UNIVERSITY OF SOUTHAMPTON
FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES
SCHOOL OF MEDICINE

The Effects of Epigenetic Modifiers on Osteogenic and Chondrogenic Differentiation of Skeletal Stem Cells

By

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ABSTRACT

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THE EFFECTS OF EPIGENETIC MODIFIERS ON OSTEOGENIC AND CHONDROGENIC DIFFERENTIATION OF SKELETAL STEM CELLS

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Tissue engineering provides hope for many intractable diseases as a treatment option and the area is currently the subject of intense investigation. Human bone marrow stromal cells (BMSCs) and human fetal femur derived cells (FC) possess the ability to differentiate into a variety of cell types of the stromal lineage including cells of the osteogenic and chondrogenic lineages. However, the process of in vitro differentiation is usually inefficient, difficult to reproduce in many cases and to date, not able to provide homogeneous bone stem cells, which is critical for tissue engineering. Epigenetic regulation of gene expression is recognized as a key mechanism governing cell determination, commitment, and differentiation as well as maintenance of those states. The main components of epigenetic control are DNA methylation and histone acetylation. During development, the epigenetic status changes as cells differentiate along specific lineages. We reasoned that epigenetic modifiers might enhance the differentiation process of BMSCs and FC towards either osteogenic or chondrogenic lineage. Cells were serum-starved for 24 hours to synchronise the cell cycle, then treated with either DNA demethylating agent 5-Aza-deoxycytidine (5-Aza-dC), or the histone deacetylase inhibitor Trichostatin A (TSA) or a combination of both. 5-Aza-dC enhanced alkaline phosphatase expression in BMSCs for 25 days in comparison to non-treated cells. Molecular characterization showed enhanced osteogenic differentiation pathways, including TGF-β and BMP pathways in addition to a number of downstream genes. Culturing 5-Aza-dC pre-treated BMSCs in pellet culture showed enhanced osteogenic matrix formation, while TSA pre-treated BMSCs and FC had enhanced chondrogenic architecture and matrix formation. Non-treated FC showed purer osteogenic matrix characterization, which was arranged in a ring conformation, surrounding a chondrogenic core, which mimics the cross section of fetal bone. In addition, FC showed greater tendency to adhere to plastic inserts, moving over them with enhancement of osteogenic matrix formation. When FC were seeded in glass tubes, they formed a monolayer that folded on itself to form a three-dimensional construct that moved over the tube base and migrated over the sidewall to the media-air interface. These constructs were larger in comparison to standard pellets and had intense osteoid formation. BMSCs cultured under the same conditions displayed enhanced matrix formation without treatment with 5-Aza-dC. Thus, TSA has been shown to enhance chondrogenic formation, while culturing the cells on a glass surface was very efficient in creating bone-like constructs. Epigenetic and physical factors should be considered in future programs for tissue engineering.

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DECLARATION OF AUTHORSHIP

I, **Ahmed Taher El-Serafi** declare that the thesis entitled **The Effects of Epigenetic Modifiers on Osteogenic and Chondrogenic Differentiation of Skeletal Stem Cells** and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
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- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
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- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- parts of this work have been published as:
    - Enhanced osteoid formation in bone marrow stromal cells and fetal derived cells in self-organizing three dimensional constructs. Poster presentation in the United Kingdom National Stem Cell Network Second Annual Science Meeting, University of Oxford, 2009.
    - 3D Tissue Engineering Models for Osteogenesis. Poster presentation in the Tissue engineering: a new dimension to animal replacement symposium, London, 2009.

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- Role of epigenetic modifiers in bone marrow stromal cell differentiation. Oral presentation in the postgraduate annual conference of the Medicine, Health and Life Sciences, University of Southampton, 2008.
- Role of epigenetic modifiers in bone marrow derived stromal cells differentiation. Poster presentation in the 3rd UK stem cell meeting: Epigenetics and Differentiation, 2008.
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Signed: ……..

Date:……..

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LIST OF ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
5-Aza-dC	5 Aza deoxycytidine
A/S	Alcian blue/ Sirius red
Acetyl-CoA	Acetyl co-enzyme A
AEC	3 amino-9-ethyl-carbazole
ALP	Alkaline Phosphatase
α -MEM	minimum essential media Eagle's alpha modifications
ANOVA	Analysis of variance
ASCs	Adult stem cells
BMSC	Bone marrow stromal cells
BMP	Bone morphogenic protein
bp	base pairs
BSA	Bovine serum albumin
bsp	Bone sialoprotein
c-myc	cellular myelocytomatosis oncogenes
Cbfa1	core binding factor alpha1
cDNA	Complementary DNA
CFU-F	Colony-forming unit-fibroblast
Collagen I	Collagen type 1
CpG	Cytosine phosphate Guanidine
D-MEM	Dulbecoo modified Eagle's medium
DC	Diffusion chambers
dH_2O	Distlled water
Dlx5	Distal-less homeobox 5
DNMT	DNA methyltransferases
DPX	Dibutyl phthalate xylene
dsRNA	Double stranded RNA
EGF	Epidermal growth factor
ESCs	Embryonic stem cells
FC	Fetal cells
FCS	Fetal calf serum
FGF	Fibroblast growth factor
gDNA	Genomic DNA
GDF	Growth differentiation factor
H	Histone
HDACs	Histone Deacetylases
HDI	Histone Deacetylase inhibitors
IGF	Insulin-like growth factor
ITS	Insulin, Transferrin & Selenium
K	Lysine
Li Cl	Lithium Chloride
LB	Luria-Bertani broth
MeCP-1	methyl-CpG-binding protein-1
MeCP-2	methyl-CpG-binding protein-2
miRNA	Micro RNA
MSCs	Mesenchymal stem cells
MZT	Monozygotic twins
NSCs	neuronal stem cells
^oC	degrees Celsius
OSF-2	Osteoblast-specific transcription factor 2
p/s	penicillin (100 U/ml) and streptomycin (100 U/ml)
P1	Passage 1
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PDGF	Platelet derived growth factor
PHEX	Phospho-regulating gene with homology to endopeptidases in the X chromosome
PPAR γ	Peroxysomes Proliferator Activated Receptors gamma
PPC	Positive PCR control
qPCR	Quantitative Polymerase Chain Reaction
RNAi	Interference RNA
rt	Reverse transcription
RTC	Reverse transcription control
rt-PCR	Conventional PCR
Runx2	Runt-related transcription factor 2
Ser	Serine
siRNA	Small interference RNA
SSA	Spiruchostatin A
SOC	Super Optimal broth with Catabolite repression
Sox-9	Sex determining region Y-box 9
SP-1	Specificity protein-1
TGF-β	Transforming growth factor beta
TGF-βR	Transforming growth factor beta receptor
TSA	Trichostatin A
UPW	Ultra pure water

Introduction

1. Introduction

1.1. Background

Regenerative medicine provides the hope of a permanent cure for many intractable diseases. Restoring the 'faulty' tissue or organ with a new, compatible and fully functional replacement would maintain self-dependency and productivity. Many scientists look at tissue engineering, as a branch of regenerative medicine, to be the next era medicine (Luyten et al., 2001). The basic principle is to isolate 'stem cells', which have the ability to differentiate to other specialized cell types, either from the patient or from equivalent fetal derived cells. After modification in the lab using specific culture conditions in order to differentiate into the desired cell type, cells are re-implanted (usually with an appropriate scaffold) into the patient to replace a diseased, failed or surgically removed tissue. Unfortunately, the process of *in vitro* differentiation of the stem cells is currently inefficient and difficult to reproduce in many cases. To date, it is not possible to produce homogeneous cell populations (Alexanian, 2007). In addition, to transfer stem cells to patients, they should be entirely differentiated. Undifferentiated stem cells may increase the risk for developing an undesired cell type including malignant transformation (Abdallah & Kassem, 2008).

Bone regeneration is needed in many instances to repair for bone defects induced by surgical removal of tumours or bone loss due to infection, trauma or congenital causes (Mankani et al., 2001; Oreffo et al., 2005). Bone grafts are used routinely in spinal fusion, corrective osteotomy and maxillo-facial reconstruction (Stiehler et al., 2008). Articular cartilage defects may be caused by trauma or congenital defects. Also ear auricle and laryngeal cartilage defects are quite common after operations such as laryngotomy and are common causes of post-operative pain. Unfortunately, cartilage has a very limited capacity for healing and considerable morbidity would follow (Meissner et al., 2008).

The current policy for the repair of bone defects is to fill the bone gap with artificial substitutes, which are clearly not physiological and whose long-

term effects are unknown (Tuusa et al., 2007). One treatment option for cartilage defects is autologous chondrocyte implantation. Cartilage biopsy is obtained arthroscopically from a minor weight bearing area either in the diseased joint or another location. The chondrocytes are extracted from the cartilage sample, expanded in culture and then implanted in the site of the primary defect. A number of problems are encountered with this technique. The biopsy method creates a new defect and may predispose to osteoarthritis in that joint (Brittberg et al., 1994). In addition, cell expansion is limited by cell senescence (Dominice et al., 1986) and progressive de-differentiation in culture (von der et al., 1977). The outcome of this technique ranges from hypercellular cartilage-like tissue that lack the chondrogenic columnar cellular organization to disorganized fibro-cartilage, which consequently cannot serve as proper functional articular cartilage (Peterson et al., 2000; Anderer & Libera, 2002).

To date, researchers have struggled to achieve 100% conversion of the stem cells towards the desired phenotypes in spite of studying signalling molecules, genetic manipulations, biomechanics and the implantation of cells within the three dimensional (3D) scaffolds (Dawson & Oreffo, 2008). Therefore, other factors may play a role in controlling the differentiation process (Oreffo et al., 2005). With this background, epigenetic regulation of gene expression is recognized as a key mechanism governing cell stemness, determination, commitment, and differentiation as well as maintenance of those states and is considered to be the sole difference between somatic cells and their embryonic origins as the genome is identical in all individual's cells (Feinberg et al., 2002; Oreffo et al., 2005; Alexanian, 2007). Modifying the epigenetic configuration may alter the control of gene expression in stem cells as well as fetal-derived cells and thus could enhance their differentiation.

1.2. Epigenetics

1.2.1. Definition

The term ‘epigenetics’ was originally used by Waddington in 1940; and defined as 'the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being' (Fuks, 2005). This concept was not clarified until the late nineteen nineties when Wolffe and Matzke set the modern definition, which was ‘the study of heritable changes in gene expression that occur without a change in DNA sequence’ (Wolffe & Matzke, 1999), or the wider definition of Bird : ‘the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states’ (Bird, 2007). The term epigenome has emerged to describe the epigenetic modifications all over the genome. Thus the epigenome controls the genome in both normal cellular processes and abnormal events (Vaissiere et al., 2008; Szyf, 2008a).

1.2.2. Methods of epigenetic control

1.2.2.1. DNA Methylation;

a. Introduction

DNA methylation is a well conserved process that occurs in prokaryotes as well as eukaryotes (Klose & Bird, 2006). In eukaryotes, it refers to the condition where a methyl group is covalently added to the carbon number five in the cytosine nitrogenous base attached to the deoxyribonucleotide skeleton of the DNA strand (Fuks, 2005; Szyf, 2008a; Szyf, 2008b), while in prokaryotes methylation takes place in cytosine as well as adenine (Klose & Bird, 2006). Not every cytosine can be methylated. Only those adjacent to guanine are targets for the methylation inducing enzymes, the *methyltransferases*. The CpG (cytidine-guanindine bound through phosphate molecule in the DNA strand) may occur in multiple repeats which are known as CpG islands (Fuks, 2005).

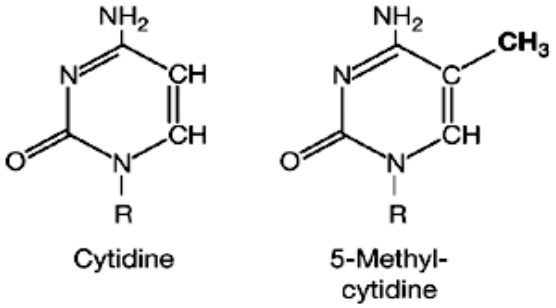


Fig.1.1. The chemical structure of the methylated and non-methylated cytidine; adapted from (Fenaux, 2005).

In 1975, DNA methylation was related to the process of X chromosome inactivation in females (Riggs, 1975). Since then, it has been used as a marker for gene silencing and extensively studied as an important mechanism of epigenetic control (Jaenisch & Bird, 2003); for example, DNA methylation is very important in the silencing of transposable elements (Walsh et al., 1998). However, over thirty years later, our understanding of DNA methylation remains largely superficial and of the methylation sites in DNA, less than 0.1% have been mapped. This has lead to criticism of the Human Genome Sequencing project in some circles for not trying to distinguish methylated cytosines from unmethylated cytosines (Schumacher et al., 2006). The Human Epigenome Project is an ongoing multicenter study that aims to map the DNA methylation all over the genome (http://www.epigenome.org/index.php).

Although the exact percentage varies in different tissues, 70-90% of available CpGs are methylated (Bird, 1986; Bird, 1999; Schumacher et al., 2006). The most important location for methylation is the gene promoters where it affects directly the gene transcription. Extensive methylation in the promoters causes repression of gene transcription (Delgado et al., 1998; Jones & Takai, 2001).

b. How does methylation occur?

DNA methyltransferases (DNMT) are a group of enzymes that catalyze the transfer of an activated methyl group from S-adenosyl methionine to the position number 5 of the cytosine ring (Schmitt et al., 1997; Bestor, 2000). Their role is to maintain the DNA methylation pattern upon cell division, in

addition to methylating the gene promoters that should be turned off during development or in response to environmental challenges (de Novo Methylation). There are currently four known DNMTs (DNMT 1, 2, 3a and 3b) (Okano et al., 1998a). DNMT1 is the maintenance methyltransferase. It has a 5- to 30- fold preference for hemi-methylated substrates (Yoder et al., 1997; Goyal et al., 2006). It exists as a component of the DNA replication complex, and thus methylates the newly synthesized DNA strand in correspondence to the template strand (Vertino et al., 2002). In addition to the epigenetic silencing of particular genes, DNMT1 supports the long-term silencing of non-coding DNA, including most of the repetitive elements (Brannan & Bartolomei, 1999; Jones & Takai, 2001; Jaenisch & Bird, 2003; Fuks, 2005). Likewise, the oocyte-specific isoform DNMT1o lacks 118 amino acids at the N terminus. It is responsible for maintaining maternal imprints during cleavage (Howell et al., 2001).

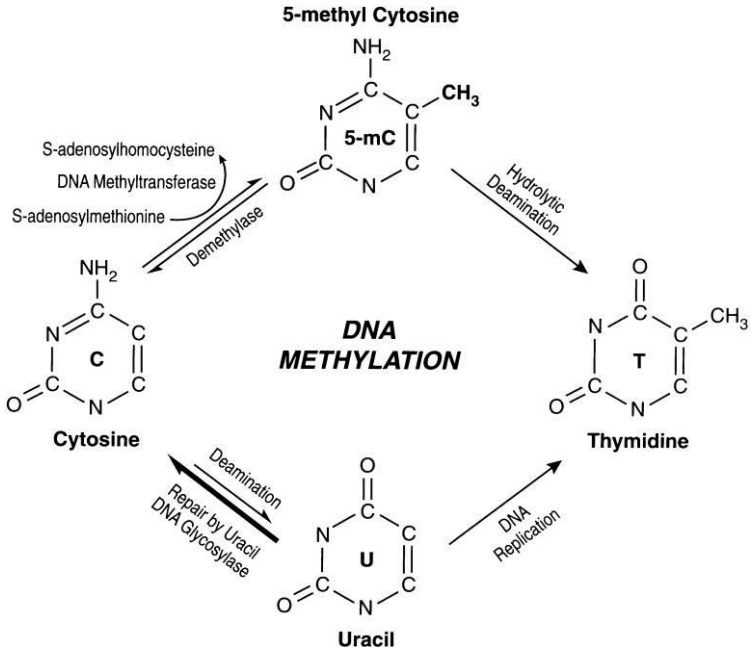


Fig.1.2. Diagrammatic representation of the possible biochemical conversions for the cytosine, including the methylation process; adapted from (Howell et al., 2001).

DNMT2, although similar in sequence and structure to the DNA methyltransferases, displays negligible evidence of transmethylase activity in biochemical or genetic tests. Deletion of the DNMT2 gene in embryonic stem cells causes no detectable effect on global DNA methylation (Okano et al.,

1998b). Alternatively, DNMT3a and DNMT3b are highly expressed in the developing embryo and are responsible for global de novo methylation after implantation (Okano et al., 1999).

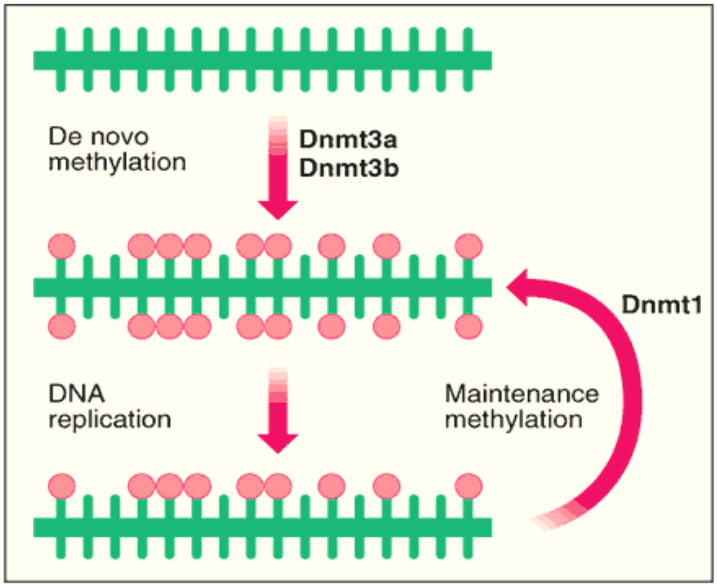


Fig.1.3. Diagrammatic representation of the action of the DNA methyltransferases on the DNA. DNMT3a&b are responsible for the de novo methylation of the non-methylated DNA. After cell division the DNMT1 'copies' the methylation pattern from the template DNA strand to the newly synthesized strand; adapted from (Bird, 1999).

A key question is how do the enzymes know where to methylate? Two theories have attempted to answer this question. The first one based on exclusion; i.e. all genes are methylated by default except the active genes (Jones & Takai, 2001). Actively transcribed DNA has a preponderance of attached transcriptional factors, giving no physical access to the methyltransferase to reach their targets. On the other hand, inactive DNA is susceptible to the methyltransferases and subsequently becomes methylated. This model was confirmed by the study of transcription factor SP1. As long as SP1 is attached to its site, no methylation could occur in the adjacent CpG sites. Removal of the SP1, leads to de novo methylation at this site (Macleod et al., 1994). However, it offers no explanation for what is happening during development as certain genes become methylated after a certain time of expression. Thus it can not be considered as the sole mechanism induced in methylation (Jones & Takai, 2001). The second theory is a methylation-

targeting mechanism steered by sequence-specific binding proteins. Thus, the methyltransferase binds with certain proteins such as histone deacetylases and other transcription repressors, and as a complex would bind to specific sequences on the DNA (Jones & Takai, 2001; Fuks et al., 2001).

C. How does demethylation occur?

Methylated genes may need to be reactivated in response to different environmental signals and thus demethylation is an important component of the DNA epigenetic control. In dividing cells, it is relatively easier to block the DNMT1 during DNA synthesis (passive demethylation). The same mechanism takes place in some instances of cell differentiation and development up to the gastrulation stage (Ramchandani et al., 1999). Conversely, the active demethylation has two possible distinct mechanisms. The first one is glycosidase dependant, which recognizes the methylated cytosine and cleaves the bond between the DNA backbone and the base. The apyrimidine deoxyribophosphate is removed and replaced with an unmethylated cytosine by the DNA repair system. At least, two enzymes have shown to have the methyl CG-DNA-glycosidase property (Zhu et al., 2000). Recently it has been shown that DNMT3a and DNMT3b - in the absence of DNMT1- recruit with the glycosylase at the CpG before demethylation took place (Kangaspeska et al., 2008). The second mechanism is a direct removal of the methyl moiety from the DNA (Ramchandani et al., 1999). Histones play an important role in the DNA demethylation. Acetylated histones would allow the enzymes to reach their substrate while deactylated (closed) histones will hinder this process. This role is especially obvious during embryonic differentiation (Weiss & Cedar, 1997; Cervoni & Szyf, 2001).

d. How does methylation affect gene transcription?

Methylation can suppress gene transcription through one of two mechanisms. The first mechanism (direct approach) is through physical interference with the binding of specific transcription factors to their recognition sites in the gene promoter area allowing improper interaction between the transcription machinery and the targeted gene (Watt & Molloy,

1988; Tate & Bird, 1993; Jones & Takai, 2001). The second mechanism (indirect approach) depends on the binding of methylated DNA to specific transcriptional repressors, which in turn suppress the gene expression. While the carboxyl terminal of DNMT-1 transfers the methyl group from the methyl carrier S-adenosylthionine, the amino terminal binds to the DNMT-1 associated protein, which has an intrinsic transcription repressor activity, in addition, it binds to the transcriptional co-repressor TSG101 (Rountree et al., 2000).

Two of the nuclear matrix proteins (methyl-CpG-binding protein [MeCP] 1 and 2) were found to be attached preferentially to the methylated cytosine and acted as gene repressors (Rountree & Selker, 1997). Within MeCP-1, there is an important component known as PCM1, which has three domains. The first is the methyl group binding domain and the other two bind to cytosine residues separated by two other bases (CXXC) (Zeleznik-Le et al., 1994; Cross et al., 1997). MeCP-2 has only two domains, one of which binds to a single methylated cytosine residue known as the methyl CpG binding domain, and the other to co-repressors such as mSin3A and histone deacetylases (Nan et al., 1997; Nan et al., 1998b). Furthermore, the presence of MeCP-2 can obstruct the binding of some transcription factors, such as SP1 into the promoter area. While MeCP-2 binds to the methylated cytosine on human leukosialin gene (which is responsible for the expression of CD 43), it binds to the SP1 at the same time inhibiting gene transcription while in the absence of methylation, SP1 binds to the promoter and initiates the transcription (Kudo, 1998; Zhu et al., 2000). DNA methylation can also inhibit RNA-polymerase elongation or impair RNA-polymerase loading (reviewed in (Vaissiere et al., 2008)).

1.2.2.2. Histone modification;

a. Introduction

Histones are five basic nuclear proteins that form the core of the nucleosome, named H2a, 2b, 3 and 4. Located outside the core, the linker histone H1 is involved in the packing of DNA (Kornberg & Lorch, 1999). 146

base pairs of DNA are wrapped around each nucleosome in two turns (Luger et al., 1997), and these nucleosomes are wrapped on each other aided by the H1 histone. With further coiling and supercoiling, the DNA length is condensed some 10,000-fold. This complicated structure would block the transcriptional machinery from approaching its target, i.e. the DNA (Grant, 2001).

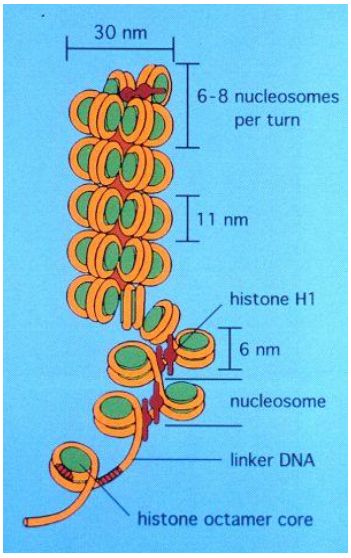


Fig.1.4. The role of histones in the packing of DNA. The nucleosomal arrangement provides a core for DNA wrapping, which is then coiled and supercoiled to compact the DNA some 10,000-fold. (http://bricker.tcnj.edu/tech/le8/nucleosome.jpg)

The N-terminal of H3 and H4 are highly charged and are tightly bound to the DNA phosphodiester backbones, specifically on the inner surface of the DNA super helix. The contact takes place once every ten base pairs where the minor groove of DNA faces inwards. Thus, the binding of DNA and histones is electrostatic in nature, rather than packing construct (Pruss et al., 1995; Kornberg & Lorch, 1999). Each core histone is composed of a structured domain and an unstructured amino-terminal 'tail' of 25-40 residues. This unstructured tail extends through the DNA gyri and into the space surrounding the nucleosomes (Kornberg & Lorch, 1999). Post-translational covalent modifications may take place in the tail region. These modifications could be in the form of methylation, acetylation, ubiquitination (Jiang et al., 2004), sumoylation (Shiio & Eisenman, 2003), phosphorylation (Jiang et al.,

2004) or ribosylation (Boulikas, 1989). Some of these modifications are consistent between species. These chemical modifications appear to determine the degree of chromatin folding as well as the interaction between histones and other proteins including the transcription machinery (Wu & Grunstein, 2000; Roth et al., 2001). Consequently, histone modifications result in considerable control on gene expression and extend the information potential of the DNA, which explains the growing interest of the 'Histone Code' (Jenuwein & Allis, 2001; Zhang & Reinberg, 2001).

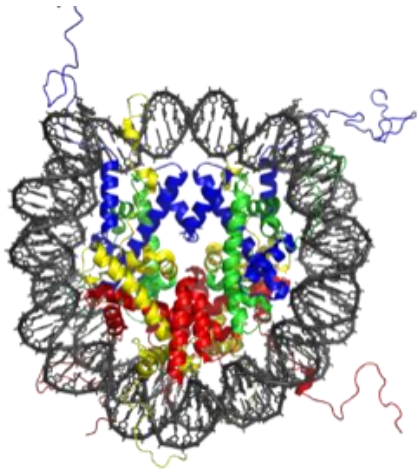


Fig.1.5. The crystal structure of nucleosome core. The histones H2A, H2B, H3 and H4 are present in the centre. The histone tails that are present at the outside are the site of different chemical modifications. (http://en.wikipedia.org/wiki/Nucleosome)

b. Histone Acetylation

Acetylation of histones has been extensively studied as one of the key regulatory mechanisms of gene expression (Grant, 2001). Histone acetylation – as well as methylation – was found to affect the RNA transcription as early as the 1960's (Allfery et al., 1964). The highly conserved lysine residue at the amino-terminal of H3 at positions 9, 14, 18 and 23, and H4 lysine 5, 8, 12 and 16, are frequently targeted for modification (Roth et al., 2001). Acetylation occurring at lysine residues neutralizes the positive charge of the histone tails, decreasing their affinity for DNA, opening the chromatin and allowing the transcriptional machinery to reach its target (Hong et al., 1993). With a similar approach, histone acetylation affects the interaction between the transcription

regulatory proteins and the DNA (Vaissiere et al., 2008). Generally, acetylation of histone is associated with gene activation (Kornberg & Lorch, 1999).

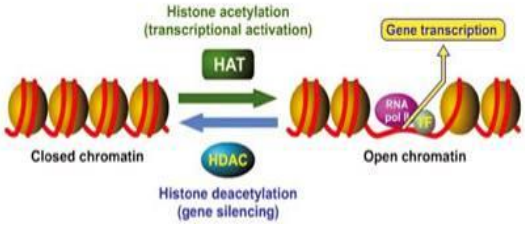


Fig.1.6. The chromatin opening up upon histone acetylation adapted from (McIntyre et al., 2007).

Many histone acetylases (Brownell & Allis, 1996; Parthun et al., 1996) and deacetylases (Taunton et al., 1996) have been described. The acetyltransferases catalyse the addition of the acetyl group from acetyl coenzyme A (acetyl-CoA) to the epsilon-amino group of specific lysine residues (it-Si-Ali et al., 1998; Kim et al., 2000), while the deacetylases reverse the reaction (Kuo & Allis, 1998). Histone deacetylases (HDACs) are eighteen enzymes in mammalian cells that are divided into two families: a) zinc metalloenzymes that catalyse the hydrolysis of acetylated specific residues on the histone tails and includes class I, II and IV HDACs and b) NAD-dependant Sir2 deacetylases which are considered as class III HDACs (Vigushin et al., 2001; Glaser, 2007).

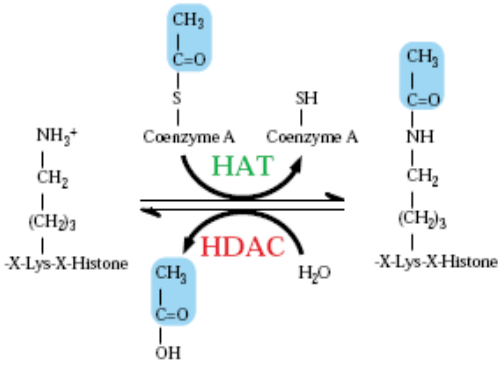


Fig.1.7. The acetylation/deactivation of histone;adapted from (Kuo & Allis, 1998).

Class I is a group of four enzymes known as HDAC1, 2, 3 and 8; this class is associated with gene expression regulation. They are expressed ubiquitously and exert their function exclusively in the nucleus (Brehm et al., 1998; Glaser, 2007). Class II is subdivided into class IIA, which includes HDAC 4, 5, 7 and 9 and class IIB that includes HDAC 6 and 10. Class II enzymes shuttle between cytoplasm and the nucleus; they use histones as well as other proteins as substrates. They are involved mainly in cell differentiation and are highly expressed in certain tissues such as heart, skeletal muscle and brain (Grozinger et al., 1999; Vigushin et al., 2001; de Ruijter et al., 2003; Glaser, 2007). Class III includes the NAD-dependant deacetylases; a group of seven enzymes that are involved in maintaining the chromatin stability. They can remove the acetyl groups from histones as well as other proteins (Kyrylenko et al., 2003). Class IV contains a single member, which is HDAC 11. It is closely related to class I thus some reviewers consider it as a member of that class. The function of HDAC 11 has not been characterized yet (de Ruijter et al., 2003; Crabb et al., 2008).

Fig.1.8. Histone acetylation effect on the wrapped DNA. The DNA (yellow) is wrapped around the histones (green). On the right end, the tails of the histones are acetylated (green dots), leading to an open chromatin. At the centre, histone deacetylase (red) is removing the acetyl groups. At the bottom, the deacetylated histone tails associate with neighbouring nucleosomes to form a compact, inaccessible chromatin; adapted from (Goodsell, 2003) .

c. Histone Methylation

Interest in histone methylation is growing due to the different effects histone methylation may induce in accordance to its position. It has been known for a long time that adding a methyl group to the histone would inhibit gene transcription by increasing the affinity of histones (basic proteins) to the

anionic DNA (Zhang & Reinberg, 2001; Rice & Allis, 2001). That is true for lysine residue K9 in H3 (H3K9) and H3K27, which methylation would mark silenced chromatin. The protein HP1, which is usually associated with condensed heterochromatin, becomes attracted to the methyl group of the H3K9. When the gene transcription is active, the methyl group at such a locus would be changed for acetyl moiety (Nakayama et al., 2001; Bannister et al., 2001; Litt et al., 2001; Li et al., 2002). In contrast, other work suggests that methylation of certain amino acids such as H3K4 and arginine 17 of histone 3 (H3R4) are associated with transcription activation (Li et al., 2002).

Furthermore, the modified lysine can be mono-, di- or tri- methylated with different functions. For example tri-methylated K36 in histone 3 (H3K36 me3) is used as a marker for gene area localization as it is usually biased towards the middle and 3′ ends of genes (Rando, 2007); and H3K4 me3 is assumed to help in the functional mapping as their attached sites are associated with the transcription initiation sites (Bernstein et al., 2005).

d. Histone phosphorylation

Similar to histone methylation, the function of histone phosphorylation differs according to their sites. H1 phosphorylation is cell cycle dependant; commencing at the S phase, increasing during the G2 phase and reaching a maximum during metaphase. H1 loses its phosphorylation status as soon as the nucleus divides (Bradbury, 1992). Furthermore, phosphorylation of H3 at serine number 10 (H3 ser 10) (Koshland & Strunnikov, 1996) and 28 (H3 ser 28) (Goto et al., 1999) occurs during chromosomal condensation and mitosis. Inappropriate H3 phosphorylation results in premature entry of the cell into mitosis and consequently cell death (Tikoo et al., 2001).

However, phosphorylation of H2B is a constant in the process of apoptosis. It is associated with apoptosis-specific nucleosomal DNA fragmentation and is now known to be an apoptotic marker (Ajiro, 2000). Phosphorylation of H2A ser 139 is consistent with DNA double strand breaks

among species within one minute after the induction of the breakage (Rogakou et al., 1999).

e. Histone Ubiquitination

Ubiquitin is a 76-amino acid protein that is *ubiquitously* distributed and highly conserved throughout eukaryotes. Ubiquitylation is proposed to have a role in signalling pathways (Di Fiore et al., 2003). Considerable evidence suggests the participation of ubiquitin in gene regulation. It is attached mainly to H2A, specifically to K 119 as 5-15% of H2A is ubiquitinated (Vijay-Kumar et al., 1987). Ubiquitination of H2B is less frequent (1%–2%) (West & Bonner, 1980).

The effect of ubiquitin on gene transcription is thought to occur through three possible mechanisms. Firstly, it may affect higher-order chromatin folding, thereby resulting in greater access of the DNA to the transcription machinery. Secondly, ubiquitination may function as a signal for the recruitment of regulatory molecules. Thirdly, the effect is through its impact on other histone modifications (Zhang, 2003). Ubiquitin acts in specific manner; it causes the transcriptional activation of some genes (Barsoum & Varshavsky, 1985; Nickel et al., 1989), and repression in others (Huang et al., 1986; Baarends et al., 1999).

f. Histone Sumoylation

SUMO stands for Small Ubiquitin Related Molecule, which is a ubiquitin-like protein of about 100 amino acids involved in post-translational modifications. H4 sumoylation is associated with repression of gene transcription through histone deacetylase recruitment (Shiio & Eisenman, 2003), blockage of acetylation and ubiquitination (Nathan et al., 2006).

g. Histone Ribosylation

The modification of histones with poly ADP-ribosylation is a direct consequence to the DNA strand breakage irrespective of the inducer

(Boulikas, 1989). Histone ribosylation would induce histone phosphorylation and subsequently cell death. Cell survival was increased by using ribosylation inhibitor (Tikoo et al., 2001).

1.2.2.3. Small RNA Control;

98% of human transcribed RNA is not translated into protein. This RNA was thought to be either functionless (Mattick, 2001), or transcriptional noise (Dennis, 2002). From this population, two types of RNA have an established epigenetic role; the micro RNA (miRNA) and the small interference RNA (siRNA). These two types are generally 21-25 nucleotides in length, and hence their collective name, the small RNA (Bartel, 2004).

The mechanism of production of the small RNA is conserved through species. It depends on the production of a double stranded RNA (dsRNA) which is subsequently processed through an enzymatic machinery to yield the small single stranded RNAs (Matzke et al., 2001). But how is this dsRNA is formed? A number of mechanisms have been suggested; (1) transcription through inverted DNA repeats, (2) simultaneous synthesis of sense and anti-sense RNAs, (3) viral replication, and (4) the activity of cellular or viral RNA–dependent RNA polymerases (Plasterk & Ketting, 2000).

The mechanism of action of the small RNA regulation of gene expression is simple. Small RNA binds their complementary mRNA and thus dsRNA is formed, recognized as foreign RNA and thus cleaved and degraded. When the matching between the small RNAs and mRNA is not perfect, while it may not be fully recognized but at least the incomplete binding blocks the translation (Mattick & Makunin, 2005).

Small interference RNA (siRNA) is generated from the interference RNA (RNAi) which was reported for the first time in C. Elegans (Fire et al., 1998). Besides this post-transcriptional epigenetic control, siRNA guides homologous DNA methylation and thus inactivates the corresponding gene (Hammond et al., 2001; Matzke et al., 2001; Reinhart & Bartel, 2002).

The interaction of miRNA unlike siRNA specifically blocks the translation of their mRNAs target, rather than inducing their degradation. The role of miRNA has been detailed in the development process (He & Hannon, 2004), including the process of stem cell differentiation (Houbaviy et al., 2003). The most interesting question is what controls these small RNAs. The answer revealed in cancer miRNA studies is: DNA methylation and histone modifications. Treatment of the cells with DNA demethylating agents and histone deacetylases inhibitors increase the miRNA expression, particularly the miR-127 which is embedded in a CpG island (Saito et al., 2006; Saito & Jones, 2006).

1.2.3. Epigenetic control

1.2.3.1. Epigenetics and development

DNA is exposed to waves of methylation and demethylation during the process of gamete formation up to fertilization and embryonic development (Jaenisch, 1997). Primordial cells undergo erasure of DNA methylation as they develop (Hajkova et al., 2002), but the epigenetic markers are restored upon gamete formation (Mayer et al., 2000; Li, 2002). After fertilization, demethylation takes place in both the male and female pro-nuclei, with the exception of imprinted genes.

Demethylation of the paternal pro-nuclei takes place faster than the maternal counterpart; which is assumed to be an active process in the paternal pronuclei but passive in the maternal pronuclei. Following which reprogramming takes place in a tissue specific manner (Niemitz & Feinberg, 2004). Disruption of any of the methyl transferases is lethal in animal models, either during the embryonic life or shortly after birth (reviewed in (Jaenisch & Bird, 2003)).

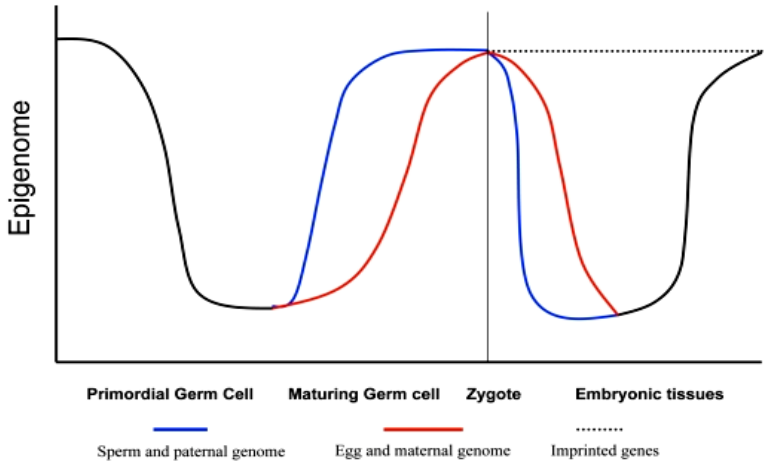


Fig.1.9. Diagrammatic representation to the epigenetic reprogramming during gametogensis and after fertilization and implantation. Primordial cells are absolutely demethylated. Methylation is restored gradually in the mature germ cells. After fertilization, another wave of demethylation takes place in both maternal and paternal genomes with exception of imprinted genes (dashed black line), being very fast in the paternal DNA (active process), which is demonstrated by the blue line, in comparison to the maternal DNA (passive process), demonstrated by the red line. DNA methylation is restored in a tissue specific form; adapted from (Niemitz & Feinberg, 2004).

Failure of this pattern of methylation programming could be the cause of the observed failure of mammalian cloning. In cloning experiments, very few animals embryos survive to birth, and those that survive either die at the post-natal period or, at best, before their normal siblings. In addition, multiple abnormalities have been reported. Although genetic factors should not be excluded as abnormalities accumulate with aging and during *in vitro* cultivation of cells, Rideout and colleagues (Rideout et al., 2001) considered the reason to be 'failure to reprogram faithfully the embryonic genes'. Thus, the nuclei of the cloned embryos as derived from adult cells lack the ability to reprogramme compared with the newly fertilized zygote.

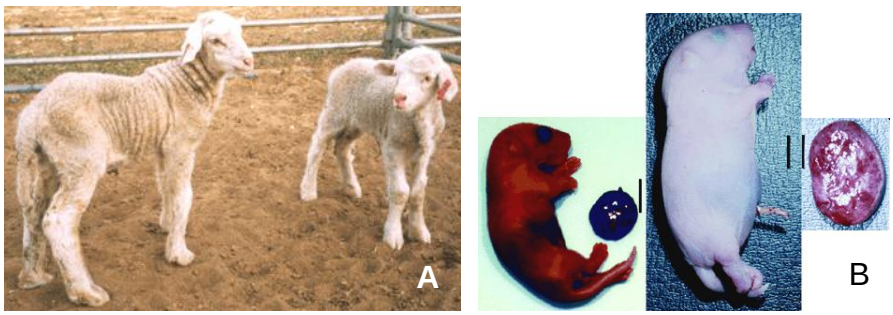


Fig.1.10. Examples of cloning abnormalities; the cloned lamb at left , adapted from (Pennisi, 2001) in (A) and the cloned pup at right, adapted from (Eggan et al., 2001) in (B) are bigger than their normal siblings with multiple abnormalities.

During the early differentiation stages, only the specific lineage gene should be kept active while other lineages genes as well as the genes responsible for self-renewal should become permanently silenced and thus the cells lose their potency. Methylation of certain CpG islands in the regulatory region of the key genes is responsible for such silencing. But before differentiation, how do stem cells in the embryo keep their potency? The answer is through an unusual histone modification pattern called the bivalent domains. Large regions of H3K27 trimethylation (repressive chromatin) harbouring small regions of H3K4 trimethylation (active chromatin) around the transcription start site and thus the cells can respond to the extracellular activating signals. The H3K4 methylation may act as a barrier to the spread of the repression marking as well as facilitating the binding of the transcription factors to the DNA upon stimulation. This possibility is further enhanced by the DNA methylation status, as the cytosine residues in the promoters of pluripotency genes are kept unmethylated. On differentiation, H3K27 methylation is removed while that of H3K4 is maintained (Bernstein et al., 2006; Agger et al., 2007; Fouse et al., 2008; Barrero & Belmonte, 2008).

1.2.3.2. Epigenetics and environmental response

The interaction between any organism and the environment is of significant importance for adaptation during life. This interaction takes place through modulation of the epigenetic status of the key genes in response to the external as well as internal signals induced by such effectors (Sheldon et al., 2000). The earliest supporting observation was in plants. The vernalization reaction (where flowering happens early in the cold weather) is believed to take place through altering the histone methylation (Bastow et al., 2004). Similar evidence has been found in mammals; feeding pregnant mice of two strains with different doses of methyl supplement diets (which would function as methyl donors and thus facilitate DNA methylation) will result in offspring with different phenotypes, i.e. fur colour in the case of Agouti mice (Wolff et al., 1998; Cooney et al., 2002).

Fig.1.11. Effect of methyl donor diets on mice colour. These mice although genetically similar, are phenotypically different due to epigenetic modification in response to maternal dietary methyl supplement; adapted from (Morgan et al., 1999).

The effect of environment on the human being is well documented and forms the basis of the 'Developmental Origin of Health and Disease' program of studies at the University of Southampton. Several studies have shown that the maternal life style and diet constituents, even before pregnancy, will affect the health of the offspring, from early childhood, adulthood and old age in an environmental compatible manner (Gluckman & Hanson, 2004; Gluckman & Hanson, 2006).

1.2.3.3. Epigenetics and disease

Epigenetics is thought to be involved in a number of diseases, including cancer(Feinberg et al., 2002). Epimutations occur when there is failure in the methylation system to preserve the methylation map, hence altering the cell characteristics, without changing the genetic code essentially (Hansen et al., 1999; Feinberg et al., 2002). Epimutagens include ionizing radiation, ultraviolet, nickel, arsenic and cadmium (reviewed in (Vaissiere et al., 2008)) and are related to a number of tissue tumours such as bladder, liver, lung, nasopharynx, skin and kidneys. Recently, smoking-induced lung cancer was related to changes in the DNA methylation pattern.

Methylation silencing of tumour suppressor genes, aberrant expression of DNMT1 or demethylation of oncogenes can lead to the conversion of a normal cell to a malignant cell. In addition, chromosomal instability and inactivation of the DNA repair system has both the genetic and epigenetic backgrounds (Esteller & Herman, 2002; Szyf, 2008a). There is also a

stringent relationship between the histone modifications and tumour formation. In addition, the recurrence of certain cancers is predicted by the presence of certain histone modifications such as H4R3 me2 in prostate cancer and increased expression of HDAC6 in breast cancer (Kurdistani, 2007). Furthermore, the prognosis of certain malignancies can be affected by the epigenetic status (Sakuma et al., 2007).

Osteoarthritis is a degenerative disease associated with progressive destruction of the articular cartilage. Inflammatory cytokines cause abnormal demethylation of the promoter of different metalloproteinases within the chondrocytes. The abnormal presence of such enzymes causes degradation of the cartilage matrix and is associated with disease progression (Roach et al., 2005; Roach & Aigner, 2007; Gibson et al., 2007; Hashimoto et al., 2007a; Cheung et al., 2008; da Silva et al., 2008).

In addition, a number of syndromes have been correlated with aberrant methylation patterns, including Rett, Prader–Willi, Angelman and Fragile-X syndromes (Oberle et al., 1991; Reis et al., 1994; Amir et al., 1999). Furthermore, the study of epigenetics has led to the hypothesis that modifications during gestation, infancy and early childhood can lead to a number of age related diseases such as cardio-vascular diseases, type two diabetes, kidney disease as well as stress response (Gluckman & Hanson, 2006).

1.2.3.4. X chromosome inactivation

One of the earliest solutions provided by epigenetics in science was the X chromosome mystery. As the male has only one X chromosome while the female has two, males would have only half the amount of the genetic information carried on that chromosome in comparison to females, which is unlikely. Many studies followed this idea and concluded that there should be a method that inhibits one whole chromosome in the females, which is widely known as dosage compensation. This is achieved epigenetically through a

cascade of CpG methylation superimposed by global histone deacetylation (Pfeifer et al., 1990; Lyon, 1999; Avner & Heard, 2001; Monk, 2002).

1.2.3.5. Epigenetics and aging

Is it aging that causes epigenetic alteration or epigenetic changes that lead to aging? This question remains unresolved. Although global genomic hypomethylation was observed especially in repeated sequences (Barbot et al., 2002) hypermethylation of specific genes could also be detected, such as the estrogen receptor gene (Issa et al., 1994). The mechanisms for both situations remain to be clarified. The importance of understanding this variable may help in explaining the difference in stem cells response between patients in terms of the age and/or environmental stresses that accumulate over the years.

1.2.3.6. Epigenetics and monozygotic twins

Monozygotic twins (MZT) originate from independent mitotic division of an early embryonic cell. These two clusters of cells lead to independent development and birth. MZT have the same genetic background. Epigenetic profiles can explain why one of a set of twins may exhibit significant health problems. While the methylation mapping sequence (Fig.1.12) between two young (3 years) MZT is very similar, it is markedly different between older (50 years) MZT (Fraga et al., 2005). The environmental challenges and life style differences may explain this discrepancy.

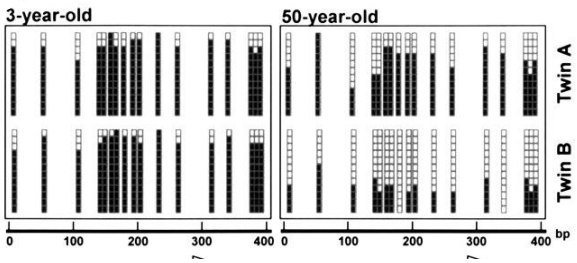


Fig.1.12. Methylation status of each CpG dinucleotide of Alu-SP. Black and white dots indicate methylated and unmethylated CpGs, respectively; adapted from (Fraga et al., 2005).

1.2.3.7. Epigenetics and hybridized animals

Hybrid animals are the offspring resulting from crossing two animals from different species but within the same family. The offspring differ in morphology although the genetic components are equal. For example when a mare is crossed with a donkey a mule is produced, whereas a stallion crossed with a donkey produces a hinny (Pennisi, 2001). Similarly, when a male lion is crossed with a female tiger, the offspring is a 'liger', which is 40% larger than its parents. Switching the parents to the opposite, a 'tigon' is produced which is much smaller than its parents. This parent-specific effect is explained by the phenomenon of genetic imprinting. Epigenetic control of certain genes to be either silenced or expressed (depending on whether the gene is of paternal or maternal origin) and DNA methylation is the major player in this context. (Pennisi, 2001)

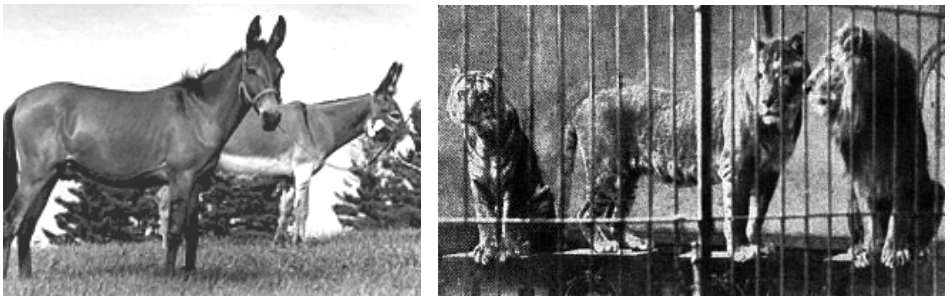


Fig.1.13. To the left, the hinny (foreground) has shorter ears, a thicker mane and tail, and stronger legs than the mule, in spite of the same genetic make up (Pennisi, 2001) (adapted from Pennisi 2001). To the right, the liger is 40% larger in size in comparison to the parents; http://sodredge.tripod.com/images/liger_08.jpg.

