterns in neuroblastoma: a molecular and immunohistochemical study establishing prognostic tools for fresh-frozen and paraffin-embedded tissues *J Clin Oncol*, **18,** 2582-2592.

Riggi N, Cironi L, Suva ML and Stamenkovic I. (2007).Sarcomas: genetics, signalling, and cellular origins. Part 1: The fellowship of TET *J Pathol*, **213,** 4-20.

Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, Milstone DS, et al. (1999).PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro *Mol Cell*, **4,** 611-617.

Schoenmakers EF, Wanschura S, Mols R, Bullerdiek J, Van den Berghe H and Van de Ven WJ. (1995).Recurrent rearrangements in the high mobility group protein gene, HMGI-C, in benign mesenchymal tumours *Nat Genet*, **10,** 436-444.

Serafini M and Verfaillie CM. (2006).Pluripotency in adult stem cells: state of the art *Semin Reprod Med*, **24,** 379-388.

Sgarra R, Rustighi A, Tessari MA, Di Bernardo J, Altamura S, Fusco A, et al. (2004).Nuclear phosphoproteins HMGA and their relationship with chromatin structure and cancer *FEBS Lett*, **574,** 1-8.

Shao D and Lazar MA. (1997).Peroxisome proliferator activated receptor gamma, CCAAT/enhancer-binding protein alpha, and cell cycle status regulate the commitment to adipocyte differentiation *J Biol Chem*, **272,** 21473-21478.

Simonsen JL, Rosada C, Serakinci N, Justesen J, Stenderup K, Rattan SI, et al. (2002).Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells *Nat Biotechnol*, **20,** 592-596.

Skubitz KM and D'Adamo DR. (2007).Sarcoma *Mayo Clin Proc*, **82,** 1409-1432.

Smas CM and Sul HS. (1995).Control of adipocyte differentiation *Biochem J*, **309 (Pt 3),** 697-710.

Thanos D and Maniatis T. (1992).The high mobility group protein HMG I(Y) is required for NF-kappa B-dependent virus induction of the human IFN-beta gene *Cell*, **71,** 777-789.

Tontonoz P, Hu E, Graves RA, Budavari AI and Spiegelman BM. (1994).mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer *Genes Dev*, **8,** 1224-1234.

Tontonoz P and Spiegelman BM. (2008).Fat and beyond: the diverse biology of PPARgamma *Annu Rev Biochem*, **77,** 289-312.

Vaziri H and Benchimol S. (1999).Alternative pathways for the extension of cellular life span: inactivation of p53/pRb and expression of telomerase *Oncogene*, **18,** 7676-7680.

Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D and Miyamoto S. (1995).Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation *Genes Dev*, **9,** 2723-2735.

Wisniewski JR and Schwanbeck R. (2000).High mobility group I/Y: multifunctional chromosomal proteins causally involved in tumor progression and malignant transformation (review) *Int J Mol Med*, **6,** 409-419.

Zhou X, Benson KF, Ashar HR and Chada K. (1995).Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C *Nature*, **376,** 771-774.