1.2.4. Epigenetic modifiers

DNA methylation and histone acetylation have been exclusively studied in relation to mechanisms of epigenetic control. Alteration of DNA methylation and histone acetylation causes a shift between active/ silent chromatin and consequently gene expression (Alexanian, 2007). Pharmacological agents have been developed to modify the epigenetic status of malignant cells and thus activate silent tumour suppressor genes (Braakhuis et al., 1988; Daskalakis et al., 2002; Emmans et al., 2004; Shang et al., 2007). These agents have been shown to enhance the mesenchymal stem cells (MSCs) trans-differentiation into putative neural cells (Alexanian, 2007). The response

of different cell types to these agents would vary according to different epigenetic make up, which originally result the interaction between the cells and their inner and/or outer environment and consequently activation and silencing of different genes. The accumulated epigenetic markings would lead to a degree of selectivity of action rather than broad or generic action and consequently could affect differentiation capacities of stem cells (Makki et al., 2008). Furthermore, different responses to such agents are expected between cells with different origins; i.e. fetal-derived cells versus bone marrow cells derived from aged donors although these cells have the same developmental origin.

1.2.4.1. Histone deacetylase inhibitors

Histone deacetylase inhibitors (HDI) are chemicals that non-competitively and reversibly inhibit histone deacetylases. HDI have a zinc binding domain and serve as substrates to chelate the zinc ion contained in the deacetylases ((Yoshida et al., 1990; Furumai et al., 2001) and reviewed in (Yurek-George et al., 2004)). The consequences of deacetylation include: a) preservation of already acetylated histones in their active state; b) a change in the balance between the histone acetylating and deacetylating enzymes resuting in increased histone acetylation (Szyf, 2008a).

Many of these inhibitors are currently involved in clinical trials as chemotherapeutic agents for many intractable diseases including systemic lupus erythrematosus (Foubister, 2003), mental disorders (Simonini et al., 2006), and various types of cancers (Crabb et al., 2008; Makki et al., 2008; Szyf, 2008a). HDI can cause cell death through other mechanisms including disruption of heat shock proteins, perturbation of NFκB pathway, stimulating apoptosis and inducing reactive oxygen species (Glaser, 2007).

A. Trichostatin A

Trichostatin A (TSA) was isolated in the 1970's from *Streptomyces hygroscopicus*, as an antifungal antibiotics, which is active against Trichophyton (Tsuji et al., 1976).

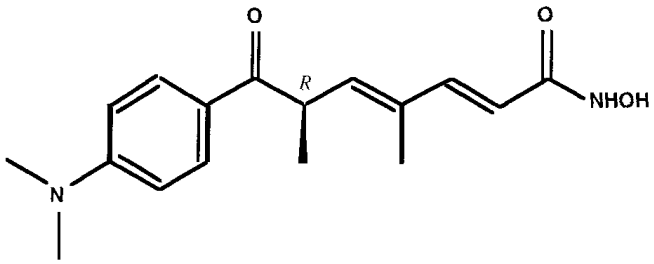


Fig.1.14. The chemical composition of TSA; (Yoshida et al., 1995).

In vitro, TSA increases histone acetylation (Yoshida et al., 1995). Induction commences two hours after application and can persist for 16 hours in cell lines (Crabb et al., 2008). As a consequence, hyperacetylated histones that are attached to the promoter regions should activate corresponding genes transcription. TSA has been proposed as a cancer chemotherapeutic as it is believed to activate tumour suppressor genes that provide a natural defence against malignant cells (Emmans et al., 2004). In addition, TSA arrests the cell cycle in both G_1 and G_2 phases (reviewed in (Yoshida et al., 1995)).

Surprisingly, TSA may also decrease the transcription and the translation of some genes; Wilms tumour gene 1 is an obvious example in this context. For yet undetermined reasons, TSA enhances the gene silencer, which consequently decreases gene expression. In addition, TSA enhances the transcription of a certain proteosome, which digests the Wilms tumour protein. The net result is marked decrease in the Wilms tumour protein (Makki et al., 2008). This harmonized action may raise the issue of selectivity. The inhibition of Wilms tumour at both the transcription and translation levels may be caused by one or more factors that selectively antagonise this protein and co-operate with the TSA or expose their target genes to the TSA. This factor could be an epigenetic modulation, a transcription factor or other regulatory molecule. Also TSA inhibits the hypoxia induction of vascular endothelial growth factor (VEGF) and hypoxia induced factor-1, while TSA induces other proteins such as p53 as TSA inhibits the hypoxia induced HDAC activity (Kim

et al., 2001). In another study, only 8 genes of 340 examined genes have enhanced transcription profile in response to TSA treatment (VanLint et al., 1996) while in a recent microarray study 0.44% of the analysed genes were affected by TSA treatment for 6 hours. Of these, 46 genes (representing 53%) were up-regulated including Siprena1 and Sox-9, while 41 genes (47%) were down regulated (Crabb et al., 2008). In addition, TSA can restore the telomerase activity in MSC transiently, overcoming the replicative senescence (Serakinci et al., 2006).

B. Spiruchostatin A

Spiruchostatin A (SSA) is a potent natural histone deacetylase inhibitor that was synthesized for the first time in 2004 by Prof. Ganesan and co-workers within the University of Southampton (Yurek-George et al., 2004). It has been isolated from *Pseudomonas* extract and shown to have ten times potency compared to TSA (Masuoka et al., 2001). Although SSA affects both class I and class II HDACs, it has 600 fold efficiency against class I and thus is considered as a class I selective inhibitor, particularly in comparison to TSA, although TSA has more rapid action. SSA arrests cells in G_2/M phase; thus, SSA has potent growth inhibitory properities and enhances the differentiation in breast cancer cells. In addition, SSA induces cell death (Crabb et al., 2008).

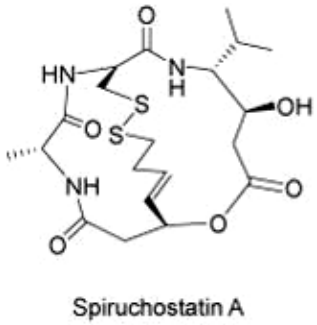


Fig.1.15. The chemical composition of SSA; adapted from (Crabb et al., 2008).

1.2.4.2. DNA demethylating agent

5 Aza deoxycytidine (5-Aza-dC) is a chemical analogue of the cytosine nitrogenous base (Christman, 2002) that is active only in dividing cells. During the S phase of the cell cycle, 5-Aza-dC is converted to a deoxynucleotide triphosphate and incorporated into newly synthesized DNA, replacing cytosine. This fraudulent base bonds covalently to the DNMT, traps the enzyme and DNA demethylation can occur; thus, 5-Aza-dC can act only on dividing cells. The half life of 5-Aza-dC in culture has been observed at 4 hours (Haaf, 1995). This epigenetic change is inherited to the next cell generation as the replication fork will proceed without the methylase (Egger et al., 2004; Szyf, 2008a).

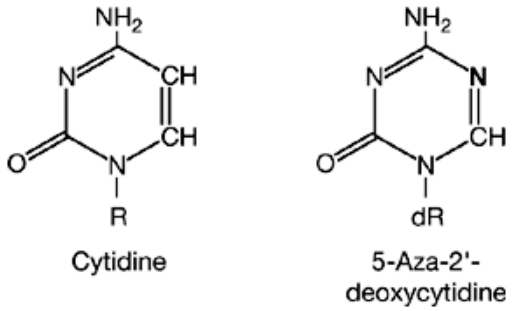


Fig.1.16. The chemical composition of 5-Aza-dC; adapted from Fenaux, 2005 (Fenaux, 2005).

Being a demethylating agent, 5-Aza-dC was proposed to activate tumour suppressor genes in tumour cells. Studies carried out in cell culture and on animals were promising especially in tumours of leukaemic origin (Daskalakis et al., 2002) and head and neck tumours (Braakhuis et al., 1988). 5-Aza-dC also enhances the response of renal cancer cell line to certain chemotherapeutic agents (Shang et al., 2007). 5-Aza-dC is currently involved in clinical trials; one of them is currently in phase II. The DNA demethylating inhibitor is being used for treating patients with oestrogen receptor negative metastatic breast cancer (http://www.mctrc.org/en/rp/studies/aza.htm). Furthermore, 5-Aza-dC has been approved by the FDA for the treatment of myelodysplastic syndromes (Kuendgen & Lubbert, 2008). Unfortunately, a recent report has documented the activation of the metastasis inducing genes as well as tumour suppressor genes in breast cancer cell lines (Ateeq et al., 2008); which could be further evidence of the relative selectivity of such agents. In cancerous

cells, the intracellular environment is modified to promote the malignant transformation as well as the progression. Thus in this particular breast cancer cell line the level of epigenetic inhibition for the metastasis induced genes is expected to be less than that of the tumour suppressor genes. Further characterization, dose response curves and animal studies will be required for these epigenetic modifiers before clinical application can be envisaged.

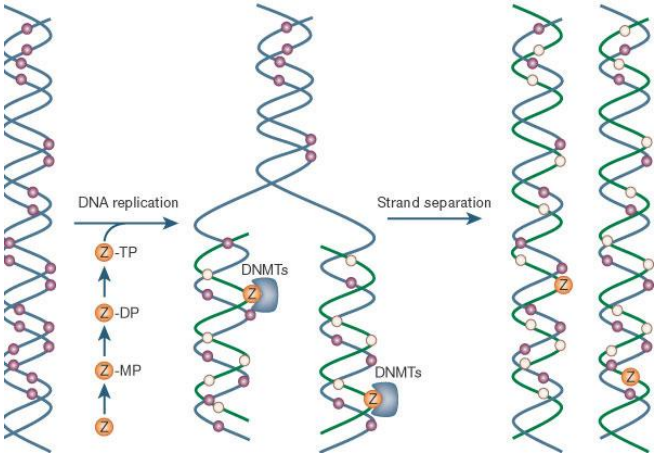


Fig.1.17. The incorporation of the 5-Aza–dC (the orange Z) into the newly synthesized DNA traps the DNMT, resulting in the absence of methylated DNA (cream circles) in comparison to the original cytosine (pink circles); adapted from (Egger et al., 2004).

5-Aza-dC has also been found to activate the fetal haemoglobin gene in sickle cell anaemia patients, increasing the level of this haemoglobin from 6.5% to 20.4% (Saunthararajah et al., 2003). Interestingly, some genes which have no methylated CpG in their promoters may be activated with 5-Aza-dC (Schmelz et al., 2005).

5-Aza-C, which is the corresponding analogue to cytidine, induces the transcription of leukosialin gene, which is known to be controlled by the transcription factor SP1 and silenced by binding of MeCP2 to the gene methylated promoter (Kudo & Fukuda, 1995; Kudo, 1998).

1.2.5. Elements of epigenetic interaction

The three components of epigenetic control interact together in a complicated and as yet not fully understood sense. It has been reported that methylated DNA is associated with deacetylated histones in the inactive areas of DNA but the order of silencing and what follows is still a question to be resolved (Eden et al., 1998; Vaissiere et al., 2008). It has been shown that DNA methylation can enhance histone modification. In addition, inactive transcription *per se* is insufficient to change the acetylation status of histone but DNA methylation is required for such change to occur, as methylated DNA will attract methylated DNA binding domain proteins such as MeCP2, which in turn recruit histone deacetylases (Jones et al., 1998; Nan et al., 1998a; Nan et al., 1998b; Stirzaker et al., 2004). While the carboxyl terminal (catalytic domain) of the DNMT1 transfers the methyl groups to the DNA; the N-terminal interacts with HDAC1 and 2 mediating histone deacetylation at the same time, resulting in gene silencing (Fuks et al., 2000; Rountree et al., 2000). As 5-Aza-dC inhibits DNA methylation and TSA inhibits histone deacetylases, MeCP2 can link between TSA and DNA methylation as well as 5-Aza-dC and histone deacetylation. Thus, indirect effects of each of the modifiers on the alternate epigenetic element could be expected. TSA is known to have a demethylating effect that is independent of cell cycle; thus, TSA can induce DNA demethylation in non-replicating cells (in contrast to 5-Aza-dC). In mammalian cells as well as in Neurospora, TSA does not induce global demethylation. Certain genes are significantly activated in a selective manner that was comparable to the effect of the DNA demethylating agents (Selker, 1998; Cameron et al., 1999; Hu et al., 2000; Dong et al., 2007; Kawamoto et al., 2007). The proposed genes are those silenced by DNA methylation then MeCP2 bound to the methyl groups followed by the recruitment of HDACs (Cameron et al., 1999; Cervoni & Szyf, 2001). Those genes showed enhanced transcription following the binding of TSA to HDAC at SP1 sites, which seems to be bound to MeCP2 (Yokota et al., 2004). Heavily silenced genes such as those on the inactive X chromosome cannot be activated by TSA alone (reviewed in (Cameron et al., 1999)).

These mechanisms support the early observation of the relation between chromatin and DNA methylation by Razin and Cedar in 1977 (Razin & Cedar, 1977). The authors found that DNA packaged in tightly bound chromatin is hypermethylated while open chromatin is associated with hypomethylation. At least some genes could be silenced on two levels: initial DNA methylation, followed by histone deacetylation (Selker, 1998; Nan et al., 1998a). This configuration may further enhance the silence state of corresponding genes as the tight interaction between non-acetylated histone tails blocks the access of DNA demethylases to reach their substrate and hence preserve the DNA methylation (Cervoni & Szyf, 2001; Detich et al., 2003). Thus DNA methylation could be considered to be in dynamic balance between the activity of demethylases and the degree of histone deacetylation (Cervoni & Szyf, 2001). Knocking out the DNA demethylase MBD2 by siRNA has abolished the corresponding effect of TSA on the DNA (D'Alessio & Szyf, 2006).

After initial histone acetylation, RNA polymerase II binds weakly to the methylated promoter, the enzyme moves along the DNA coding regions, which enhances DNA demethylation and histone acetylation, possibly through opening up the chromatin or physically recruits DNA demethylases such as MBD2. Following the DNA demethylation, histones methylases would be recruited to add the marker of actively transcribed genes: H3K4 trimethylation and the serine 5 phosphorylation in RNA polymerase II (D'Alessio & Szyf, 2006).

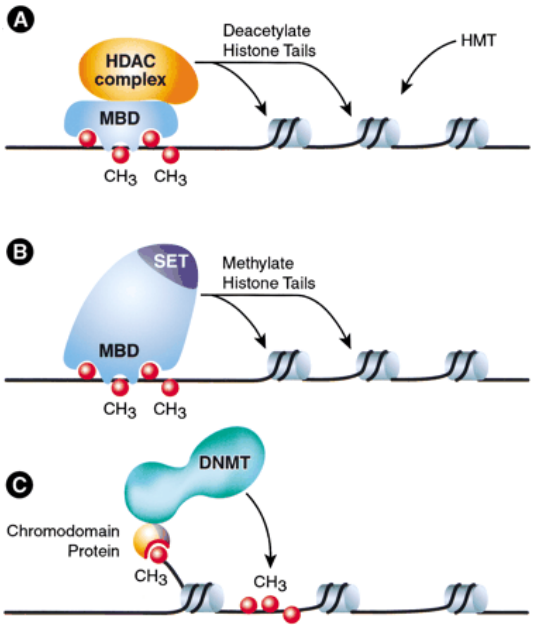


Fig.1.18. Diagrammatic representation of the models proposed for the interaction between different elements of epigenetic control. Models depicting the relationship between DNA methylation, histone deacetylation, and histone methylation: The first model (A) is the DNA methylation induced histone deacetylation as MBDs recruit HDAC complex to deacetylate histone. The second (B) is DNA methylation induced histone methylation and the third (C) Methylated histone tails recruits DNMTs to silence the DNA by methylation; adapted from (Zhang & Reinberg, 2001).

Alternatively, DNA methylation can be induced by histone methylation, particularly the methylation of H3K9. Furthermore, histone methyltransferase can also target specific position in the DNA and consequently silence a particular gene. DNA methylation in turn reinforces histone modifications (Egger et al., 2004; Vire et al., 2006; Meissner et al., 2008). MeCP2 may provide the link also between the DNA and histone methylation as it is associated with a histone methyltransferases that methylates H3K9 (Fuks et al., 2003). RNAi in yeast and plants retains another level of control over the DNA methylation and histone modifications (Egger et al., 2004). Although the hierarchical order of events is still debatable (Vaissiere et al., 2008), the epigenetic layering of silencing may be modelled with initial DNA methylation, reinforced by the histone deacetylation and then followed by histone methylation and both histone modifications would reinforce the DNA methylation (Cameron et al., 1999; Stirzaker et al., 2004).

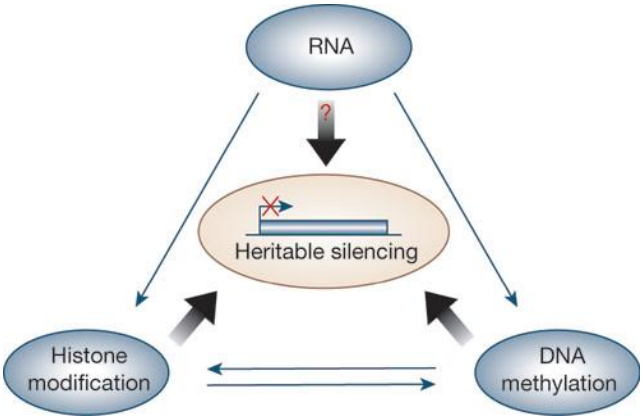


Fig.1.19. The possible interaction between the three elements of epigenetic control; adapted from (Egger et al., 2004).

1.3. Stem Cells

1.3.1. Introduction

The term "stem cells" is used to describe any cell, regardless of its source, that display two characteristics; a capacity for self-renewal and the ability to differentiate into more specialized cells. The capacity of these cells to divide is maintained throughout life (Hauschka et al., 1983); (Barry & Murphy, 2004). Asymmetrical cell division *in vivo* generates one daughter cell that retains stem cells characters and a daughter cell that can clonally expand and/or differentiate (Bianco et al., 2006). Stem cells have been observed in a number of different organs and tissues and contribute to postnatal growth as well as maintaining tissue homeostasis by replacing lost cells due to injury, death or turnover (Plasterk & Ketting, 2000; Luyten et al., 2001).

Stem cells can be classified according to their ability to differentiate into various specialised cell types, i.e. their potency. Totipotent cells have total potency to develop into any cell type, including the extraembryonic tissue and this term is applied to the early days of the embryonic life, before the formation of blastocyst. Pluripotent cells have the potency to differentiate into any cell type derived from the three germ layers: ectoderm, mesoderm and endoderm. The inner cell mass of the early embryo and its derivative, the embryonic stem cells, are the classic example of pluripotent cells. Multipotent

cells develop into a more restricted lineage, for example the hematopoietic cell differentiates into red blood cells, white blood cells, platelets,…. This group includes the adult stem cells; bone marrow derived cells have the ability to differentiate into many cell types of mesenchymal origin as well as few extra-mesenchymal populations. Oligopotent cells have the ability to differentiate into few cell types, which are usually known as progenitors. Also the vascular stem cells have the ability to differentiate into endothelial as well as smooth muscle cells. Unipotent cells produce only one cell type but keep their character of self-renewal such as skin stem cells (http://en.wikipedia.org/wiki/Stem_cell#Potency_definitions).

From a regenerative medicine perspective, the cell types that are involved in most of current studies are the embryonic stem cells and adult stem cells (from various origins) in addition to fetal derived cells.

1.3.2. Embryonic stem cells

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of the blastocyst of the early mammalian embryo. Those cells are capable of unlimited proliferation *in vitro*. The source of these cells is an issue of debate. Currently, they are usually obtained from the donated frozen embryos that were produced by *in vitro* fertilization and which are no longer required (Thomson et al., 1998; Frankel, 2000).

The transcription factors Oct-4 (Niwa et al., 2000) and Nanog (Chambers et al., 2003; Mitsui et al., 2003) are considered as markers for ESCs and their expression is maintained as long as the cells remain undifferentiated. Although the ESCs have a great potential for differentiation to almost any type of cells, their use faces significant challenges ranging from the ethical issues to the compatibility between the donor and recipient of these cells upon the usage in tissue regeneration. In addition the embryonic cells can form teratomas following *in vivo* transplantation (Orkin & Morrison, 2002). The first clinical trial with ESCs will take place in 2009 for patients having spinal cord injury in order to evaluate their safety (Wadman M., 2009) .

However, ESCs are also promising in the field of drug discovery and testing. ESCs can provide a source of limitless cells, which upon differentiation, could be an excellent *in vitro* screen model to study new drugs. In addition, the effect of drugs on ESCs differentiation could indicate the safety of such drugs during pregnancy (Reubinoff et al., 2000).

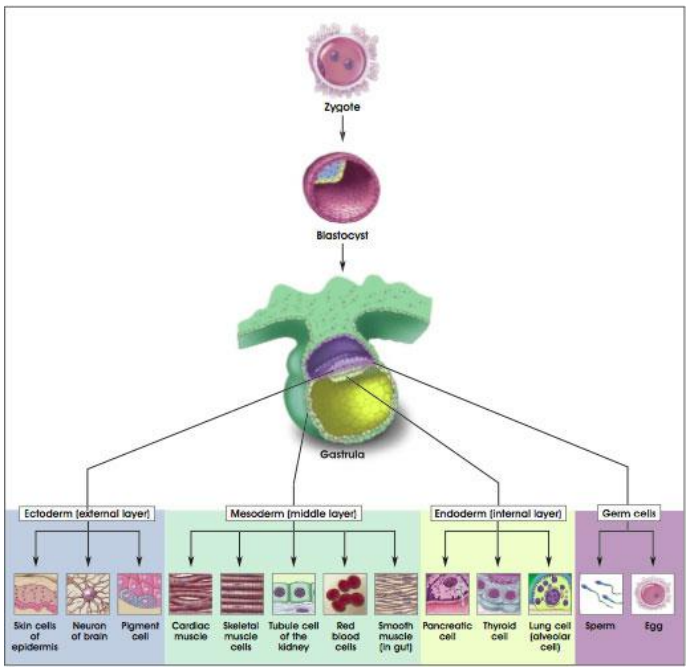


Fig.1.20. Embryonic Stem Cell differentiation lineages
(http://stemcells.nih.gov/info/scireport/chapter1.asp)

1.3.3. Adult stem cells

Adult stem cells (ASCs) are present in almost every tissue and are responsible for the tissue regeneration and repair processes (Minguell et al., 2001). Their differentiation potential exceeds the tissues they are located in to other tissue types, which is known as trans-differentiation (Alexanian, 2007). One subset of the ASCs are the mesenchymal stem cells (MSCs). This term was first used by Caplan in 1991, as this cell population is originally derived from the mesenchyme (Caplan, 1991). MSCs are defined as colonogenic, non-haematopoietic stem cells that present in the bone marrow and are able to differentiate into multiple mesoderm-type cell lineages e.g. osteoblasts, chondrocytes and adipocytes. Some groups suggest these cells, somewhat controversially can differentiate into non-mesodermal lineages e.g.

endothelial-cells (Reyes et al., 2002), neuronal-like cells (Kassem et al., 2004), beta pancreatic cells (Chen et al., 2004), hepatocyte (Petersen et al., 1999; Sgodda et al., 2007), different respiratory epithelium types (Krause, 2008a; Krause, 2008b) and multiple skin cell types (Fathke et al., 2004; Sasaki et al., 2008).

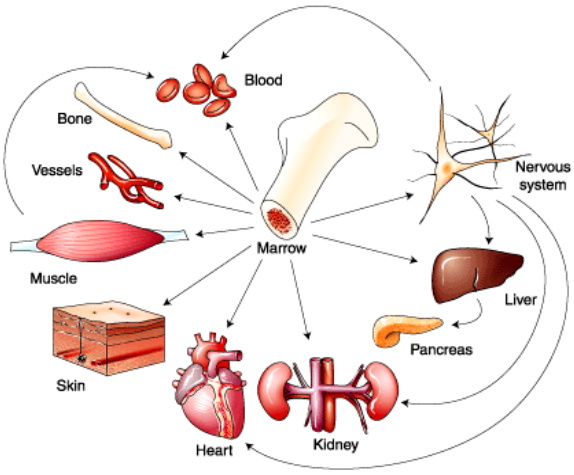


Fig.1.21. The differentiation potential of bone marrow derived mesenchymal stem cell; adapted from (Holden & Vogel, 2002).

The ability of the MSCs to differentiate into multiple cell types and the relative ease with which they can be isolated, cultured, proliferated and, possibly re-implanted suggests therapeutic applications for these cells in regenerative medicine (Friedenstein et al., 1968; Erenpreisa & Roach, 1999; Bianco et al., 2001). Bone marrow is thought to be the central reservoir of stem cells, and, more precisely, the stroma and hence the term bone marrow stromal cells (BMSCs); from which the cells can reach the circulation and then home into peripheral tissues ‘ niches’ and acquire more restricted commitment towards differentiation (Zvaifler et al., 2000). BMSCs are a minor component of the post-natal bone marrow, compared to the predominant cells of the haematopoietic lineage. On average MSCs are thought to represent one cell every 100,000 nucleated cells in bone marrow (Connolly et al., 1989; Prockop, 1997). Cells with similar differentiation potential have been identified in peripheral blood, umbilical cord blood, synovial membranes and deciduous teeth, supporting the principle of replenishement from the stores ‘the bone marrow stroma’ (reviewed in (Kassem et al., 2004) and (Sasaki et al., 2008)). More than 90% of these cells exist at the G0/G1 phases of the cell cycle

(Conget & Minguell, 1999). When BMSCs are plated as a single cell suspension, they adhere rapidly to the plate and form a fibroblastic like colony of fusiform cells, called by Friedenstein (Friedenstein, 1976) the colony forming unit fibroblast (CFU-F). There remains continuous debate over the *in vivo* potential of single colonies to differentiate into cells of extraskeletal lineage. Only 30% of the colonies are reported in some studies to be multipotential (Abdallah & Kassem, 2008). Other related terms are skeletal stem cells (Bianco et al., 2008), unrestricted somatic stem cells (Kogler et al., 2004) or as recommended by the International Society for Cytotherapy: multipotential mesenchymal stromal cells (Dominici et al., 2006). The broader term bone marrow stromal cells (BMSCs) will be used in this thesis, while skeletal stem cells will be used to include the BMSCs and the fetal femur derived multipotential cells.

Another rich source of active MSCs is umbilical cord blood. Although preterm cord blood is even richer than term blood, both tissues provide adherent cell layers that can be differentiated into various lineages (Erices et al., 2000; Goodwin et al., 2001). In addition, adherent and colonogenic cells were isolated from the blood of adult human as well as many kinds of animals (Kuznetsov et al., 2001).

1.3.3.1. *In vivo* adult stem cells differentiation

In vivo, ASCs derived from certain origins can give rise to completely distinctive cells. For example ASCs derived from the brain can give rise to the hematopoteic lineage provided that these cells are transferred into the haematopoietic system, i.e. the bone marrow (Bjornson et al., 1999). On the other hand, intravenous injection of labelled BMSCs has indicated the conversion of these cells into neural phenotype upon reaching the brain (Brazelton et al., 2000) and suggest a role in wound repair when reached the skin (Sasaki et al., 2008). The presence of stem cells in the blood stream has been reported in humans especially after growth factor mobilization (Fernandez et al., 1997; Kuznetsov et al., 2001).

These responses by the ASCs to the surrounding environment can be partially explained by the cell niche. The definition of the stem cell niche is ‘a specific location in a tissue where stem cells can reside for an indefinite period of time and produce progeny cells while self-renewing’ (Ohlstein et al., 2004). It is also ‘the functional and anatomic location in which stem cells are maintained typically in quiescent state, prior to division and generation of proliferating, repopulating differentiated cell populations (Tare et al., 2008). The niche contains stem cells throughout the organism life as stem cells renew themselves on division, while the more specialized cells and the progenitor cells leave the niche as soon as they start to develop (Watt & Hogan, 2000; Ohlstein et al., 2004).

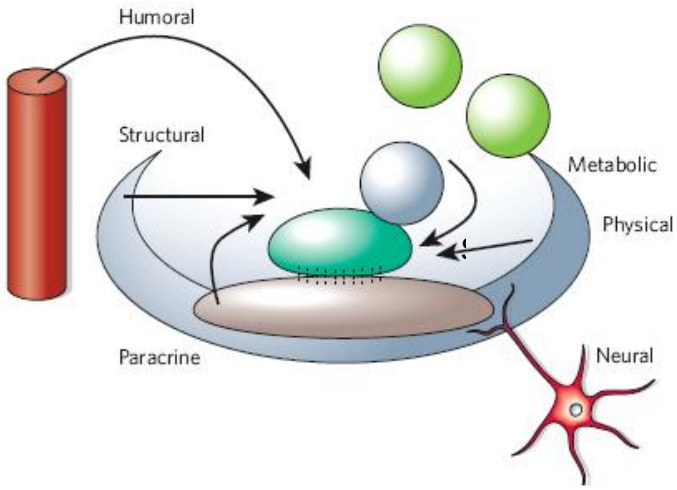


Fig.1.22. Elements of the microenvironment that affect the stem cell differentiation in the niche. Stem cells differentiation is affected by autocrine, paracrine and endocrine messengers as well as physical constrains and neural factors; adapted from (Scadden, 2006).

The niche is an interactive structural unit that hosts the stem cells, which will react with and respond to the microenvironment messengers formed by the surrounding cells as well as humoral, neuronal, metabolic and physical factors. The result of this interaction is the regulation of stem cell development in the direction of tissue homeostasis while at the same time preventing the excessive stem cell growth into cancer (Moore & Lemischka, 2006; Scadden, 2006). It is not only the stem cells that require the persistence of a niche, cartilage cells, which are highly specialized also show diverse fate

according to where they are located. Chondrocytes located at the bone ends i.e. articular cartilage, are adapted to their supportive function by the secretion of various components of the extracellular matrix and maintains the matrix integrity, while those located at the epiphyses are associated with the longitudinal growth of the bones (Archer & Francis-West, 2003).

Stem cell fusion is an important phenomenon. MSCs were thought to be targeted to the organ that needed regeneration. Entering the new niche, they would divide and differentiate. Instead, MSCs may fuse with a specialized cell (e.g. liver) and thus a polyploid cell will result. This hybrid cell may undergo a reduction division after that and produce normal and healthy cells with the characteristics of the more specialized cells. The safety of these polyploid cells, however is uncertain (Medvinsky & Smith, 2003). In another study, MSCs were cultured with heat shocked small airway epithelial cells. The MSCs were differentiated to replace the injured cells, giving both the morphological and molecular characters of these epithelial cells; 4% of the differentiated cells express the MSCS reporter (GFP) and 25 % of this subpopulation were binucleated (Spees et al., 2003).

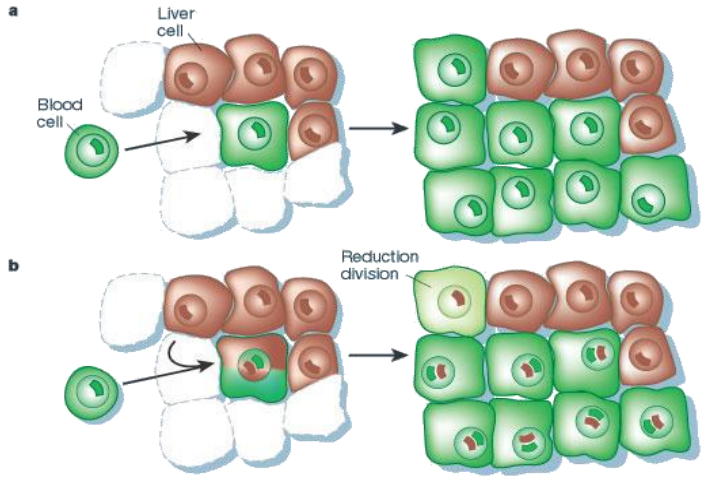


Fig.1.23. Possible fate for stem cells approached the liver. a. is direct conversion of the circulating stem cell upon reaching the hepatic niche. b. is the cell fusion with the formation of hybrid cell and underwent reduction division; adapted from (Medvinsky & Smith, 2003).

To address the frequency of this unusual occurrence of cell fusion and its effect on cell differentiation, murine neuronal stem cells (NSCs) have been

co-cultured with human endothelial cells. A subpopulation has been shown to express the endothelial markers and have acquired the capacity to form blood capillaries with no evidence of fusion. Meanwhile co-culturing the same NSCs with fixed endothelial cells (fixation prevents cells fusion) suggested NSCs differentiate into endothelial - like cells (Wurmser et al., 2004). In addition neuronal differentiation can be obtained directly from MSCs in co-culture either with living or fixed neuronal stem cells in neural induction media (Alexanian, 2007). Thus although cell fusion is an important phenomenon in terms of stem cell research and possible future applications in regenerative medicine, the plasticity of these cells is in no doubt.

1.3.3.2. *In vitro* adult stem cells differentiation

BMSCs (as a subset of adult stem cells) can differentiate in culture into osteoblasts, chondrocytes, adipocytes, myoblasts and reticulocytes, with an apparent degree of plasticity and interconversion; (Rideout et al., 2001; Barry & Murphy, 2004; Bianco et al., 2006; Kern et al., 2006; Mirmalek-Sani et al., 2006a). This differentiation may reflect what happens *in vivo*. BMSCs are settled in a niche of the microvasculature in the trabecular bone. In addition to their location between the bone spicules, isolated cell populations may contain osteoprogenitors (Manolagas & Jilka, 1995). This explanation supports the relative ease with which the cells differentiate towards the osteogenic lineage relative to other stromal lineages. The ability of bone marrow stromal cells to differentiate into adipocytes is also not surprising. Fatty conversion of the marrow in old age is well known (Rice & Allis, 2001) while chondrogenic differentiation is an important step in the fracture repair, namely during callus formation (Brownell & Allis, 1996).

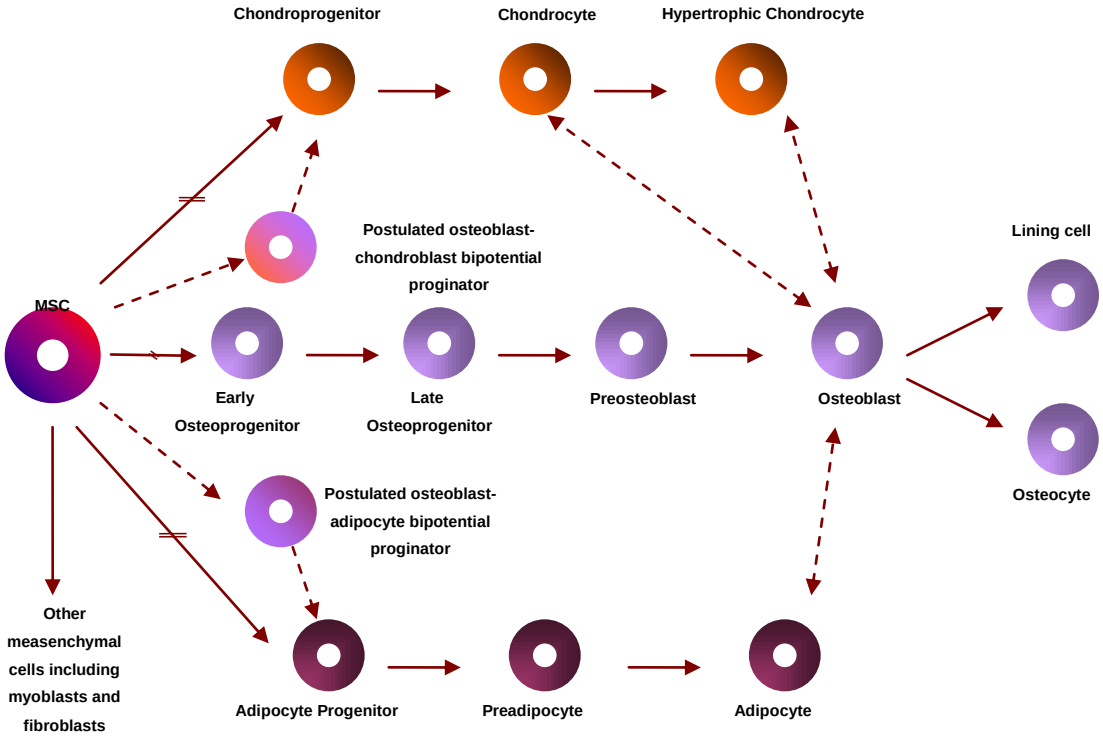


Fig.1.24. The differentiation lineage of the bone, cartilage and fat cells adapted from (Aubin, 1998).

BMSCs offer great potential over the differentiated cells for tissue repair and for clinical application. These cells are easier to use in terms of isolation and expansion. BMSCs lack senescence (to a certain extent) and they have great potential to differentiate and maintain their differentiation phenotype (Luyten et al., 2001). In addition, using autologous (patient's own cells) preparation would be recognized by the immune system as self-cells. The long-term stability of the differentiated MSCs has yet to be determined, however as there is a risk that the great differential potential of these cells may give rise to heterotopic tissue formation (Gilbert, 1998). The differentiation potential of BMSCs *in vitro*, the differentiation stimuli as well as the differentiation markers are summerized in table 1.

1.3.3.3. Adult stem cell markers

As mentioned in section 1.3.3. stem cells constitute a tiny percentage of nucleated cells in the bone marrow. Thus, the purification of the stem population of the marrow cells to contain a homogeneous population of MSCs remains a significant research challenge (Bianco et al., 2001).

Initially, the BMSCs were identified by a simple non-specific method, based on the adherence to the plastic surface of tissue culture flasks. Contamination with hematopoietic cells and cellular heterogenicity are unavoidable (Friedenstein et al., 1968; Rideout et al., 2001; Bianco et al., 2006; Mirmalek-Sani et al., 2006a; Abdallah & Kassem, 2008). Over the years, many groups have made strenuous efforts to find a cell surface marker for the mesenchymal stem cells. This search revealed a number of markers, such as CD 44^+ and Stem cell antigen (Sca-1), but unfortunately these markers remain non-specific (Ryan et al., 2005; Abdallah & Kassem, 2008). Data from different studies suggest the cell surface molecular profile for a mesenchymal stem cell should be $CD34^-$ $CD45^-CD14^-$ $CD13^+$ $CD29^+$ $CD44^+$ $CD49a^+$ $CD63^+$ $CD90^+$ $CD105^+$ $CD106^+$ $CD146^+$ $CD166^+$ (Bianco et al., 2006).

Meanwhile, the use of a single specific antibody which would enrich the cell population to homogeneously either with stem cells or with one of the early progenitor lineages remains elusive (Abdallah & Kassem, 2008). For instance, *Stro-1* surface antigen is a cell-surface glycoprotein found on the mesenchymal precursor cell. The expression of the Stro-1 is higher in the stromal cells than the haematopoietic cells. The murine IgM monoclonal antibody Stro-1 binds to approximately 10% of the human bone marrow mononuclear cells, greater than 95% of them are nucleated erythroid precursors. Stro-1 antibody does not react with committed progenitor cells (colony-forming unit granulocyte-macrophage, erythroid bursts or mixed colonies).

Fibroblast colony-forming cells are present exclusively in the Stro-1^+ enriched population. Stro-1^+ cells have been shown to display a capacity for developing into bone and cartilage cell populations (Simmons & Torok-Storb, 1991; Gronthos et al., 1994; Dennis et al., 2002; Mirmalek-Sani et al., 2006a).

Hop-26 is another antibody that reacts with early osteoprogenitor cells in the bone marrow, even before the alkaline phosphatase (ALP) enzyme activity could be detected. It binds to 0.59 ± 0.27% of the nucleated marrow cells. The antibody does not react with those cells in culture on reaching

confluence or the differentiated primary cells or cell lines such as MG63 or SAOS2 (Joyner et al., 1997).

Table 1. Differentiation potential of bone marrow-derived mesenchymal progenitors *in vitro*: stimuli, molecular, and cellular Markers (Minguell et al., 2001).

Differentiation to:	Stimuli	Terminal phenotype identification markers	
		Molecular	Cellular
Adipocytes	• Dexamethasone + isobutilmethylxanthine • Dexamethasone + isobutilmethylxanthine + indomethacin + insulin • Dexamethasone + insulin	PPARγ 2 C/EBPß aP2 Adipsin Leptin Lipoprotein lipase	Cytoplasmic lipid droplet accumulation
Chondrocytes	TGFß3 + ascorbic acid TGFß1 + ascorbic acid	Cbfa-1 (in hypertrophic) Collagen types II and IX Aggrecan COMP	Matrix enriched in proteoglycans and collagen types II, VI,IX and XI
Osteoblasts	Dexamethasone + ß-glycerophosphate + ascorbic acid	Cbfa-1 Bone/liver/kidney alkaline phosphatase Bone sialoprotein Osteopontin Osteocalcin Collagen type I	Mineralized matrix formation
Tenocytes	BMP12	Collagen type II Proteoglycans	Improved biomechanical properties of implanted tendon
Skeletal muscle cells	5-Azacytidine	MyoD Myf 5 and 6 MEF-2 Myogenin MRF4 Myosin	Multinucleated contractile cells
Smooth muscle cells	PDGF-BB	ASMA Metavinculin Calponin h-Caldesmon Later smooth muscle actin	n.d.
Cardiac muscle cells	bFGF	GATA 4 and 6 Cardiac troponin I and C Sarcomeric-actin Slow twitch myosin ANP	n.d.
Astrocytes	DMSO + dexamethasone	Glial fibrillary acidic protein Intermediate filament	Integration into neonatal brain
Oligodendrocytes Neurons	PDGF + EGF + linoleic acid	Galactocerebroside Neurofilament Tubulin BIII Synaptophysin	

The *SB-10* antibody reacts with CD 166 antigen that presents on the surface of the MSCs and is lost once the cells start to develop along the osteogenic lineage and express ALP (Bruder et al., 1997; Bruder et al., 1998).

SH-3 and SH-4 antibodies recognize two different epitopes on CD 73 (Barry et al., 2001). They are present on the BMSCs and do not react with other marrow cells or osteoblasts (Haynesworth et al., 1992); meanwhile CD 73 antigen is widely expressed on other cell populations such as the lymphoid tissues (Yamashita et al., 1998).

CD146 is a relatively recent marker that has been shown to separate a colony forming cell population from the human bone marrow. They can differentiate into osteogenic lineage in addition; they are able to organize themselves as hematopoietic sinusoids in animal models, which mimic the natural architecture of the bone marrow cavity. These cells display self-renewal after being removed from the animal, although this was not obtained in all colonies (Sacchetti et al., 2007).

To date, immunoselection can only purify the cologenic fraction of stem cells, which could be separated easily with plastic adherence. No studies have to date shown that the immunologically purified population is biologically different from classical CFU-F cultures or that more CFU-Fs could be obtained. On the contrary, a recent study has shown that for long term culture there is no preference of the enriched cell populations over the cells selected by the classical plastic adherence (Sacchetti et al., 2007; Bianco et al., 2008). However, in a very important trial, a panel of 22 markers has been used to analyze MSCs from different sources as well as human fibroblastic cell lines and none of them could differentiate between the two cell types. Furthermore, this study compared the molecular expression and the protein production; none of the up-regulated genes or proteins were specific (Wagner & Ho, 2007). Thus, currently MSCs are identified by their phenotypical, physical and functional characters (Sasaki et al., 2008).

1.3.4. Fetal derived Cells

Fetal cells (FC) are those cells that have been obtained from the digestion and expansion of fetal tissues obtained from early aborted fetuses (Mirmalek-Sani et al., 2006b) up to the placentas from full term caesarean section delivery (Kaviani et al., 2002). Cells from different fetal and extra-fetal tissues are probably an active and potential resource for tissue engineering (reviewed in (Montjovent et al., 2004)). The differentiation potential of the placental mesenchymal core cells (33 to 35 weeks) is comparable to the amniocytes (16 to 21 weeks) (Kaviani et al., 2002) . Placental core cells in turn express the embryonic stem cell marker Oct-4 even at full term and have the potential to differentiate to the three germ layers *in vitro* (Miki et al., 2005). Human femoral bone- derived cells as well as articular chondrocytes have the ability to differentiate into various mesenchymal cells lineages including bone, cartilage and fat as well as the extra-mesenchymal neural cells. These cells appear to be at an early differentiation stage that may be able to differentiate to other cell types either with or without prior dedifferentiation (Cui et al., 2006; Mirmalek-Sani et al., 2006b). Fetal cells have short doubling time in comparison to adult cells, allowing rapid expansion of cells from single donor to potentially many recipients (Montjovent et al., 2004; Mirmalek-Sani et al., 2006b). These cells may provide an ideal model for studying the natural development in humans and new clinical approaches (Ostrer et al., 2006).

1.3.5. Growth factors involved in the differentiation process

Many growth factors are involved in osteogenesis and chondrogenesis processes. They induce different transcription factors as well as various key genes, acting together in harmony, with variable involvement, at different time points. The balance between these factors is crucial for maintaining the bone integrity as well as fine-tuning of the differentiation of MSCs towards their target lineage.

1.3.5.1. Transforming growth factor beta (TGF-β) superfamily

TGF-β superfamily includes groups of multi-functional proteins that affect the growth and differentiation of BMSCs as well as formation of the extracellular matrix (Nakamura et al., 1997; Mackay et al., 1998). Bone morphogenic proteins (BMP) are a subgroup of this superfamily that includes more than thirty members and consists about one third of the TGF-β superfamily (Ducy & Karsenty, 2000). BMP2 is one of the earliest expressed growth factors after fertilization. BMP2 is known to be a major driving factor for osteogenesis (Katagiri et al., 1994; Nakamura et al., 1997). It enhances the transcription and the translation of the osteogenic master transcription factor Runx2 either directly or through the induction of Dlx5, which leads to the upregulation of various bone related proteins, such as osteopontin, bone sialoprotein, osteocalcin, alkaline phosphatase, and type I collagen (Lecanda et al., 1997; Karsenty et al., 1999; Spinella-Jaegle et al., 2001; Lee et al., 2005). BMP2 also induces the zinc finger transcription factor 'osterix' which is another key transcription factor in the osteogenesis process (Nakashima et al., 2002). BMP2 was found to support the transition of mesenchymal progenitors to chondroblasts, and promote proliferation and hypertrophy of chondrocytes (Macias et al., 1997; Shukunami et al., 1998). In a time course study in rats, BMP2 induced initially ectopic cartilage formation after 7 days of initial application and then bone formation after 14 days (Wang et al., 1990). BMP2 also induced the bone formation from non-osteogenic precursors (Wang et al., 1993; Ji et al., 2000).

BMP3, which is also known as growth differentiation factor 10 (GDF-10), augments the osteo-inductive effect of BMP2 in mice and rats but is not osteoinductive. BMP3 was associated with differentiation of immature chondrocytes during osteogenic development as well as fracture repair (Fujimoto et al., 2001; Kaihara et al., 2003). BMP3 knockout mice had twice as much trabecular bone compared to control and was suggested that BMP3 has an inhibitory effect on BMP2 through the activation of TGF-β pathway. Thus BMP3 may have a modulating role on other BMPs (Bahamonde & Lyons, 2001) as it enhances the osteogenic differentiation while negatively

regulates the bone density. BMP4 is closely related to BMP2 and its function is related to the neural as well as skeletal development (Leong & Brickell, 1996). BMP5 expression marks some of the mesenchymal condensations such as the genital tubercle, the sternum, the thyroid cartilage, the tracheal cartilagenous rings and the vertebrae (Ducy & Karsenty, 2000). BMP6 is a potent regulator of osteoblast differentiation. The expression of BMP6 is detected before the BMSCs differentiation to the osteogenic lineage (Friedman et al., 2006). In addition; BMP6 is essential for chondrocyte development to the hypertrophic stage (Eames et al., 2003). A delay in ossification has been noticed in BMP6 knocked out mice as BMP2 compensated for its deficiency (Solloway et al., 1998). BMP2 and 6 as well as the BMP receptors are induced during osteogenic differentiation of the MSCs (Elabd et al., 2007).

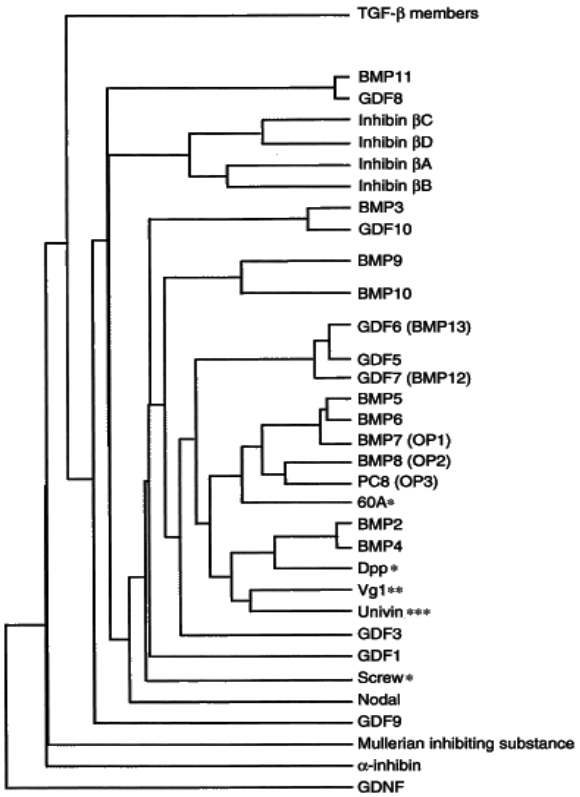


Fig.1.25. Phylogenetic relationships between bone morphogenic protein family members; adapted from Ducy and Karsenty, 2000 (Ducy & Karsenty, 2000).

BMPs as members of TGF-β family exert their functions as dimers. Some heterodimers, such as BMP2/BMP6 and BMP2/BMP7, have shown 5-10 times potency compared to BMP2 homodimers (Israel et al., 1996).

Although BMPs have a role in the differentiation of MSCs , the dose, duration and method of delivery are yet to be determined before clinical application (Luyten et al., 2001).

The TGF-β members display a range of biological activities that control cell proliferation, migration and differentiation in addition to the regulation of extracellular matrix deposition (Proetzel et al., 1995). TGF-β has diverse effects on cell proliferation depending on the cell type. TGF-β inhibits the cyclin dependant kinases and the *c-myc* gene, which leads to the arrest of the human osteosarcoma cell line MG63 as well as mouse keratinocytes in the G1 stage. In contrast, TGF-β upregulates the same gene in mouse embryo derived AKR-2B cell line (Leof et al., 1986; Alexandrow & Moses, 1995; Alexandrow et al., 1995; Matsuyama et al., 2003). TGF-β1 and 2 are present in the bone marrow stroma at a concentration of 0.1 mg/kg dry weight. In addition, TGF-β members have divergent effects on bone formation. They are powerful stimulators of bone formation *in vivo* induced by subcutaneous injection adjacent to bone surface (reviewed in (Mundy et al., 2001)). However, TGF-β1 opposes the action of BMP2. TGF-β1 inhibits the activity of the early osteogenic marker ALP without affecting its mRNA expression, while directly inhibits the expression of the late developmental marker osteocalcin as well as the BMP2 induced calcification. Thus, TGF-β1 antagonizes both the early and late effects of BMP2 (Katagiri et al., 1994). In addition, TGF-β1 enhances the BMP2 induction of Runx2 as both factors act on the same responsive element in the Runx2 promoter. Runx2 mRNA is induced after one hour of stimulation but this effect rapidly declines as Runx2 itself acts negatively on the same promoter region (Lee et al., 2000; Spinella-Jaegle et al., 2001). Thus TGF-β1 may enhance the early development of osteogenic lineage but it will have negative effect on the developed and/or late developing cells. For example TGF-β1 inhibits matrix mineralization (Heberden et al., 1998; Spinella-Jaegle et al., 2001). TGF-β 1, 2 and 3 are expressed by the human articular chondrocytes and are essential for suppression of cartilage maturation (Zuscik et al., 2004). TGF-β 3 appears more important in the early differentiation process of endochondral bone

formation (Schmid et al., 1991) in addition to its important role in the active growth and differentiation of chondroblasts (Millan et al., 1991) as well as the chondrogenic differentiation of MSCs in micromasses (pellet culture system) (Mackay et al., 1998) .

TGF-β binds to extracellular receptors which have two different types of intracellular serine/theronine kinases, termed type I and II receptors. Upon ligand binding, the two receptors form a dimer and the constitutively active Type II receptor activates the intracellular domain of the type I receptors which is either ALK1 in endothelial cells or ALK5 in other cells (reviewed in (Matsuyama et al., 2003)). This is followed by activation of the Smad pathway as well as Smad-independent MAP kinase pathways (Derynck & Zhang, 2003). TGF-β activates Smad 2, 3 and 4 while BMPs activate Smad 1, 5 and 8 (Nakao et al., 1997; Miyazono et al., 2001). Phosphorylated Smad 2 and 3 form a heterodimer that would complex with Smad 4. The whole complex is then translocated into the nucleus to activate gene transcription (Nakao et al., 1997). Although Smad 2 and 3 have distinct targets according to their DNA binding region, both of them play a role in the process of differentiation (Zhang et al., 1999; Garcia-Campmany & Marti, 2007).

TGF-β inhibits BMP3b production, which is induced by BMP2 during osteogenesis induction (Hino et al., 1999). Interestingly, 5-aza-cytidine induces a shift of the signalling dominance in articular chondrocytes, from TGF-β pathway to BMP pathway. 5-aza-cytidine represses Smad 2 and 3 while enhances the expression of Smad 1 and 5; in addition 5-aza-cytidine induces smurf2, which catalyses Smad 2 and 3 as well as TGF-β type I receptor ubiquitination and degradation (Zuscik et al., 2004). 5-aza-cytidine upregulates the oncoproteins Ski/Sno. These proteins bind to the Smad 2, 3 and 4 complexes and consequently inhibit their function; in addition Ski/Sno recruit histone deacetylase which would cause transcriptional repression (Liu et al., 2001; Wu et al., 2002).

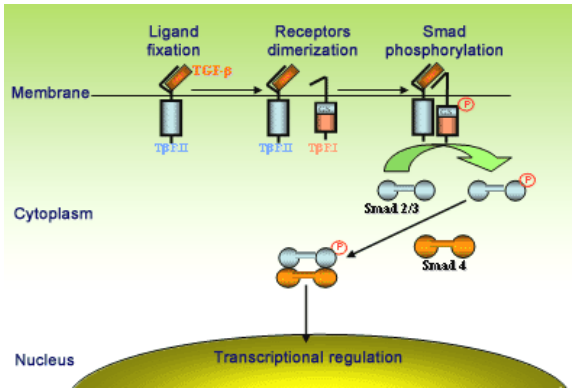


Fig.1.26. TGF-β pathway; as the ligand binds to the receptor, type I and II receptors dimerize and the intracellular domains phosphorylated Smad 2 and 3 which combines with Smad 4 and translocate into the nucleus to activate gene transcription. (http://tgfbetafaresse.ifrance.com/pages/pathway.html)

TGF-β illustrates the interaction of growth factors, transcription factors and epigenetic modifiers. The collagen I promoter is induced by TGF-β, at least in part, through inducing SP1. TGF-β induced Smads binds to the promoter of collagen I, which has three TGF-β response elements. Thus, the physical interaction of SP1 with the Smad molecules is mandatory for the gene activation (Inagaki et al., 1994; Zhang et al., 2000). TSA inhibits collagen I synthesis at the level of gene transcription. The signalling pathway was not affected but the induction of SP1 by TGF-β had not occurred. This effect was reversed by SP1 transfection (Ghosh et al., 2007). Furthermore, SP1 is essential for the TGF-β receptor II expression and 5-Aza-dC enhanced the production of this receptor through increasing its mRNA transcription as well as the protein level of SP1 (Venkatasubbarao et al., 2001).

Different members of the TGF β superfamily are expressed at different time points during bone repair and thus have different roles in the differentiation process. BMP2 and GDF8 are maximally expressed on the first day after the fracture while GDF5, TGF-β 2 and TGF-β 3 are maximally expressed on days three to seven when collagen II expression reaches its peak during the cartilaginous stage of the repair. On day 14 and up to day 21 the expression of BMP3, BMP4, BMP7 and BMP8 is restricted when the resorption of calcified cartilage and osteoblastic recruitment takes place. Interestingly, TGF-β 1, BMP5, BMP6 and GDF-10 were constitutively expressed from day 3 to day 21 (Cho et al., 2002; Gerstenfeld et al., 2003).

1.3.5.2. Wnts

Wnts are secreted proteins that act in the same cell in different ways according to their concentration (de et al., 2004). The Wnt signalling pathway is involved in the early embryonic organogenesis as well as fracture repair. In contrast to the BMP and FGF pathways, which affect the differentiation of both osteogenic and chondrogenic lineage, the Wnt pathway inhibits chondrocyte differentiation and promotes bone formation (Day et al., 2005; Tsiridis & Giannoudis, 2006). This canonical pathway is transduced through inhibiting the phosphorylation of the β-catenin protein, which accumulates in the cytoplasm, translocate to the nucleus and consequently activates target genes (reviewed in (Day et al., 2005)).

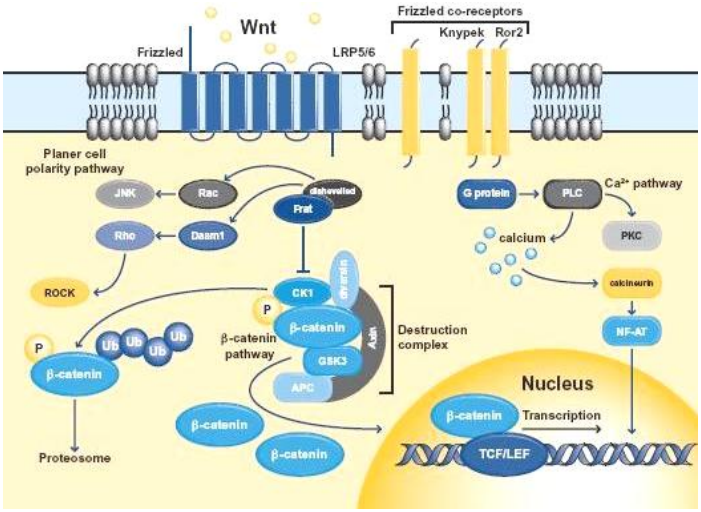


Fig.1.27. Wnt pathway; as the ligand binds to the ftizzled receptor, a series of events takes place in the cytoplasm, with final inhibition of β-catenin phosphorylation. Unphosphorylated β-catenin accumuates in the cytoplasm and then translocated to the nucleus to activate target genes. (http://www.abcam.com/cms/displayImage.cfm?intImageID=17993)

Activating the Wnt pathway in human MSC has been shown to oppose the effect of osteogenic media at low concentration of the stimulus; neither the earlier marker ALP nor the late occurring mineralization was induced and the cells kept their stem cell-like character. This effect is reversed by higher concentrations of Wnt pathway proteins or agonists (de et al., 2004).

1.3.5.3. Fibroblast growth factor

Fibroblast growth factor (FGF) is actually a family of growth factors that play a critical role in the bone and cartilage development (Mansukhani et al.,

2000; Ornitz & Marie, 2002). FGF receptor 2 is expressed in the developing limbs, while the expression of receptor 1 and 2 persists in the perichondrium and periosteum. TGF-β as well as FGF2 can upregulate FGF2 transcription which induces osteogenesis through TGF-β pathway activation (Nakamura et al., 1995; Matsuyama et al., 2003). FGF induces proliferation of bone precursor as well as immature osteoblasts while it causes apoptosis of mature cells (Dunstan et al., 1999; Mansukhani et al., 2000). Both FGF-1 and 2 enhanced the proliferation of bone precursor after two weeks of intravenous injection in young and old rats (Mayahara et al., 1993). FGF-1 doubled the bone density in overctomized rats and induced extensive new bone formation after six months of the surgery (Dunstan et al., 1999). FGF2 enhanced osteocalcin expression and inhibited collagen I expression in osteoblasts (Ornitz & Marie, 2002). In addition, the FGF pathway activates Sox-9 as a part of the chondrogeneis differentiation of stem cells (reviewed in (Tare et al., 2008)). Thus, FGF plays a role in both osteogenesis and chondrogenesis through activation of other factors such as TGF-β and Sox-9 respectively.

1.3.5.4. Platelet derived growth factor

Platelet derived growth factor (PDGF) enhances the chemotaxis and division of many cells of mesenchymal origin including the periodontal ligament cells (Boyan et al., 1994). In addition, PDGF enhanced the osteoblast proliferation, migration and differentiation when loaded in chitosan sponge (Park et al., 2000). PDGF is regulated by TGF-β in a biphasic model, as low concentration results in upregulation while high concentration decreases the transcription of PDGF (Leof et al., 1986; Seifert et al., 1994; Matsuyama et al., 2003).

1.3.5.5. Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a potent angiogenic inducer that is responsible for the vascular invasion of the hypertrophic cartilage, which is followed by ossification (Gerber et al., 1999; Colnot & Helms, 2001). Osteoblasts secret VEGF during the fracture repair under the influence of BMP2 and Runx2 as angiogenesis is critical to support the newly

formed bone segment. The vascular invasion is facilitated by the secretion of matrix metalloproteinases 2, 14 and 23 in addition to the metalloproteinase 9 in the later stages of repair (reviewed in (Tsiridis & Giannoudis, 2006)).

1.3.5.6. Insulin-like growth factors

Insulin-like growth factors (IGF) are responsible for the initial events for bone remodelling and enhance the proliferation of osteoblast precursors. IGF-I and II stimulate bone cell mitogenesis and consequently collagen synthesis *in vivo* and *in vitro*. Knock-out experiments have shown the importance of both factors in the bone development as well as ossification (reviewed in (Mundy et al., 2001)).

1.3.5.7. Epidermal growth factor

Epidermal growth factor (EGF) has been shown to enhance the osteogenic differentiation of adipose tissue derived-stem cells through inducing the transcription of BMP2, BMP6 as well as BMP receptors. The osteogenic differentiation induced by the endogenous BMP panel (which is not induced) was superior to that induced by exogenous supplement of BMP2 (Elabd et al., 2007).

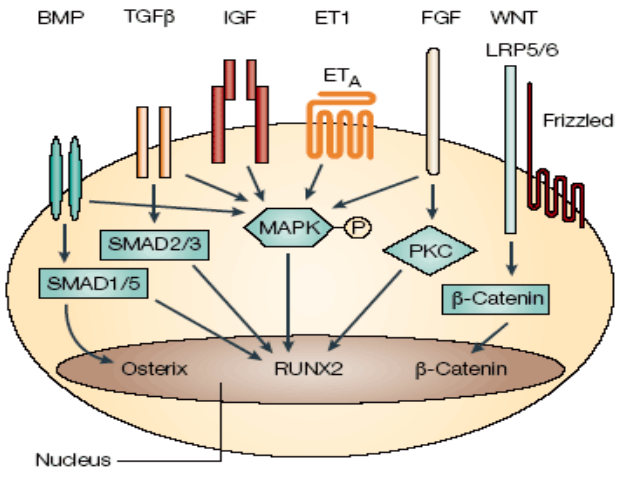


Fig.1.28. Diagrammatic representation of the main growth factor pathways, showing the key intracellular mediators such as Smad 1 and 5 with BMP, Smad 2 and 3 with TGF-β, protein kinase C (PKC) for FGF and β-catenin for Wnt pathway. p38 mitogen-activated protein kinase (MAPK) is a common target for BMP, TGF-β, insulin growth factors, endothelin-1 (ET1) and FGF. Runx is a downstream target for Smads, MAPK and PKC. Osterix is activated by BMP pathway (Logothetis & Lin, 2005).

1.3.6. Transcription factors involved in the differentiation process

1.3.6.1. Runt-related transcription factor 2; *Runx2*

Runx2, is also known as core binding factor alpha1 (Cbfa1) or osteoblast-specific transcription factor 2 (OSF-2), is the key transcription factor involved in the osteogenesis process (Komori et al., 1997). Runx2 is required for the early commitment of mesenchymal stem cells to differentiate into the osteogenic lineage. It regulates several osteogenesis specific genes including collagen I, bone sialoprotein, osteopontin and osteocalcin; hence the direct action of Runx2 is on maturation of osteoblast (Komori et al., 1997; Ducy et al., 1997). Homozygous Runx2 knockout mice typically die immediately after birth. These mice display a complete absence of endochondral or intramembranous bone formation. Heterozygous mice have hypoplastic bone formation. Runx2 was found to be constitutively expressed in stromal cell cultures but not in the non-cultured CFU-Fs (Satomura et al., 2000), which provides further evidence of the osteogenic commitment of this cell population. Runx2 is stimulated by different members of the TGF-β superfamily as well as FGFs and consequently activates the downstream osteogenic genes as well as other growth factors including the VEGF; hence Runx2 is pivotal in the commitment of mesenchymal cells along the skeletal cell lineage (Zelzer et al., 2001).

1.3.6.2. Distal-less homeobox 5; *Dlx5*

Dlx5 is another key regulatory transcription factor in bone induction. It is expressed predominantly during the late stages of bone differentiation enhancing the development of the mature bone phenotype (Ryoo et al., 1997). Dlx5 is induced by BMP2 and BMP4 and, in turn, stimulates the expression of Runx2, which consequently induces different osteogenic genes and enhances ALP activity. Dlx5 is not induced by ascorbic acid, dexamethasone, FGF-2 or other members of the TGF-β superfamily. Furthermore, TGF-β1 blocks the transcription of Dlx5 as TGF-β1 induces *c-jun* component of the AP-1 protein, which antagonise the BMP2 induction of Dlx5 (Lee et al., 2003).

1.3.6.3. Sex determining region Y-box 9; *Sox-9*

Sox-9 is a transcription factor that is expressed during skeletal development at the site of chondrogenic differentiation and is required for all stages of chondrocyte differentiation, except hypertrophy, whenever Sox-9 is switched off (Wright et al., 1995a; Ng et al., 1997a; Akiyama et al., 2002a). Sox-9 is a direct activator of the cartilage extracellular matrix proteins, such as collagen II (Bell et al., 1997; Ng et al., 1997a; Lefebvre et al., 1998), collagen XI (Bridgewater et al., 1998), collagen IX (Zhang et al., 2003) and aggrecan (Lefebvre et al., 1998). Sox-9 mutant (deleted) cells could not form cartilage and subsequent endochondral bone ossification (Bi et al., 1999; Akiyama et al., 2002b). The Sox-9 promoter has two SP1 sites, which play an important role in Sox-9 transcription. The inflammatory mediators responsible for osteoarthritis, Interlukin-1 and tumour necrosis factors, caused marked suppression of Sox-9 transcription through - at least for the interleukin - inhibition of SP1 binding to its particular site. In contrast, Sox-9 expression is up-regulated by FGF, which may play an important role in chondrogenesis (Murakami et al., 2000a; Murakami et al., 2000b; Piera-Velazquez et al., 2007). Sox-9 is also a down stream mediator of BMP2 and BMP4 signalling in bone cells (Zehentner et al., 1999). This transcription factor is also involved in the proliferation and differentiation of many extraskeletal tissues, including the central nervous system, neural crest, intestine, pancreas, testis, and endocardial cushions (Akiyama et al., 2005).

1.3.6.4. Specificity protein-1; *SP1*

SP1 is a ubiquitously expressed zinc finger transcription factor that is responsible for gene activation. SP1 is involved in mediating environmental stimuli, for example the gastrin gene activation by the epidermal growth factor (Merchant et al., 1995) and collagen I expression in response to oncostatin M (Ihn et al., 1997). SP1 recognizes a specific sequence (5’ GGGCGG 3’) in the promoter of the corresponding genes (Lania et al., 1997). SP1 activity is affected by the epigenetic status of the genes promoters. Methylated CpG adjacent to SP1 site attracts MeCP2, which binds to the SP1 and inhibits its activity (Kudo, 1998). Nevertheless, the carboxyl terminal of SP1 is a target

for HDAC1. SP1-HDAC1 complex repress the transcription of the target genes. This action is reversed by the effect of TSA, which binds to the HDAC1 and allowing co-activators such as E2F1 to bind to the carboxyl terminal of SP1 mediating its gene activating function (Doetzlhofer et al., 1999). 5-Aza-dC enhances the SP1 protein level, which stimulates the expression of the TGF-β receptors in different cancer cell lines (Venkatasubbarao et al., 2001). The SP1, mecp2, HDAC1, 5-Aza-dC and TSA interaction may provide another level at which epigenetic modifiers interact with the transcriptional mechanisms and possibly the differentiation process.

1.3.6.5. Osterix

Osterix is a zinc finger transcription factor that has great resemblance to the SP family, also known as SP7 (Milona et al., 2003). Osterix knock out mice have inadequate bone formation or mineralization. Osterix promotes the differentiation of osteogenic condensations and the maturation of osteoblasts while inhibiting the chondrogenic differentiation (Nakashima et al., 2002; Kaback et al., 2008). Although the presence of osterix and Runx2 together is essential for bone development, it is still unclear if osterix is induced by Runx2 or directly by BMP2 (Nishio et al., 2006; Kaback et al., 2008).

1.3.7. Markers of osteogenesis and chondrogenesis

1.3.7.1. Nucleostemin

Nucleostemin is a protein located in the nucleoli of stem cells as well as many cancer cell lines. It is a marker for undifferentiated BMSCs and its function is related to the regulation of cell proliferation through the tumour suppressor genes in late S and G2 phases of the cell cycle. When stem cells differentiate into more specialized cells, the level of nucleostemin has been shown to decrease markedly (Tsai & McKay, 2002; Kafienah et al., 2006).

1.3.7.2. Cellular myelocytomatosis oncogene; *c-myc*

c-myc is a nuclear DNA-binding phosphoprotein that is positively correlated with DNA synthesis and cell proliferation. It has a special

importance in the differentiation of the early mammalian embryos with special emphasis on the first few cleavages of the fertilized egg (Paria et al., 1992).

1.3.7.3. Collagen

Collagen is the most abundant protein in animals, makes up about 90-95% of the organic content of bone ; thus collagen is the main constituent of the bone matrix (Miller, 1984). At least ten different genes for collagens have been identified. Each is encoded for one of the three chains that make up the collagen triple-helix molecule, resulting in many subtypes of collagens. Different tissues have different collagen subtypes distribution. Collagen I is found predominantly in bone while collagen II is present mainly in cartilage (Miller, 1984). Collagen makes up two thirds of the dry weight of articular cartilage, with collagen II the main components, while collagen III, VI, IX - XII and XIV are present as well. Collagen III is detected in normal as well as osteoarthritic articular cartilage where it co-localizes with type II as a minor but regular component, while collagen X is present only in the thin layer of calcified cartilage at the bone attachment surface, as well as in hypertrophic chondrocytes in the growth plate (Linsenmayer et al., 1991; Eyre, 2002). Collagen III is concentrated in the bone in Haversian canal surface, around the hematopoietic cells, at the bone-periosteal interface and at the tendon-periosteal attachment. Collagen III consists 6% of the total collagen produced by bone cells (Reddi et al., 1977; Keene et al., 1991). Collagen III is formed earlier in the bone matrix and then its rate declines and the rate of collagen I synthesis increased. This switching is evident from a developmental perspective as well as in the fracture repair process (Reddi et al., 1977; Steinmann & Reddi, 1980).

1.3.7.4. Osteonectin

Osteonectin is one of the most abundant non-collagenous proteins in bone (Delany et al., 2003). Osteonectin has been shown to display a wide range of functions related to its role in inhibiting different growth factors such as TGF β, platelet derived factor and vascular endothelial growth factor. Thus it can be related to regulation of cell proliferation and modulation (Bradshaw &

Sage, 2001). Osteonectin null mice have shown a key role of osteonectin in osteoblast formation, maturation and survival (Delany et al., 2003). Osteonectin is detected in all osteogenic lineage from early osteoprogenitor cells to osteocytes (Hirakawa et al., 1994).

1.3.7.5. Osteopontin

Osteoponetin is another abundant non-collagenous protein in bone and adds physical strength to the extracellular matrix through binding to various proteins. Although being of less importance during the bone development, osteoponetin is necessary for bone remodelling (Denhardt & Noda, 1998).

1.3.7.6. Bone sialoprotein-II; *bsp-II*

Bsp-II is a non collagenous protein that function as the main nucleator factor for hydroxyapetite crystal formation (Hunter & Goldberg, 1994). Bsp-II is induced in newly formed osteoblasts, not the immature precursors (Bianco et al., 1991).

1.3.7.7. Phospho-regulating gene with homology to endopeptidases in the X chromosome; *PHEX*

PHEX is a cell surface metalloprotease that is involved in the regulation of phosphate metabolism (Quarles, 2003), which is expressed mainly in bone and teeth. PHEX was found in osteoblasts, osteocytes, and odontoblasts but not in osteoblast precursors, suggesting a level of maturity (Ruchon et al., 2000).

1.3.7.8. Osteocalcin

Osteocalcin is expressed at high levels only in differentiated osteoblasts (Hauschka et al., 1983) and odontoblasts, the tooth counterpart of osteoblasts (Akiyama et al., 2005). Osteocalcin is among the most abundant non-collagenous bone matrix proteins and forms 1-2% of total protein of bone (Gerstenfeld et al., 2003) . Osteocalcin appears in newly developing bone about the same time as the initial mineral phase matures into hydroxyapatite (Price et al., 1981). Osteocalcin function is related to the inhibition of

hydroxyapatite crystallization and thus slows down bone mineralization (Romberg et al., 1986). A recently discovered function is that osteocalcin plays a role in the regulation of insulin and adiponectin secretion by pancreatic beta cells and adipocytes repectively. These functions give the skeleton an endocrine function in glucose tolerance (Lee et al., 2007).

1.3.7.9. Alkaline Phosphatase (ALP)

ALP is an enzyme that is produced in bone during the developmental process. ALP is a membrane-anchored protein that modified through complex steps and finally transported to the cell membrane; these intracellular events are thought to be the target of the TGF-β1 inhibition of ALP activity (Spinella-Jaegle et al., 2001). ALP splits pyrophosphate, which is an inhibitor of mineralization, to provide inorganic phosphate, which is required as part of the mineralization process. ALP is used *in vitro* as a marker to follow cell development along the osteogenic lineage (Holtorf et al., 2005; Mirmalek-Sani et al., 2006b). The ALP activity typically becomes significantly higher in BMSCs cultured in osteogenic conditions compared to cells cultured in basal conditions after 6 days, though activity decreases after 12 days and is not detectable at 18 days when bone nodules are calcified (Akbari et al., 2003).

Although these markers should be expressed at the molecular level and the protein level in differentiating skeletal cells, studies to date have not been able to show all of them. The standard is ALP activity in fixed cells, while in molecular levels, BMP2, osteoponetin and bone sialoprotein-II are the only markers that significantly change during the differentiation of BMSCs in osteogenic conditions (Frank et al., 2002). BMP2 can induce the expression of both osteopontin and bone sialoprotein-II, which together organize the extracellular environment for new bone formation (Lecanda et al., 1997).

1.3.8. Applied Perspective of Regenerative Medicine

The use of stem cells as the basis of regenerative medicine has already taken place in few cases. One example was the construction of a segment of the mandible. Subtotal mandibulectomy for ablative tumour

surgery had created a defect more than 7 cm in size. Three dimensional computer tomography helped in the design a titanium mesh, which was then filled with ten bone mineral blocks coated with BMP7 to act as a scaffold. 20 ml of non-selected bone marrow aspirate - from the patient's iliac crest - were mixed with natural bone mineral of bovine origin prior to transplantation. The titanium mesh cage was then implanted into a pouch of the right latissimus dorsi for 7 weeks. Afterwards the transplant with an adjoining part of the muscle containing the adjoining artery and vein was excised and fitted into the defect with blood vessels anastomosis. Bone developed at the site of defect and the patient was then able to undertake some mastication (Warnke et al., 2004).

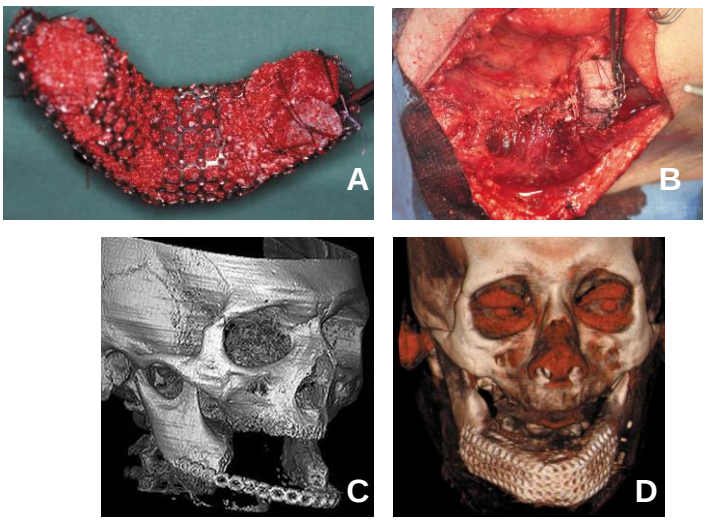


Fig.1.29. Construction of a segment of the mandible using regenerative medicine approach. Initially 3D computer tomography was done to estimate the size of the defect (C), then a titanium mesh cage was designed and filled with bone mineral blocks infiltrated with recombinant human BMP7 and bone-marrow mixture (A), implantation into right latissimus dorsi muscle (B) and finally implanted into the defect (D); adapted from (Warnke et al., 2004).

The advantage of the BMSCs is their low immunogenicity. Human BMSCs have been shown to remain competent in a xenogenic model. Intraperitoneal injection of human BMSCs in fetal lamb (in utero) was associated with the presence of mature cells carrying the human antigens in most of the tissues and organs in 28 of the 29 of the study sheep for the whole study duration (13 months). The injection of cells took place either before or after the development of the immune system (Liechty et al., 2000).

This immune competence could be - at least partially - because human BMSCs express class I human leukocyte antigen, but not class II (Pittenger et al., 1999a).

Fetal neuron cells were transplanted to Parkinson's patients under local anathesic. The cells transferred to each patient had been extracted from one fetus aged 7-8 weeks post conception. No immunosuppression regimen was implemented in any of the patients. The final results after more than a year of follow up were a) survival of the cells and b) improvement of patients below 60 years old in terms of radiological evidence and clinical manifestations (Freed et al., 2001).

Strategic directions in tissue engineering include the understanding of cellular machinery, biomarkers, cell-environment interactions and assembling complex tissues. The strategy ambition is to achieve broad clinical success of tissue engineering by the year 2021 (Johnson et al., 2007).

1.4. Aim of the study

The aim of this study was to improve osteogenic and chondrogenic differentiation of human skeletal stem cells in order to understand skeletal tissue formation and possibly, provide new stratiges for tissue regeneration.

1.5. Objectives of the study

1- To explore the use of epigenetic modifiers on the differentiation of skeletal stem cells along the osteogenic and chondrogenic lineages.

2- To study the effects of a 3D environment on the skeletal cells differentiation process.

3- To compare the differentiation potential in 3D environment on the fetal derived cells and adult derived BMSCs as sources of skeletal stem cells.

MATERIALS AND METHODS

2. Materials and Methods

2.1. Samples

2.1.1. Bone marrow stromal cells

Bone marrow stromal cells were isolated from bone marrow samples obtained from patients who were undergoing hip replacement as a consequence of fracture of the neck of femur or osteoarthritis. All samples were used with the approval of the Southampton Hospital Ethics Committee and after obtaining the patient's consent. During hip replacement operation, the bone medulla is drilled in order to place the metal prosthesis. A cancellous bone segment is removed from the marrow cavity, which is a rich source of bone marrow cells including the stromal cells. Cells were separated from the bone segment by repeated shaking with Dulbecco modified Eagle's medium (D-MEM; Invitrogen Ltd, cat. no. 10829-018) until the colour of the bone segment changed from red to white, due to the depletion of red blood cells. The cell suspension was centrifuged at 250g for 4 minutes at 4 ^{o}C. The pellet of cells was re-suspended in minimum essential media Eagle's alpha modifications (α-MEM; Sigma-Aldrich Ltd, cat. no. M0644). Cells were cultured in α-MEM, 10% fetal calf serum (FCS; Invitrogen, cat. no. 10106169) and 100 U/ml penicillin and 100 U/ml streptomycin (p/s; Sigma-Aldrich Ltd, cat. no. P4333). Media were changed on all samples twice weekly and cultures were kept under 5% CO_2 at 37^{o}C in humidified air. At confluence, cells were washed twice with phosphate buffered saline (PBS; Fisher Scientific, cat. no. BR014G). To separate the cells from the plastic, they were incubated with trypsin for 5 minutes at 37 ^{o}C. The trypsin stock (Sigma-Aldrich Ltd, cat. no. T4174) was diluted as 500 mg in 100 ml of PBS. 1 ml of diluted trypsin was used to replace 5 ml of medium. The enzyme activity was deactivated by 10% FCS in D-MEM. Cells were centrifuged, re-suspended and cultured again. BMSCs in passage 1 (P1) were utilized in all experiments.

2.1.2. Fetal derived cells

FC were obtained from aborted fetuses with ethical approval under the recommendations of the Polkinghorne Committee, 1989. The recommendations included obtaining separate clinical and research consent,

the lack of financial or commercial incentive for the donor, and the need for the research consent to be acquired by individuals remote from the planned laboratory experiments.

The fetal femur was dissected and all attached tissues removed. Cells were obtained as an explant from the primary bone or after digestion with collagenases (Mirmalek-Sani et al., 2006b). The femurs were incubated overnight with 5 mg/ml collagenase B (Roche Diagnostics Ltd., cat. no. 1-088 831) in α-MEM at 37 ^{o}C, then the cells were centrifuged and separated from the remaining bony structures by 70 μm cells strainer (VWR, cat. no. 402031604). Cells were cultured under 5% CO_2 at 37^{o}C in humidified air.

2.1.3. Primary Chondrocytes

Primary chondrocytes were used as positive control in studies of chondrogenic gene expression. Chondrocytes were isolated from the articular cartilage covering the femoral head, which had been removed during hip arthroplasty and donated for research. Articular cartilage was isolated from areas distant from the weight bearing area. Small pieces (~3 mm^3) were incubated at 37^{o}C with trypsin for 30 minutes, followed by hyaluronidase type I-S from bovine testes (Sigma-Aldrich Ltd, Cat. no. H3506) at concentration of 451 units/ml for 15 minutes and then collagenase B (Roche Diagnostics, cat no. 11088807001) of 15 units/ml for 17 hours and finally deactivated in media containing 10% FCS. Sample was centrifuged at 250*g* at 4 ^{o}C for 4 minutes. The pellet was washed with PBS and used for DNA and RNA extraction and subsequent PCR studies.

2.2. Cell Culture

2.2.1. Monolayer cell culture conditions

Basal conditions consisted of α-MEM, 10% FCS and 100 U/ml p/s. Osteogenic conditions included the basal conditions with addition of 100μM ascorbate-2-phosphate (Sigma-Aldrich Ltd, cat. no. A8960) and 10nM dexamethasone (Sigma-Aldrich Ltd, cat. no. D4902). Chondrogenic conditions

comprised of α MEM with 100 U/ml p/s, 10ng/ml TGF β3 (VWR, cat. no. PF073-2), 10 µM dexamethasone, 100 µM ascorbate-2-phosphate and 1 X insulin, transferrin & selenium (ITS; Sigma-Aldrich Ltd, cat. no. I3146) at the concentrations of 1.0 mg/ml insulin from bovine pancreas, 0.55 mg/ml human transferrin and 0.5 µg/ml sodium selenite.

2.2.2. Pellet Culture

Cells in the living body do not exist in monolayers, rather the cells have spatial conformation, which allow cell-cell and cell-matrix interaction and consequently enhance the differentiation process. The pellet culture system (Mackay et al., 1998; Pittenger et al., 1999b; Lee et al., 2001; Tare et al., 2005) is a model for three dimensional cell culture. Pellet culture followed initial culture in monolayer, in order to obtain enough cells and to introduce possible treatments. Confluent cells in monolayer were washed twice with PBS, incubated with collagenase IV (Sigma-Aldrich Ltd, cat. no. C5138) at the concentration of 0.2 mg/ml for 45 minutes at 37^{o}C which facilitated the disintegration of the extracellular collagen secreted by the cells, followed by another incubation for 5 minutes with trypsin. Both enzymes were added at 1 ml/ 25 cm^{2} of the flask surface area. The action of both enzymes was stopped 10% FCS in D-MEM. By the action of both enzymes, cells were obtained as individual cells rather than a monolayer sheet. Cells were passed through a 70 µm cells strainer to remove any remaining cell clamps, counted and then centrifuged at 250g for 4 minutes. Equal number of cells were transferred to 30 ml concave-end polystyrene universal tubes (Greiner Bio-One Ltd., cat. no. 201152), and incubated with 1 ml of media. Tubes were centrifuged at 400 g for 10 min and then incubated at 37^{o}C, 5% CO_2 with loose caps to allow gas exchange. The cells formed a sphere within 24 to 48 hours. The pellets became firmer after about a week. Media were changed three times per week until the end of the culture period.

2.3. Histological studies

2.3.1. Pellet Fixation

At the end of the culture period, the pellets were washed twice with excess PBS and then fixed with 4% paraformaldhyde and kept at 4^{o}C for at least 24 hours. Pellets were then dehydrated in increasing concentrations of ethanol (50%, 90% and 100%) for 30-60 minutes on a roller-mix, followed by staining with Eosin Y (Fisher Scientific UK, cat. no. 21298-0250) for 5 minutes. Eosin Y, an acidic dye, stains the basic areas of cytoplasm and is usually used as a counterstain as 0.5% in methanol. Eosin Y gave the pellets an orange- red colour that facilitated the visualization of the small pellets. The dehydration of the pellets continued with 100% ethanol (60 minutes), then 50% ethanol and 50% chloroform (60 minutes). Pellets were processed through 100% chloroform for 90 minutes twice, followed by wax embedding. The wax blocks were kept at 4 ^{o}C, then sections were cut at 7µm and mounted on superfrost glass slides (Fisher Scientific UK, cat. no. MNJ-700-010N).

2.3.2. Fixation of cells in monolayer

To stain for ALP activity or to immuno-stain for collagen I, cells were washed twice with PBS, and then 95% ethanol added. The cells were incubated at -20 ^{o}C for 15 minutes, then the ethanol was removed and plates were left to dry. When dry, PBS was added to the wells and plates were kept in 4^{o}C.

2.3.3. Histological staining

For dewaxing and hydration, sections were passed through Histoclear (Fisher Scientific UK, cat. no. H/0468/17), a xylene derivative, for 7 minutes twice, then through decreasing concentration of methanol (Fisher Scientific UK, cat. no. M400017) - from 100% to 50% (2 minutes each) into water. Staining for proteoglycan-rich cartilage matrix was carried out using 0.5% Alcian blue and 1% Sirius red for collagenous matrix (A/S staining). The first staining step was nuclear staining with Weigert's haematoxylin (Fisher Scientific UK, cat. no. H001046) for 10 minutes, followed by a wash in running

water bath and differentiation in Acid/Alcohol (20 ml of concentrated hydrochloric acid (VWR, cat. no. 101256J) in 2 litres of 50% methanol) to remove non-specific staining, followed by another rinse for 5 minutes in running water. The Alcian blue stain was prepared by dissolving 1.5 gm of Alcian blue 8X (Fisher Scientific UK, cat. no. 40046-0100) in 300ml of dH_2O with 3 ml acetic acid (Fisher Scientific UK, cat. no. A/0400/PB17). Sections were stained in Alcian blue for 10 minutes followed by quick rinse in running water. Then they were stained in 1% molybdophosphoric acid (Fisher Scientific UK, cat. no. M565048) in dH_2O, for 10 minutes and then directly into Sirius red stain for 60 minutes, which was prepared by adding 0.3 gm Sirius red F3B (Gurr-BDH, cat. no. 34149) in 100ml saturated picric acid (Gurr-BDH, cat. no. 29554) and 200 ml of dH_2O. Sections were rinsed in running water and dehydrated in increasing concentrations of methanol followed by Histoclear. Slides were mounted with dibutyl phthalate xylene (DPX)(LISON, 1954) (Fisher Scientific UK, cat. no. M/D110/08).

Safranin O (Sigma-Aldrich Ltd, cat. no. S2255) is a cationic dye that binds to the polyanionic proteoglycan in the cartilage matrix (Rosenberg, 1971). Sections were de-waxed and hydrated. Nuclei were stained with Weigert's haematoxylin for 10 minutes as above and counter-stained with 0.001% fast green (Gurr-BDH, cat. no. 34030) for 5 minutes followed by a dip in 1% acetic acid then stained with Safranin O (0.1% in dH_2O) for 5 minutes, which stained the proteoglycan with an orange-red colour. Slides were mounted with DPX.

The Von-Kossa stain was used to identify mineralized matrix, which stained black. Sections were de-waxed and hydrated, treated with 1% Silver Nitrate (Sigma-Aldrich Ltd, cat. no. S6506) and exposed to ultraviolet light for 20 minutes, then rinsed in running water prior to incubation in 2.5% Sodium Thiosulphate (Gurr-BDH, cat. no. 302364F) for 8 minutes. The sections were counterstained with Alcian blue for 1 minute, rinsed and stained with Van Giessen stain, which is 180mg Acid Fuchsin (Sigma-Aldrich Ltd, cat. no. F8129), 100 ml saturated picric acid and 100 ml distilled water, for 5 minutes.

Van Giessen stain is a counter-stain that identifies collagen and give a purple colour. Finally, sections were mounted in DPX.

2.3.4. Immuno-staining

Immuno-staining is useful procedure for localizing the distribution of specific proteins as well as for the comparison of the amount of specific proteins between different groups. Sections were dewaxed and hydrated. The endogenous activity of hydrogen peroxidase was quenched by incubating the sections with 3% Hydrogen Peroxide (H_2O_2, Sigma-Aldrich Ltd, cat. no. H1009) for 5 minutes. Non-specific binding was blocked by 5 minutes incubation with 1% bovine serum albumin (BSA; Sigma-Aldrich Ltd, cat. no. A3294) in PBS. Then sections were incubated with the diluted primary antibody overnight at a pre-determined concentration at 4^{o}C. On the next day, sections were rinsed in running water and kept for 5 minutes in each of the wash solutions: (1) high salt solution, which was 23.35 gm NaCl (Sigma-Aldrich Ltd, cat. no. S7653), 6.05 gm Tris (Sigma-Aldrich Ltd, cat. no. T1503), 0.5 ml of 0.05% Tween (Sigma-Aldrich Ltd, cat. no. P1504) in 1000 ml of dH_2O; (2) low salt solution, which was 8.7 gm NaCl, 6.05 gm Tris, 0.5 ml of 0.05% Tween in 1000 ml of dH_2O and (3) Tris solution, which was 6.05 gm Tris, 0.5 ml of 0.05% Tween in 1000 ml of dH_2O. The pH was adjusted to 8.5 for the three wash solutions. The next step was incubation for 60 minutes with the corresponding biotin labelled- secondary antibody, followed by washing in running water and the three salt solutions. Extravidin peroxidase (Sigma-Aldrich Ltd, cat. no. E2886) was applied for another 60 minutes, followed by the washes. Finally, the substrate was prepared in stages. Initially 0.01 gm of 3 amino-9-ethyl-carbazole (AEC; Fisher Scientific UK, cat. no. 14787-0050) was added to 1250 µl of dimethylformamide (VWR, cat. no. 23469.298). From that solution, 500µl was added to 5 µl of 30% H_2O_2 and 9500 µl acetate buffer (0.3 ml acetic acid and 0.7 mg Sodium Acetate (Sigma-Aldrich Ltd, cat. no. S7899) in 70 ml dH_2O). Slides were covered with the substrate for 10 minutes and the reaction was terminated in running water and counter-stained in Light green (Fisher Scientific, cat. no. 22977-0100) and Alcian blue, one minute each. The Light green stain was prepared by dissolving 0.6 gm light green,

0.6 ml acetic acid and 300 ml dH_2O. Slides were mounted using glycerol (Sigma-Aldrich Ltd, cat. no. GG1).

For all antibodies, positive controls (sections of rat femur) were used for the validation of the immuno-staining, as most of the used antibodies cross-react with both human and rat antigens. Negative controls were sections that had been processed in all the immuno-staining steps, without adding the primary antibody, in order to determine the non-specific staining. All antibodies were diluted with 1% BSA in PBS.

Type I collagen (LF67 (Fleischmajer et al., 1990), a generous gift from Dr Larry Fisher, NIH, Bethesda, USA; 1:300), Type II collagen (Calbiochem cat. no. 234187; 1:500), von Willebrand factor (vWF; Dako, cat. no. A0082; 1:200), Osteonectin (LF8(Pacifici et al., 1990), a generous gift from Dr Larry Fisher, NIH, Bethesda, USA; 1: 200), BSP(Chen et al., 1991)(a generous gift from Dr Jaro Sodek, MRC Group in Periodontal Physiology, Toronto, Canada; 1:200) and Sox-9 (Chemicon cat. no. AB5535; 1:150) are polyclonal antibodies raised in rabbits. The secondary antibody was an anti-rabbit IgG biotin-conjugated antibody (DAKO, cat. no. E0432; 1:200). Osteocalcin was assayed using a monoclonal antibody (Abcam, cat. no. ab13418; 1:100), that had been raised in mice and the secondary antibody was biotin-conjugated anti-mouse IgG (Sigma-Aldrich Ltd, cat. no. B7264; 1:100).

Pre-treatment with hyaluronidase type I-S from bovine testes (Sigma-Aldrich Ltd, cat. no. H3506 451 units/ml, used as 0.8 mg/ ml of 1% BSA in PBS for 20 minutes at 37^{o}C) was used for collagen II immuno-staining to expose the isotope. Antigen retrieval was undertaken before Sox-9 immuno-staining by boiling the slides in 0.01 M citrate buffer (Sodium citrate, Sigma-Aldrich Ltd, cat. no. S1804, in dH_2O) for 5 minutes in a microwave on the highest power. Slides were then placed immediately in distilled water to cool and then into PBS at room temperature.

2.4. Molecular biology techniques

2.4.1. DNA and RNA extraction

DNA and RNA were extracted using 'All Prep DNA/RNA Mini Kit' (Qiagen, cat. no. 80204). This kit used a column –dependant method to purify the genomic DNA and total RNA simultaneously (Fig.2.1). The cells were harvested with trypsin, which was deactivated by 10% FCS in D-MEM. Cells were then centrifuged for 4 min at 250g to remove the media. The cell pellet washed thoroughly with PBS, centrifuged again and PBS was removed. The pellet was de-bulked by vortex with the lysing buffer and the pellet particles passed through a 0.6 mm gauge needle attached to an RNase free syringe 5 times at least, to ensure homogenization. The cell lysate was loaded into the All Prep DNA spin column, where DNA selectively bound to the column while was kept in 4 ^{o}C until the next step. The lysate was then passed through the RNeasy spin column where RNA was selected. DNase (Qiagen, cat. no. 79254) was added before the final washes to remove any possible contamination of genomic DNA (gDNA). RNA and DNA were eluted separately. RNA was eluted in RNase free water, while DNA was eluted in TE buffer.

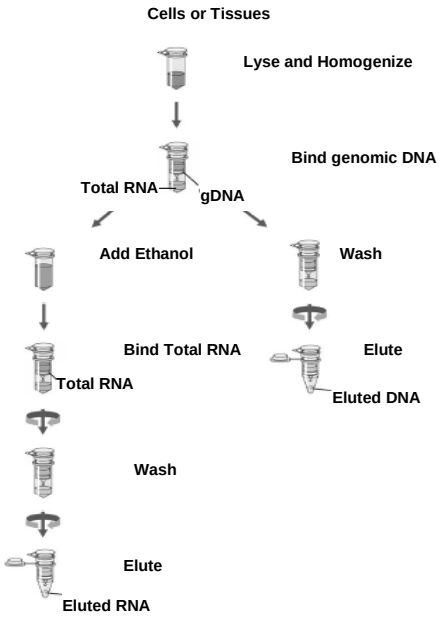


Fig.2.1. Diagrammatic representation of the DNA/RNA extraction scheme by 'Qiagen All Prep DNA/RNA Mini Kit'. (http://www1.qiagen.com/literature/handbooks/literature.aspx?id=1000529)

2.4.2. cDNA synthesis

Reverse transcription (RT) into complementary DNA (cDNA) was carried out immediately after RNA extraction. 14µl of RNA was denatured at 70^{o}C for 5 minutes. Then 7.5 µl of the master mix (prepared from Promega® reagents) was added directly into each denatured RNA tube. The master mix consisted of 1.0 µl of AMV reverse transcriptase in 4.0 µl of 5X specific buffer (cat. no. M5108) with 2.0 µl of nucleotide mix (cat. no. C1141), 1.0 µl oligo-dT primer (cat. no. C1101), 1.0 µl random primers (cat. no. C1181) and 0.5 µl ribonuclease inhibitor (cat. no. N2111). The reaction started with initial denaturing of the hot-start polymerase for two minutes at 94 ^{o}C, followed by synthesis of the complementary strand at 42 ^{o}C for an hour and then the temperature was raised to 95 ^{o}C for four minutes to destroy the enzyme and thus terminate the reaction.

2.4.3. The conventional polymerase chain reaction (RT-PCR)

The PCR reaction was carried out with 23 µl of Platinum® PCR SuperMix (Invitrogen, cat. no. 11306-016), 1µl of cDNA and 0.5 µl of each primer to achieve a final concentration of 400 µmol. Cycling conditions were initial heating at 94^{o}C for 2 minutes to activate the DNA polymerase enzyme (hot start), followed by cycles of 90^{o}C for cDNA denaturation, 55 ^{o}C for 1 minute for annealing with the primers and 72^{o}C for 2 minutes for the extension of the newly synthesized DNA. Thirty cycles were applied for all primers pairs except collagen IX and XI (35 cycles). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Primers for Sox-9, Sox-9- promoter and c-MYC were designed using the DNASTAR Lasergene 6 software. Other primers (shown in table 2) were available for use within the Bone and Joint group.

PCR products were mobilized by electrophoresis on 1% agarose gel, prepared as 1 gm of agarose (Invitrogen Ltd. cat. no. 15510-027) in 100ml dH_2O and visualised by 5 µl ethidium bromide (Sigma-Aldrich Ltd. cat. no. E1510) as this is a fluorescent dye that intercalates between the double

stranded DNA, and can be visualized by Ultraviolet light (Rigby et al., 1977). The electric current was set to 100 mV, 5 mA for 45 minutes.

Table 2. List of primers used in RT-PCR

Gene	Primer Sequence	Amplicon
Aggrecan	F: 5' ccc gct acg acg cca tct g 3' R: 5' cgg gcg gta gtg gaa gac g 3'	427bp
ALP	F: 5' cgc cgc cta cgc cca ctc 3' R: 5' cgt tca ccg ccc acc acc 3'	759 bp
c-*myc*	F : 5' cca tag cag cgg gcg ggc act ttg 3' R: 5' ctc gtc gtc cgg gtc gca gat gaa 3'	569 bp
Collagen I	F: 5' acc cac cga cca aga aac c 3' R: 5' ggc acc atc caa acc act g 3'	525 bp
Collagen II	F: 5' aga tgg ctg gag gat ttg atg 3' R: 5' cca gga gca cca gca gat 3'	732 bp
Collagen IX A1	F: 5' gcc cag cca gac cac cga cga g 3' R: 5' ggc ctg gat atc ctg agc gac ctg 3'	494 bp
Collagen XI A2	F: 5' ggg ccg acc tgt ccg ctt cct gt 3' R: 5' tgg ggt cct ggc tct ggg gtt cct 3'	458bp
GAPDH	F: 5' cga gat ccc tcc aaa atc aa 3' R: 5' atc cac agt ctt ctg ggt gg 3'	330 bp
Nucleostemin	F: 5' taa cca agc gtg tga agg caa aga aga atg 3' R: 5' caa gat gta ggg gga tgg caa tag taa 3'	579 bp
Osterix	F: 5' ttc tcc cat tct ccc tcc ctc 3' R: 5' ggg agc aaa gtc aga tgg gta gg 3'	751
PPAR-γ	F: 5' cca gaa agc gat tcc ttc ac 3' R: 5' cca aca gct tct cct tct cg 3'	633 bp
RUNX2	F: 5' cca taa ccg tct tca caa atc c 3' R: 5' ggt agt gag tgg tgg cgg 3'	656 bp
Sox-9	F: 5' gcg ccc cca gcc cca cca t 3' R: 5' agc cgc tcc gcc tcc tcg acg aa 3'	424 bp

2.4.4. The real time polymerase chain reaction

Real time PCR has made the quantitative PCR assay more reproducible, precise and sensitive (Frank et al., 2002). The system used was the 'Applied Biosystems 7500' with Power SYBR® Green PCR Master Mix (Applera UK, cat. no. 4368702). PCR assays were performed in 25µl using 12.5µl of Power SYBR® Green PCR Master Mix, 2.5µl each of forward and reverse primer (final concentration of 500nM for GAPDH and β-Actin, 300nM

for Sox-9 and 1µM for Runx2 and ALP), 6.5µl of ultra pure water and 1µl of sample cDNA per reaction. cDNA concentration was 100ng/µl, measured by the Thermo Scientific NanoDrop^{TM} 1000 Spectrophotometer. Cycling conditions were 50^{o}C for 2 min, 95^{o}C for 10 min, 45 cycles of 95^{o}C for 15 s and 60^{o}C for 1 min. Each reaction was performed in triplicate. Dissociation curves were obtained at the end of each run.

The quantification was performed using $2^{-\Delta\Delta}$Ct method (Livak & Schmittgen, 2001). This method depends on normalizing the cycle number (Ct) of the gene of interest to that of a housekeeping gene in the same sample at certain cut-off level to determine ΔCt value. Then one of the samples was set as a reference and all samples are subtracted from this particular sample to obtain ΔΔCt. The reference ΔΔCt will be zero. The final results are calculated as $2^{-\Delta\Delta}$Ct and represented as means and standard deviation, while the reference result will be one.

Table 3. List of real time primers

Gene	Primer Sequence	Amplicon
ALP (Abdallah et al., 2006)	F: 5' acg tgg cta aga atg tca tc 3' R: 5' ctg gta ggc gat gtc ctt a 3'	475 bp
β-Actin (Pedersen et al., 2001)	F: 5' tgt gcc cat cta cga ggg gta tgc 3' R: 5' ggt aca tgg tgc cgc cag aca 3'	433 bp
GAPDH	F: 5' cca ggt ggt ctc ctc tga ctt c 3' R: 5' tca tac cag gaa atg agc ttg aca 3'	108 bp
Runx2 (Abdallah et al., 2006)	F: 5' tct tca caa atc ctc ccc 3' R: 5' tgg att aaa agg act tgg tg 3'	230 bp
Sox-9 (Tew & Hardingham, 2006)	F: 5' ccc caa cag atc gcc tac ag 3' R: 5' gag ttc tgg tcg gtg tag tc 3'	101 bp
Sox-9 Promoter	F: 5' gga gtt gga gag ccg aaa gc 3' R: 5' tct ccg cgc cac tga agt 3'	59 bp

2.4.5. Restriction enzyme methylation study

The methylation sensitive restriction enzyme method (Pogribny et al., 2000; Moore et al., 2006) was used to analyze DNA methylation status. Restriction enzymes cut the DNA at a specific sequence. The methylation sensitive restriction enzymes can only cut the DNA when the cytosine base in

this defined sequence is not methylated. To determine the candidate enzymes, the promoter sequence was plotted into the restriction mapper website (http://www.restrictionmapper.org/) and compared against all known methylation sensitive restriction enzymes. In order to achieve restriction digestion a defined amount of PCR product was incubated with corresponding units of the enzyme in the appropriate buffers for 16 hours. The reaction was terminated by destroying the enzyme by overheating, followed by PCR.

For Sox-9, 10 µl of DNA (conc. 200ng/ µl) was incubated with enzyme Aci I (New England BioLabs, cat. no. R0551S) and 3.5 µl buffer 3 or 2 units of HPY 99I (New England BioLabs, cat. no. R0615S) and 3.5 µl buffer 4 with 0.35 µl BSA for 16 hours at 37^{o}C. The enzyme was deactivated at 65 ^{o}C. Negative controls were incubated without the enzyme.

2.4.5.a. Semi quantitative method

This method depends on the PCR protocol described in section 2.4.2, followed by gel electrophoresis. The presence of a band denotes failure of digestion, while no-bands means complete digestion of the PCR product. Hence, the absences of bands denote the absence of DNA methylation at the site cleaved by the enzyme. The advantage of this method is that larger amplicons can be used than for the quantitative method (see below).

The Sox-9 primers (F: 5' ccc tgg gac tgc tgt gct gtg att 3', R: 5' cgg cgg ctc ggg gac tcg 3') covered 405 bp (Fig.2.2). The DNA sequence was sensitive for two methylation sensitive restriction enzymes. The first was Aci I enzyme which recognize and cut at sequences C⌶CGC and GGC⌶C (where⌶ is the site of cleavage). There were 6 CpG sites cut by this enzyme in the segment. The second was Hpy99I which recognized and cut at the sequence⌶ CGWCG and GCWGC⌶(where W is either A or T) and only one CpG site was sensitive for this enzyme in the studied DNA segment.

2.4.5.b. Quantitative method

This method has been designed in our lab (Hashimoto et al., 2006) given the heterogeneous nature of methylation in any group of cells, especially stem cells during and after differentiation (Noer et al., 2006) .

The method depends on the combining the methylation sensitive restriction enzyme method with real time PCR technology to detect the digestion products. PCR primers were designed for real time PCR, using ABI® Primer Express software V 3.0 and spanned 59 base pairs (Fig.2.2). This DNA region contained two sensitive sites for the Aci I enzyme.

For quantification of the percentage of methylation, fully methylated DNA (Chemicon, cat. no. S7821) and non-methylated DNA, produced by the Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare Life Sciences, cat. no. 25-6600-30) were used. This DNA amplification kit amplifies the original DNA (conc. 10 ng/ µl) using non-methylated cytosine bases, which should produce non-methylated DNA. Master mix provided in the kit was incubated at 30 ^{o}C with previously denatured DNA (at 95 ^{o}C for 3 minutes) for 90 minutes. Mixing the methylated and non-methylated DNA produced a standard curve of different amounts of methylated DNA, which were 0, 20, 40, 60, 80 and 100%.

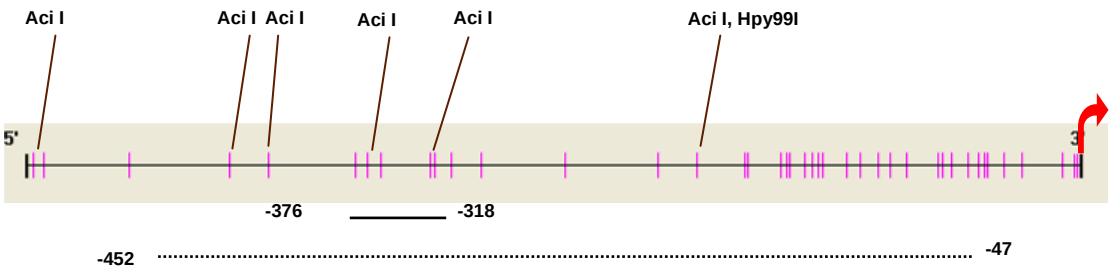


Fig.2.2. The CpG map for the Sox-9 promoter. Each longitudinal line represents a CpG site. The curved arrow shows the transcription initiation site. The solid bar indicates the promoter area covered by the real time primer pair and the dotted bar shows the product of the conventional primer pair. The CpG sites that are affected by the methylation sensitive restriction enzyme are labelled with the enzymes names.

2.5. Biochemical Analysis

2.5.1. Alkaline phosphatase assay

ALP activity was determined by two methods: cytochemistry as well as quantitative enzyme activity. Cells were fixed with ethanol (section 2.3.2). Fixed cells were incubated in the dark at 37^{o}C with Napthol As-Mx Phosphate (Sigma-Aldrich Ltd, cat. no. 855) diluted 25 times in dH_2O and mixed with 0.24 mg/ml Fast Violet B salt (Sigma-Aldrich Ltd, cat. no. F1631) for 60 minutes. The reaction was stopped by rinsing the wells with distilled water. Cells with ALP activity typically displayed intense red staining.

For biochemical quantification, Triton X (0.001%) (BDH, cat. no. 306324N) was added to the fixed cells and exposed to three freeze/thaw cycles, with scraping of the monolayer in between. Cells lysate were used to quantify the DNA content as well as the ALP activity. ALP activity was determined by incubating lysed cells for a recorded time with 100 µl of ALP buffer (Sigma-Aldrich Ltd, cat. no. A9226) plus 1 mg/ml 4-Nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich Ltd, cat. no. P4744) at 37^{o}C. 4-Nitrophenol solution (Sigma-Aldrich Ltd, cat. no N7660) was titrated in 0, 10, 50, 100 and 200 nmol/ml as a standard curve. The reaction was stopped by adding 100 µl 1 M sodium hydroxide (VWR, cat. no. 102525P) into the samples as well as the standards. The colour produced was read at the wavelength of 405 nm. The results were corrected for the DNA content of each well. Enzyme specific activity was expressed as nmol PNPP/hr/ng DNA.

2.5.2. DNA Picogreen Assay

DNA was quantified using the Picogreen dsDNA Assay (Invitrogen Ltd, cat. no. P7581). Samples were compared to a standard curve with known values, prepared by diluting herring sperm DNA (BDH, cat. no 100403-24-5) with TE Buffer (Promega UK Ltd., cat. no V4281). The standard curve included DNA at concentrations of 20, 100, 250, 500, 750 and 1000 ng/ml. TE buffer was set as blank. Samples were run in triplicates. Fluorescence intensity was measured at 485 nm (excitation) and 530 nm (emission).

2.6. Statistics

Statistical analysis was performed using SPSS V14.0 and Microsoft Excel 2003. Unpaired T test was used to determine the p-value and consequently the significant difference between two groups while ANOVA (analysis of variance) was used among several groups. A p-value of <0.05 was considered significant. Diagrams were produced by Microsoft Excel 2003 software. Bar chart with error bars were used to represent the average with standard deviations of different data sets.

Doubling time was calculated by subtracting the log of the initial cell number from the log of the final cell number. The result was multiplied by the constant 3.32 and divided by the time difference in hours. The inverse of that value represents the number of hours per cell doubling (http://www.scientistsolutions.com/t3035-doubling+time.html based on (Davis J, 2002)).

THE EFFECT OF 5 AZA DEOXYCYTIDINE ON THE DIFFERENTIATION OF BMSCS IN MONOLAYER CULTURES

3.1. Differentiation of BMSCs along the osteogenic and adipogenic lineages does not occur in all cells

BMSCs have the potential to differentiate into a number of mesenchymal lineages including osteogenic, chondrogenic and adipogenic lineages. However, the extent of such differentiation *in vitro* as well as the purity of the differentiated population remains questionable (Alexanian, 2007).

3.1.1. Hypothesis

Only a subpopulation of the cultured BMSCs change their morphology and gene expression, while the rest of the cells maintain the same characteristics as cells cultured under basal conditions.

3.1.2. Methods

BMSCs were isolated from an 87 year old male as detailed in section 2.1.1. Cells at passage one were divided and cultured under basal (control), osteogenic (section 2.2.1) and adipogenic conditions. The adipogenic conditions involved culturing the cells in monolayer with two alternate types of media. The first, complete adipogenic media, consisted of α-MEM with 3 g/L glucose (Sigma-Aldrich Ltd, cat. no. G7528), 10% FCS, with 0.5mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich Ltd, cat. no. I5879), 1µM Dexamethasone, 10µg/ml Insulin as Insulin, Transferrin, Selenium Supplement and 100µM Indomethacin (Sigma-Aldrich Ltd, cat. no. I7378). The second, incomplete adipogenic media consisted of α-MEM with 3 g/L glucose, 10% FCS, 10µg/ml insulin as insulin, transferrin and selenium supplement. The complete media was added for three days, then removed and cells washed twice with PBS, then the incomplete media was added for 24 hours. This cycle was repeated three times. RNA was extracted (section 2.4.1.), gene expression was assayed by PCR (section 2.4.2.), and PCR products were visualized by ethidium bromide incorporation followed by agarose gel electrophoresis.

3.1.3. Results

Cells cultured under osteogenic conditions showed increased ALP expression (Fig 3.1), although there was no apparent up-regulation of other osteogenic markers such as Runx2 or collagen I, or repression of the stem cell markers nucleostemin or *c-myc*. Cells cultured under adipogenic conditions produced fat globules in a few cells as evidenced by light microscopy (Fig 3.2). The cells expressed the adipogenic marker peroxisome proliferator activated receptor gamma (PPAR γ), which was completely absent in the control cells. In addition, the expression of the osteogenic marker ALP was observed in control as well as in cells cultured under adipogenic conditions.

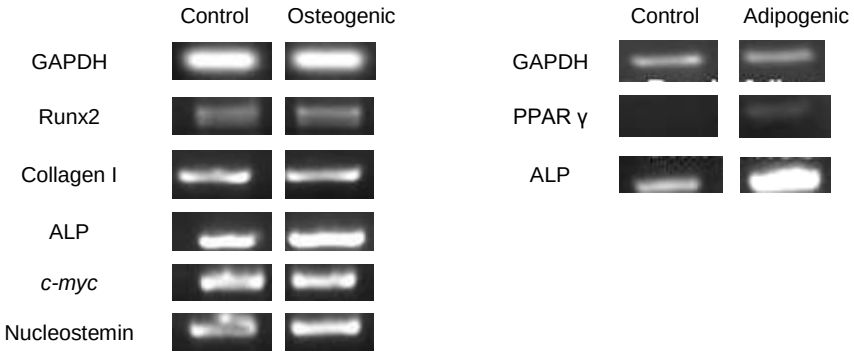


Fig 3.1. Gel electrophoresis for PCR products of cDNA of cells cultured under osteogenic and adipogenic conditions in comparison to control. The expression of GAPDH, Runx2, collagen I, *c-myc* and nucleostemin was comparable in cells cultured under osteogenic conditions and controls. Osteogenic conditions displayed enhanced expression of ALP. Adipogenic conditions showed up-regulated PPAR γ which was absent in control cultures, while enhanced the ALP expression was observed at the same time.

3.1.4. Discussion

These initial results indicate that BMSCs have: a) osteogenic potential even when cultured under basal or adipogenic conditions, b) respond incompletely to the culture conditions and c) maintain their stem cell characteristics. This potential of BMSCs has also been reported in 1991 by Caplan (Caplan, 1991). The incomplete conversion of these cells could be explained by the heterogenicty of the population of the bone marrow stromal cells and their natural propensity to develop along the osteogenic lineage

under the culture conditions employed, although diverse responses of different cells cannot be excluded (Abdallah & Kassem, 2009). Epigenetic silencing of certain developmental genes could be an important factor that prevents cellular differentiation in response to culture conditions; thus removing this silencing signal may help to enhance the differentiation of BMSCs.

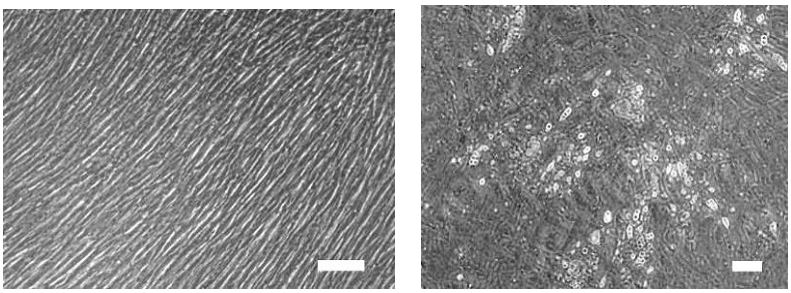


Fig 3.2. Photomicroscopy of the bone marrow stromal cells cultured under (A) basal conditions and (B) adipogenic conditions. Lipid droplets are evident in some cells, suggesting adipogenic differentiation in (B). This morphology was not present in all cells in (B), but was completely absent in (A).

Adipogenesis was used in this experiment as easily visualized evidence confirming the differentiation ability of BMSCs as well as the inefficiency of the differentiation protocols. Adipogenesis was not studied any further as it was outside the focus of this thesis.

3.2. 5-Aza-dC induced BMSCs to express a number of osteogenic and chondrogenic genes

DNA methylation is an important epigenetic mechanism for gene silencing during development (Jaenisch, 1997); thus, it may have a role in rendering the key genes unresponsive to culture conditions and consequently interfering with the differentiation of BMSCs. The effect of the DNA methylation inhibitor 5-Aza-dC therefore was investigated.

3.2.1. Hypothesis

5-Aza-dC is incorporated into the DNA of dividing cells, where it inhibits methylation, which may result in widespread activation of genes, including

osteogenic and chondrogenic genes, thus rendering the cells more responsive to culture conditions and consequently enhancing differentiation.

3.2.2. Methods

BMSCs were cultured in basal conditions or osteogenic conditions with and without prior treatment with 5-Aza-dC. DNA and RNA were extracted as detailed in section 2.4.1. Gene expression of both osteogenic and chondrogenic genes was analysed by RT-PCR (section 2.4.2.). Primary isolated chondrocytes were used as a control for chondrogenic gene expression. gDNA was used to determine the DNA methylation status of some CpG sites (Fig. 2.2) within the promoter of Sox-9 (the chondrogenic master transcription factor) using semi-quantitative methylation sensitive restriction enzyme analysis (section 2.5.4) with enzymes ACi I and Hpy 99.

3.2.3. Results

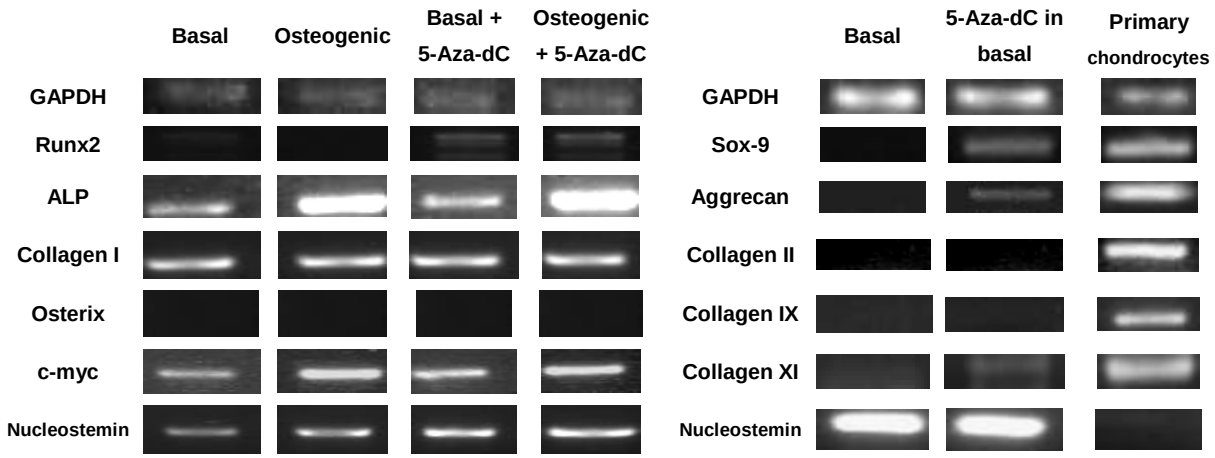


Fig. 3.3. Gel electrophoresis for PCR products of cDNA for cells cultured under basal and osteogenic conditions with and without prior treatment of 5-Aza-dC. 5-Aza-dC induced Runx2, Sox-9, aggrecan and collagen XI. ALP was induced under osteogenic conditions irrespective of 5-Aza-dC pre-treatment. No effect on the stem cell markers nucleostemin or *c-myc* was observed.

There was evidence of discrete osteogenic enhancement by 5-Aza-dC (Fig 3.3) as evidenced by induction of Runx2, the osteogenic master gene. ALP expression was observed in the controls, but was markedly enhanced under osteogenic conditions. 5-Aza-dC treatment resulted in a modest up-regulation of ALP under both basal and osteogenic conditions. Interestingly,

under basal conditions, 5-Aza-dC did not enhance ALP to the same extent as occurred under osteogenic conditions. The expression of collagen I or osterix was not up-regulated by 5-Aza-dC in any of the culture conditions examined. Unexpectedly, nucleostemin and *c-myc* expression were not downregulated as the cells differentiate.

The chondrogenic master gene Sox-9 was induced by 5-Aza-dC as well as the downstream chondrogenic genes aggrecan and collagen XI, although the expression of such genes was less than that observed in primary isolated chondrocytes. Despite being Sox-9 targets, collagen II and IX were not up-regulated. Collagen II in particular is the most abundant type of collagen in cartilage and it is expressed in developing chondrocytes. The expression of nucleostemin was unaffected by the 5-Aza-dC treatment and was completely absent in primary chondrocytes, as would be expected in fully differentiated cells.

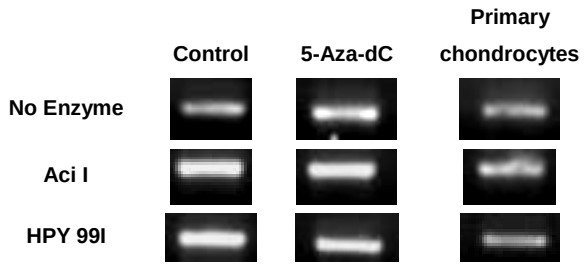


Fig. 3.4. PCR results for gDNA digestion by the methylation sensitive restriction enzymes. The presence of bands indicating the presence of methyl groups at the relevant CpG sites.

The effect of 5-Aza-dC on Sox-9 induction raised the question of whether the induction of expression by 5-Aza-dC was due to DNA methylation changes. To examine this further, gDNA was incubated with the methylation sensitive restriction enzymes Aci I or HPY 99I. By using this method, the methylation status of the Sox-9 promoter (mapped in Fig 2.2) following 5-Aza-dC treatment could be compared with the primary isolated chondrocytes. No differences were observed between the groups (Fig 3.4).

3.2.4. Discussion

Although BMSCs have the potential to differentiate along various stromal cell lineages, not every cell responded to the stimuli to the same extent. Some cells were refractory to treatment and maintained their stem cell-like characteristics. This result was supported by the persistence of nucleostemin and *c-myc* expression in all groups in spite of the increased ALP expression under osteogenic conditions and the induction of Runx2 and Sox-9 in the 5-Aza-dC treated groups. Other markers may be expected to be up-regulated either directly through promoter demethylation or indirectly by Runx2 or Sox-9. The absence of collagen I, II and IX as well as osterix induction can be explained by the absence of suitable culture conditions to promote further development. Runx2 affects osteogenic genes in a time-dependent manner and it is possible that the differentiating BMSCs in this particular study were at an early stage of their osteogenic development (Ducy et al., 1999). Similarly, the absence of collagen up-regulation in response to Sox-9 could be explained by an early stage of differentiation. Although Sox-9 is well defined as a transcription factor that plays a pivotal role in chondrogenesis (Wright et al., 1995b; Ng et al., 1997b; Akiyama et al., 2002b), it is also expressed in many other tissues (Akiyama et al., 2005).

Thus, the up-regulation of Sox-9 could be due to a mechanism that is unrelated to DNA methylation. Alternatively, the methylation sensitive restriction enzymes used in this study may not have targeted the crucial CpG sites in the Sox-9 promoter. Another consideration is that the percentage of differentiated cells was not sufficient to demonstrate differences in the methylation pattern. It is possible, therefore that a homogeneous subpopulation of the BMSCs may be more appropriate for both the methylation studies and for tissue regeneration as a whole.

3.3. 5-Aza-dC and TSA have negligible effects on the differentiation of Stro-1$^+$ enriched cell population

Stro-1$^+$ cells are a subpopulation of the BMSCs that present mainly in the stromal fraction of bone marrow whose osteogenic and chondrogenic potentials have been demonstrated (Dennis et al., 2002). The effects of the DNA demethylating agent 5-Aza-dC, the histone deacetylase inhibitor TSA and the combination of both agents on the differentiation potential of Stro-1$^+$ cells has yet to be determined.

3.3.1. Hypothesis

Stro-1$^+$ cells have the characteristics of osteogenic precursor cells. Changing the epigenetic profile of these cells using the modifiers alters the gene expression profile and consequently the differentiation lineage. 5-Aza-dC activates genes that are silenced by DNA methylation, while TSA activates genes that are silenced by histone deacetylation, which might affect DNA methylation as well. It is proposed that the combination of both factors will have an additive effect, especially on genes that have been silenced by two layers of epigenetic silencing.

3.3.2. Methods

Stro-1$^+$ cells were isolated from the bone marrow stromal cells by immuno-selection, using magnetic cell sorting (Howard et al., 2002). Cells were isolated from bone marrow (section 2.1.1.), suspended in 25 ml of media and laid over 20 ml of Lymphoprep (Axis-Shield Diagnostic Ltd, cat. no. 1114547), and centrifuged at 800*g* for 40 minutes at 18^{o}C in order to separate the red blood cells according to the density gradient at the bottom of the tube, i.e. below the lymphoprep. The buffy coat, containing the mononuclear cells, was separated and cells were incubated in the blocking buffer containing human albumin serum and bovine fetal serum for 30 minutes on ice, in order to block non-specific binding sites. Cells were then incubated with the Stro-1 monoclonal IgM antibodies (undiluted culture supernatant from the Stro-1 hybridoma provided by Dr J.Beresford, University of Bath) on ice for 60 minutes. Cells were then mixed with magnetically labelled rat anti-mouse IgM

micro beads (Miltenyi Biotec., cat. no. 130047301) for 15 minutes at 4^{o}C. Labelled cells were transferred to the Macs column (Miltenyi Biotec., cat.no.130041306) and placed in the magnetic field (Miltenyi Biotec., cat.no.130043102). The column was washed three times with Macs buffer to remove the non-labelled cells. Labelled cells were then removed from the magnetic field and washed again to obtain Stro-1^{+} cells.

Passage 2 cells were used for these experiments. The cells were treated with either 5-Aza-dC three times per week, a single application of the histone deacetylase inhibitor TSA or TSA initially followed by 5-Aza-dC three times per week, starting 72 hours after the TSA application. The known toxicity of the epigenetic modifiers limited their application to the described regimen. The concentration of 5-Aza-dC was 1µmol and of TSA 100 nmol. The cells were cultured for 4 weeks in basal conditions. No specialized media was used in this experiment, as the aim was to investigate the effects of the modifiers on gene expression of both osteogenic and chondrogenic genes. At the end of culture, RNA was extracted (section 2.4.1) and cells were fixed with ethanol and immuno-stained for collagen I.

3.3.3. Results

Stro-1^{+} cells, irrespective of treatment, expressed the osteogenic master gene Runx2 as well as the osteogenic marker ALP (Fig.3.5.) while collagen I expression was inhibited by TSA and relatively downregulated in the combined treatment group.

Sox-9 expression was present in the basal group although expression was not up-regulated by 5-Aza-dC, in contrast to non-selected BMSCs (section 3.2.3). In contrast, TSA inhibited Sox-9 expression, while the combined treatment displayed an intermediate expression between basal expression and TSA inhibition. The expression of aggrecan and collagen XI was similar in all groups while collagen IX was absent in all treated groups. Nucleostemin, a marker of stemness, was highly expressed in all groups and unaffected by any treatment examined. The increased intensity of GAPDH

with TSA treatment has been previously shown for GAPDH as well as other house keeping genes such as β-Actin and 18S rRNA (Mogal & Abdulkadir, 2006).

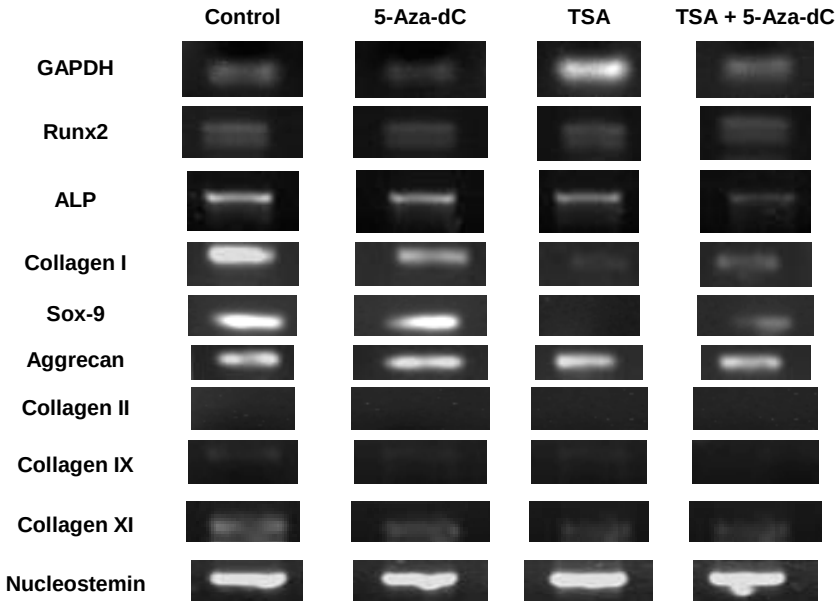


Fig. 3.5. Gel electrophoresis for PCR products of Stro-1^{+} cells cultured under basal conditions with and without 5-Aza-dC and/or TSA treatment. TSA induced GAPDH and inhibited collagen I. The expression of nucleostemin was comparable across all groups.

Immuno-staining for collagen I (Fig.3.6) revealed distinct staining in the control group and the 5-Aza-dC treated group. The staining was mainly inside the cells, suggesting an earlier stage of maturity, although staining can be found sparsely in the intercellular spaces. The TSA treated group was negative for collagen I expression while cells treated with combined treatment reflected an intermediate expression pattern.

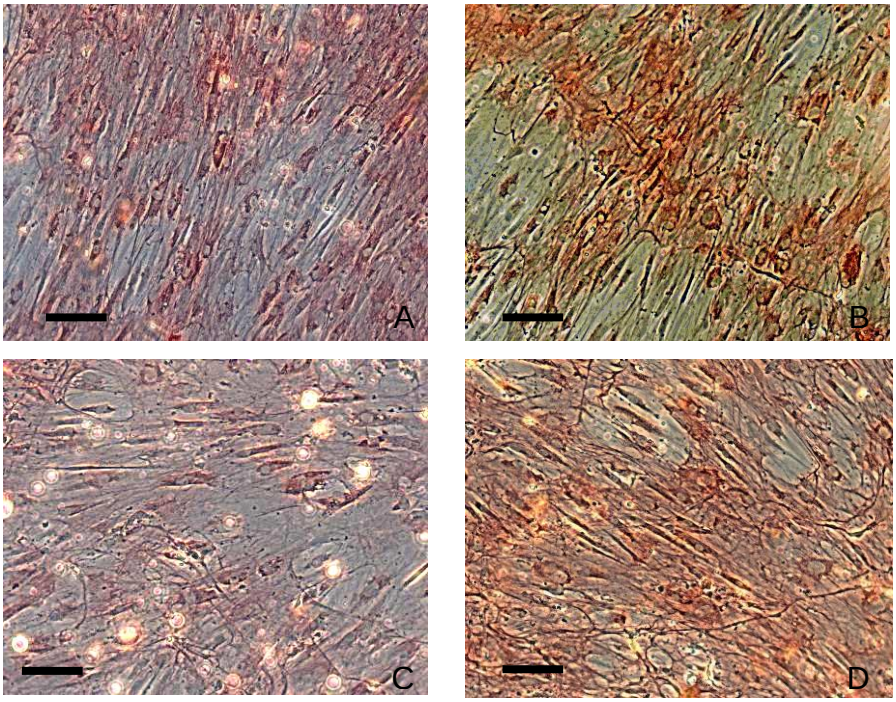


Fig.3.6. Photomicroscopy of collagen I immuno-staining of Stro-1$^+$ enriched population cultured in basal condition with no treatment (A), and treatment with 5-Aza-dC (B), TSA (C) and combination of treatments (D). The most intense staining was achieved with 5-Aza-dC while TSA inhibited collagen I and the treatment combination had an intermediate degree of staining. Scale bars = 100 μm.

3.3.4. Discussion

The gene expression profile confirmed the osteogenic potential of Stro1$^+$ cells, even when cultured in basal conditions. The Stro1$^+$ cell population could be refractory to the epigenetic modifiers, since the only effects were seen with TSA treatment. The downregulation of collagen I and Sox-9 by TSA was unexpected, as positive effects on gene expression by TSA were expected. A possible reason for this downregulation could be that under these conditions TSA activates a silencer or a repressor protein (Ghosh et al., 2007).

Alternatively, Stro1$^+$ cells may already be committed to the osteogenic lineage (Gronthos et al., 1994), as suggested by the persistence of Runx2 and ALP, which would mean that the epigenetic modifiers were not required for further activation of osteogenic genes. Interestingly although up-regulation of collagen I was absent, 5-Aza-dC enhanced staining for collagen I. In contrast, TSA inhibited collagen I expression at both the molecular and

histological level. Such an inhibitory effect has been shown by Ghosh et al, 2007 and was thought to be due to inhibition of the transcription factor SP1, which is required for collagen I expression. TSA induced GAPDH; an effect in agreement with previous findings (Mogal & Abdulkadir, 2006). The inhibition of collagen I and induction of GAPDH confirmed the effectiveness of TSA in this treatment regimen.

Furthermore, this study has shown that the effects of the 5-Aza-dC treatment varied depending on the cell population, as 5-Aza-dC induced Runx2 in BMSCs but did not upregulate the same gene in the Stro1^{+} selected populations. 5-Aza-dC may have removed an inhibitor or induced a stimulant in BMSCs. Thus, the Stro1^{+} cell population may not be the optimal cell type for testing the differentiation potential of epigenetic modifiers. The same treatment regimen could affect the differentiation of BMSCs in different ways, which has been shown for the 5-Aza-dC with respect to osteogenesis-related genes. The response of BMSCs to epigenetic modifiers, in particular with respect to chondrogenic genes has yet to be determined.

3.4. TSA and 5-Aza-dC up-regulate chondrogenic genes differentially in BMSCs

Stro1^{+} cells represent only 10% of the BMSCs population (section 1.3.3.3) and have the proprieties of osteoprogenitors. The effect of the epigenetic modifiers on the unselected population could be different from the Stro1^{+} subpopulation.

3.4.1. Hypothesis

Epigenetic modifiers have a different effect on unselected BMSCs compared with BMSCs enriched Stro1^{+} populations. The genes silenced by DNA methylation are activated by 5-Aza-dC, while those silenced by histone deacetylation are activated by TSA. The combination of both modifiers has synergistic effects.

3.4.2. Methods

Passage 1 BMSCs were used in this study. The cell culture conditions and treatment protocol were as described for the Stro1^{+} study (section 3.3.3). Cells were cultured in basal conditions to observe the direct effects of the epigenetic modifiers. Sox-9 was one of the genes of interest. gDNA was used for assessment of methylation study of the promoter of Sox-9 using a quantitative real time PCR based method (Hashimoto et al., 2007b) (section 2.5.4.b). Primers were designed to cover the area from -318 to -376 in the promoter region, which included two CpG sites that could be cleaved by the methylation sensitive restriction enzyme Aci I. This area was selected (with the primer design software) to be the most appropriate. The gene map is shown in fig. 2.2.

3.4.3. Results

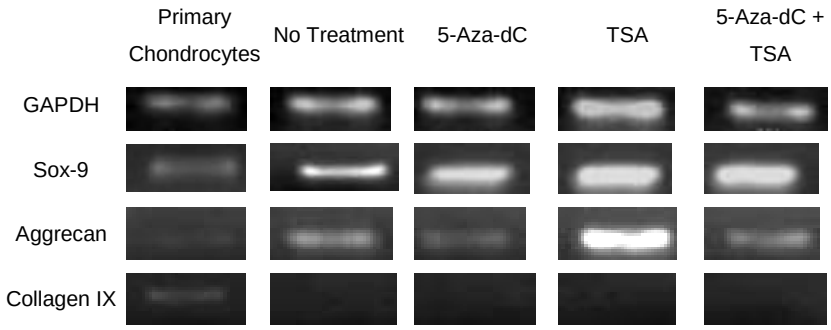


Fig. 3.7. The PCR products as shown on 1% gel electrophoresis. Sox-9 was induced by all epigenetic modifiers. TSA has up-regulated both GAPDH and aggrecan.

RT-PCR results (Fig.3.7) indicated that the housekeeping gene GAPDH was expressed in all groups, with relative up-regulation in the TSA treated group. The chondrogenic marker Sox-9 was expressed in all groups; with up-regulation with 5-Aza-dC, TSA and in combination. Aggrecan was also expressed in all groups; with highest expression after TSA treatment, and an enhanced expression with combined treatment. Collagen IX was expressed only in primary chondrocytes (positive control for chondrogenic genes).

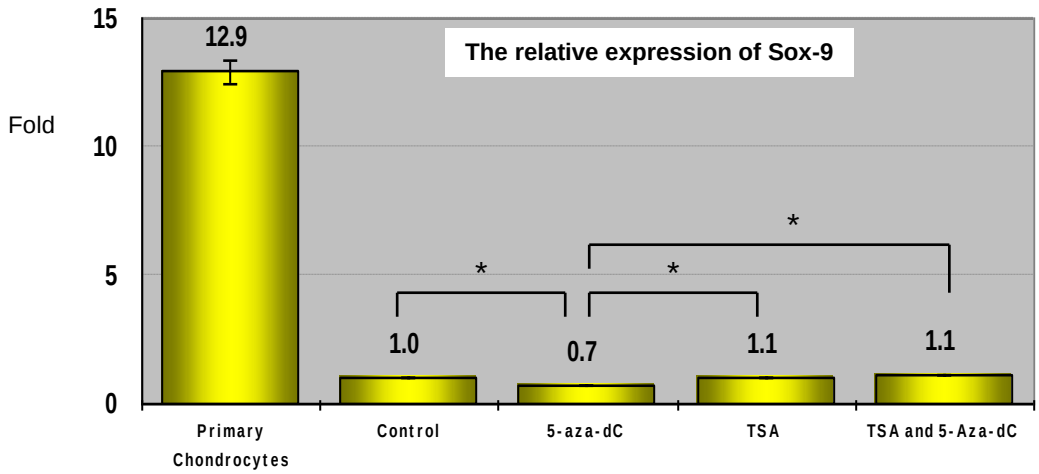


Fig. 3.8. The relative expression of Sox-9, corrected to the housekeeping gene GAPDH, in the study groups and primary chondrocytes. The study groups display comparable expression of the transcription factor while the primary chondrocytes displayed a 12.9 fold expression in comparison to the control. Anova test was significant. * p-value <0.05. Standard deviation bars were too small to be presented in most of the groups.

Sox-9, the chondrogenic master transcription factor, was induced in all treatments. Conventional PCR showed no obvious difference between the expression pattern in different groups; thus, real time PCR was used for relative comparison (Fig.3.8). BMSCs cultured without treatment has been set as reference; i.e. set to one. The Anova test showed significant statistical difference for the study groups (p-value < 0.001). While the primary chondrocytes displayed 12.9 fold higher expression in comparison to the reference, the TSA and combined treatment displayed similar relative values to the reference. 5-Aza-dC pre-treated cells showed Sox-9 expression only 0.7 time of the control. The expression in this group was statistically significant lower across other groups.

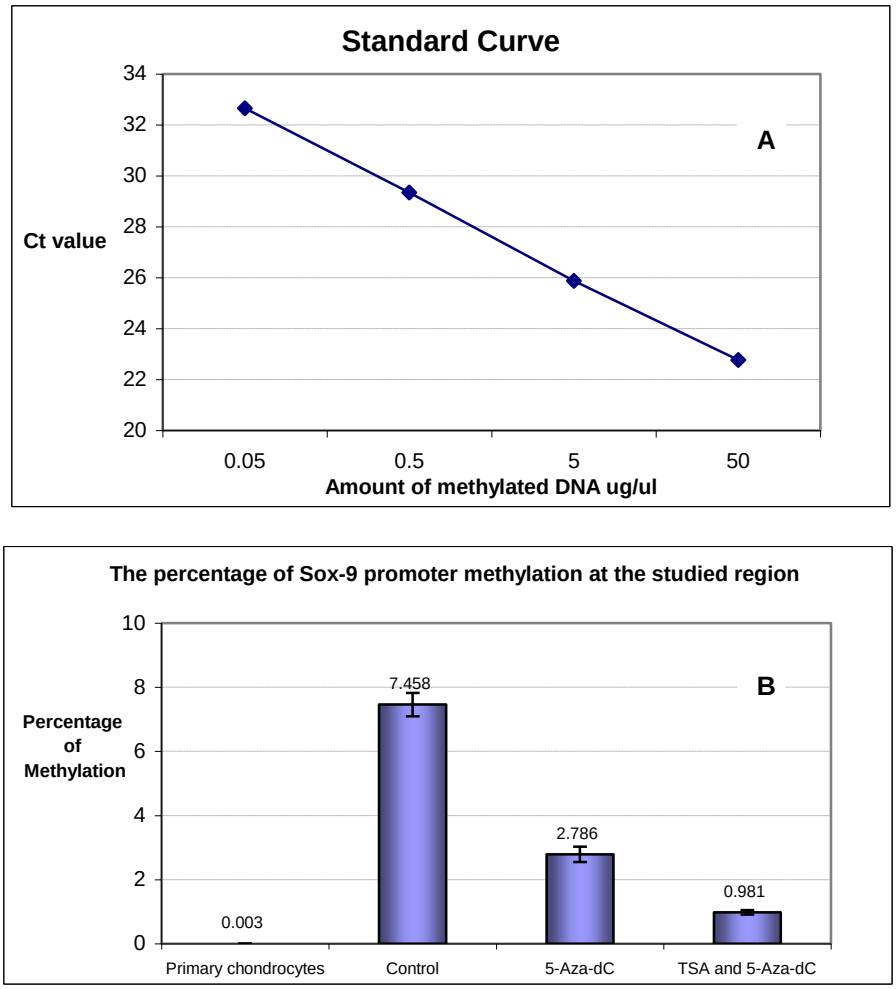


Fig.3.9. Quantitative assessment of the Sox-9 promoter methylation. (A) The standard curve illustrates the relation between the amount of methylated DNA in µg/µl to the mean of the C_t value (assayed in triplicates). Standard deviation presented as error bars. (B) The percentage of methylation of studied region of Sox-9 promoter. The methylation level was decreased with 5-Aza-dC treatment and lowered even further by the addition of TSA. Primary chondrocytes were almost completely demethylated. Anova test and t-test showed significant differences between the groups (p-value <0.001).

Quantification the methylation status of the Sox-9 promoter (Fig.3.9) at the two CpG sites -324 and -358 was performed using the real time standard curve method to detect the percentage of methylation after incubation with methylation sensitive restriction enzymes (Hashimoto et al., 2007b). The standard curve ranged from non-methylated DNA to fully methylated DNA. The control group displayed the highest methylation (7.5%); the 5-Aza-dC treated group methylation level was about one third of the basal group (2.8%), the combined TSA and 5-Aza-dC treatment was one third that of the 5-Aza-

dC treatment alone (1%) while the primary cartilage group displayed negligible methylation (0.003 %).

3.4.4. Discussion

BMSCs are a heterogeneous population of cells that express both osteogenic and chondrogenic markers. The cells responded to the two epigenetic modifiers in different ways. The changes in expression were modest, possibly due to the heterogenicity of the cells or the incomplete response of the cells to such modifiers. The up-regulation of aggrecan with TSA could be an early marker of TSA-induced chondrogenic differentiation.

There was a narrow range of methylation levels observed in all groups. Only two CpG sites (-324 and -358) were investigated. It is not known whether these sites were important for the epigenetic regulation of Sox-9. The comparable Sox-9 expression in all cultured groups could be related to the absence of significant differences in the methylation status of the gene promoter. Although there was a statistical significance in the percentage of methylation observed between the study groups, the actual difference range was narrow and such differences may not have physiological significance. The increased demethylation in the TSA (+ 5-Aza-dC) treated groups was in agreement with the Selker study in Neurospora (1998) (Selker, 1998). The author found that TSA-associated DNA demethylation could by explained by either hyperacetylation of the methylated regions or hyperacetylation elsewhere in the genome affecting the methyltransferases. This TSA-induced effect together with the up-regulation of aggrecan supports the notion that TSA stimulated the differentiation of the chondrogenic cell lineage.

The initial evidence for 5-Aza-dC enhancement of osteogenesis in BMSCs (section 3.2.) was demonstrated at the molecular level. To provide additional evidence, the effects on ALP activity were also investigated.

3.5. 5-Aza-dC enhances and preserves ALP activity in BMSCs cultured under osteogenic conditions

ALP activity is the one of the most widely used markers for osteogenesis (Holtorf et al., 2005; Mirmalek-Sani et al., 2006b). An enhanced activity of ALP would suggest that the transcript was translated into the protein and was able to exert its function, which indicating the differentiation of the cell towards the osteogenic lineage.

3.5.1. Hypothesis

BMSCs pre-treated with 5-Aza-dC display enhanced ALP activity in comparison to controls.

3.5.2. Methods

BMSCs, obtained from a 73 years old male, were cultured in basal conditions in monolayer until 50% confluence, and then serum was removed for 24 hours to synchronize cell division. The media were changed to osteogenic media (day 0) and 5-Aza-dC was added for three successive days at final concentration of 1μmol. Cultures were continued for 25 days. Cell groups were harvested twice weekly, assayed biochemically and/or stained for ALP activity.

3.5.3. Results

A gradual increase in the ALP activity (Fig.3.10) was observed in both treated and non-treated groups up to day 21. BMSCs pre-treated with 5-Aza-dC displayed enhanced ALP activity, evident by staining from day 14, while a statistically significant difference could be detected from day 21. At day 25, the difference in activity was profound. Under both methods, pre-treated cells displayed almost twice the activity of controls. In addition, no statistical difference could be found between non-treated cells at days 21 and 25.

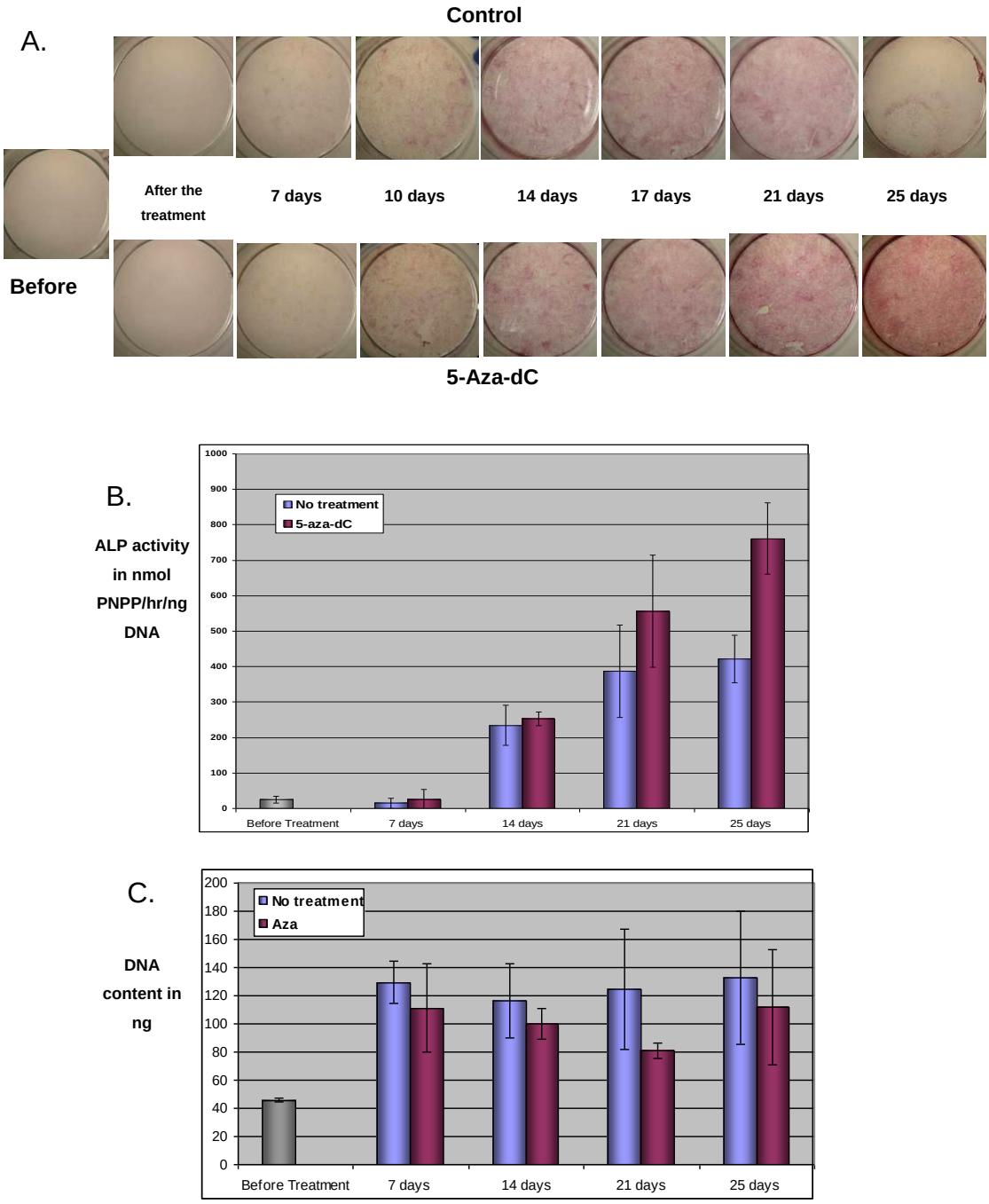


Fig.3.10. ALP staining (A) and activity (B) of BMSCs cultured in osteogenic conditions with or without initial treatment of 5-Aza-dC. Gradual increase in the intensity of ALP staining (A) in both the control and 5-Aza-dC pre-treated cells over 21 days. At 25 days, cells pre-treated with 5-Aza-dC showed a further increase in staining. The DNA content (C) showed no significant difference at any time points examined. The quantitative enzymatic activity, (B) expressed as nmol PNPP/hr/ng DNA, was significantly different as tested by ANOVA test.

Cell death in the controls, on day 25, was excluded by comparison with DNA content. There was no significant difference between the DNA content in the controls compared to 5-Aza-dC pre-treated cells or the control cells on

day 25 or any other time points. The absence of a significant difference in the DNA content of different groups starting from day seven (by ANOVA test) indicates the absence of proliferation, which may confirm the progressive induction of ALP with 5-Aza-dC pre-treatment.

3.5.4. Discussion

The increased ALP activity by 5-Aza-dC beyond 21 days is consistent with an increased stimulation of osteogenic differentiation. In addition, such enhancement continued beyond that obtained in controls. Long-term culture often resulted in loss of phenotype, possibly due to DNA hypermethylation (Allegrucci et al., 2007). 5-Aza-dC pre-treatment may prevent this, which may explain the sustained ALP activity.

The continuous increase of ALP activity in 5-Aza-dC pre-treated cells and not in controls cannot be explained by the maturity of the cells as ALP activity decreased with maturation. During long bone development, ALP activity can be found very early and is known to decrease with osteogenic matrix mineralization (Chentoufi et al., 1993). In monolayer cell culture, matrix formation was not obtained even with 5-Aza-dC pre-treatment. Thus, the effect of 5-Aza-dC in enhancing ALP activity may be extended over a longer time frame.

3.6. Molecular characterization of 5-Aza-dC pre-treated BMSCs cultured in osteogenic media for 25 days

The enhanced and sustained activity could denote the activity of a panel of genes, rather than individual genes, which may be responsible for transcription and translational changes, including ALP. Thus, activation of an osteogenesis pathway is more likely than activation of a single gene (ALP) by chance. A screening of gene transcription at that time point could confirm this hypothesis and provide clues about the genes affected by 5-Aza-dC in BMSCs cultured under osteogenic conditions.

3.6.1. Hypothesis

5-Aza-dC activates genes involved in osteogenic differentiation.

3.6.2. Methods

BMSCs, obtained from a 73-year-old male, were cultured in osteogenic conditions for 25 days with and without initial treatment with 5-Aza-dC (see section 3.4). At day 25, DNA and RNA were extracted. An osteogenic pathways array (SABioscience, cat. no. PAHS-026) was used to compare the expression of 84 osteogenic related genes (table 4). PCR arrays technology combines the ability of arrays to scan large number of relevant genes together with real time quantitative PCR accuracy. The osteogenic PCR array contains groups of genes involved in osteogenic growth, differentiation, matrix formation and mineralization and as well as cartilage condensation. The RNA was reverse transcribed to cDNA using the RT^2 first strand kit (SABioscience, cat. no. C-03), which was optimized for the PCR array system. According to the manufacturer's instruction, 1µg of RNA was added to 2.0 µl of the 5X gDNA elimination buffer. The mixture was made up to final volume of 10 µl and incubated at 42 ^{o}C for five minutes in the thermal cycler then chilled on ice for one minute. The reverse transcription (RT) cocktail contained 4 µl of 5x RT buffer, 1 µl of the mixture of primers and external control, 2 µl RT enzyme and 3 µl RNase-free water. 10 µl of the RT cocktail was added to the 10 µl RNA mixture after chilling and incubated at 42 ^{o}C for 12 minutes. The reaction was stopped by heating at 95 ^{o}C for 5 minutes. 91 µl of ultra pure water (UPW) was mixed well with cDNA and the tubes were kept on ice. The PCR array master mix included 1350 µl 2X SABioscience RT2 qPCR Master Mix (SABioscience, cat. no. PA-012-12), 108 µl cDNA and 1242 µl UPW. Using a multi-channel pipette, 25 µl of the master mix (containing the cDNA) were dispensed into each well of the PCR array plate. The array consisted of a 96 well PCR plate. Primers pairs were dispensed into corresponding wells and represented 84 target genes; shown in table 4. An Applied Biosystems 7500 PCR system was used for the reaction. Cycling conditions included an initial 10 minutes at 95 ^{o}C to activate the DNA polymerase followed by 40 cycles of

95 ^{o}C for 15 seconds and 60 ^{o}C for 1 minute. Dissociation curves were obtained.

The osteogenic PCR arrays contained 5 house keeping genes including beta-2-microglobulin, GAPDH, β Actin, Ribosomal protein L13a - which is a ribosomal protein that plays a role in the regulation of cell proliferation and is essential for cell survival (Chen & Ioannou, 1999) - and Hypoxanthine phosphoribosyltransferase 1 which is a key enzyme in the purine metabolism. In addition, other controls were used to monitor the technical quality, including:

a) control for genomic DNA contamination, which detects any gDNA that has not been removed during the RNA extraction process. The cut-off C_t for the gDNA control set by the manufacturer is 35. Thus, any sample that had C_t value for gDNA contamination control less than 35 should not be assayed.

b) Positive PCR control (PPC). These wells contain a pre-dispensed artificial DNA sequence with its corresponding primers. The aim is to detect the efficiency of the PCR reaction in general, including the master mix, the machine, carry-over contamination and handling errors; in addition any impurity of the RNA samples would have an effect. PPC C_t should be in the range of 18 to 22.

c) Reverse transcription control (RTC), which determines the efficiency of reverse transcription process and estimates the efficiency of the reverse transcription by the first strand kit (SABioscience, cat. no. C-03). These wells contain primers for a special template that should be synthesized as a control during the first strand synthesis. The difference between the average C_t for the RTC and PPC should be less than 5 cycles.

Table 4. The gene list of the osteogenic PCR array

GeneBank	Description	Gene Name
NM_001622	Alpha-2-HS-glycoprotein	A2HS/AHS
NM_000478	Alkaline phosphatase, liver/bone/kidney	ALP
NM_016519	Ameloblastin (enamel matrix protein)	AMBN
NM_001143	Amelogenin, Y-linked	AMGL/AMGY
NM_001154	Annexin A5	ANX5/ENX2
NM_199173	Bone gamma-carboxyglutamate (gla) protein (osteocalcin)	BGP/PMF1
NM_001711	Biglycan	DSPG1/PG-S1
NM_006129	Bone morphogenetic protein 1	PCOLLAGENC/
NM_001200	Bone morphogenetic protein 2	BMP2A
NM_001201	Bone morphogenetic protein 3	BMP3A
NM_130851	Bone morphogenetic protein 4	BMP2B
NM_021073	Bone morphogenetic protein 5	MGC34244
NM_001718	Bone morphogenetic protein 6	VGR/VGR1
NM_001742	calcitonin receptor	CRT/CTR
NM_000072	CD36 molecule (thrombospondin receptor)	CHDS7/FAT
NM_001797	Cadherin 11, type 2, OB-cadherin	CAD11
NM_000493	Collagen, type X, alpha 1(Schmid metaphyseal chondrodysplasia)	COL10
NM_080629	Collagen, type XI, alpha 1	CO11A1/COLLAGENL6
NM_004370	Collagen, type XII, alpha 1	COLLAGEN12A1L
NM_021110	Collagen, type XIV, alpha 1	UND
NM_001855	Collagen, type XV, alpha 1	FLJ38566
NM_000088	Collagen, type I, alpha 1	OI4
NM_000089	Collagen, type I, alpha 2	OI4
NM_001844	Collagen, type II, alpha 1	ANFH/AOM
NM_000090	Collagen, type III, alpha 1	EDS4A
NM_000091	Collagen, type IV, alpha 3	TUMSTATIN
NM_000093	Collagen, type V, alpha 1	COLLAGEN5A1
NM_000095	Cartilage oligomeric matrix protein	EDM1/EPD1
NM_000758	Colony stimulating factor 2	GMCSF
NM_000759	Colony stimulating factor 3	G-CSF/GCSF
NM_000396	Cathepsin K	CTS02/CTSO
NM_004407	Dentin matrix acidic phosphoprotein	Dmp1
NM_014208	Dentin sialophosphoprotein	DFNA39/DGI1
NM_001963	Epidermal growth factor	HOMG4/URG
NM_005228	Epidermal growth factor receptor	ERBB/ERBB1
NM_031889	Enamelin	ADAI/AIH2
NM_000800	Fibroblast growth factor 1 (acidic)	AFGF/ECGF
NM_002006	Fibroblast growth factor 2 (basic)	BFGF/FGFB
NM_005247	Fibroblast growth factor 3 (murine mammary tumour virus integration site (v-int-2) oncogene homolog)	HBGF-3/INT2
NM_015850	Fibroblast growth factor receptor 1	BFGFR/CD331
NM_000141	Fibroblast growth factor receptor 2	BEK/BFR-1
NM_002019	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	FLT/VEGFR1
NM_002026	Fibronectin 1	CIG
NM_004962	Growth differentiation factor 10	BMP3b
NM_000201	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	BB2/CD54
NM_000618	Insulin-like growth factor 1	IGFI
NM_000875	Insulin-like growth factor 1 receptor	CD221/IGFIR
NM_000612	Insulin-like growth factor 2	INSIGF
NM_181501	Integrin, alpha 1	CD49a/VLA1
NM_002203	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	BR/CD49B

GeneBank	Description	Gene Name
NM_002204	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	CD49C/GAP-B3
NM_000632	Integrin, alpha M (complement component 3 receptor 3 subunit)	CD11B/CR3A
NM_002211	Integrin, beta 1 (fibronectin receptor, beta polypeptide)	CD29/FNRB
NM_004897	Multiple inositol polyphosphate histidine phosphatase, 1	DKFZp564L2016/HIPER1
NM_002425	Matrix metallopeptidase 10 (stromelysin 2)	SL-2/STMY2
NM_004530	Matrix metallopeptidase 2 (gelatinase A)	CLG4/CLG4A
NM_002424	Matrix metallopeptidase 8 (neutrophil collagenase)	CLG1/HNC
NM_004994	Matrix metallopeptidase9 (gelatinase B)	CLG4B/GELB
NM_002448	Msh homeobox 1	HOX7/HYD1
NM_003998	Nuclear factor of kappa light polypeptide gene enhancer in B-cells1	DKFZp686C01211/EBP-1
NM_002607	Platelet-derived growth factor alpha polypeptide	PDGF-A/PDGF1
NM_000444	Phosphate regulating endopeptidase homolog, X-linked	HPDR/HPDR1
NM_004348	Runt-related transcription factor 2	AML3/CBFA1
NM_005505	Scavenger receptor class B	CD36L1/CLA-1
NM_001235	Serpin peptidase inhibitor	AsTP3/CBP1
NM_005900	SMAD family member 1	BSP1/JV4-1
NM_005901	SMAD family member 2	JV18/JV18-1
NM_005902	SMAD family member 3	DKFZp686J10186/HSPC193
NM_005359	SMAD family member 4	DPC4/JIP
NM_000346	SRY (sex determining region Y)-box 9	CMD1/CMPD1
NM_003154	Statherin	STR
NM_012143	Tuftelin interacting protein 11	TIP39
NM_000660	Transforming growth factor, beta 1	CED/DPD1
NM_003238	Transforming growth factor, beta 2	TGF-beta2
NM_003239	Transforming growth factor, beta 3	TGF-beta3
NM_004612	Transforming growth factor, beta receptor I (activin A receptor type II-like kinase)	AAT5/ACVRLK4
NM_003242	Transforming growth factor, beta receptor II	AAT3/FAA3
NM_000594	Tumour necrosis factor	DIF/TNF-alpha
NM_020127	Tuftelin 1	TUFT1
NM_000474	Twist homolog 1	ACS3/BPES2
NM_001078	Vascular cell adhesion molecule 1	CD106
NM_000376	Vitamin D (1,25- dihydroxyvitamin D3) receptor	NR1I1
NM_003376	Vascular endothelial growth factor A	VEGF/VEGF-A
NM_003377	Vascular endothelial growth factor B	VEGFL/VRF
NM_004048	Beta-2-microglobulin	B2M
NM_000194	Hypoxanthine phosphoribosyltransferase 1	HGPRT/HPRT
NM_012423	Ribosomal protein L13a	RPL13A
NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	G3PD/GAPD
NM_001101	Actin, beta	PS1TP5BP1
SA_00105	Human Genomic DNA Contamination	HIGX1A
SA_00104	Reverse Transcription Control	RTC
SA_00104	Reverse Transcription Control	RTC
SA_00104	Reverse Transcription Control	RTC
SA_00103	Positive PCR Control	PPC
SA_00103	Positive PCR Control	PPC
SA_00103	Positive PCR Control	PPC

Two PCR array plates were assayed for each sample. The data was uploaded into SABioscience analysis website (http://www.superarray.com/pcr/arrayanalysis.php). This data analysis tool, which is specific for PCR array data sets, depends on using the $2^{-\Delta\Delta}$Ct method and provides a robust analysis of the data for that number of genes. The control group was assigned as the reference and thus the results represented the changes of gene expression in BMSCs pre-treated with 5-Aza-dC to non-treated cells.

Data were finally presented as fold regulation; i.e. when 5-Aza-dC pre-treated BMSCs had equal or higher expression than the non-treated cells. The reference was set to one, while when the opposite was true, it would be present in the negative direction and thus the control would be presented as minus one. The fold regulation system has the advantage of demonstrating the relative down-regulation in an easily illustrated manner that aids in comparison. Two-fold up or down regulation was set as the cut-off level.

3.6.3. Results

From the 84 osteogenesis-related genes, only 19 showed more than a two fold regulation change. The affected genes could be divided into two categories. The first included the transcription and growth factors that drive the cells towards the osteogenic lineage while the second included typical osteogenic markers.

In the first group (Fig.3.11), up-regulation of different members of the TGF-β pathway such as TGF-β1, TGF-β2 and Smad3 was observed and downregulation (more than 10 fold) of TGF-β receptor 2 (TGF-βR2), which was constitutively expressed and active. Although the activation of the TGF-β pathway would enhance osteogenesis, TGF-βR2 downregulation might inhibit such an effect.

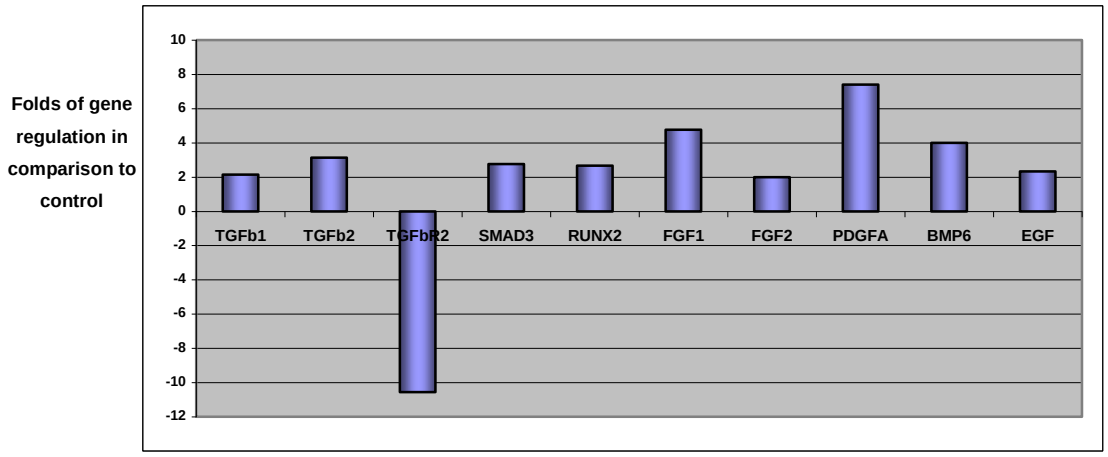


Fig. 3.11. Fold regulation of key growth and transcription factors affecting osteogenesis. TGF-β1 and 2, Smad 3, FGF-1 and 2, PDGFA and BMP6 as well as Runx2 were up-regulated in 5-Aza-dC pre-treated cells (over two fold) in comparison to controls. TGF-β receptor 2 was markedly downregulated.

There was also activation of the PDGF, FGF1 and FGF2, which could induce osteogenesis through the activation of TGF-β pathway (Leof et al., 1986; Nakamura et al., 1995). Thus, the up-regulation of TGF-β may be either a direct effect of 5-Aza-dC or an indirect effect via the activation of other pathways. From the BMP family only one member, BMP6, a powerful inducer of osteogenesis, was up-regulated (Solloway et al., 1998), although EGF has been shown to enhance osteogenesis through the up-regulation of BMP2, BMP6 as well as BMP receptors (Elabd et al., 2007).

Both TGF-β and BMP pathways would enhance osteogenesis through the activation of Runx2 (two-fold up-regulated). These results indicate that 5-Aza-dC was not specific to certain pathways, rather it up-regulated a number of pathways that could enhance the osteogenesis as a final result.

In the second group (the osteogenic marker genes), several genes were up-regulated by 5-Aza-dC pre-treatment including the fibrillar collagens, collagen III and collagen V. These collagens are found in bone together with collagen I (Fig.3.12). In addition, many fibrillar-associated collagens (FACIT collagens) that interact with collagen I such as collagen XII and XIV were up-regulated.

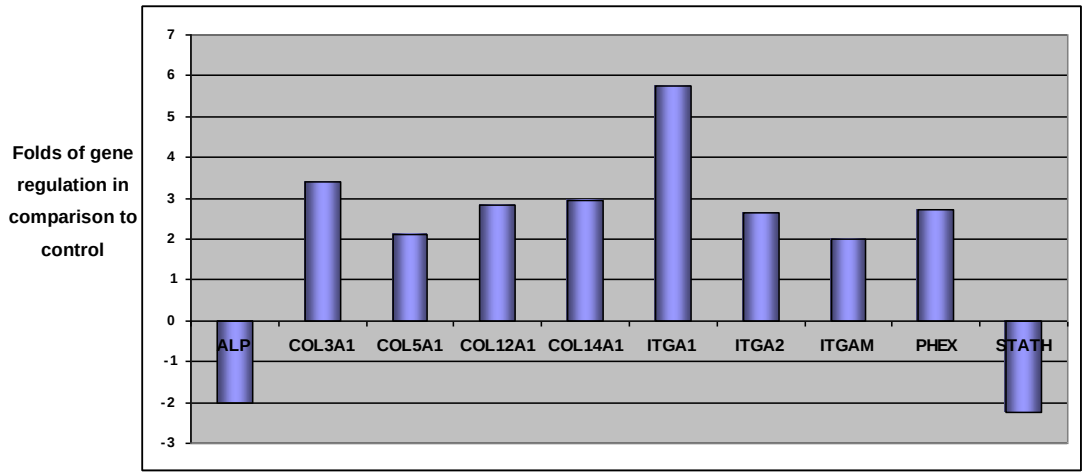


Fig. 3.12. Fold regulation of osteogenic related genes. Collagen 3,5,12 and 14, integrin A 1, 2 and M as well as PHEX were up-regulated in the 5-Aza-dC pre-treated cells over two folds in comparison to controls. ALP and statherin were downregulated.

Integrins are transmembrane receptors that mediate the interaction of extracellular matrix with the intracellular signalling (Moritz et al., 1994). Integrin alpha 1 (ITGA1), ITGA2 and ITGAM, which is a part of ITGA3, were up-regulated by 5-Aza-dC treatment. PHEX, the extracellular metalloprotease involved in the phosphate metabolism was also up-regulated. ALP and statherin (a cell surface protein that mediates hydroxyapetite crystal formation and attachment in bone and teeth (Stayton et al., 2003)) were just below the two-fold down regulation.

3.6.4. Discussion

The induction of different genes by 5-Aza-dC was not global over all examined genes, in keeping with the data in sections 3.2, 3.3 and 3.4. Only 16 genes of 84 genes (20.2%) were up-regulated while 3 genes were downregulated (3.6%). Menendez et al (2007) have studied the genes responsive to 5-Aza-dC treatment in an ovarian cancer line using a microarray approach. From 47,000 transcripts, only 465 (1%) were up-regulated and 366 (0.8%) were down-regulated (Menendez et al., 2007). Their data is in agreement with this study in: a) not all genes responded to 5-Aza-dC stimulation and b) a number of genes were downregulated by the DNA methylation inhibitor. They anticipated that the downregulation may have resulted from access of gene repressors or negative regulatory factors to the

hypomethylated promoter. This induction has also been shown for TSA treatment (Makki et al., 2008).

PCR array results showed enhancement by 5-Aza-dC of a number of growth factors that may act in synergy to induce osteogenesis, either directly or through interacting with each other. This fine-tuning and possible complementary role may be responsible for the enhancement of osteogenesis found with 5-Aza-dC. No single growth factor was identified as the main osteogenic inducer as all the up-regulated factors are known to contribute to osteogenesis. This study suffers from the limitation that it was carried out at a single time point during the differentiation process and from our knowledge of the fracture repair process, as a model of post-natal development, the expression profile of growth factors, transcription factors as well as osteogenic lineage genes will vary at different time points (Cho et al., 2002; Tsiridis & Giannoudis, 2006).

The final effect of all induced growth factors should be the up-regulation of the osteogenic master transcription factor Runx2. Only a modest 2 fold up-regulation of Runx-2 was observed. The reason for such a limited increase in Runx2, the absence of up-regulation of other BMP members as well as type I collagen could be that many of the cells cultured in osteogenic conditions had already differentiated to the osteogenic lineage. Thus, no further up-regulation by 5-Aza-dC was possible. Alternatively, the genes may have been expressed in an earlier time frame. The increase in ALP activity (shown in section 3.4.4) adds support to this explanation, although if 5-Aza-dC enhanced the osteogenic potential of the cells, the mRNA of the osteogenic related genes, including the ALP, may have been stabilized by other factors and translated. The discrepancy between ALP transcription and activity has been shown (Diefenderfer et al., 2003).

The induction of collagen type III and type V could be a direct effect of TGF-β1 stimulation, which has been also shown by Bertelli et al (1998). In their system, they studied the effect of TGF-β1 in the tubulointerstitial fibrosis

of the kidney and could illustrate the direct induction of both types of collagens by this growth factor, at the molecular as well as the protein level. The authors did not investigate the effect of TGF-β1 on other collagen types but reported that TGF-β1 has also inhibited the proteases involved in collagen lysis (Bertelli et al., 1998).

The induction of different collagens, as well as different integrins by 5-Aza-dC, may denote more physiological differentiation of 5-Aza-dC pre-treated BMSCs along the osteogenic lineage. Integrins are receptors for many collagen types (Eble et al., 1993). Immuno-selection of $ITGA1^{+}$ cells enhanced the differentiation potential of BMSCs into the three mesenchymal lineages (Rider et al., 2007). TGF-β induced ITGA2 in the human osteosarcoma cell line MG63, which was followed by changes in surrounding collagenous conformation (Riikonen et al., 1995). ITGA3, as a receptor of fibronectin, was up-regulated during early osteoblast differentiation but downregulated in later stages including during the mineralization step (Moursi et al., 1997). Thus, the cocktail of induced growth factors may have produced a more physiological developmental process *in vitro*.

The up-regulation of PHEX (the cell surface metalloprotease that regulates phosphate metabolism (Quarles, 2003)) and downregulation of statherin (a cell surface protein that mediates the hydroxyapatite crystal formation (Stayton et al., 2003)) suggests that cells were at an early state of maturation that may initiate calcification but not hydroxyapatite crystallization and attachment. The absence of β-glycerol phosphate in the media may also have prevented mineralization at this very early stage of maturity.

As shown in the previous results, 5-Aza-dC affected various genes. This was to be expected, as the epigenetic modifiers used are not specific agents. The osteogenic lineage genes were the main active group of genes in this system, which may provide a degree of selectivity to the action of 5-Aza-dC. This selectivity could be explained as follows: many genes were activated, but according to the osteogenic media, the osteogenic genes were

kept free from silencing mechanisms inside the cells, while genes related to other lineage were silenced. As shown in section 3.2, 5-Aza-dC had no effect on osteogenic induction whereas Sox-9 expression was enhanced under basal conditions. In addition, 5-Aza-dC pre-treated BMSCs cultured in osteogenic conditions showed an absence of chondrogenic lineage genes by PCR arrays, such as Sox-9, collagen II, collagen XI and serpin peptidase inhibitor.

A second possibility is that genes related to the BMSCs' origin have a low level of silencing, which could be activated by 5-Aza-dC. Multilayering of silencing has been shown by Cervoni and Szyf, 2001 (Cervoni & Szyf, 2001) (section 1.2.5). More likely, the effect of 5-Aza-dC is a combination of both theories; i.e. 5-Aza-dC was incorporated into easily accessible genes, which displayed a low level of silencing, and the up-regulatory effect was evident and persistent in response to the cell culture conditions.

The PCR array used in this study was only specific for osteogenic genes. The activation of non-osteogenic genes, however, should not be excluded or ignored, as 5-Aza-dC is not an osteogenic specific agent. Thus, this modifier should be studied extensively in terms of different genes expression with special emphasis on proto-oncogenes, before determining the possibility for utilization in human-related applications.

The absence of BMP2 up-regulation by 5-Aza-dC was unexpected as BMP2 is considered one of the most potent osteogenic growth factors. It is possible that BMP2 was up-regulated by the osteogenic conditions alone to the maximum induction level. Thus we propose that adding BMP2 to 5-Aza-dC may provide additional enhancement of osteogenesis and will examine this in the following sections.

3.7. No difference in the CpG methylation status of collagen III promoter could be detected

Collagen III is one of the genes up-regulated by 5-Aza-dC treatment (section 3.6). The promoter of collagen III is well-characterized (Bertelli et al., 1998). Only seven CpG sites are present in the whole promoter (1375 bp). To determine whether increased expression correlated with demethylation of the DNA, methylation status was investigated.

3.7.1. Hypothesis

5-Aza-dC causes loss of the methylation at specific CpG sites in the collagen III gene.

3.7.2. Methods

Sodium bisulfite deaminates non-methylated cytosine residues, while it has no effect on methylated cytosines (Wang et al., 1980). Deaminated cytosine (uracil) will be replicated to Thymine during PCR amplification and thus all non-methylated cytosines would be sequenced as Thymine (fig 1.2).

DNA bisulphite modification was carried out using EZ DNA Methylation-Gold™ Kit (Zymoresearch, cat.no.D5005). gDNA was diluted to the concentration of 25 ng/µl. 130 µl of the conversion reagent were added to 20 µl of gDNA and incubated in thermal cycler at 98 ^{o}C for 10 min, 64 ^{o}C for 150 min and 4 ^{o}C to hold. The samples were then centrifuged with 600 µl of the binding buffer in a Zymo-Spin™ column at 13000 rpm for 30 sec, followed by 100 µl wash buffer, which has also been centrifuged at the same speed and time. 200 µl of desulphonation buffer were added, incubated at room temperature for 20 min, spun down and washed twice with 200 µl wash buffer. Finally, the DNA was eluted with 15 µl elution buffer.

Primers were prepared for the bisulphite modified DNA using the software Methyl Primer Express® Software v 1.0 (Applied Biosystems, cat. no. 4376041). Semi-nested PCR were used to obtain higher accuracy. One reverse primer and two forward primers were designed. Two sets of primers

were designed in order to span the whole promoter area. The primers location is shown in Fig.3.13. and the primers sequence in table 5. PCR reaction was performed according to the protocols detailed in section 2.4.2.

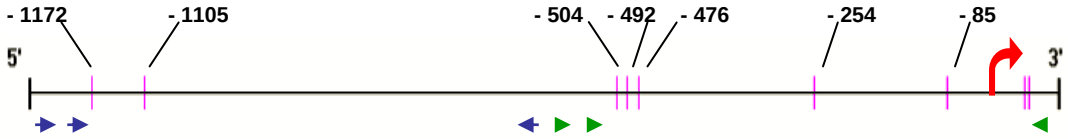


Fig. 3.13. The promoter map of collagen III promoter. The position of the first set of primers was marked with arrows, arranged as the outer forward primer, inner forward primer and reverse primer. The position of the second set is marked with arrowheads and had the same arrangement. Each vertical line demonstrates a CpG site while the transcription start site is marked by the curved arrow.

PCR products were purified using QIAquick PCR Purification Kit (Qiagen, cat. no. 28104). According to the manufacture instructions. 125 µl of buffer BP was added to the PCR products, which were in 25 µl and then transferred to the provided column and centrifuged at 13000 rpm for 1 min. The column membrane was washed with 750 µl buffer PE and finally the DNA was eluted in 50 µl of buffer EB. 10 µl aliquots were used for electrophoresis.

Table 5. Collagen 3 bisulphite modification primers list

Primer	Sequence	Amplicon
1st set		
Reverse	5' CACAAATTTCTTTAACAAACCAA 3'	
Outer Forward	5' GGAAGTGATTTTTATTAATTGGTG 3'	632 bp
Inner Forward	5' GGGGAGAATTTTATGTGTATAGGT 3'	582 bp
2nd set		
Reverse	5' CAAATTATTCCCTACCCTTCAA 3'	
Outer Forward	5' TTTGTTTTTTGATATTTGTTTGAA 3'	603 bp
Inner Forward	5' AATTTTTAGGATTTAGGGTGGG 3'	574 bp

The purified PCR products were cloned using pGEM® T Vector Systems (Promega, cat. no. A3610). This plasmid contains a β-lactamase coding region, which provides resistance to penicillin, and a T7 RNA polymerase transcription initiation site, used for sequencing at the end of experiment (Fig.3.14). The DNA insert interrupts the galactosidase gene, which means that any cells that have successfully combined with the plasmid do not exhibit the characteristic blue colour normally produced by X-Gal.

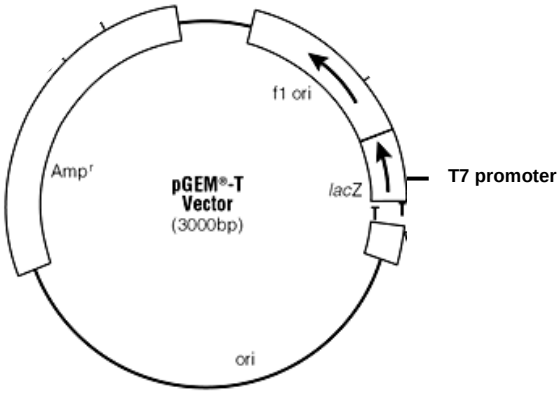


Fig. 3.14. Diagrammatic representation of the plasmid of pGEM® T Vector.
(http://www.promega.com/figures/popup.asp?fn=0356va)

Purified PCR products were ligated to the plasmid according to the manufacturer's instructions. 5 µl of 2X Rapid Ligation Buffer were added to one µl of the plasmid (50 ng) and 1 µl of the DNA Ligase (3 Weiss units/µl) and mixed with 3 µl of the PCR products and incubated at room temperature for 1 hour. After the incubation, 2 µl of the ligation reaction was added to 50 µl of JM109 high efficiency competent cells and incubated on ice for 20 minutes, followed by heat shock at 42^{o}C for 45 sec and 2 minutes incubation on ice. 950 µl room of Super Optimal broth with Catabolite repression (SOC) medium (Invitrogen, cat. no. 15544034) was then added and incubated at 37^{o}C on horizontal shaker set at 150 rpm for 90 min. Then 100 µl of the transformation reaction was plated on Luria-Bertani broth (LB) - agar. LB media was prepared previously with 10g tryptone (Sigma-Aldrich, cat. no.T9410), 5g yeast extract (Sigma-Aldrich, cat. no. Y1625),10g NaCl (Sigma-Aldrich, cat. no. S7653) and made up to one litre with distilled water followed by filter sterilization. 7.5 gm of agar (Sigma-Aldrich, cat. no. A5054) was added to 500 ml of the LB media to produce LB-Agar. After autoclaving, LB-Agar was poured into sterile Petri dishes and supplemented with ampicillin 50 µg/ml (Sigma-Aldrich, cat. no. A5354) and X-Gal 40 mg/ml (Promega, cat. no. V3941). The Petri dishes were cooled overnight in 4^{o}C. Transformed cells cultured on LB-agar Petri dishes were incubated overnight at 37 ^{o}C and on the following day, 10 white colonies were selected and incubated together for 24 hours.

After 24 hours, cultures were centrifuged, media removed and plasmids purified using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, cat.no. K210011). Pellets were resuspended in 250 µl of the resuspension buffer R3 then 350 µl of the lysis buffer L7 added and the tubes incubated at room temperature for 5 min. Buffer N4 (350 µl) was added to the tubes and centrifuged for a further 10 min at 13000 rpm. The supernatant was loaded into the provided column and centrifuged for 1 min, washed with 500 µl buffer W10, incubated for one min at room temperature and centrifuged again. Another wash with 700 µl buffer W9 followed and the columns centrifuged twice to dry the membranes. Plasmid DNA was eluted with 75 µl of TE buffer. The DNA concentration was determined and samples were sent for sequencing to MWG sequence (www.eurofinsdna.com/home).

3.7.3. Results

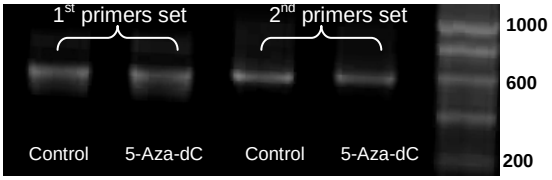


Fig. 3.15. PCR products of the two samples after the nested PCR.

PCR was carried out for the bisulfite modified DNA using specially designed primers. Two reactions were performed for each sample according to the two primers sets. Electrophoresis of the PCR products showed bands of the expected size, which confirmed the efficiency of the bisulfite conversion (Fig.3.15).

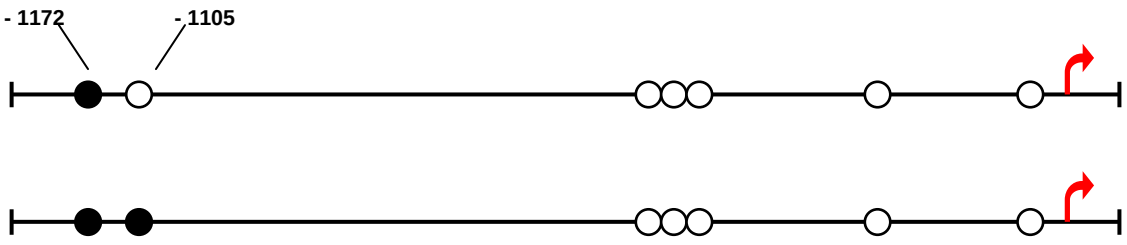


Fig. 3.16. The promoter map of collagen III in control (top) and 5-Aza-dC (bottom) pre-treated cells. Each circle represented one CpG site. Open circle represented a non-methylated CpG, while closed circle represented a methylated CpG. One CpG was methylated in control, while the same CpG as well as the adjacent site were methylated in 5-Aza-dC pre-treated cells. The curved arrow indicates the transcription start site.

After cloning and purification of the plasmid containing the DNA insert, samples were sequenced. In the control sample, only one CpG (at -1172 bp) was methylated (Fig 3.16); i.e. was sequenced as cytosine, while on the 5-Aza-dC pre-treated cells, two methylated CpGs were found (at -1172 bp and -1105 bp).

3.7.4. Discussion

Since CpGs in the promoter of collagen III were mostly un-methylated, the promoter was already primed for transcription. Hence, DNA methylation is not the main player in the regulation of the transcription of this gene and direct incorporation of 5-Aza-dC is not the main inducer of collagen III up-regulation. In addition, the presence of a methylated CpG position in the pre-treated cells that was not methylated in the control may emphasis that this CpG was not crucial for its transcription. Hence, other regulatory mechanisms are important for collagen III, which may highlight the important role of the growth factors induced by 5-Aza-dC and reported by the PCR arrays. In the Menendez et al microarray study (2007), promoters of up-regulated genes were analysed by bisulphite sequencing and it was found that not all promoters were hypomethylated in response to the 5-Aza-dC treatment. The bisulfite sequencing results support the conclusions presented in the previous section that 5-Aza-dC may exert its effect through the up-regulation of the osteogenesis-related growth and transcription factors and, in turn, these factors induce other osteogenic genes. This observation may also address the issue of 5-Aza-dC selectivity, because if the incorporation were random, no methylated cytosines should theoretically be found. The factors that govern the incorporation of the 5-Aza-dC and consequently its effects either in this system or in Menendez et al, 2007 remain to be identified.

3.8. 5-Aza-dC induces ALP activity more than BMP2

BMP2 is the major growth factor inducing osteogenesis. Osteogenic PCR arrays showed that BMP2 was not up-regulated by the 5-Aza-dC. As BMP2 can also be induced in the osteogenic control, the addition of supplementary BMP2

to the 5-Aza-dC pre-treated cells could provide further enhancement of osteogenesis. This study compared alkaline phosphatase activity, as a marker of osteogenic development, between cells cultured in basal, osteogenic and BMP2 plus osteogenic conditions, with and without prior treatment of 5-Aza-dC.

3.8.1. Induction of ALP activity using different concentrations of BMP2

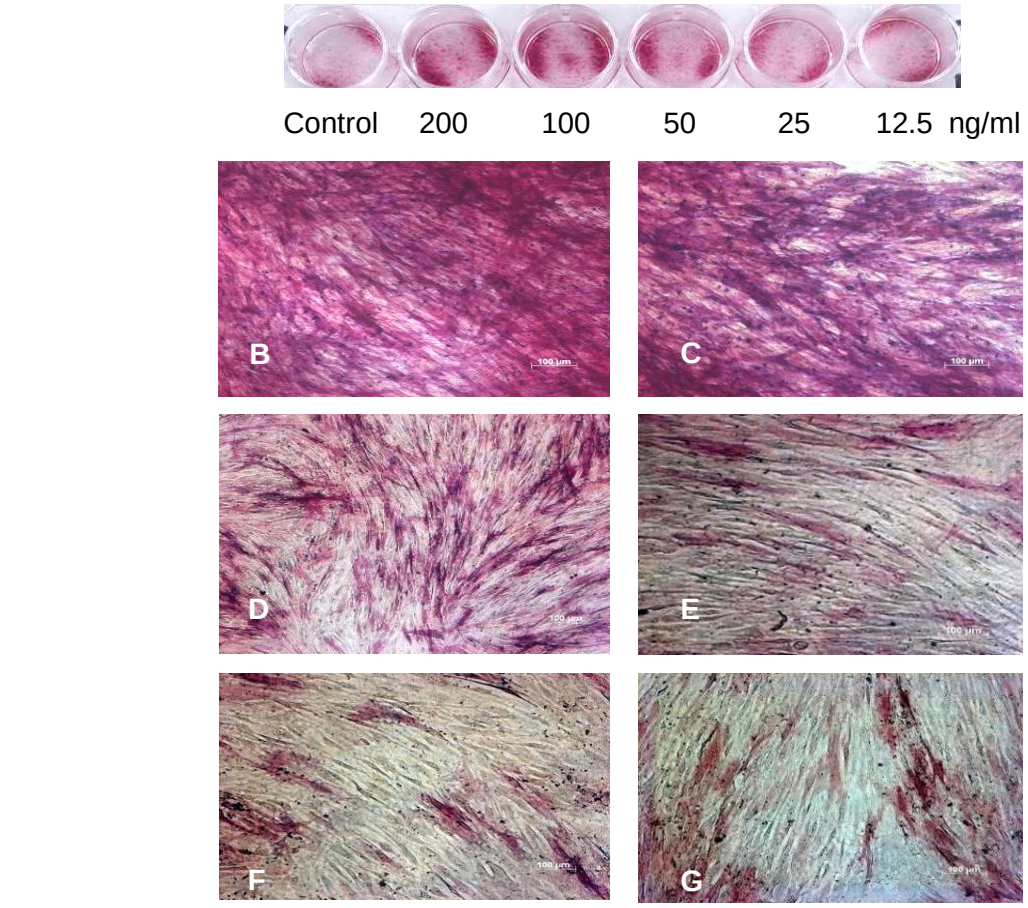
Different concentrations of BMP2 were applied to BMSCs in order to determine the appropriate concentration that enhanced ALP activity as a marker of osteogenesis.

a) Methods

BMSCs, obtained from a 67 year old male, were cultured at low density ($5X10^4$) in basal conditions. After 72 hours when the cells were 70% confluent, the media was changed to 2% FCS and BMP2 was added to achieve final concentrations of zero (control), 12.5, 25, 50, 100 and 200 ng/ml. Cells were incubated at 37^oC, 5% CO_2 for 48 hours before fixation with 95% ethanol. ALP activity was determined by both staining and biochemical assay corrected to the total DNA concentration.

b) Results

The treatment of BMSCs with different concentrations of BMP2 resulted in a significant statistical difference of ALP expression in BMSCs treated with 100 and 200 ng/ml BMP2 (Fig.3.17). The biochemical assay results were confirmed visually by ALP staining (Fig.3.17.A-G). The concentration of 100 ng/ml was selected for further experiments. BMP2 at the dose of 12.5 ng/ml had significantly lower ALP activity compared with control. Low BMP2 has been shown to have an inhibitory effect on bone formation in an *in vitro* study (Jeppsson et al., 1999).



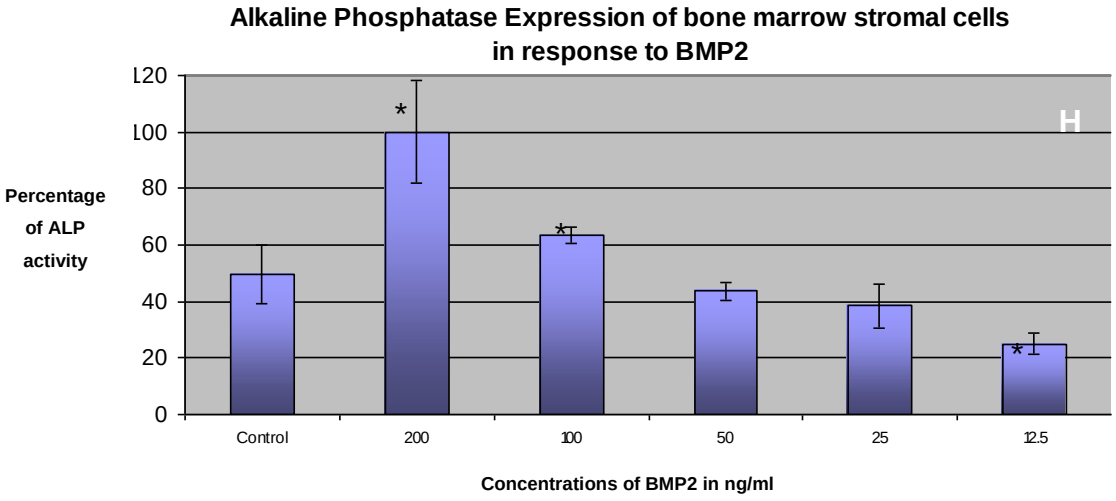


Fig.3.17. Alkaline phosphatase staining. (A) An overview of all groups. Micrographs of the individual groups, (B) 200 ng/ml, (C) 100 ng/ml, (D) 50 ng/ml, (E) 25 ng/ml, (F) 12.5 ng/ml and (G) was the control; i.e. no BMP2 was added. (H) ALP activity quantitation in all study groups corrected to the amount of DNA. The ALP expression of BMSCs treated with 200 ng/ml BMP2 was set as 100% for comparison. The figures represent the relative expression with the standard deviation bars (samples assayed in triplicates). * p-value < 0.05 in comparison to control.

3.8.2. Comparison of BMP2 induction of osteogenesis with and without pre-treatment with 5-Aza-dC

a) Hypothesis

5-Aza-dC has an additive effect on BMP2 induction of osteogenesis of BMSCs as 5-Aza-dC can induce growth and transcription factors that have osteogenic inducing potential.

b) Methods

BMSCs, obtained from a 60-year-old male, were cultured under basal conditions until 50% confluence, and then serum starved for 24 hours followed by 5-Aza-dC treatment (1µmol) for three days. Treated and untreated cells were cultured in osteogenic media with and without 100 ng/ml BMP2. Cells were also cultured in basal conditions as controls (fig.3.18).

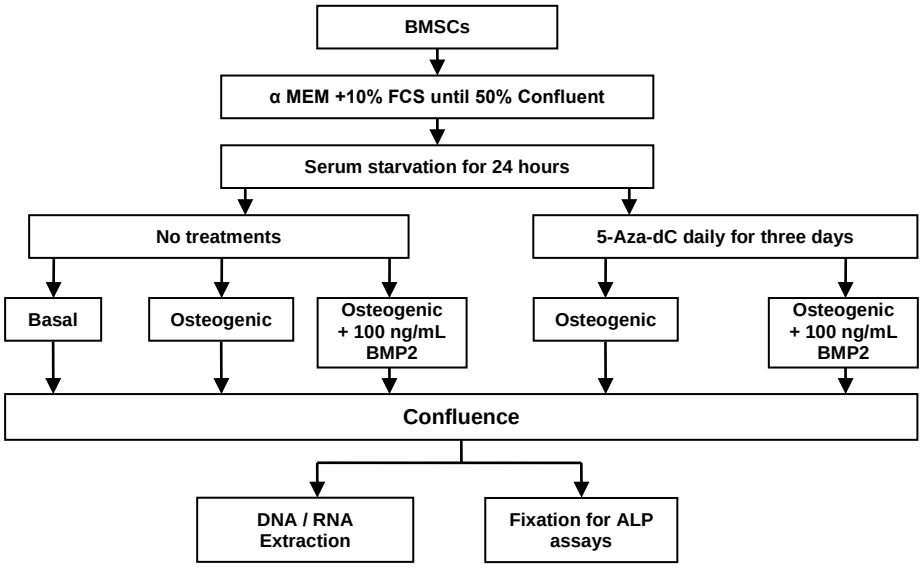


Fig.3.18. Schematic representation of the study groups.

After four days, cells were over-confluent and some cells were fixed for ALP assays. RNA and DNA were extracted at the same time point. Cells cultured under basal conditions with BMP (200 ng/ml) were considered as the positive control for the PCR study as BMSCs cultured under such conditions displayed the highest alkaline phosphatase activity in the dose response curve

(Fig.3.17). Runx2 and ALP expression were quantified by real time PCR. The expression of these genes was corrected to the house-keeping gene β-Actin.

c) Results

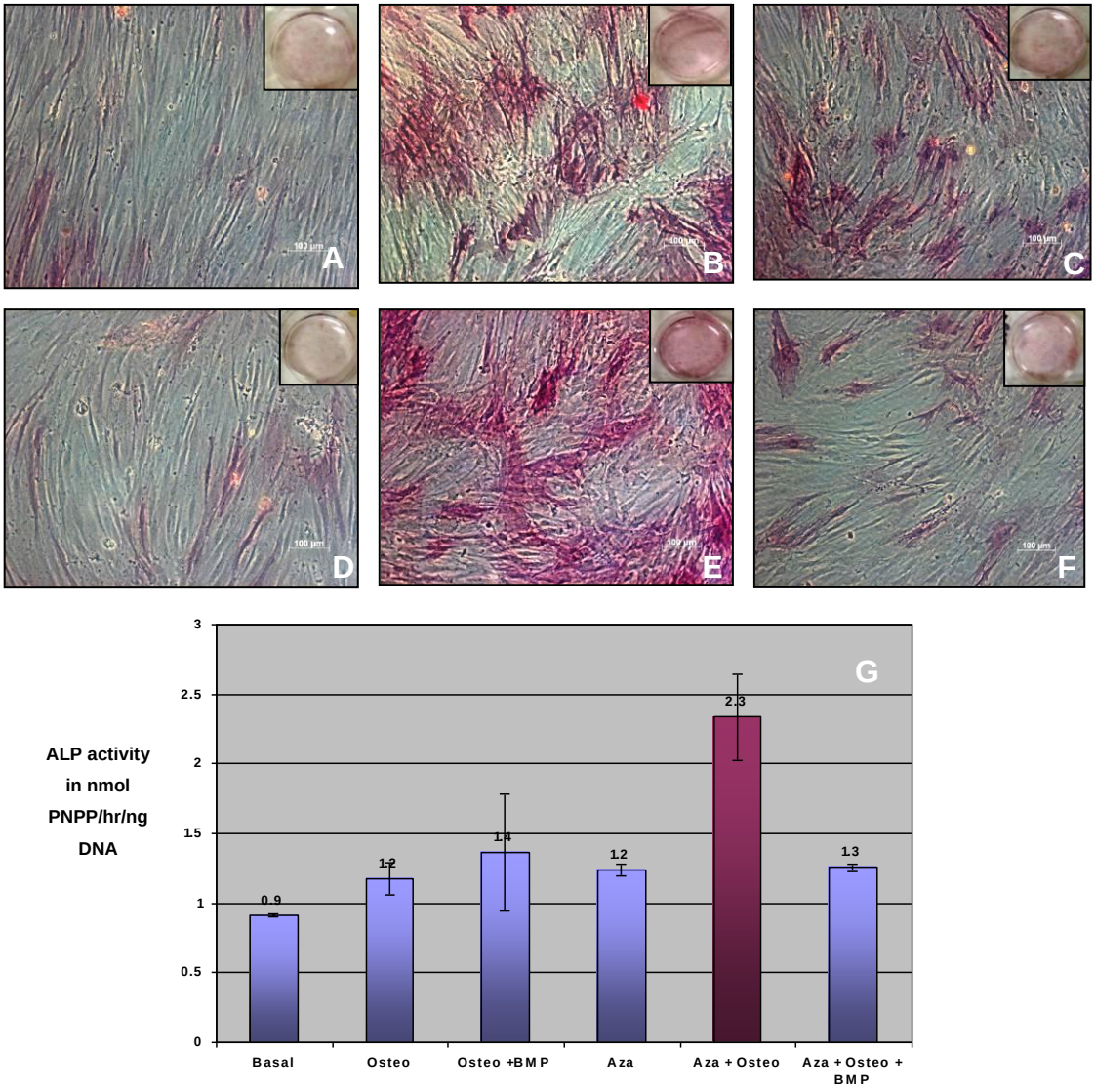


Fig. 3.19. Alkaline phosphatase activity in different groups presented by staining (A-F) and quantitative assay in nmol/hr/ng DNA with standard deviation bars. (A) Basal, (B) Osteogenic media alone, (C) Osteogenic conditions with BMP2, (D) Basal previously treated with 5-Aza-dC, (E) Osteogenic conditions previously treated with 5-Aza-dC and (F) Osteogenic conditions with BMP2 previously treated with 5-Aza-dC. (G) Alkaline phosphatase quantification in all study groups expressed in nmol/hr/ng DNA. ANOVA test was significant. (samples run in triplicates).

5-Aza-dC pre-treated cells, cultured under osteogenic conditions, displayed the highest ALP activity (Fig. 3.19.), while activity in other groups

were found not to be significantly different (cells in basal conditions, osteogenic conditions, osteogenic conditions plus BMP2, 5-Aza-dC pre-treatment in basal conditions and 5-Aza-dC in osteogenic conditions plus BMP2).

Runx2 is the master transcription factor that is critical for osteoblast differentiation (Karsenty et al., 1999). Real time PCR analysis (Fig. 3.20) showed slight differences between the basal control, osteogenic control and cells cultured in osteogenic control pre-treated with 5-Aza-dC. Significant differences were found between the pre-treated cells and both control groups. Adding 5-Aza-dC increased Runx2 expression 3.7-fold, which was not statistically significant from the maximum expression level obtained in this study, i.e. cells treated with 200 ng/ml BMP2. Thus, 5-Aza-dC has enhanced significantly Runx-2 expression in both the control group and BMP2 treated BMSCs.

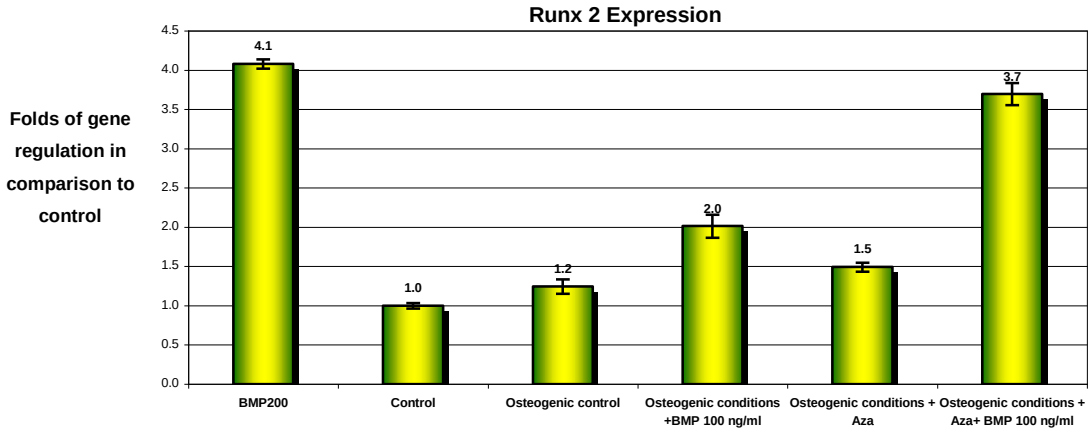


Fig. 3.20. Runx2 expression in the different groups. Bars represented the fold increase in the relative quantification with standard deviation. Samples were run in triplicates. ANOVA test showed significant statistical difference.

The ALP expression pattern (Fig. 3.21.) differed from that of Runx2. Osteogenic conditions up-regulated ALP significantly, in comparison to the basal conditions with and without 100 ng/ml BMP2. 5-Aza-dC failed to upregulate ALP either in osteogenic conditions alone or osteogenic conditions containing 100 ng/ml BMP2 as no significant difference could be found between these groups.

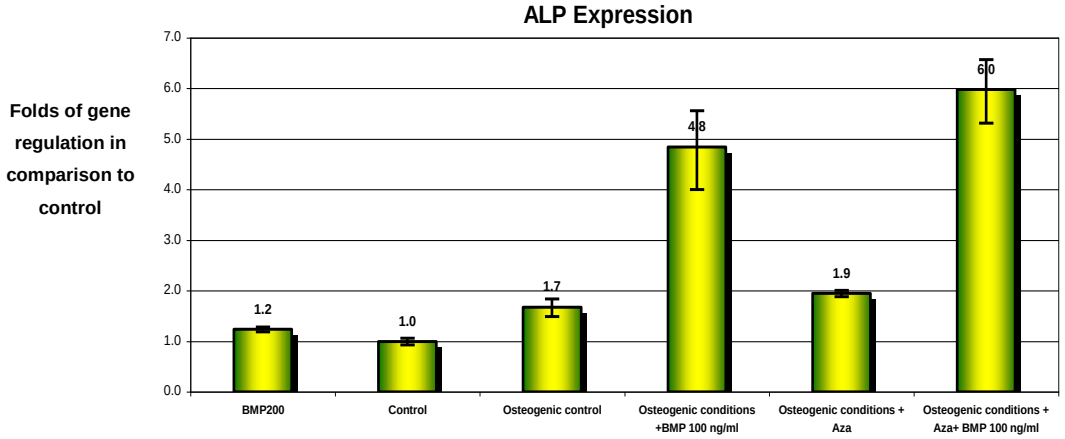


Fig. 3.21. ALP expression in the different groups presented as fold expression with standard deviation bars (n=2 in triplicates). ANOVA test was statistically significant.

d) Discussion

Contrary to the hypothesis proposed, the maximum induction for alkaline phosphatase activity was produced by the cell group treated initially with 5-Aza-dC and cultured under osteogenic conditions. Surprisingly, the addition of BMP2 into the culture conditions decreased the ALP activity and was comparable to the induction by BMP2 without further treatment with 5-Aza-dC. This visual evidence was further confirmed by quantification of ALP activity.

5-Aza-dC has been shown to induce different growth factors. The simple addition of BMP2 may be unable to add further to the induction of the corresponding pathway. External BMP2 could have only activated the BMP2/Smad pathway, counteracting other pathways and neutralising the effect of 5-Aza-dC. In this study, there was a discrepancy between the ALP expression and activity observed (section 3.5.5 for details). In addition, this study showed the variable effect of BMP2 at different concentrations as, for example BMP2 at the low dose of 12.5 ng/ml significantly decreased the ALP activity (Lee et al., 2003).

The results provide further evidence that 5-Aza-dC enhances osteogenesis at the molecular level. There was no difference in Runx2 expression between cells cultured in basal conditions and those in osteogenic conditions. These effects were extended to osteogenic matrix formation, which is shown in the next chapter.

3.8. Conclusions:

The differentiation of BMSCs along the osteogenic lineage does not occur under the classical culturing conditions. Epigenetic modifiers have been shown to change the gene expression profile in BMSCs and, to lesser degree, in Stro1^{+} enriched populations. 5-Aza-dC enhanced the osteogenic process in BMSCs, providing that the cells were cultured in osteogenic conditions. 5-Aza-dC stimulated the induction of osteogenesis *in vitro* in comparison to the addition of growth factors to media. A possible explanation is that 5-Aza-dC induced a panel of growth and transcription factors (section 3.5.), which could co-ordinate to stimulate downstream osteogenic genes. The enhancement of osteogenesis was shown by increased ALP activity in the treated cells as well as the persistence of this activity over a longer time frame.

THE EFFECT OF EPIGENETIC MODIFIERS ON THE DIFFERENTIATION OF BMSCS IN THE PELLET CULTURE SYSTEM

4.1. Introduction

5-Aza-dC treatment had been shown to enhance osteogenic differentiation. However, chondrogenic differentiation, which is the other important event in skeletogenesis, could not be examined in monolayer culture because of the complexity of matrix deposition and arrangement of the chondrocytes in lacunae embedded in the matrix. To investigate this issue, the pellet culture model (Lee et al., 2001; Tare et al., 2005) was used.

4.2. The effect of epigenetic modifiers on the differentiation potential of BMSCs cultured in 3D culture using chondrogenic media

The pellet culture system has been shown to be useful in culturing chondrocytes as well as BMSCs under chondrogenic conditions to examine cartilage formation. The attachment and interaction of the cells within a three-dimensional environment has been shown to enhance matrix formation and favoured differentiation (Mackay et al., 1998; Pittenger et al., 1999b; Lee et al., 2001; Tare et al., 2005). This study compared the differentiation potential of BMSCs in the pellet culture under chondrogenic conditions with and without prior treatment with the DNA demethylation inhibitor 5-Aza-dC, the histone deacetylase inhibitor TSA and in combination.

4.2.1. Hypothesis

5-Aza-dC and TSA enhance chondrogenic differentiation in comparison to cells in basal conditions while the combined treatment of TSA and 5-Aza-dC have synergistic effects and further enhance chondrogenesis.

4.2.2. Methods

BMSCs were obtained from an 81-year-old male. Cells were treated with TSA (100 nM) once, 5-Aza-dC (1 μM) three times per week. The combined treatment consisted of an initial treatment with TSA for 48Hs followed by 5-Aza-dC three times per week. After 4 weeks, cells were processed into pellet cultures at a density of 9×10^5 cell/pellet (as shown in Fig. 4.1).

The pellets were cultured for a further four weeks in 1 ml of chondrogenic media in 25 ml universal tubes. After fixation and processing for histology, sections were stained with Alcian blue/Sirius red (A/S) to distinguish between collagen rich osteogenic matrix (Sirius red positive), and the proteoglycan-rich chondrogenic matrix, which stains with Alcian blue. In addition staining with Safranin O provided further confirmation of a proteoglycan-rich matrix.

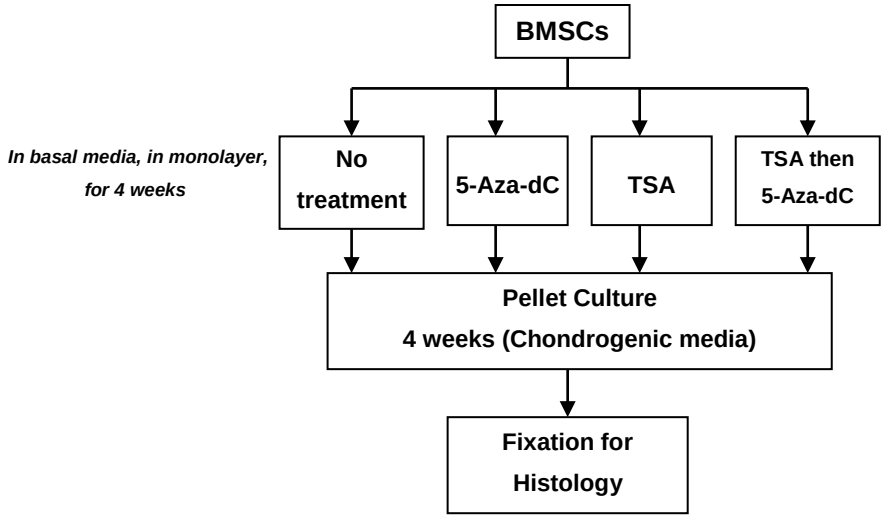


Fig. 4.1. Schematic representation of the study.

4.2.3. Results

In control cultures, Alcian blue staining (Fig.4.2.A) and Safranin O (Fig.4.2.E) staining demonstrated the presence of a proteoglycan rich matrix particularly in the central two thirds of the pellet. The outer third was stained with Sirius red, consistent with a bone-like matrix. This was confirmed by positive immuno-staining for osteocalcin (Fig.4.2.I) and collagen I (Fig.4.2.M). Areas of necrosis were observed in the centre, possibly due to difficulty in the diffusion of nutrients and/or oxygen across the pellet.

Pellets of cells pre-treated with 5-Aza-dC showed negligable chondrogenic matrix formation as demonstrated by the absence of Alcian blue (Fig.4.2.B) and Safranin O staining (Fig.4.2.F). Instead, the matrix was positive for Sirius red (Fig.4.2.B) and collagen I (Fig.4.2.N) and a few cells were immuno-positive for osteocalcin (Fig.4.2.J). This suggested osteogenic differentiation in these pellets despite use of the chondrogenic culture

conditions and the absence of serum. The localization of collagen I was observed to co-locate with the Sirius red staining.

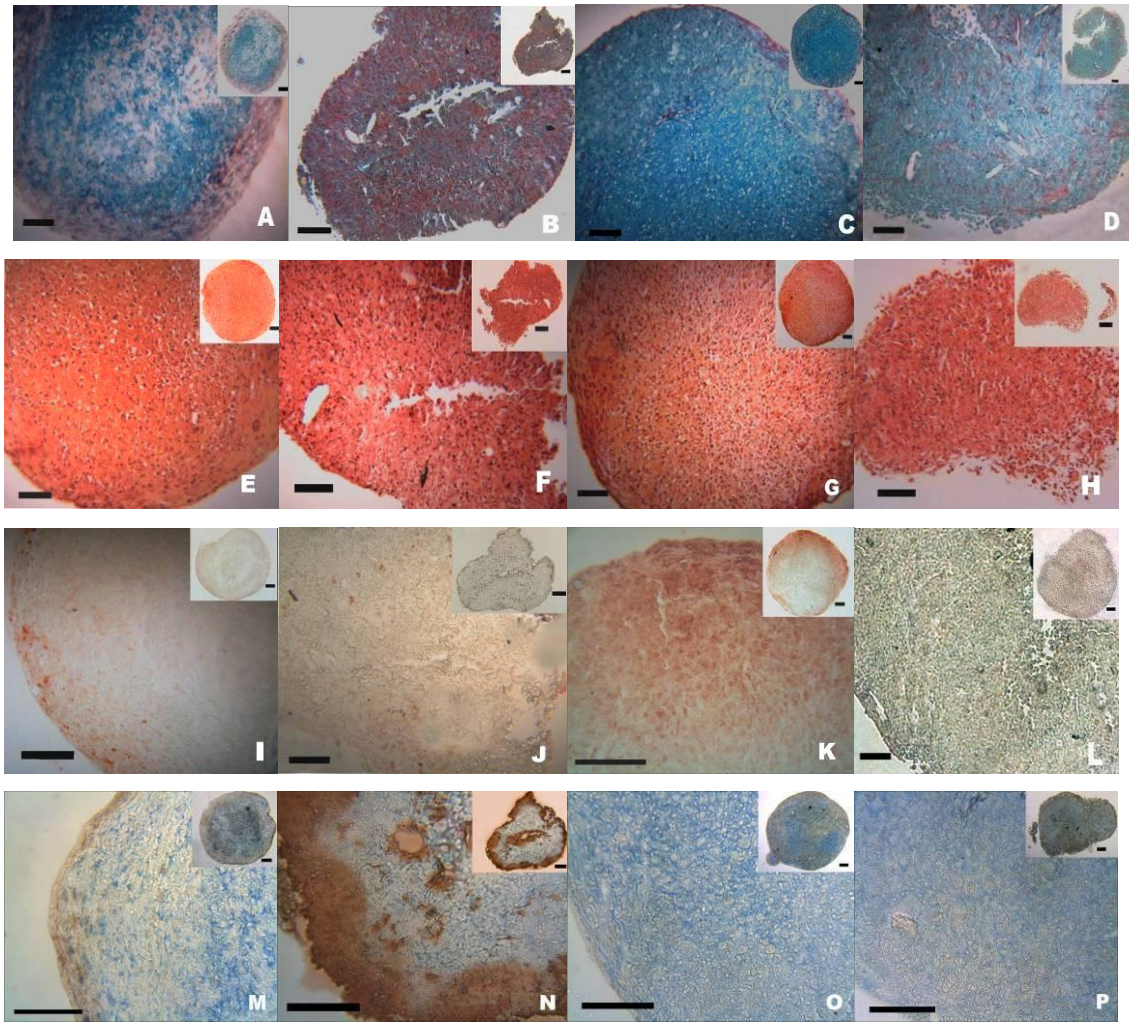


Fig 4.2. Matrix characterization of the pellets. A, E, I, M and Q originally cultured under basal conditions. B, F, J, N and R, treated with 5-Aza-dC. C, G, K, O and S treated with TSA. D, H, L, P and T, treated initially with 5-Aza-dC then with TSA. A-D: Alcian blue/Sirius red. E-H: Safranin O. I-L: Osteocalcin immuno-staining. M-P: Collagen I immuno-staining. Enhanced osteogenesis in the 5-Aza-dC pre-treated cells, shown by Sirius red, collagen I and von Giesen stain. Enhanced chondrogenesis evident in TSA pre-treated cells shown by Alcian blue and Safranin O staining. Scale bars = 100 µm.

In the TSA treated group, extensive proteoglycan formation was observed across the pellets, evidenced by a deep blue colouration of sections after A/S staining (Fig.4.2.C), orange red areas in the Safranin O staining (Fig.4.2.G) as well as absence of collagen I immuno-staining (Fig.4.2.O). Modest osteocalcin expression was observed in small portion of the pellet (Fig.4.2.K). The pellets of combined 5-Aza-dC and TSA pre-treatment showed an intermediate response between the two groups, namely a mixed osteogenic

and chondrogenic matrix as illustrated with A/S (Fig.4.2.D), Safranin O (Fig.4.2.H), osteocalcin (Fig.4.2.L) and collagen I (Fig.4.2.P).

4.2.4. Discussion

The presence of a proteoglycan rich matrix in the pellets of the control group demonstrated that BMSCs respond to chondrogenic conditions in a 3D culture system by differentiation along the chondrogenic pathway. These conditions appear to mimic, in part, the *in vivo* situation in terms of maintaining cells in contact with each other, which has been shown to be a requisite to maintain the chondrogenic phenotype (Mackay et al., 1998; Lee et al., 2001). In addition, this study provided evidence that an inhibitor of DNA methylation or histone deacetylation can affect differentiation of BMSCs. The effect of these modifiers persisted through the monolayer and into pellet cultures. All the pellets were cultured under the same conditions but differed in their initial treatment, which affected subsequent cell differentiation. Thus, initial reprogramming may occur in the cells in response to epigenetic treatment. The osteogenic characteristics of the pellets formed from 5-Aza-dC pre-treated cells were apparent even though the pellets were cultured in chondrogenic media devoid of any osteogenic factors and in the absence of serum. This suggests that the transcriptional pathways that were induced in monolayer culture by 5-Aza-dC continue to be active within the pellet system. TSA stimulated chondrogenic matrix production whilst inhibiting the osteogenic phenotype. In contrast, 5-Aza-dC appeared to stimulate osteogenesis and suppress chondrogenesis. When both modifiers were used, an intermediate phenotype was observed. These results suggest a relative selectivity of these agents. The induction of osteogenesis and chondrogenesis by epigenetic modifiers needs further characterization, for example, the effect of TSA in osteogenic media will need to be addressed. In addition, the optimum effective dose of 5-Aza-dC and TSA remains to be determined.

4.3. Dose response study of the epigenetic modifiers on the osteogenic and chondrogenic differentiation potential of BMSCs within the pellet culture model

A wide range of epigenetic modifiers at varying concentrations have been used in different studies with marked discrepancies between reported concentrations as related to efficacy and toxicity. The response of cells varied according to many factors including cell types and culture conditions. Hence, dose responses were used to determine the most suitable dose in the current experimental model. 5-Aza-dC was not studied in chondrogenic conditions as this agent has been shown not to induce chondrogenic matrix formation (section 4.1).

4.3.1. Hypothesis

Matrix formation is enhanced by specific concentrations of epigenetic modifiers.

4.3.2. Methods

BMSCs from two patients were obtained for this study; the first, from a 92-year-old female was used for the 5-Aza-dC and TSA dose responses in osteogenic conditions and the second, from a 45-year-old female was used for TSA in chondrogenic conditions as well as Spiruchostatin A (SSA) in both conditions. Cells were treated after reaching 50% confluence and serum-fasting for 24 hours. 5-Aza-dC was added to attain 10 nM, 100 nM, 1 μM and 5 μM concentrations, while TSA dose concentrations (for osteogenic response) were 4 nM, 20 nM, 100 nM and 500 nM on a daily basis for 10 days. When cells reached confluency in monolayer, they were transferred into pellet culture. In a second experiment for both osteogenic and chondrogenic dose responses, TSA was added at 25nM, 50 nM, 100 nM and 200 nM for the first three days after the serum removal. SSA was added at 15 nM, 30 nM, 60 nM and 120 nM. SSA was added only once in these studies.

Cells were counted before transfer to pellet culture and were seeded at a density of 5 X 10^5 cells /pellet as the higher cell number used in section 4.1 displayed areas of necrosis at the centre of the pellet. Pellets were cultured in osteogenic or chondrogenic conditions for four weeks; pellets were then fixed in 4% paraformaldhyde for matrix characterization and in 85% ethanol for alkaline phosphatase activity analysis.

4.3.3. Results

a) BMSCs tolerate epigenetic modifiers to variable extents

Before transferring cells to pellets, a cell count was performed. The number of cells was inversely related to the concentration of the modifiers. With 5-Aza-dC (Fig. 4.3), there was negligible difference in cell number at the lowest concentration (10nM) examined. In contrast, cell numbers decreased by 50% at 100nM and to around 30% at 1 μM and 5 μM 5-Aza-dC. This could have been as a consequence of toxicity, the inverse relationship between the differentiation and proliferation or, most likely, a combination of both.

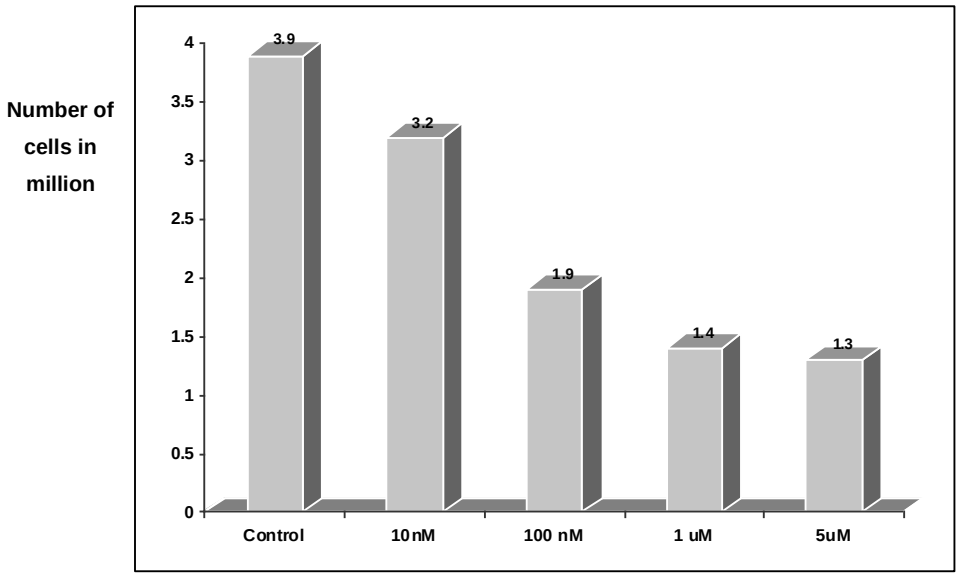


Fig. 4.3. Cell number after 10 days in culture with daily-added 5-Aza-dC. 5-Aza-dC inversely affected cell numbers in a dose dependent manner.

The inverse relationship between cell numbers at the end of the monolayer culture and TSA doses was even more striking (Fig. 4.4). Even at the lowest dose (4 nM) cell numbers decreased by 50% of control, while at 100

nM cell numbers decreased to about 25%. At 500 nM cell number fell to about 10% of the control cell count (0.3 million cells). These cells failed to form a pellet and were excluded from the results. The inverse relationship between TSA dose and cell numbers may be due to toxicity as well as the well known effect of the TSA in arresting the cell cycle in G1 and/or G2 stage (Yoshida & Beppu, 1988).

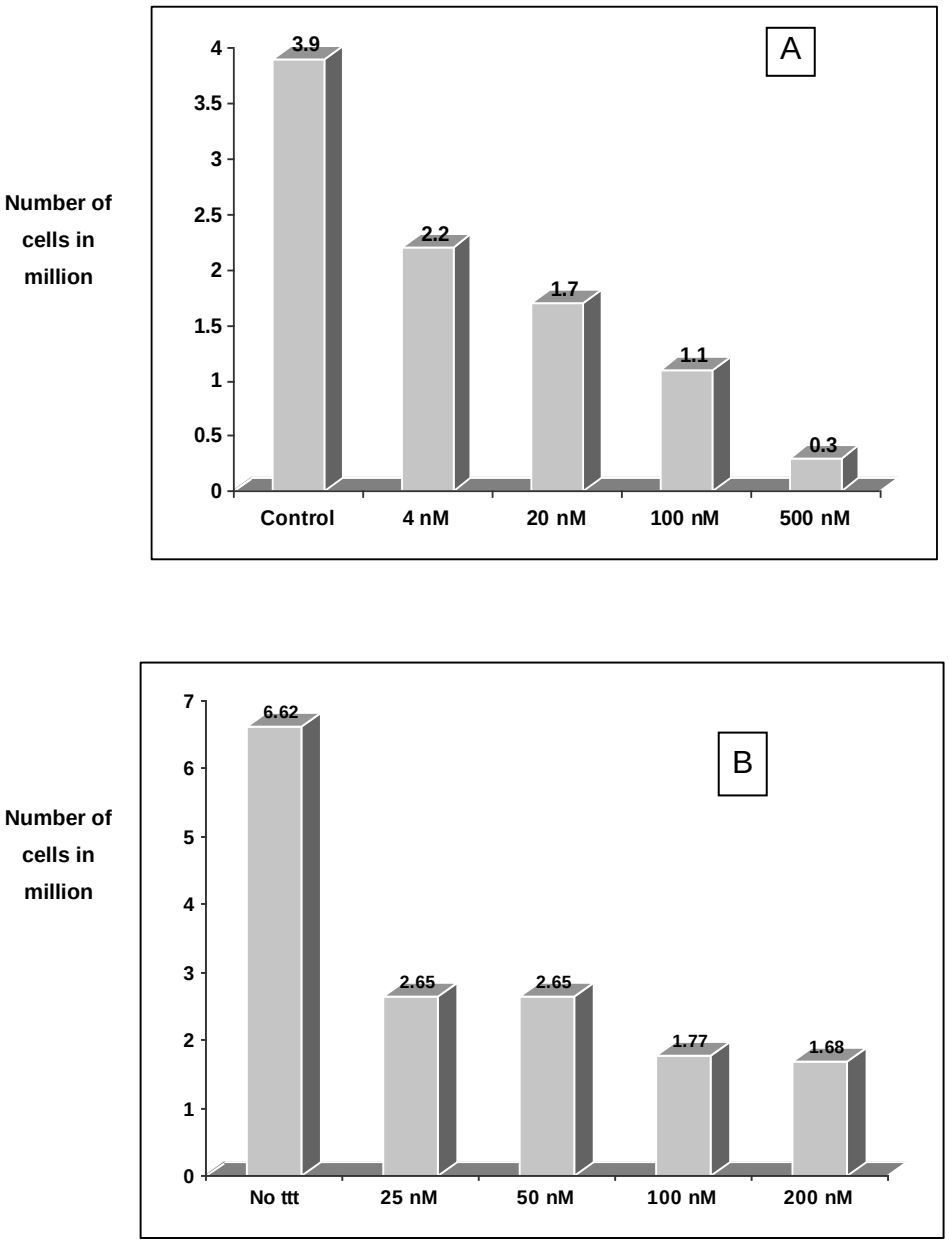


Fig. 4.4. Cell counts: in (A) after 10 days with TSA added daily and in (B) after 6 days with initial three days treatment. TSA was more toxic than with 5-Aza-dC. TSA affected the cells in a dose dependant manner.

In view of the toxic response of high doses of TSA, concentrations were reduced in the following study, i.e. TSA dose response in chondrogenic conditions: three days of treatment were followed by another three days in basal conditions without treatment until the cells became confluent. The rationale was to decrease the toxic effects of TSA, increase the number of recovered cells and shorten the initial monolayer culture prior to 3D culture. The range of doses was changed to exclude the lowest dose (4 nM) and the highest dose (500 nM) of TSA.

In comparison to the controls, at 100 nM, 28 % of cells (Fig.4.4) survived the first treatment regimen (10 doses) and 27% the second regimen (3 doses). The toxic effect of 100 nM TSA was similar in the two treatment regimens. Regression analysis of both dose responses was comparable. R^2 for first dose response was 0.54 and 0.47 for the second (Fig.4.5).

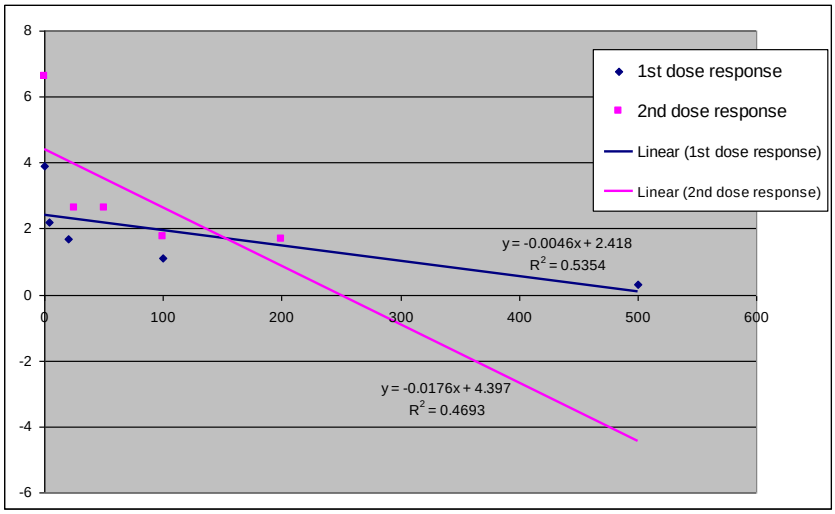


Fig. 4.5. Regression analysis of the TSA treated cells in the first dose response (10 treatments of TSA) and the second dose response (3 treatments of TSA). The R^2 were closely related.

SSA was highly toxic in these studies. Addition of this modifier on a daily base proved cytotoxicity in initial trials. SSA had a long lasting effect for over 24 hours (personal communication with Dr. A. Ganesan). Thus in this dose response study, the treatment was added only once and media were changed 3 days later. At this stage, many of cells had died and detached from the plastic surface. The cytoplasm of most of the remaining cells had contracted and the

cells had acquired a distinct fibroblastic morphology (Fig 4.6), not observed with 5-Aza-dC or TSA treated cells.

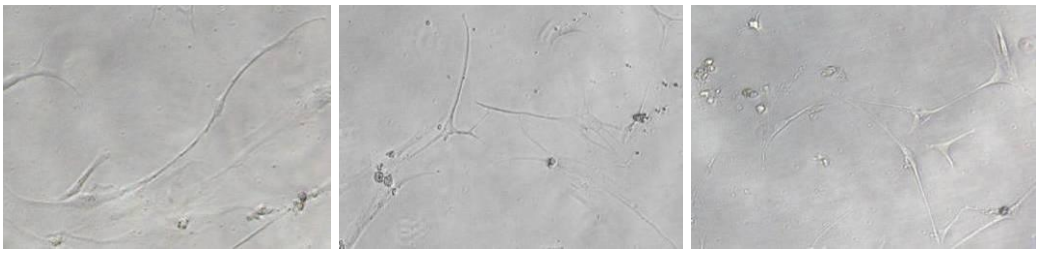


Fig. 4.6. The effect of SSA treatment on BMSCs after a single application. Many cells detached from the plastic. The cytoplasm of the remaining cells was observed to be markedly shrunken and the cells displayed a fibroblastic morphology.

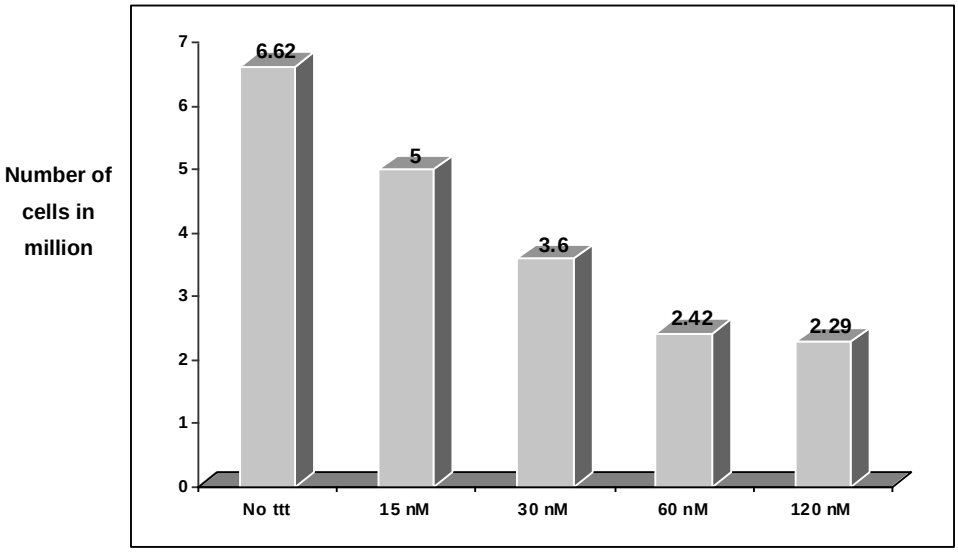


Fig. 4.7. Cell counts after 15 days in culture in basal conditions treated once with SSA. Cell number was inversly related to the treatment dose.

SSA treated BMSCs needed an extended culture period to recover and reach confluence. At the lowest dose (15 nM), cells needed two weeks to become confluent in comparison to the TSA lowest dose (25 nM), which required less than a week. Cells, that survived the initial toxic effect of the SSA, proliferated without any further treatment in basal conditions and maintained their cell numbers. The cell count at confluence (Fig.4.7) exceeded TSA comparable treatments (Fig.4.5.B). SSA treated cells may have acquired an enhanced potential to proliferate upon recovery. In addition, the fibroblastic

nature of the cells may have allowed more space for cell divisions before reaching confluency.

b) 5-Aza-dC osteogenic response

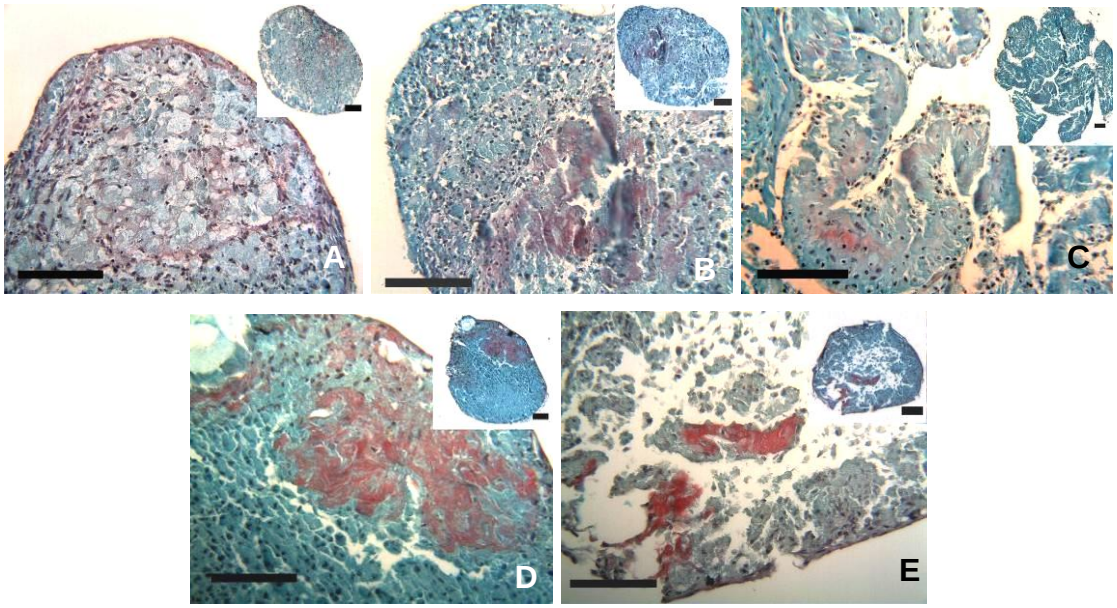


Fig. 4.8. Alcian blue/ Sirius red staining of pellets cultured with various doses of 5-Aza-dC during the initial monolayer stage. (A) control with minimal collagenous matrix formation present. (B) 10 nM and (C) 100nM with modest matrix formation while (D) 1μM induced a maximal matrix formation not only dispersed across the pellet but also intensely localized to one pole of the pellet. (E) 5 μM 5-Aza-dC showed a discrete area of intense matrix formation. The rest of the pellet was negative. Hence, the 1 μM dose was chosen for studying the effects of 5-Aza-dC on BMSC differentiation. Scale bars = 100 μm.

Alcian blue /Sirius red staining clearly showed the maximum collagenous matrix formation induced by 5-Aza-dC at 1 μM concentration (Fig 4.8.D). Areas of homogeneous Sirius red staining were located at the pole of the pellet, while the rest of the pellet showed disperse filamentous red staining, corresponding to collagen organization. Other doses showed inconsistent matrix formation that was comparable to the control group. 5 μM 5-Aza-dC had minimal localized collagenous matrix, which stained intense red (consistent with the staining of osteoid matrix).

Alkaline phosphatase is one of the earliest markers of osteogenic differentiation. The pellets treated with 10 nM 5-Aza-dC showed low activity

(Fig. 4.9). The 100 nM pellets had low activity while the higher doses 1 and 5 μM were negative.

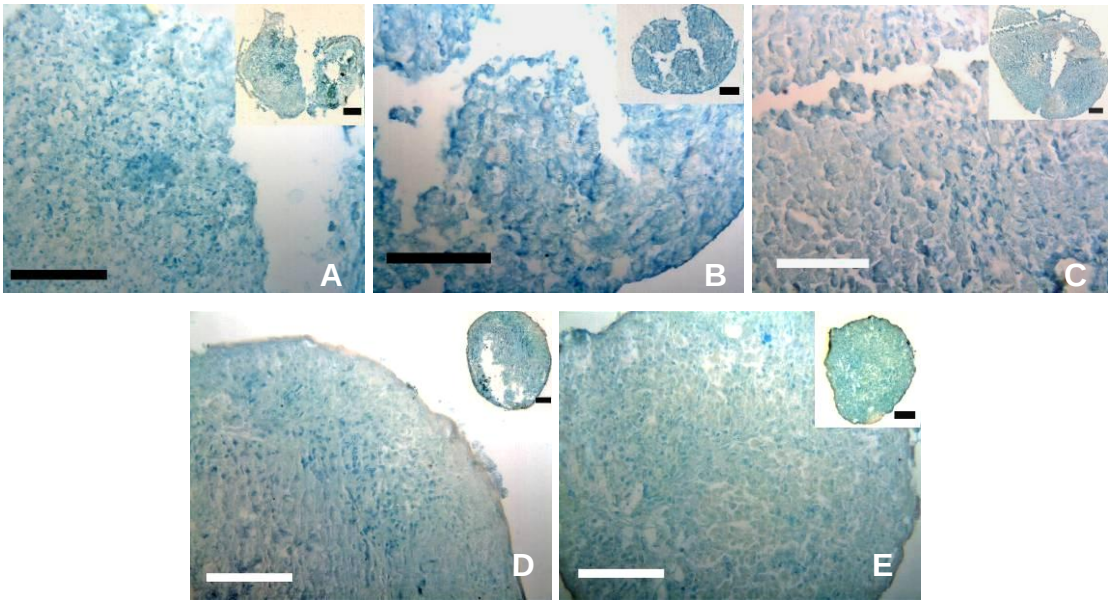


Fig. 4.9. Alkaline phosphatase staining following treatment with 5-Aza-dC. (A) control; no ALP activity could be found. (B) 10 nM and (C) 100nM a few cells displayed weak ALP activity , being more in (C). while (D) 1μM and (E) 5 μM were negative. Scale bars = 100 μm.

c) TSA osteogenic response

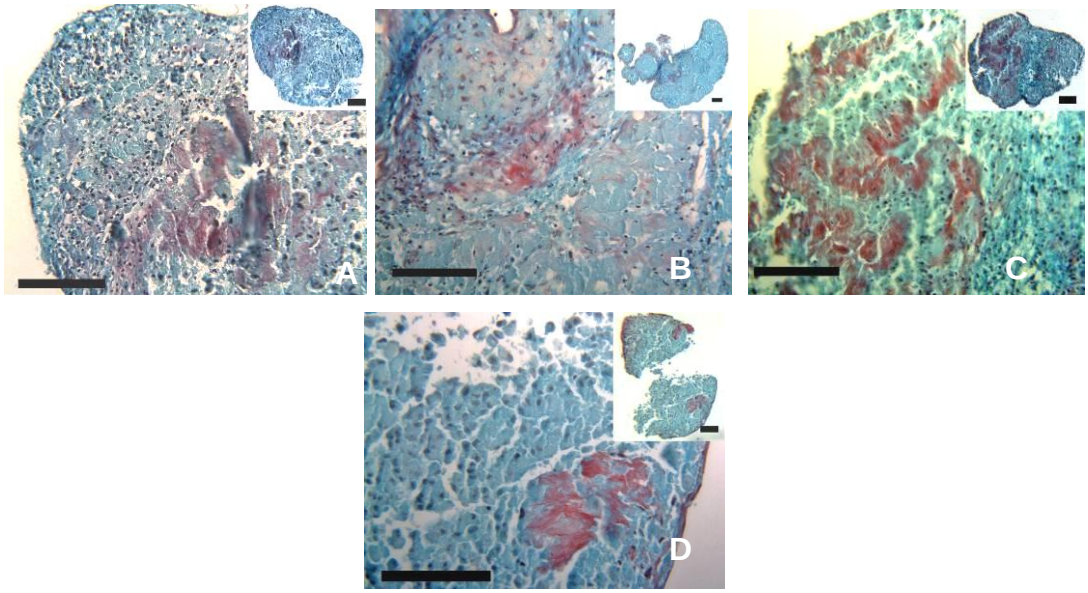


Fig. 4.10. Alcian blue/ Sirius red staining following TSA addition. In (A) control and (B) 4 nM, there was minimal collagenous matrix formation. (C) 20nM and (D) 100 nM had osteogenic matrix induction, although this was not comparable to matrix formation obtained with 5Aza-dC at 1μM.
Scale bars = 100 μm.

Collagenous matrix formation was minimal with 4 nM TSA and comparable to control pellets. The maximum induction of osteogenic matrix was observed at 20 nM and 100 nM, although matrix formation was not homogeneous or intense. Although TSA induced some collagenous matrix formation, it was not comparable to that induced by the 5-Aza-dC especially at the dose of 1 µM TSA.

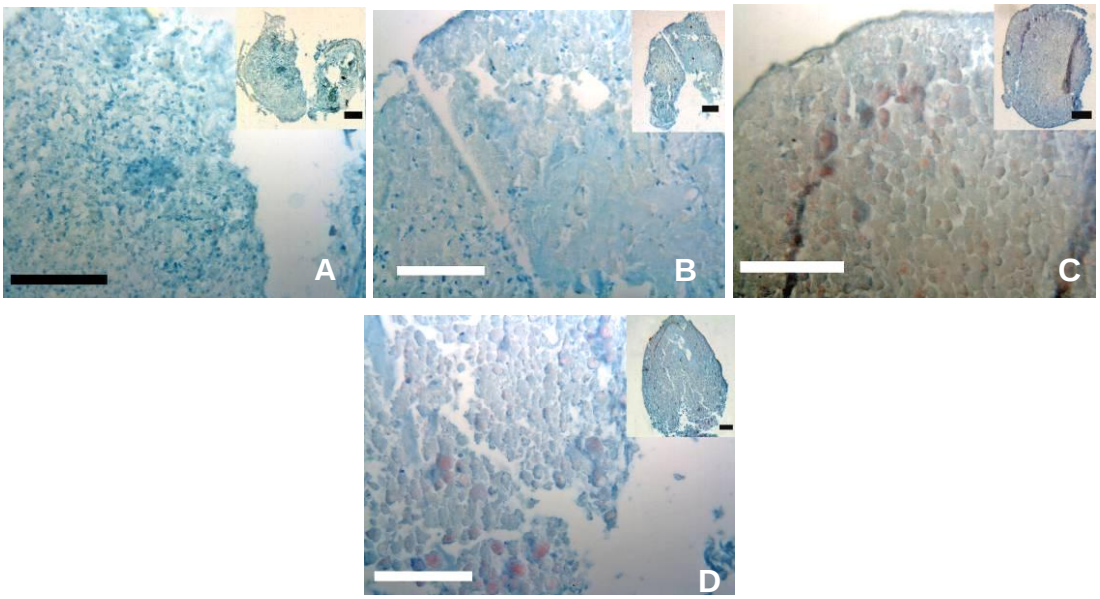


Fig.4.11. ALP staining following TSA treatment. In (A) the control; no ALP activity could be observed (B) TSA at the dose of 4 nM cells were faintly stained, while (C) 20nM and (D) 100nM have well defined positive cells. Scale bars = 100 µm.

Modest ALP activity was observed at the lowest concentration of TSA treatment (4nM), compared to 5-Aza-dC at 10nM and 100nM. Distinct positive cells could be observed at 20nM and 100nM TSA. ALP staining was localized to cell cytoplasm and not in the matrix.

d) SSA osteogenic response

The BMSCs treated with SSA that survived, recovered and proliferated in monolayer culture, and were able to form matrix in the pellet culture. SSA induced collagenous matrix formation in comparison to control cultures at the lowest tested dose 15nM. At 30 nM SSA, maximal induction was observed with over 60 % of the pellet positive for Sirius red stain. Higher concentrations (60 and 120 nM) showed less matrix formation.

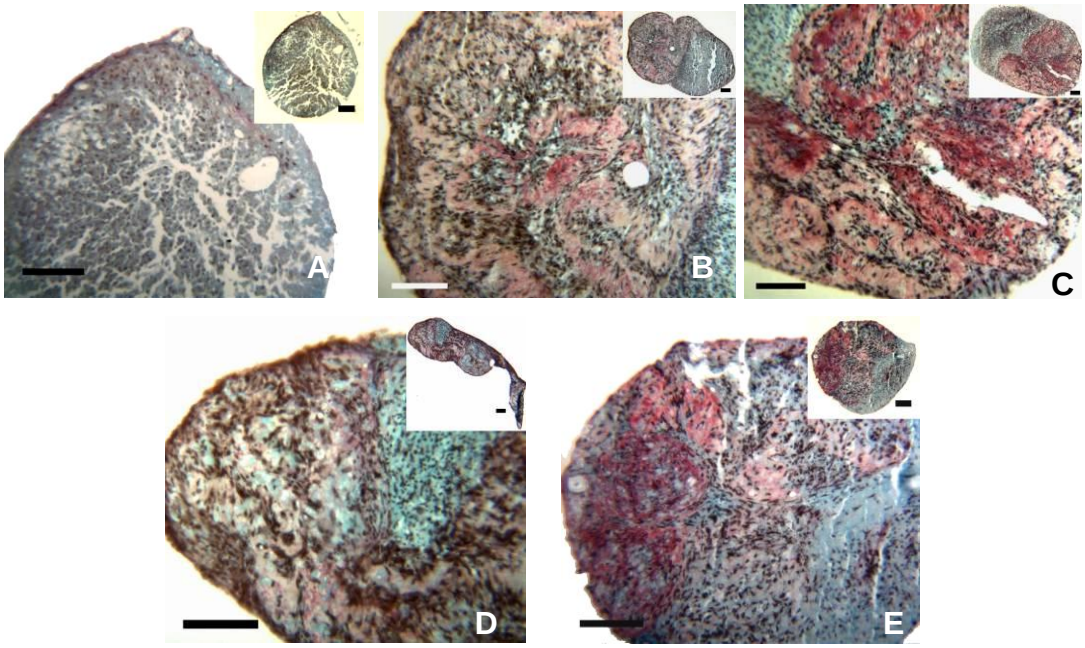


Fig. 4.12. Alcian blue/ Sirius red staining after SSA treatment. In the control (A) minimal collagenous matrix was formed while 15 nM SSA (B) induced collagenous matrix formation. Further enhancement occurred with 30nM SSA (C) as collagenous matrix covers more than half of the pellet. 60nM (D) and 120nM (E) SSA were associated with decreased matrix formation. 30nM SSA produced maximal dose has the maximum induction of collagenous matrix formation. Scale bars = 100 µm.

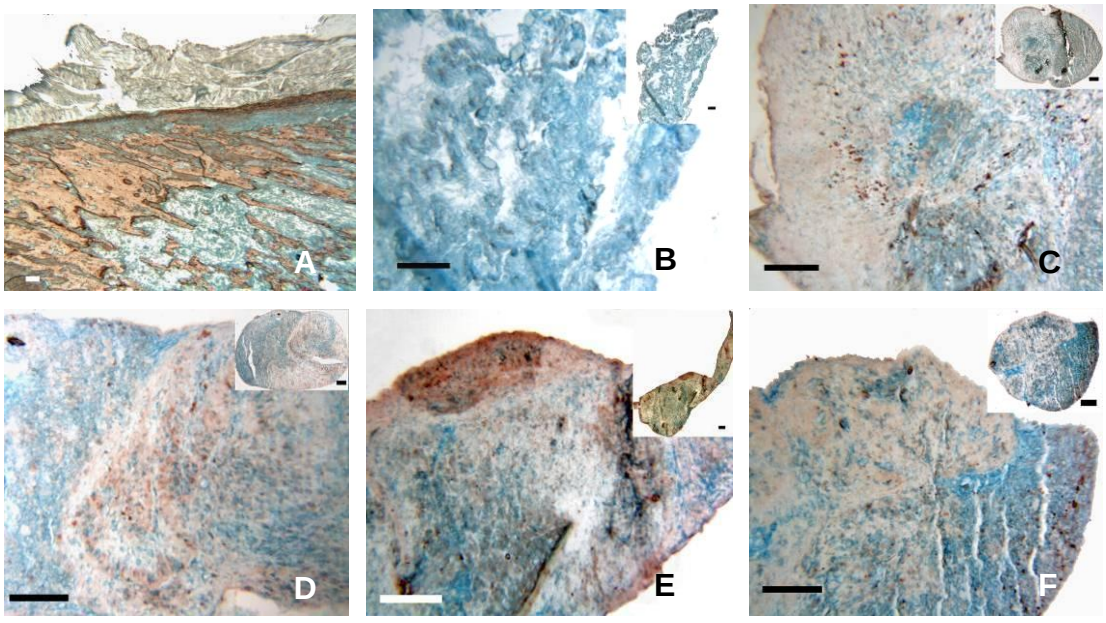


Fig. 4.13. Collagen I immuno-staining after SSA treatment. Pellets were cultured in osteogenic conditions. (A) Positive control (a section in a rat femur); collagen I stains the periosteum as well as the bone trabecula. B. Negative control. In all doses 15nM (C), 30 nM (D), 60 nM (E) and 120 nM (F). Weak positive staining co-localized with Sirius red staining. No differences observed across the treatment groups. Scale bars = 100 µm.

However, immuno-staining for the two fibrilar collagens, collagen I (predominant in bone) and collagen II (predominant in cartilage) showed that collagen I immuno-staining (Fig.4.14) of the pellets was weak and failed to correspond to the intensity of Sirius red staining. There were positive areas in all sections that represent a small proportion of the Sirius red stained areas. The intensity of staining was similar in all pellets, irrespective of the SSA concentration and was not comparable to that of 5-Aza-dC treated samples.

For further characterization of the collagenous matrix, immuno-staining for type II collagen was carried out. As a positive control, sections of rat femur were stained, as the antibody crossreacts with type II collagen of rat origin. Cartilage matrix of the growth plates showed positive staining, as expected, in the chondrocytes within lacunae as well as the extracellular space; i.e. the matrix. For negative controls, sections of pellets were used, in the absence of primary antibody.

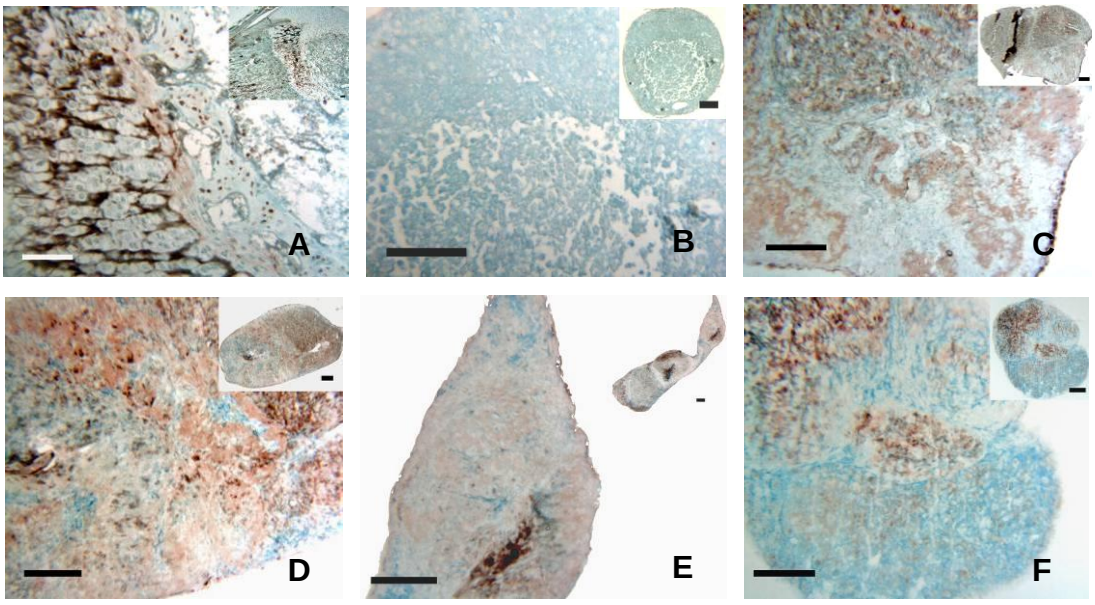


Fig. 4.14. Collagen II immuno-staining after SSA treatment. Pellets were cultured in osteogenic conditions. (A) Positive control (a section in rat femur growth plate); collagen II stained the matrix surrounding the chondrocytes. (B) Negative control. Collagen II stained the corresponding areas of Sirius Red with parallel intensity. Staining with 30nM (D) was more intense compared to 15nM SSA(C). Less staining was observed at 60nM (E) and the 120nM (F) concentration. Scale bars = 100 μm.

Unlike collagen I, the intensity of collagen II staining was comparable to the intensity of Sirius red staining in different sections (Fig.4.15). Collagen II was evident at 15nM SSA, reached maximal expression with 30 nM SSA, and decreased again at higher concentration. The areas in each section were comparable to that of the Sirius red staining. Thus, the collagenous matrix was composed mainly of collagen II.

e) TSA chondrogenic response

The chondrogenic differentiation of BMSCs is illustrated by two features; 1) the formation of cartilagenous matrix, which is a mixture of proteoglycans and collagens. Type II collagen is the main collagen type in cartilage. It is fibrillar in nature, stains only with Sirius red if the proteoglycans are insufficient (Smith et al., 2003); and 2) the chondrogenic morphology indicated by chondrocytic cells within lacunae.

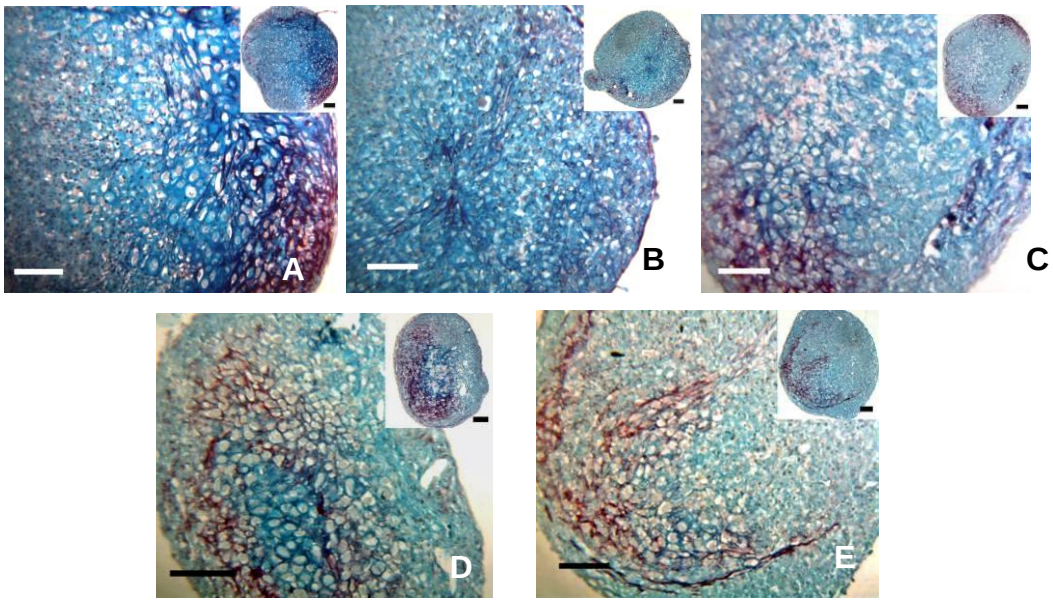


Fig. 4.15. Alcian blue/ Sirius red staining after TSA addition; pellets were cultured in chondrogenic conditions. In the chondrogenic control (A), one pole of the pellet displayed a cartilage-like morphology. The 25 nM TSA (B) showed even less matrix formation in comparison to control. With the 50 nM TSA (C) there was increased matrix formation compared to control but not as much as at 100nM TSA (D) where over 80% of the pellet displayed a chondrogenic matrix with lacunae architecture. The architecture was preserved at 200nM TSA (E) but with much less matrix formation.
Scale bars = 100 μm.

In the chondrogenic control, a confined area of matrix formation together with cells within lacunae was observed (Fig. 4.15). The total area showing this architecture was about 20% of the pellet. At the lowest concentration examined (25nM), there was no increase in chondrogenic induction compared to the control, which was probably because this dose of TSA was unable to induce the key genes, even though TSA modulates cell number. Increasing the dose to 50 nM TSA, matrix formation was enhanced with more lacunae formation observed. Further enhancement of both chondrogenic architecture and matrix formation was evident at 100 nM TSA. In these pellets, the cartilage-like matrix occupied more than 80% of the section. Lacunae were abundant and cell nuclei (stained dark grey to black with haematoxylin) could be observed within. Lacunae were surrounded with matrix stained for proteoglycan and collagens. The highest concentration (200 nM) TSA was associated with decreased cartilage matrix, which was confined to one pole of the pellet.

In the chondrogenic control as well as the lower doses of TSA (25 nM and 50 nM) collagen II was located within the cells, which may indicate an early stage of maturity, i.e. cells were synthesizing the protein although not secreting collagen into the extracellular space. At the higher doses examined (100 nM and 200 nM), the staining was mainly in the matrix. Some cells were also observed to synthesize the protein, as evidenced by the scattered intracellular positive staining. The lacunae architecture was preserved as evidenced by the filamentous collagen II matching the staining observed using A/S staining.

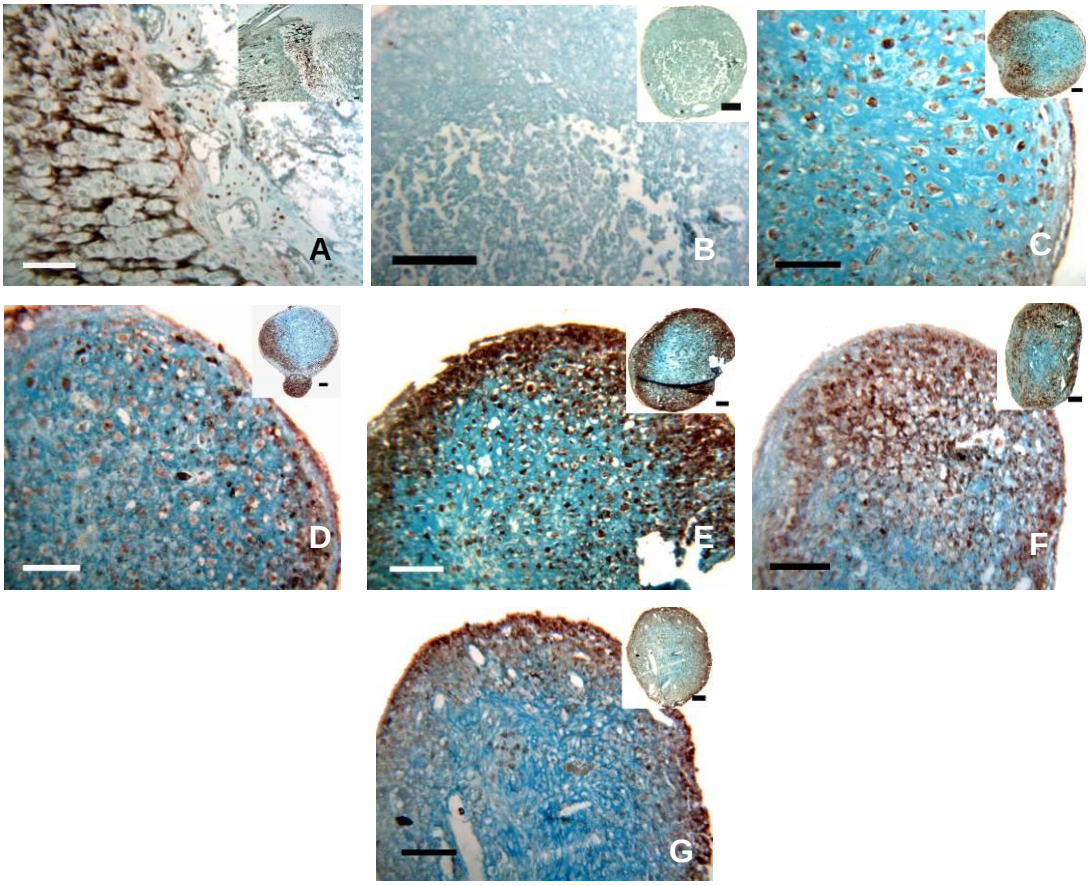


Fig. 4.16. Immuno staining for collagen II after TSA treatments of pellets in chondrogenic conditions. (A) positive control: rat femur, (B) negative control. In the chondrogenic control(C), 25nM TSA (D) and 50 nM (E), there was positive intracellular staining, which may indicate an early developmental stage, while with 100nM TSA (F) and 200nM TSA (G) the staining was mainly in the matrix and filamentous in nature, which is the physiological location of collagen II. In addition, in 100nM TSA (F) the staining was observed to match the lacunae architecture found in the A/S stain. Scale bars = 100 μm.

f) SSA chondrogenic response

A comparable protocol of treatment to that used in the osteogenic study (section 4.2.4.d) was employed. No matrix formation could be detected in any examined dose. In addition, no chondrogenic architecture could be detected.

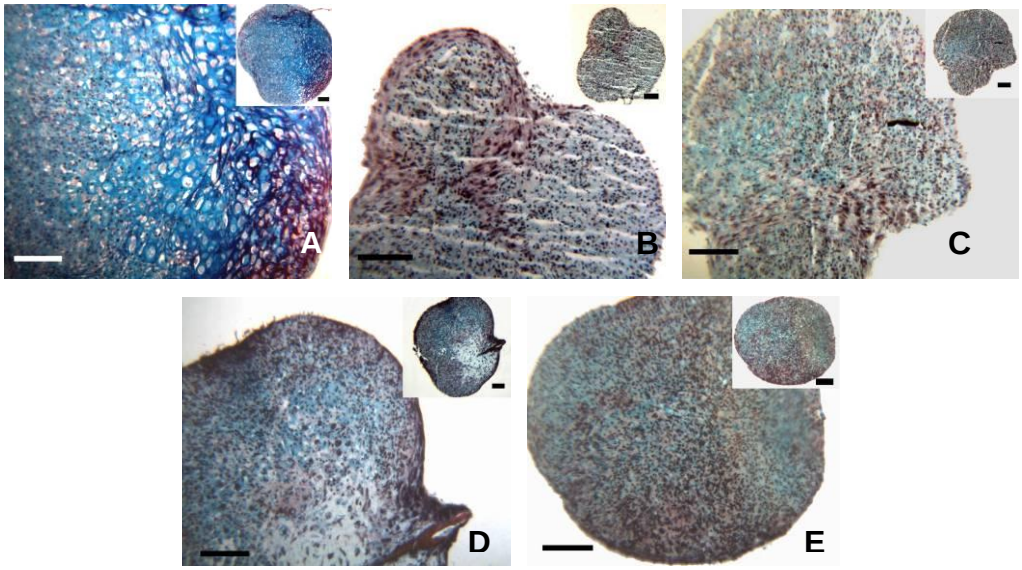


Fig. 4.17. Alcian blue/ Sirius red staining after SSA treatment; pellets were cultured in chondrogenic conditions. In A. the chondrogenic control. All tested doses showed negligable matrix formation of either collagenous (Sirius red positive) or proteoglycan rich (Alcian blue positive) matrix; at 15 nM (B), 30 nM (C), 60 nM (D) or 120 nM (E) SSA treatments. Scale bars =100 μm.

4.3.4. Discussion

The dose response studies provide a useful approach in determining the most suitable concentration for enhancing the differentiation of BMSCs in three-dimensional aggregates, i.e. pellet culture. The ideal concentration must balance the toxicity of the epigenetic modifiers with their effect on differentiation. 5-Aza-dC induced osteogenic matrix formation results, which confirmed the initial data within section 4.2. The optimal osteogenic response was obtained when BMSCs were treated with 1μM 5-Aza-dC and then cultured in pellets in osteogenic media (with no further 5-Aza-dC treatment). The absence of ALP staining was in agreement with the absence of ALP gene induction shown in the previous PCR studies, but in contrast to the enhanced the ALP activity observed in monolayer culture for up to 25 days (section 3.6). It is possible that an early induction of the gene by 5-Aza-dC was subsequently downregulated. The doses of 1 μM and 5 μM may have induced further differentiation of the pellets along the osteogenic lineage. Thus, although 5-Aza-dC reduced cell number at a dose of 1μM, the surviving cells were the most responsive to differentiation induction, while a higher dose (5μM) may have resulted in toxicity and affected the metabolism or differentiation ability of the cells without causing

cell death. Based on these results, 1μM 5-Aza-dC was used for subsequent experiments.

The failure of TSA treated cells to form a pellet at 500 nM cannot be explained only by the low cell number as some studies have used lower number of cells to form pellets (Ong et al., 2006). TSA at this concentration may have affected some genes that are related to cell contact or extracellular receptors or alternatively acetylated intracellular proteins or growth and transcriptional factors (Huang et al., 2005). Cells treated with lower concentrations formed pellets and showed distinct ALP activity. Staining was mainly within cells rather than the matrix, suggesting a state of immaturity, as ALP is produced initially within the cells then excreted into the extracellular spaces. The presence of ALP activity, but the absence of uniform matrix formation, may indicate that although the cells were developing along the osteogenic lineage, they were at an early state of differentiation. This suggests that the DNA demethylase 5-Aza-dC, but not the histone deacetylase TSA, pushes cycling cells towards the osteogenic lineage. In contrast, TSA appeared to have a powerful chondrogenic induction potential. All the tested concentrations displayed a gradual increase in the amount of cartilage matrix formed and a distinct chondrogenic morphology. TSA at a concentration of 100 nM induced the formation of a proteoglycan and collagen II rich matrix with chondrocyte-like cells located in lacunae. This morphology resembles, in part, primary cartilage. TSA thus increased the efficiency of chondrogenesis over standard chondrogenic media.

SSA was observed to be toxic to BMSCs and had unexpected effects, such as the activation of collagen II under osteogenic conditions. This could be considered a disadvantage from the physiological perspective. Consequently, the possible use of SSA for osteogenic regeneration would not be recommended. Since SSA induced collagen II in osteogenic conditions, enhanced chondrogenesis would be expected when culturing the pellets under chondrogenic conditions. However, this could not be shown. The absence of matrix formation under the chondrogenic conditions culture with all SSA concentrations suggested that SSA would not be ideal for chondrogenic tissue

engineering. The toxic effect of SSA on BMSCs may explain the reduction of matrix formation below that of controls in chondrogenic media, whereas the presence of fetal calf serum in osteogenic medium may have stimulated the recovery of the cells and thus formation of the matrix. The complete absence of any proteoglycan-rich matrix under either chondrogenic or osteogenic conditions was intriguing as SSA induced heterogeneous collagenous matrix formation under osteogenic conditions whilst it failed to induce similar or comparable matrix under chondrogenic conditions. This absence of collagenous matrix would reduce the probability of collagen II selective induction by SSA. The difference between the two conditions was the presence of serum in the osteogenic conditions and the ITS and TGFβ3 in the chondrogenic conditions. Further studies are required to determine the possible effects and/or interactions between these factors and SSA. SSA has not been fully characterized and to the best of our knowledge, this is the first study to use this molecule to examine BMSCs differentiation. However, in view of the negative findings, no further experiments were undertaken using SSA with BMSCs.

To conclude, although all the epigenetic modifiers induced collagenous matrix formation to variable degrees, 5-Aza-dC displayed maximal osteogenic induction at a concentration of 5-Aza-dC 1 μM. TSA induction at the range between 20 nM and 100nM may need further characterization, while TSA clearly induced chondrogenic matrix formation at a concentration of 100 nM TSA.

4.4. Refining the optimal conditions for epigenetic modifiers

Based on the previous results, the optimal combination of modifier and culture media were explored further; i.e. 5-Aza-dC and TSA in osteogenic conditions and TSA in chondrogenic conditions.

4.4.1. Hypothesis

Cells from a single patient treated in monolayer with 1 μM 5-Aza-dC or 100 nM TSA and cultured as pellets in osteogenic conditions produce

osteogenic matrix, while cells treated with 100 nM TSA in monolayer and cultured in pellets in chondrogenic conditions have chondrogenic characteristics and produce chondrogenic matrix.

4.4.2. Methods

BMSCs were isolated from a 66-year-old male. Cells were treated according to the previously used protocol (section 4.3.2). In brief, passage 1 BMSCs were cultured under basal conditions until 50% confluency. At this stage, cells were washed twice with PBS, and the media changed to serum free media. The cells were incubated at 37^{o}C and 5% CO_2 for 24 hours, then washed in PBS and α-MEM with 10% FCS was added. 5-Aza-dC (1μM) was added to the first study group, TSA (100nM) to the second group and no treatment for the control group. The modifier treatments were added for three successive days, after which culture was continued under basal conditions until confluence was reached.

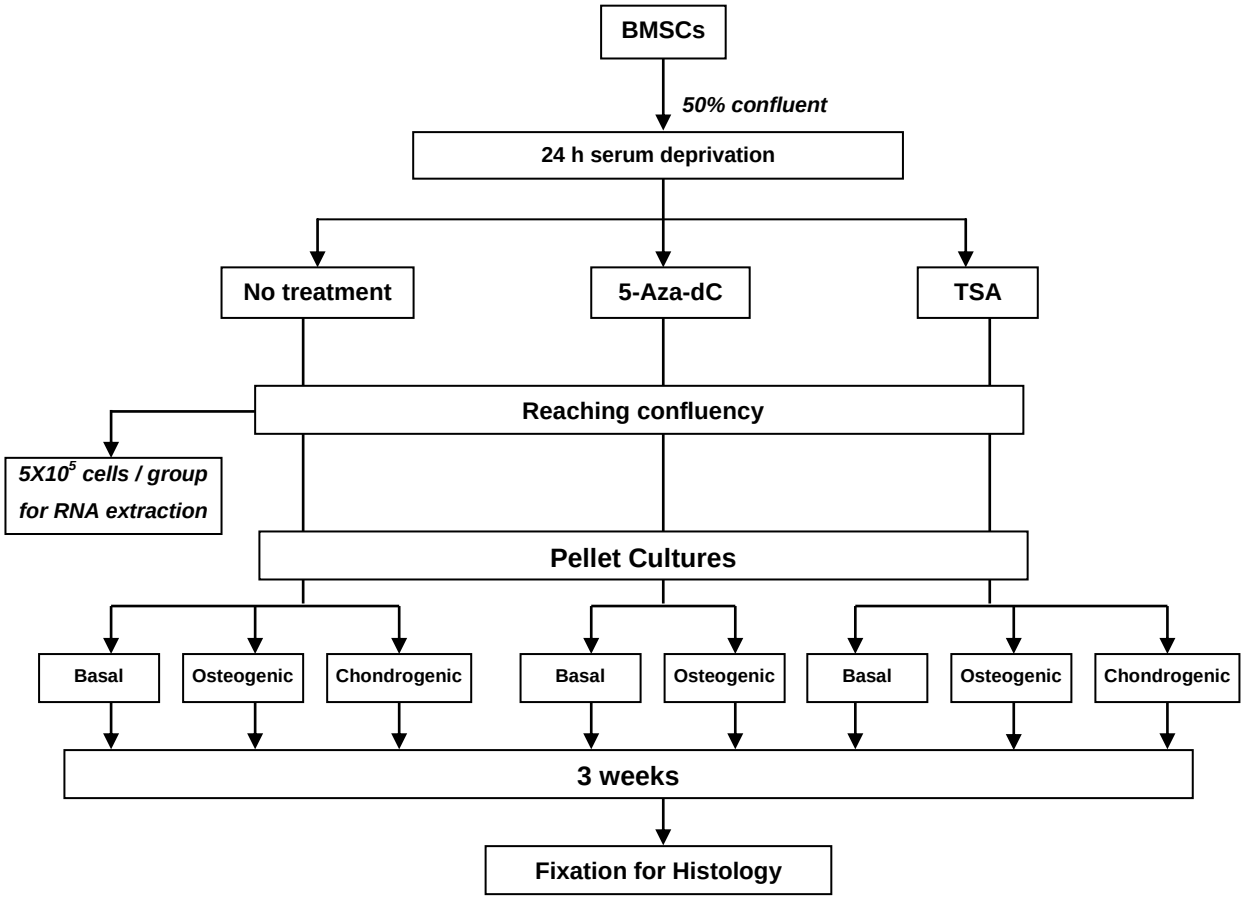


Fig. 4.18. Diagrammatic representation of study groups and plan.

At this stage, cells were harvested. $5X10^5$ cells were extracted for DNA and RNA. Runx2 and ALP expression was quantified using real time PCR. Remaining cells were cultured in pellet culture ($5X10^5$ cells/pellet) for 21 days. The first subgroup of pellets was cultured under basal conditions for each treatment group. The second subgroup was cultured under osteogenic conditions. The third subgroup was cultured under chondrogenic conditions for the TSA and no treatment groups (Fig. 4.18).

The pellet cultures were maintained for three weeks, after which pellets were fixed for histology, the matrix was characterized using Alcian blue / Sirius red, Safranin O, and Von Kossa stains. For further characterization, immuno-staining was carried out for collagen I, collagen II and Sox-9.

4.4.3. Results

a) 5-Aza-dC and TSA induced Runx-2 and ALP expression

At the end of the monolayer culture stage under basal conditions, significant induction of the osteogenic master gene Runx-2 with both 5-Aza-dC and TSA was observed, although no significant difference was found between the treated groups.

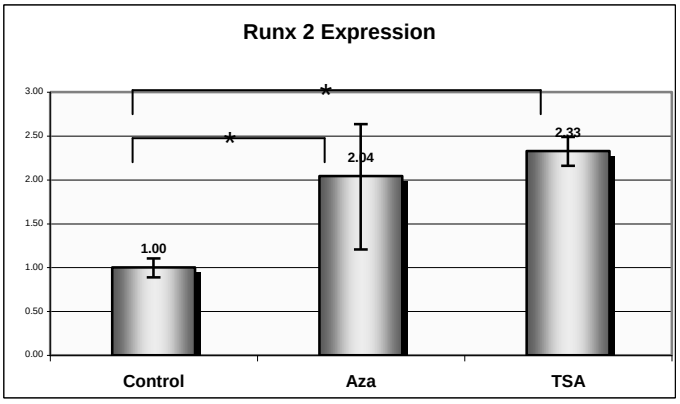


Fig. 4.19. Runx2 expression in the different groups. Bars represent the fold increase in expression (means ± standard deviations). Samples were run in triplicates. * p-value < 0.05.

The ALP expression pattern displayed similar trends, although the increase in ALP expression by 5-Aza-dC was not statistically significant. No statistical significance was found between 5-Aza-dC and the control or TSA.

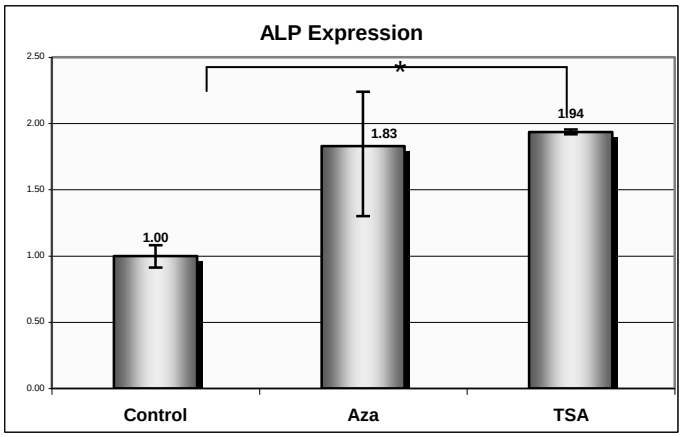


Fig. 4.20. ALP expression in the different groups. Bars represent the fold increase in expression (means ± standard deviations). Samples were run in triplicates. * p-value < 0.05.

b) Epigenetic modifiers induced osteogenic and chondrogenic matrix formation in pellets

Although no significant differences in gene expression could be found between 5-Aza-dC and TSA at the end of the monolayer culture, matrix staining showed marked differences between the groups. The pellets of 5-Aza-dC pre-treated cells cultured under osteogenic conditions (fig.4.21.E) displayed enhanced osteogenic matrix formation compared to the no treatment basal (fig.4.21.A), no treatment osteogenic (fig.4.21.B), TSA osteogenic (fig.4.21.G) and 5-Aza-dC basal (fig.4.21.D) groups. While the pellets for TSA cells cultured under chondrogenic conditions (fig.4.21.H) showed enhanced chondrogenic matrix formation compared to the no treatment basal (fig.4.21.A), no treatment chondrogenic (fig.4.21.C) and TSA basal groups (fig.4.21.F). The cells in the TSA chondrogenic groups were present within lacunae, which is the typical morphology of cartilage *in vivo*.

The chondrogenesis observed was further confirmed by Safranin O staining. The staining was negative for all groups except the chondrogenic control (fig. 4.22.C) and TSA chondrogenic group. The staining was more intense and uniformly distributed in the latter (fig.4.22.H).

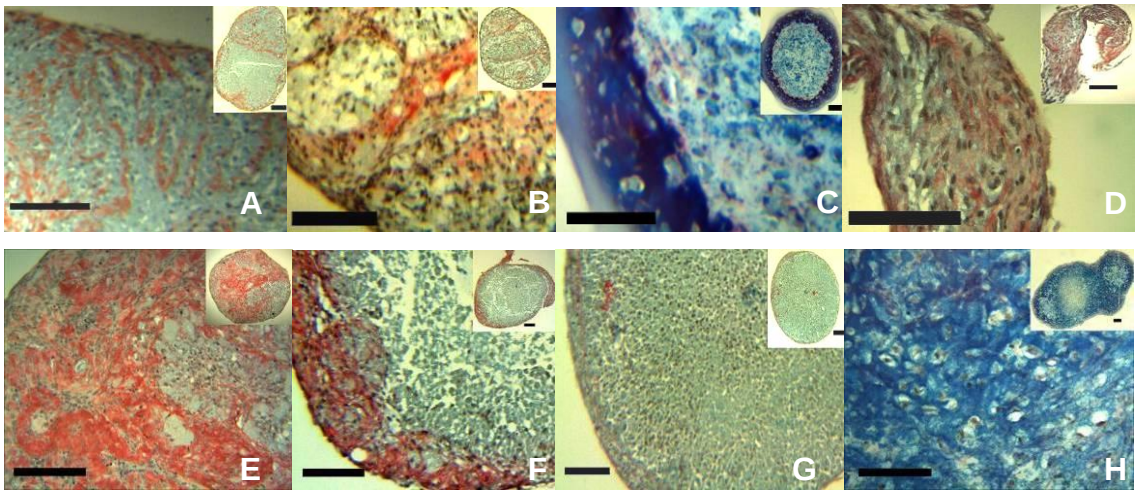


Fig. 4.21. Matrix staining of the pellets with Alcian blue and Sirius red. (A) no treatment group in basal conditions. (B) no treatment group in osteogenic conditions. (C) no treatment group in chondrogenic conditions. (D) 5-Aza-dC treated cells in basal conditions. (E) 5-Aza-dC treated cells in osteogenic conditions. (F) TSA treated cells in basal conditions. (G) TSA treated cells in osteogenic conditions. (H) TSA treated cells in chondrogenic conditions. Enhanced osteogenic matrix formation was observed with cells pre-treated with 5-Aza-dC and cultured as pellets in osteogenic conditions (E), while chondrogenic matrix formation was enhanced in TSA pre-treated cells, cultured as pellets under chondrogenic conditions (H). Scale bars = 100 µm.

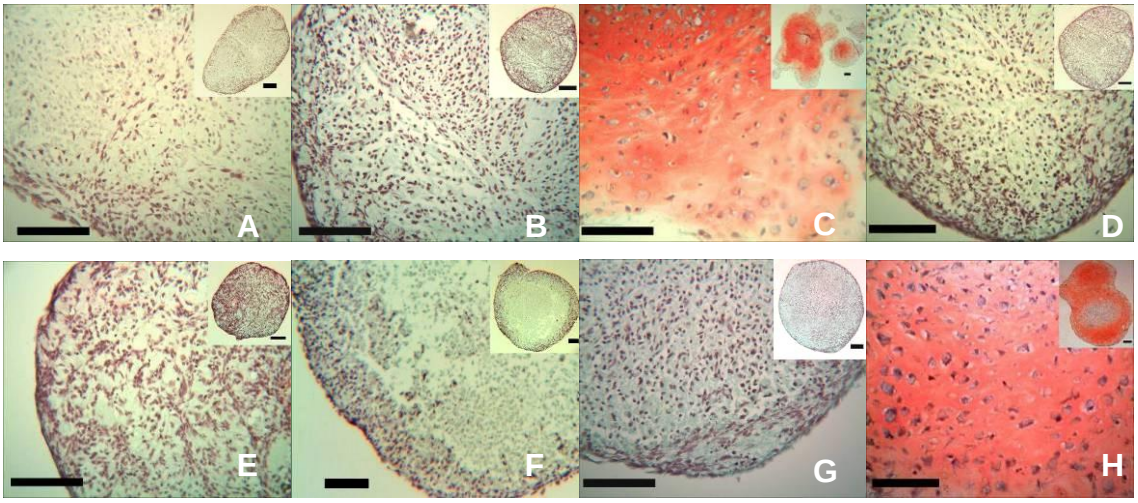


Fig. 4.22. Matrix staining of the pellets with Safranin O. (A) no treatment group in basal conditions. (B) no treatment group in osteogenic conditions. (C) no treatment group in chondrogenic conditions. (D) 5-Aza-dC treated cells in basal conditions. (E) 5-Aza-dC treated cells in osteogenic conditions. (F) TSA treated cells in basal conditions. (G) TSA treated cells in osteogenic conditions. (H) TSA treated cells in chondrogenic conditions. Safranin O staining was positive only in pellets cultured in chondrogenic conditions, displaying a more intense and uniform for the cells pre-treated with TSA.
Scale bars = 100 µm.

Negligable von Kossa staining was present in all groups. The counter stains (Alcian blue and Van Giessen) provided further confirmation of the

previous data. There was a demonstrable increased staining observed with Alcian blue in the TSA chondrogenic group (fig.4.23.H) and less staining with Van Giessen stain (collagen stain) in comparison to the chondrogenic control (fig.4.21.C and fig 4.23.C), suggesting the advantage of TSA pre-treatment for chondrogenic differentiation. Furthermore, the 5-Aza-dC osteogenic group (fig.4.23.E) displayed the most intense von Giessen stain of the different groups examined. This together with absence of Alcian blue stain supported the notion that initial treatment with 5-Aza-dC stimulated osteogenic differentiation.

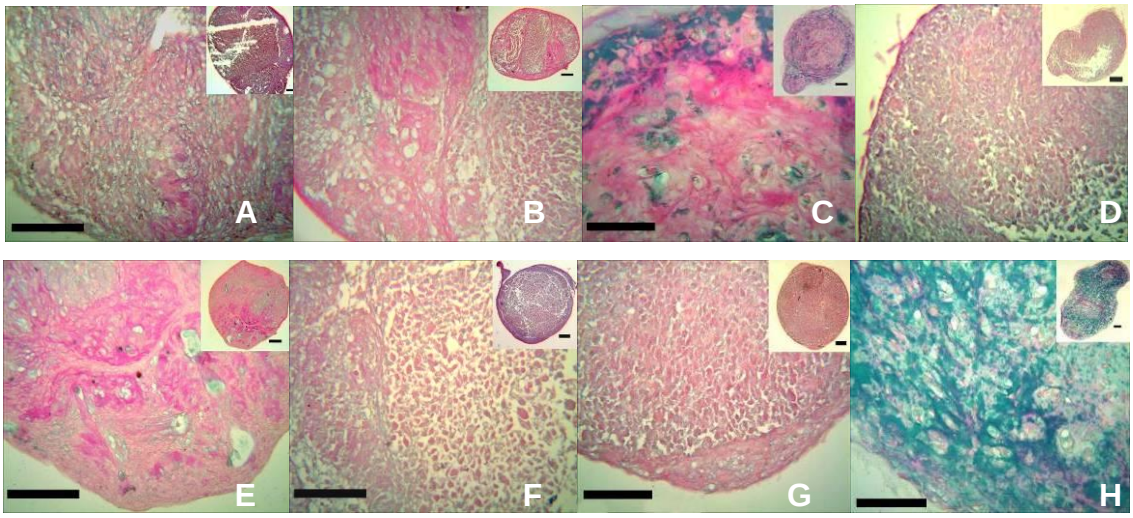


Fig. 4.23. Matrix staining of the pellets with Von Kossa stain , counter-stained with Van Giessen and Alcian blue. (A) no treatment group in basal conditions. (B) no treatment group in osteogenic conditions. (C) no treatment group in chondrogenic conditions. (D) 5-Aza-dC treated cells in basal conditions. (E) 5-Aza-dC treated cells in osteogenic conditions. (F) TSA treated cells in basal conditions. (G) TSA treated cells in osteogenic conditions. (H) TSA treated cells in chondrogenic conditions. The positive van Giessen counter stain in (E) and Alcian blue counter stain in (H) provided further confirmation of the previous results. Scale bars = 100 µm.

Immuno-staining provided further characterization of the matrix. The sections for the no-treatment group cultured in basal media were considered as baseline control for protein expression. Collagen I immuno-staining was undertaken for the no-treatment cells cultured under osteogenic conditions and the cell groups treated with 5-Aza-dC. Collagen II and Sox-9 immuno-staining was undertaken for no treatment cells cultured under chondrogenic conditions and the TSA treated cell groups.

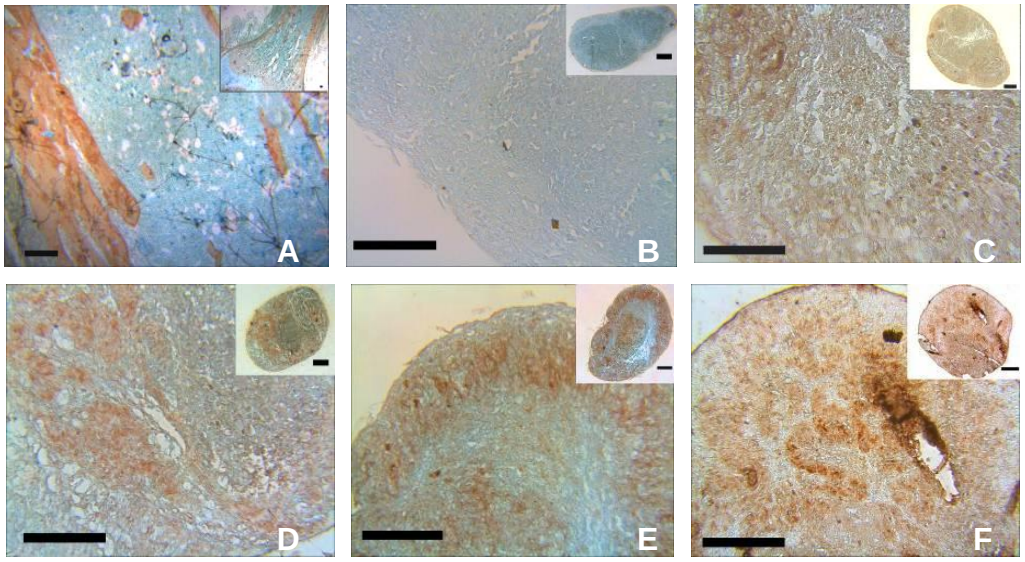


Fig. 4.24. Collagen I Immuno-staining of the pellets. (A) positive control (rat femur). (B) negative control. (C) no treatment group in basal conditions. (D) no treatment group in osteogenic conditions. (E) 5-Aza-dC treated cells in basal conditions. (F) 5-Aza-dC treated cells in osteogenic conditions, which dispayed the most intense staining. Scale bars = 100 μm.

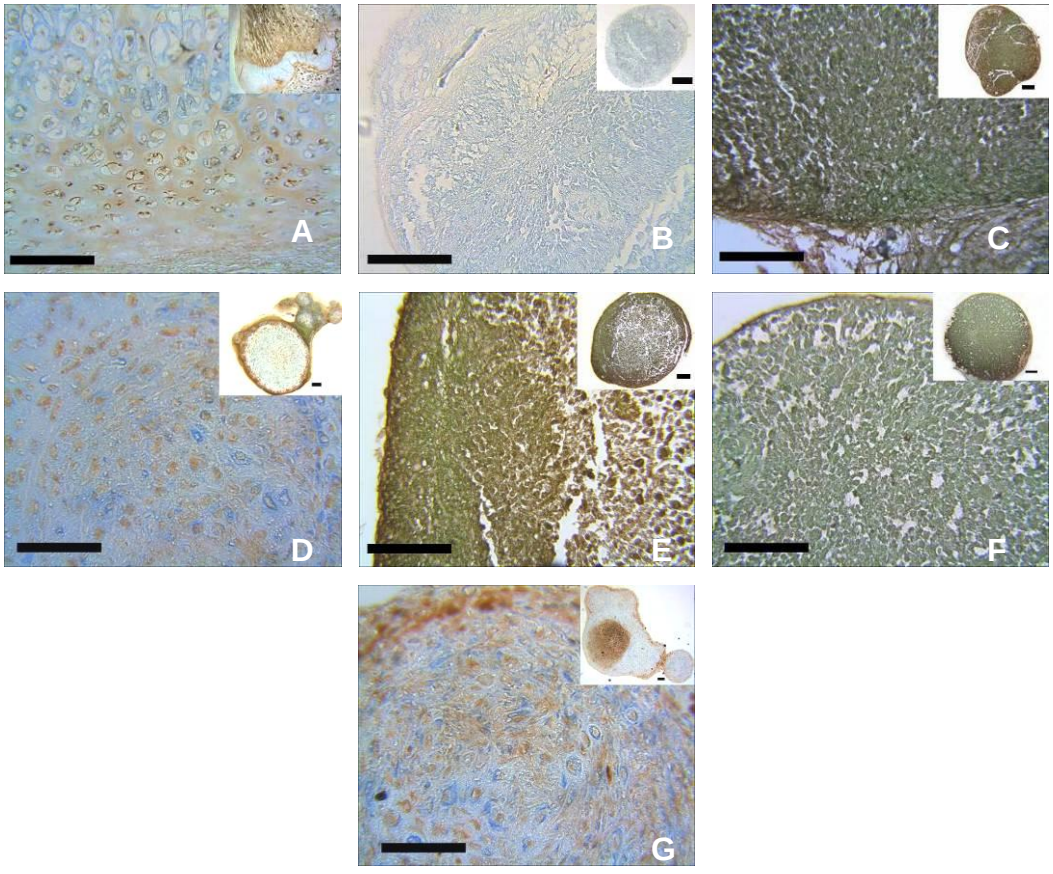


Fig. 4.25. Collagen II Immuno-staining of pellets. (A) positive control (rat femur). (B) negative control. (C) no treatment group in basal conditions. (D) no treatment group in chondrogenic conditions. (E) TSA treated cells in basal conditions. (F) TSA treated cells in osteogenic conditions. (G) TSA treated cells in chondrogenic conditions, displaying the most intense collagen II staining, especially in comparison to the chondrogenic control (D). Scale bars = 100 μm.

The positive control (rat femur) for collagen I staining showed extensive staining of the femur shaft (Fig.4.24.A). Staining was completely absent in the negative control (Fig.4.24.B). There was distinct and definitive matrix staining in the 5-Aza-dC treated cells cultured under osteogenic conditions (Fig.4.24.C). Staining was noted across the pellet with distinct expression in the matrix. This group had uniform staining across the pellet compared to the other studied groups, which showed some degree of staining.

Collagen II positive control stained the femur growth plate cells and matrix (Fig.4.25.A), but staining was completely absent in the negative control (Fig.4.25.B). Negligible staining was observed for the no treatment cell group cultured under basal conditions (Fig.4.25.C) or TSA treated cells cultured under osteogenic conditions (Fig.4.25.F). The TSA treated cells cultured under basal conditions acquired a degree of background staining (Fig.4.25.E). Conversely, non-treated cells cultured under chondrogenic conditions displayed distinctive staining for collagen II inside the cells with modest amount of matrix staining (Fig.4.25.D). The staining was enhanced in the TSA pre-treated cells cultured under the same conditions, with collagen II staining observed inside the cells as well as in the matrix.

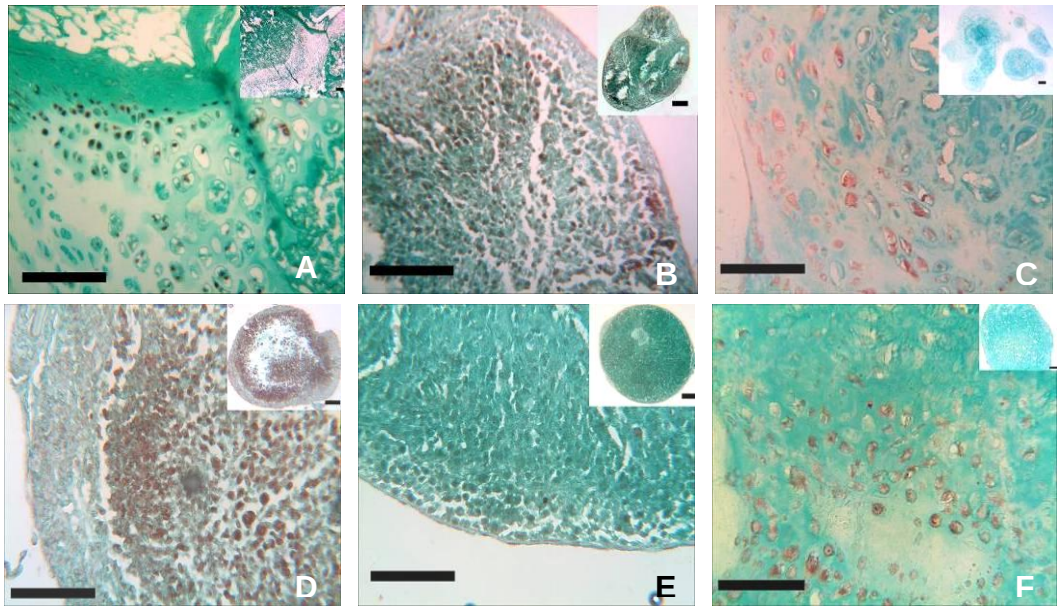


Fig. 4.26. Sox-9 Immuno-staining of the pellets. (A) positive control (rat femur). (B) no treatment group in basal conditions. (C) no treatment group in chondrogenic conditions. (D) TSA treated cells in basal conditions. (E) TSA treated cells in osteogenic conditions. (F) TSA treated cells in chondrogenic conditions. Scale Bar = 100 μm.

The localization of the transcription factor Sox-9 was comparable to that of collagen II. The positive control showed a distinctive nuclear staining (Fig.4.26.A). Similar but fainter staining was found in the non-treated cells cultured under chondrogenic conditions (Fig.4.26.C). More intense staining could be noticed in TSA treated cells cultured under chondrogenic conditions (Fig.4.26.F). In the control cell group cultured under basal conditions and the TSA treated cells cultured under basal conditions, non-specific cytoplasmic staining in addition to sporadic nuclear staining was observed. TSA treated cells cultured under osteogenic conditions were negative for Sox-9.

4.4.4. Discussion

The current studies confirmed and extended the results of the dose response studies. The ability of the BMSCs to differentiate along the osteogenic and chondrogenic lineages upon culture in the corresponding culture conditions (section 3.1) was confirmed. In addition, Runx-2 and ALP expression levels in the cells following treatment in monolayer was in agreement with the results obtained in sections 3.2, 3.4, 3.6 and 3.7. 5-Aza-dC up-regulated Runx-2 slightly, while the ALP showed a similar non-significant trend. Thus after treating the cells with either of the agents, no obvious molecular evidence could be found for lineage modulation. The importance of pellet culture as another cell culture method based on providing contact between the cells in 3D aggregates was evident. The effect of the epigenetic modifiers treatment in the beginning of the monolayer phase of the experiment was displayed at the end of the pellet cultures several weeks later. As the cells were treated when they were 50% confluent, the alteration of the epigenetic status caused by the modifiers was expected to be inherited to the daughter cells. Based on the definition of epigenetics (Wolffe & Matzke, 1999), inheritance is a crucial characteristic, demonstrated in this culture system. Although pellet culture itself, was known to enhance matrix formation, epigenetic modifiers were shown in these studies to enhance both the quantity and quality of that matrix.

In addition, this study demonstrates the ability of the demethylating agent 5-Aza-dC and the histone deacetylase inhibitor TSA to enhance osteogenic or chondrogenic differentiation respectively. Furthermore, the regimen of treatment

used here enhanced differentiation more than the regimen used in section 4.2, which was a single application of TSA and three applications of 5-Aza-dC per week along the monolayer phase. 24 hours serum deprivation followed by three days of treatment was sufficient to drive the cells towards their differentiation lineages. The effect of TSA on cells that were subsequently cultured in pellet form under osteogenic conditions was negligible. These results suggest that demethylation of certain genes was required for osteogenic development whereas histone acetylation of other genes drove the chondrogenesis. The induction of osteogenic and chondrogenic matrix was obtained in the *in vitro* system. The study of this system *in vivo* would be useful to study the consistency of the epigenetic modifiers effect on the pellets *in situ*.

***4.5. In vivo* study of pellets of BMSCs pre-treated with epigenetic modifiers**

The *in vivo* subcutaneous implantation model provides an environment for culturing cells or implants in different conditions than is possible using the *in vitro* incubation system. Pellets are exposed to a range of physical and chemical challenges carried by the host blood, which potentially mimic what occurs during the natural developmental process.

4.5.1. Hypothesis

Matrix formation by pellets is further enhanced by culturing the pellets *in vivo*, following pre-treatment with epigenetic modifiers.

4.5.2. Methods

BMSCs were obtained from a 75 years old female and were cultured after the first passage in monolayer to 50% confluency. Thereafter cells were cultured for 24 hours without serum followed by treatment for three consequent days with 5-Aza-dC, TSA or PBS as control. Cells were moved to the pellet culture system at confluence. Control cells were cultured in either osteogenic or chondrogenic conditions, while 5-Aza-dC treated cells were cultured in osteogenic conditions and TSA treated cells were cultured in chondrogenic

conditions. The pellet cultures were continued for 21 days and then the pellets then placed in diffusion chambers (DC) in immuno-compromised mice.

DC are constructed using completely inert materials. Each chamber is composed of a Plexiglas® ring with an outer diameter of 14 mm, inner diameter of 10 mm and 2 mm thickness (Millipore, cat. no. PR00 014 00) with two membranes of mixed cellulose esters, 13 mm in diameter with 0.45 µm pores size (Millipore, cat. no. HAWP01300) fixed on the ring with special cement (Millipore, cat. no. XX70000072). The components of the DC were sterilized by UV exposure overnight and assembled in two phases. First, the ring was glued to a membrane using cement, the pellets added, and the other side membrane was attached. The whole structure became stable immediately. DC were incubated overnight in 2 ml of corresponding media under 37^{o}C and 5% CO_2 and implanted subcutaneously in the flanks region of athymic mice.

These membranes allow fluids to move in and out the chamber to nourish the cells and eliminate waste products, while the diameter of membrane pores inhibits the migration of host cells into the chambers. Thus, development of BMSCs could theoretically be enhanced by physiological triggers present in the body fluids, such as mitogens, growth factors and/or physical factors as the pressure and vibration created during the movement of the mice (Bab et al., 1986).

After 28 days, the experiment was terminated by exposing the mice to rising CO_2, followed by translocation of the upper cervical vertebrae. The chambers with surrounding tissues were excised. Pellets were extracted from the diffusion chambers, fixed and processed for histological studies.

4.5.3. Results

DC were completely enclosed in subcutaneous tissue. Vascularisation was evident on the inner side of the subcutaneous pouch in all samples, and was most prominent adjacent to chambers containing 5-Aza-dC pre-treated pellets (Fig.4.27). Although the diffusion chambers were intact, many pellets

were lost. Inside the chambers, there was serum-like fluid filling the whole chamber.

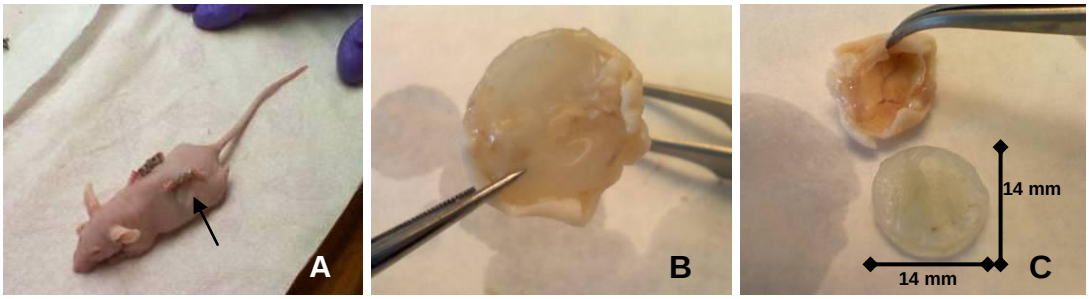


Fig. 4.27. The *in vivo* study. (A) The arrow indicates the diffusion chamber implanted under the skin of the flank region on both sides. After 28 days, the diffusion chambers were removed (B). The enclosed sac was opened (C) and vascularisation observed on the inner side. The diffusion chambers - shown on the bottom of panel (C) were intact. Pellets were extracted and fixed for histology.

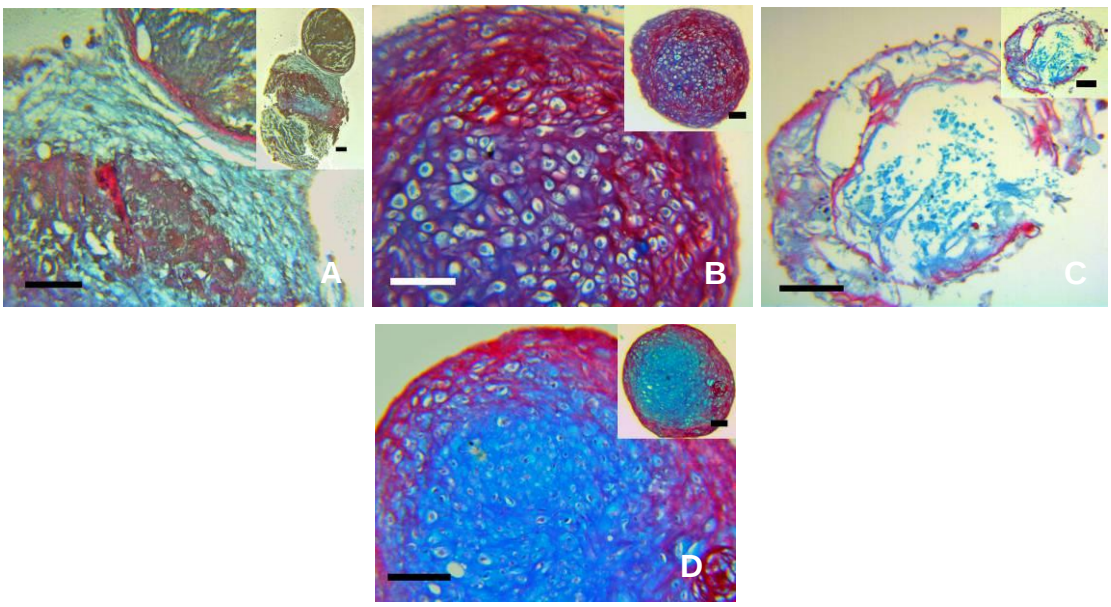


Fig. 4.28. Alcian blue/ Sirius red staining for BMSCs pellets cultured for 28 days inside diffusion chambers *in vivo*. Pellets were cultured initially for 21 days *in vitro* in osteogenic conditions (A), chondrogenic conditions (B), osteogenic conditions pre-treated with 5-Aza-dC (C) and chondrogenic conditions pre-treated with TSA (D). The subcutaneous implantation did not enhance the osteogenic response either in the control or in the 5-Aza-dC pre-treated group, although bright collagenous filaments in the latter group were observed. The chondrogenic controls displayed the architecture of cells located within lacunae surrounded by collagenous and proteoglycan matrix. Proteoglycans were markedly increased across the whole section in the cells pre-treated with TSA. In addition, the cells were smaller. Deep thanks for Dr. Janos Kanczler for doing the Animal work. Scale Bar = 100 µm.

A/S staining showed poor osteogenic matrix formation in the control group (Fig. 4.28.A). Matrix synthesis was enhanced within 5-Aza-dC pre-treated group with filamentous collagen located on the periphery of the pellets (Fig.

4.28.B). The chondrogenic controls showed the distinct morphology of large cells within lacunae, surrounded by collagenous and proteoglycan matrix, resembling hypertrophic cartilage (Fig. 4.28.C). Pellets of TSA pre-treated cells (Fig. 4.28.D) displayed extensive proteoglycan expression with small cells located with lacunae, similar in part, to the epiphyseal cartilage.

4.5.4. Discussion

A relatively poor matrix was observed within the osteogenic pellets (irrespective of treatment) which may be explained by the location of the implant, as the DC were implanted subcutaneously. Thus, a period of time would be needed to establish an appropriate blood supply to the DC. Osteogenesis is inhibited by low oxygen tension (D'Ippolito et al., 2006). The relative hypoxia may have acted as a physical factor that affected the differentiation process. This explanation was further confirmed by the chondrogenic differentiation, as low oxygen tension is required (Hansen et al., 2001).

Thus although the subcutaneous implantation of diffusion chambers enhanced chondrogenic differentiation, the advantage of TSA pre-treatment was that chondrocytes remained as small epiphyseal cells and did not appear to progress to hypertrophic chondrocytes. In addition, this model may point at the importance of the oxygen tension on the differentiation of BMSCs.

4.6. Conclusions

The effects of epigenetic modifiers on the differentiation of the BMSCs were confirmed using pellet culture, a 3D model of tissue culture. Cells treated with epigenetic modifiers in monolayer changed their phenotype in the pellets with altered corresponding matrix. The effect of epigenetic modifiers was obvious only after pellets were cultured in specialized media. A degree of selectivity of the modifiers was observed with 5-Aza-dC promoting osteogenic differentiation in osteogenic media. In contrast, TSA enhanced chondrogenic differentiation when pellets were cultured in chondrogenic media. These results

may provide a step towards deriving purer differentiated cell populations, derived from the patient's own bone marrow stromal derived cells, with the potential to improve bone and cartilage regeneration.

NOVEL MODELS OF PELLET CULTURE

5.1. Introduction

Fetal derived cells (FC) were obtained from the cartilagenous femurs of 8-10 week fetuses. These cells are known to have great potential for *in vitro* expansion as well as differentiation into the osteogenic, chondrogenic and adipogenic lineages (Mirmalek-Sani et al., 2006b). FC have not been studied, to the best of our knowledge, in 3D osteogenic cultures. In addition, their differentiation capacity in response to the epigenetic modifiers was unknown. Such responses might help to confirm the effects of these modifiers on other multipotential cell types.

5.2. FC response to the epigenetic modifiers

5.2.1. Hypothesis

FC differentiate to the osteogenic or chondrogenic lineage in the corresponding media. 5-Aza-dC enhances the ALP activity in monolayer and matrix formation in pellets when cultured in osteogenic conditions, while TSA enhances matrix formation in pellets cultured in chondrogenic conditions.

5.2.2. Methods

FC were isolated from an 8.5 weeks post conception fetal femur. FC were used in this study at passage two. FC were treated using the same protocols described before (section 4.3.2.) either with 5-Aza-dC or SSA followed by pellet culture in osteogenic conditions or with TSA for subsequent pellet culture in chondrogenic conditions **(5 pellets / group)**; see fig.5.1. In addition, FC were cultured in monolayer in osteogenic conditions with and without 5-Aza-dC or SSA for 30 days, then the cells were fixed and stained for ALP activity.

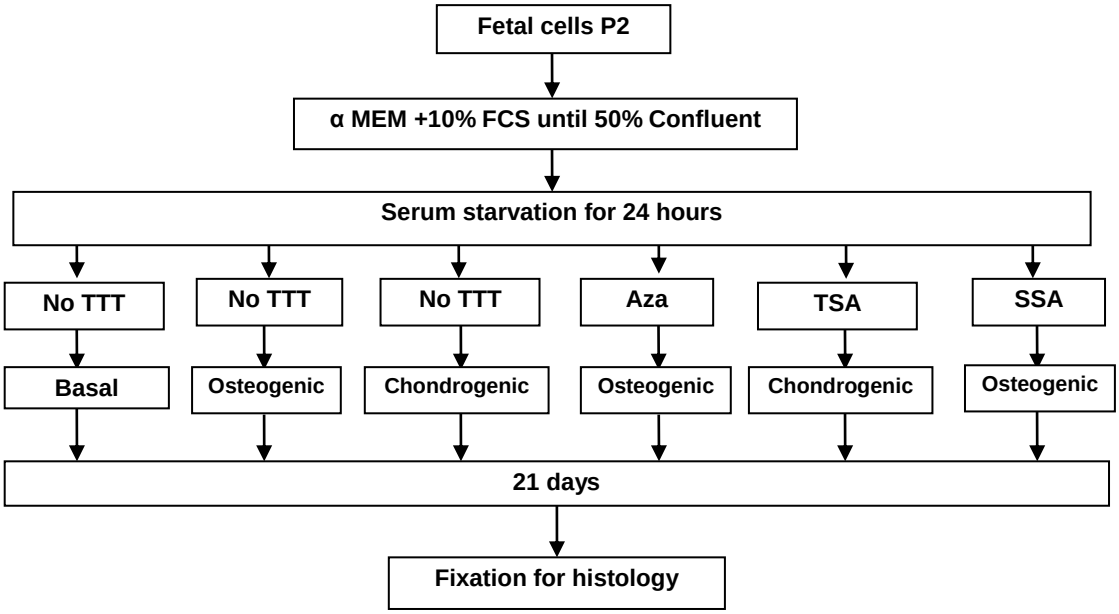


Fig. 5.1. Diagrammatic representation of the study phases and groups.

5.2.3. Results

a. FC recovery from the epigenetic modifiers was comparable to BMSCs

FC were counted at the end of the monolayer stage. The epigenetic modifiers reduced the cell numbers with the effect of 5-Aza-dC < TSA < SSA (Fig.5.2). The cell count of TSA pre-treated cells represented only 60% of the control, while FC pre-treated with SSA was less than 16%. The cell count of 5-Aza-dC pre-treated cells was 84% of the controls.

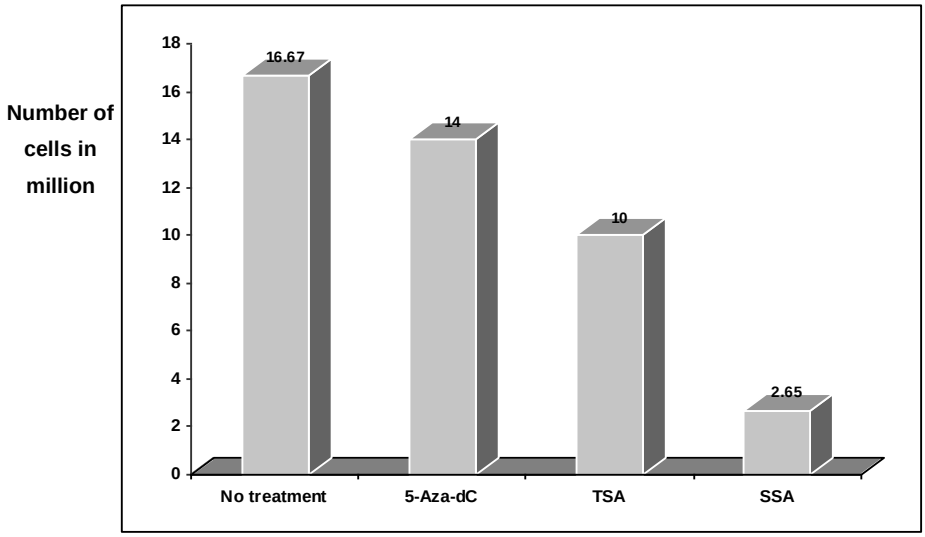


Fig. 5.2. Cell numbers in millions of FC treated with different epigenetic modifiers. The maximum toxicity was found in SSA pre-treated cells.

b. Epigenetic modifiers reduced ALP activity of FC

Cells that were cultured in osteogenic conditions without any initial treatment with epigenetic modifiers showed the highest induction of ALP activity (Fig.5.3.B). Intense staining could be observed in almost every cell. FC pre-treated with 5-Aza-dC (Fig.5.3.C) had ALP activity that was comparable to the basal controls (Fig.5.3.A) while cells pre-treated with SSA displayed even less activity (Fig.5.3.D).

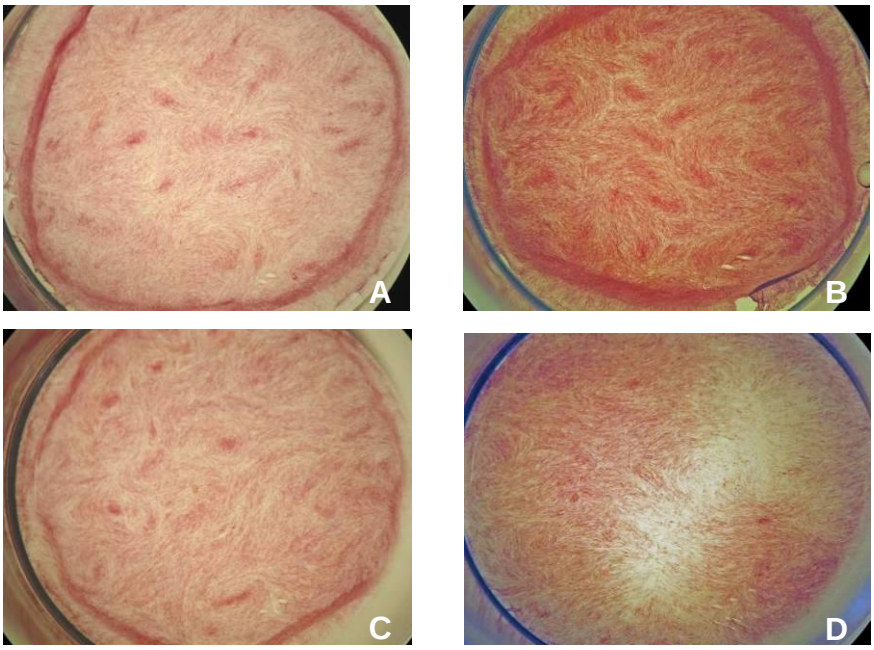


Fig.5.3. ALP activity of FC cultured in basal conditions (A), osteogenic conditions without previous treatment (B), with initial treatment with 5-Aza-dC (C) or SSA (D). Cells cultured in osteogenic conditions without treatments showed highest ALP activity compared to the basal conditions or to cells pre-treated with either epigenetic modifiers.

c. Pellets in osteogenic conditions differentiated into a configuration similar to fetal bone cross section, while TSA was essential for chondrogenic differentiation

All pellets cultured in basal and osteogenic conditions displayed the same morphology of a Sirius red stained ring, surrounded by an ill-defined cellular layer while the centre of the ring was composed of an area with cartilage-like morphology and small dense compact cells. This configuration was not present in the pellets cultured in chondrogenic conditions. Pellets cultured as osteogenic control (without any pre-treatment, Fig 5.4.B) and those pre-treated with 5-Aza-dC (Fig.5.4.D) showed this configuration. Pellets cultured

in basal conditions displayed a similar morphology, although the thickness of the collagenous band was less and the ring was eccentric (Fig.5.4.A). The cells pre-treated with SSA had the same arrangement although with fainter Sirius red staining (Fig.5.4.F). This arrangement was very similar to that of a cross section of a fetal femur (Fig.5.4.H). Illustration was drawn based the longitudinal section of an 8 week post-conception fetal femur (Mirmalek-Sani et al., 2006b).

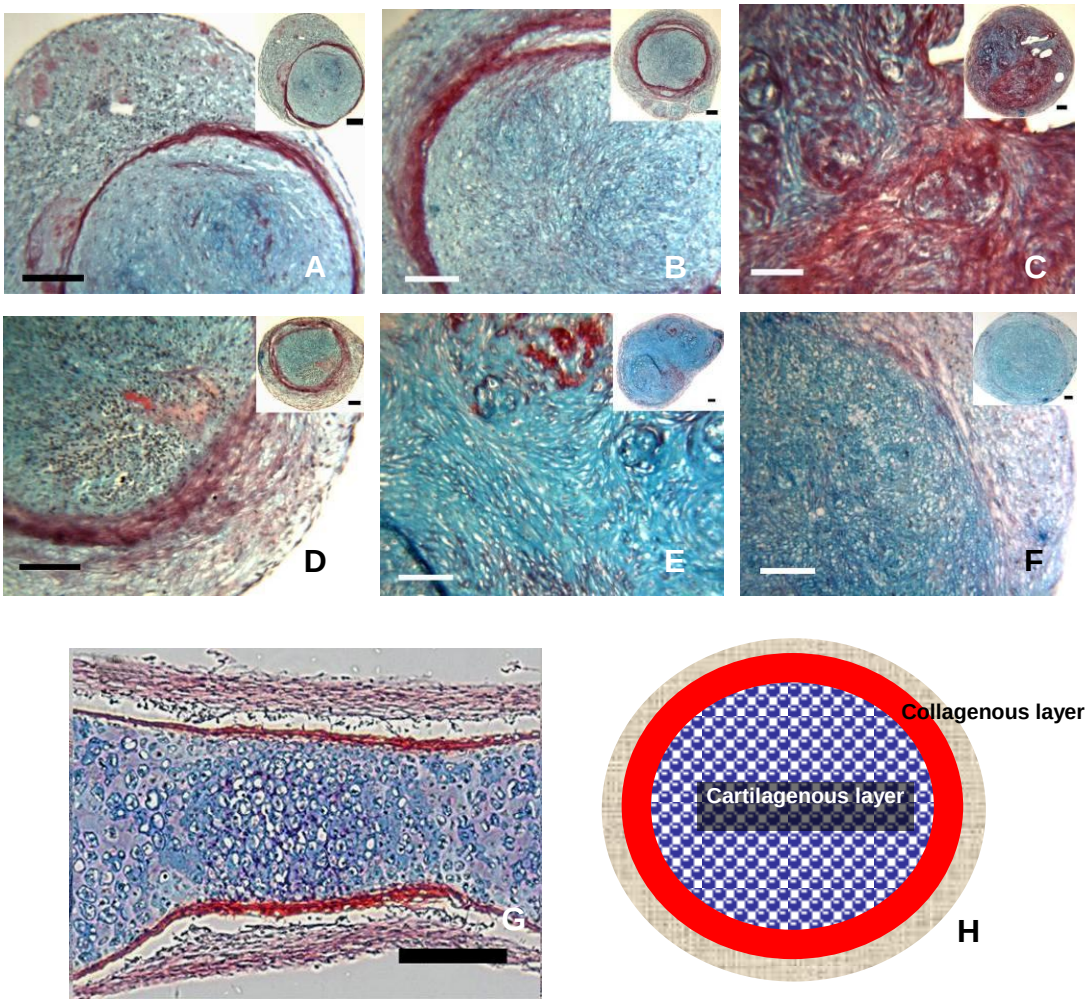


Fig. 5.4. Alcian blue/ Sirius red staining for FC; pellets were cultured in basal conditions (A), osteogenic conditions (B) and 5-Aza pre-treated cells in osteogenic conditions showed the morphology of a ring of Sirius red positive staining (D), being comparable in the osteogenic control and 5-Aza-dC pre-treated pellets. This morphology matches the diagrammatic cross section (H) of an 8 week old fetal femur (G) published earlier by our group (Mirmalek-Sani et al., 2006b). Pellets cultured in chondrogenic conditions (C) have intense collagenous matrix formation throughout the pellet. When pre-treated with TSA, this matrix was proteoglycan-rich (E). Cells pre-treated with SSA and cultured in osteogenic conditions (F) had negligable amount of collagenous matrix that kept the ring-like morphology. Scale Bar (A-F) = 100 μm and for (G) = 200 μm.

Cells cultured in chondrogenic conditions showed profound matrix formation that was mainly collagenous in nature, as shown by the Sirius red staining, with negligable proteoglycan as shown by Alcian blue staining (Fig.5.4.C). The chondrogenic matrix had no specific configuration. Pre-treatment with TSA markedly enhanced the formation of a proteoglycan rich matrix (Fig.5.4.E).

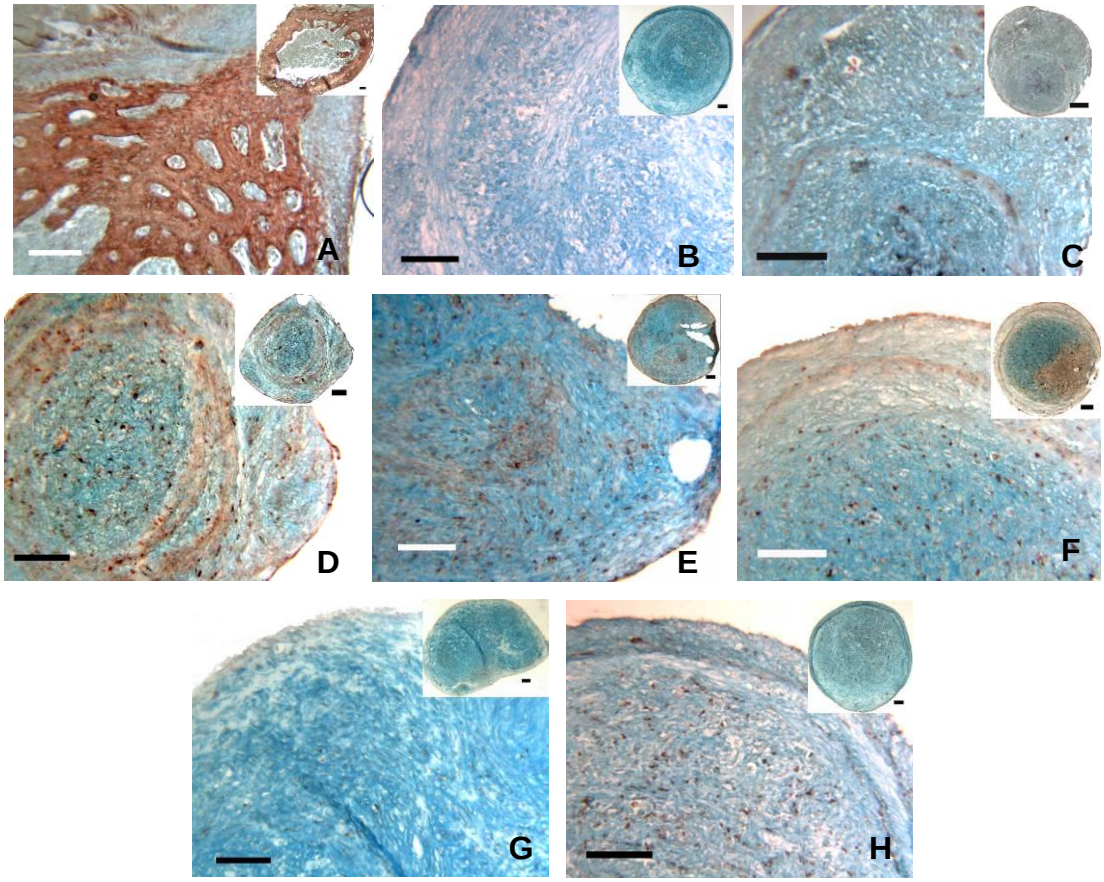


Fig. 5.5. Collagen I immuno-staining of Fetal pellets. (A) Positive control (a section of rat femur); collagen I deeply stained the periosteal bone. (B) is a negative control; there was a complete absence of staining. The ring structure in basal control (C), osteogenic control (D), 5-Aza-dC pre-treated cells in osteogenic conditions (F) and SSA pre-treated cells in osteogenic conditions (H) was positively stained, as were scattered cells. Staining was most intense in the osteogenic control (D). The chondrogenic control (E) had dispersed positive staining in the matrix as well as inside the cells, while the cells pre-treated with TSA and cultured in chondrogenic conditions (G) were almost completely negative for collagen I and no stain could be observed in the matrix. Scale bars = 100 μm.

The collagenous matrix formed by the pellets was characterized by collagen I, collagen II and collagen III immuno-staining. The collagen I antibody stained the ring structure in all pellets with varying intensity. The intensity of

collagen I was comparable to Sirius red staining in the osteogenic control (Fig.5.4.D) while the intensity of the immuno-staining in 5-Aza-dC pre-treated pellets did not correlate with the Sirius red staining. In contrast, cells pre-treated with TSA and cultured in chondrogenic conditions did not express collagen I (Fig.5.5.G), while those, which received no previous treatment, had produced a small amount of collagen I (Fig.5.5.E). Thus, TSA enhanced the physiological characteristics of cartilage collagen in chondrogenic pellets. Cells pre-treated with SSA also displayed weak staining for collagen I.

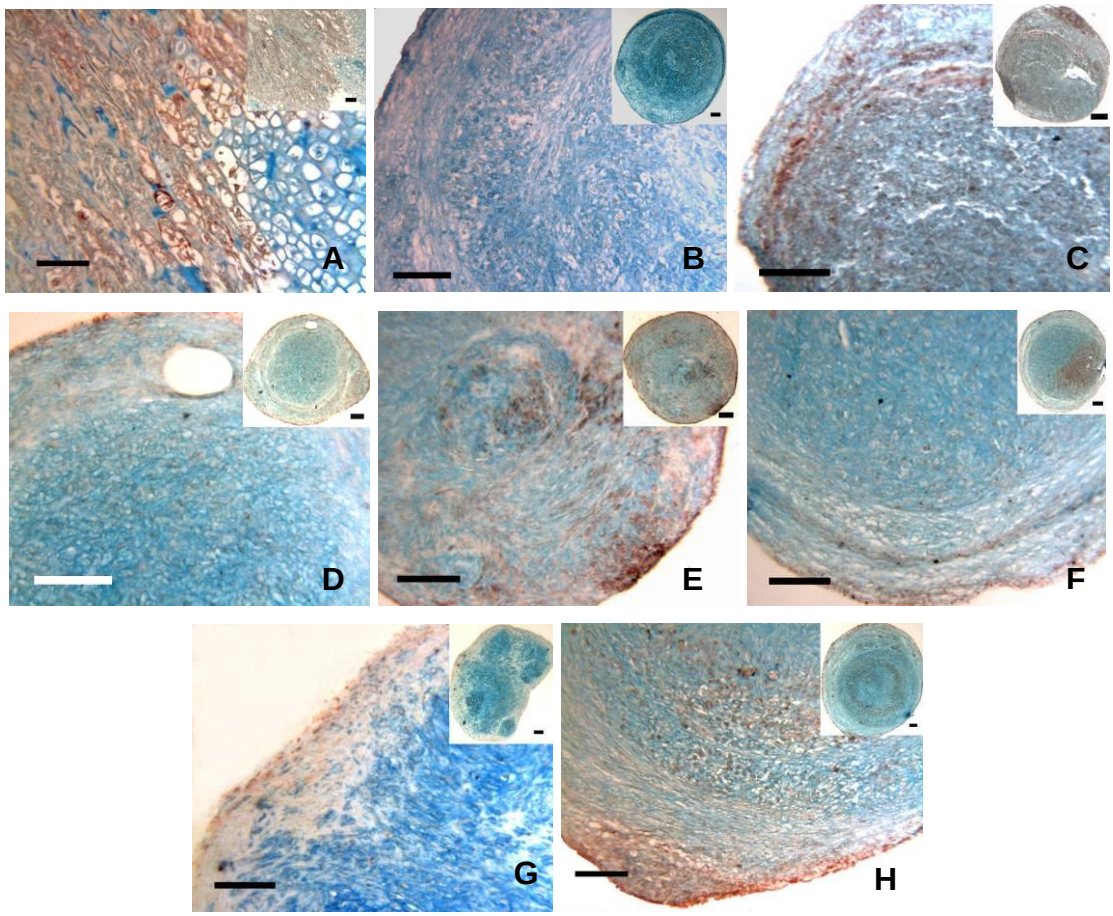


Fig. 5.6. Collagen II immuno-staining of Fetal pellets. (A) Positive control (a section of rat femur); collagen II strongly stained the growth plate. (B) Negative control. The ring structure in basal control (C), 5-Aza-dC pre-treated cells in osteogenic conditions (F) and SSA pre-treated cells in osteogenic conditions (H) was positive for collagen II, as well as inside some cells, while the osteogenic control (D) showed negligible collagen II staining. The chondrogenic control (E) has widespread positive staining in the matrix as well as inside the cells while the cells pre-treated with TSA and cultured in chondrogenic conditions (G) displayed less expression of collagen II. Scale bars = 100 μm.

Collagen II, the most abundant type of collagen in cartilage, was produced in pellets cultured under chondrogenic conditions. The relative decrease in immuno-staining in the centre of the TSA pre-treated pellets (Fig.5.6.G) could be due to the coverage of the collagen II epitope by the extensive proteoglycan formation in TSA pre-treated pellets (Smith et al., 2003), marked by the Alcian blue staining in Fig.5.4.E. Pellets cultured in osteogenic conditions with initial treatment with 5-Aza-dC and SSA as well as pellets cultured in basal conditions expressed variable degrees of collagen II in the Sirius red positive ring. Positive staining was also observed inside many cells. The rings of the osteogenic control pellets were almost completely negative (Fig.5.6.D). Staining was positive inside some scattered cells in the chondrogenic centre of the pellet.

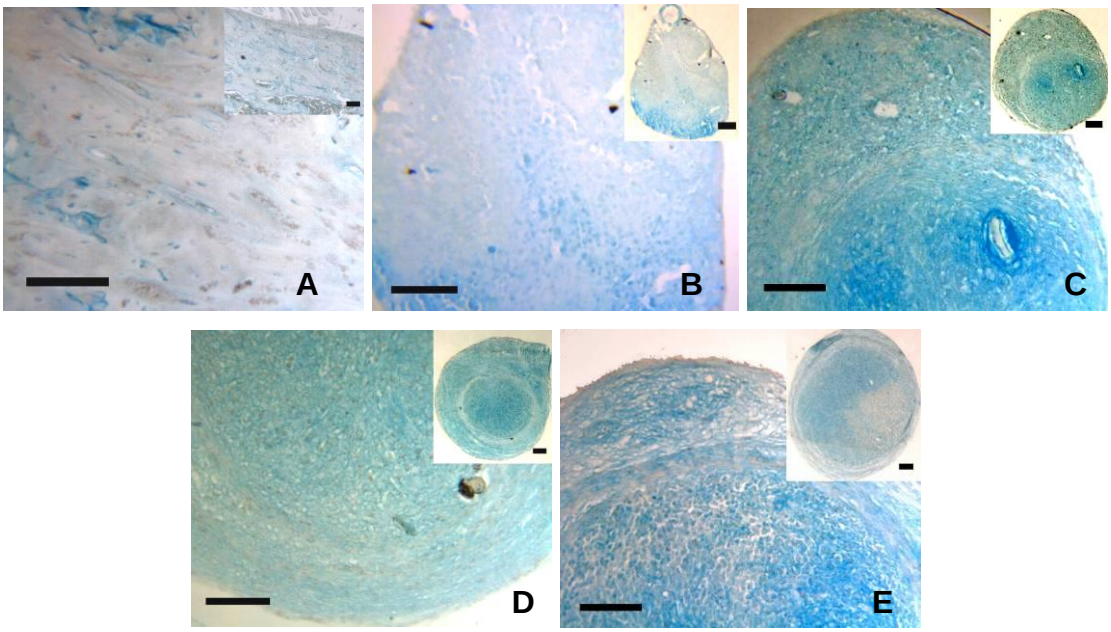


Fig. 5.7. Collagen III immuno-staining of Fetal pellets. (A) Positive control (a section of rat femur); collagen III stained the periosteal bone where it is physiologically located in combination to collagen I. (B) was negative control. (C) Basal control and (D) Osteogenic control has very weak staining while the 5-Aza-dC pre-treated pellet (E) showed intense staining. Scale bars = 100 μm.

Collagen III is the main type of collagen in the connective tissue of the skin. It is also found in bone between collagen I fibres. As shown previously with the PCR arrays, collagen III was up-regulated by 5-Aza-dC in BMSCs cultured in monolayer (section 3.6.3.). The results of the immuno-staining in FC confirmed the molecular findings in BMSCs. Pellets from cells previously treated

5-Aza-dC showed the most intense staining of collagen III (Fig.5.7.E) while pellets cultured under basal conditions had negligible collagen III in the collagenous ring (Fig.5.7.C). The osteogenic control (Fig.5.7.D) had less staining than the 5-Aza-dC pre-treated pellet, and thus was comparable to the rat femur (Fig.5.7.A).

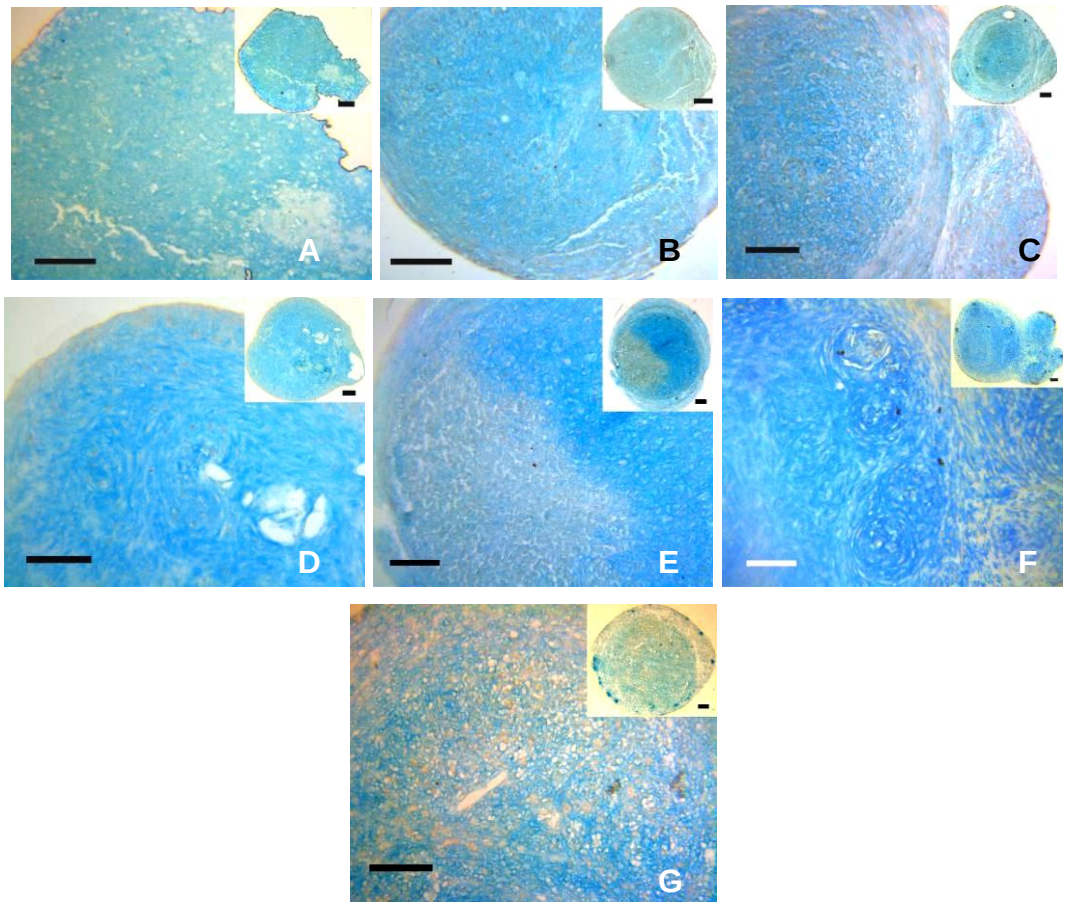


Fig. 5.8. Nucleostemin immuno-staining of Fetal pellets. (A) Negative control. Scattered cells showed intranuclear staining in the basal pellets (B), while a large proportion of cells were positive in the osteogenic control (C) either inside or outside the ring structure. The high-density cellular pole inside the ring of the pellets pre-treated with 5-Aza-dC (E) was stained with the nucleostemin antibody in addition to scattered cells all over the pellet. There was a perfect match between the highly compacted cell population and the intranuclear staining. Pellets cultured under chondrogenic conditions either as control (D) or pre-treated with TSA (F) showed the least staining being negative throughout the pellet except for a few cells on the periphery. Surprisingly, the SSA pre-treated pellet (G) displayed the most intense staining that was present throughout the pellet. Scale bars = 100 µm.

Nucleostemin is a marker of early progenitor cells and is found normally inside the nucleolus. Nucleostemin expression was expected to decrease during differentiation. The immuno-staining for nucleostemin showed a wide

spectrum of expression, ranging from almost negative in some pellets, which were those cultured in chondrogenic conditions, irrespective of initial treatment (Fig.5.8.D and F), to highly positive intranuclear staining in the osteogenic groups. The osteogenic control (Fig.5.8.C) and the 5-Aza-dC pellets (Fig.5.8.E) showed staining in many cells all over the sections but especially in the dense small cells that were located inside the osteogenic matrix ring. SSA treated cells had the most intense staining. This could be, in part, due to the bigger size of the nuclei in these cells (Fig 5.8.G).

5.2.4. Discussion

The fetal cells used in this experiment were derived initially from fetal bone (typically 8-11 weeks post-conception), which was composed mainly of a cartilaginous cell population that retained many of the stem cell characteristics. The potential of FC to differentiate into the osteogenic and chondrogenic lineages was confirmed in this study and was in agreement with Mirmalek-Sani et al, 2006. Mirmalek-Sani et al described the fetal femur at the age of 8 weeks post-conception to consist of a bone shaft that was stained with Sirius red and was surrounded by a fibrous periosteum. The bone collar enclosed a cartilagenous core that stained with Alcian blue. Thus, the cross section of *in vivo* developing bone would consist of an outer fibrous ring, followed by a middle bone ring and a central cartilaginous core. This configuration was the same as that obtained in the fetal pellets cultured in the basal and osteogenic conditions, irrespective of the treatment with any epigenetic modifier. These initial results may indicate the natural tendency of these cells to proliferate and differentiate along the osteogenic lineage. The pellet culture may have provided the FC with a three dimensional environment where cells, in the outer surface, were exposed to the stretch forces due to the expansion of the cells in the centre and the mechanical force applied following media change. In addition, these cells were in direct contact with the internal surface of the culture tubes and were exposed to higher oxygen tension in comparison to the cells located in the centre of the pellet. All these factors may have created an *in vivo* like environment for the differentiation of FC into such configuration. Osteogenic culture conditions were sufficient to enhance ALP activity in monolayer as well

as osteogenic matrix formation in the pellets. This may indicate the absence of silencing mechanism in the genes of the osteogenic lineage.

FC retained their stem cell-like character during the differentiation to their natural target and thus retained the expression of nucleostemin in both the osteogenic control and 5-Aza-dC pre-treated cells. The small-cell population that was present inside the collagenous ring was positive for nucleostemin, which may indicate that those cells have kept their stem cell-like character and have not yet differentiated. This would be expected from a stem cell-like population; i.e. a capability to differentiate into specific lineages and at the same time renews themselves. SSA pre-treated pellets displayed more expression of nucleostemin, while the expression of collagen was reduced in the FC pellets cultured in basal conditions. These results suggest SSA induced FC to revert into an earlier differentiation stage, irrespective of culture conditions.

FC with 5-Aza-dC pre-treatment were small and compacted. The comparable cells pre-treated with SSA pellets were larger and with large nuclei and thus showed more intense stain. The presence of fewer cells and less matrix with SSA may be due to the SSA toxicity and/or keeping their stem cell-like nature. The absence of osteogenic driving factors in the basal control may explain the presence of fewer nucleostemin positive cells, which matches the faint collagenous ring and the poorly defined matrix.

FC were induced by chondrogenic conditions to lose their stem cell character, including the expression of nucleostemin and to differentiate into chondrocyte-like cells; i.e. cells in lacunae surrounded by a collagenous and proteoglycan-rich matrix. This explanation was supported by the fact that TSA was needed to enhance the properties of the chondrogenic differentiation, possibly by activating epigenetically silenced genes or inactive pathways.

Thus, the effects of epigenetic modifiers on FC differentiation were not the same as for BMSCs. While the effect of TSA was similar in both types of cells, 5-Aza-dC was not needed for optimal osteogenic induction.

The formation of the bone-collagenous ring required further investigation. The first question was how this ring has formed over time and thus a time course study of the FC osteogenic pellets was carried out.

5.3. FC pellets gradually form an osteogenic ring over four weeks of culture

5.3.1. Methods

FC were cultured in monolayer until confluence, and then transferred to the pellet culture in osteogenic conditions. Five pellets were harvested every 7 days for the whole culture period, which was 28 days. Pellet sections were stained for A/S and collagen I as well as non-collagenous proteins osteonectin, BSP and osteocalcin.

5.3.2. Results

A collagenous ring was evident from day 7 onwards. At day 7, the ring was formed of Sirius red positive fibres, which enclosed small cells within an ill-defined matrix (Fig.5.9.A). The population of small cells was comparable to those shown in section 5.2.3. The same cells and matrix pattern were evident on day 14, when the ring fibres were thicker and adherent to each other (Fig.5.9.B). On day 21, the ring increased further in thickness and was well defined (Fig.5.9.C). Two populations of cells were present in the pellet centre, the same small compacted cells, which occupied the periphery of the ring and cartilage-like cells, i.e. cells in lacunae and surrounded by a proteoglycan rich matrix, in the centre. The same configuration was present on day 28, when the ring was observed to be even thicker and crinkled (Fig.5.9.D). The small cell population had regressed in favour of the chondrogenic architecture in the centre, which became broader and stained more intensely with Alcian blue.

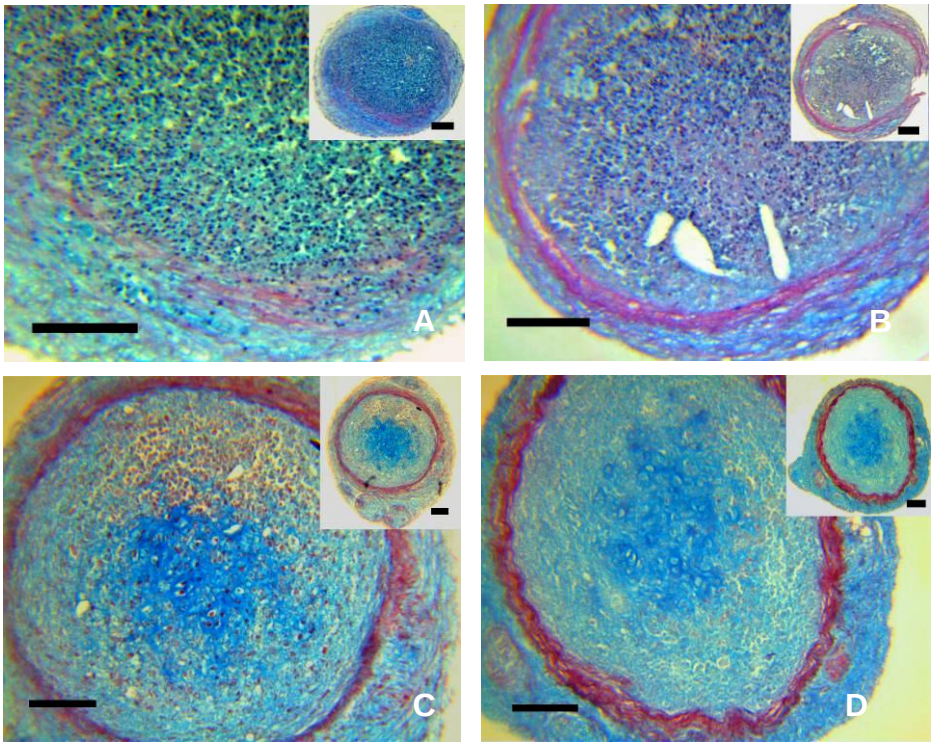


Fig. 5.9. Time course of fetal pellets fixed after 7 days (A), 14 days (B), 21 days (C) and 28 days (D). The width and the intensity of the staining of the rings gradually increased over the time course of study. Chondrogenic matrix was found in the centre of the pellets fixed at 21 days (C) and the proteoglycan density was increased at 28 days (D). Scale bars = 100 μm.

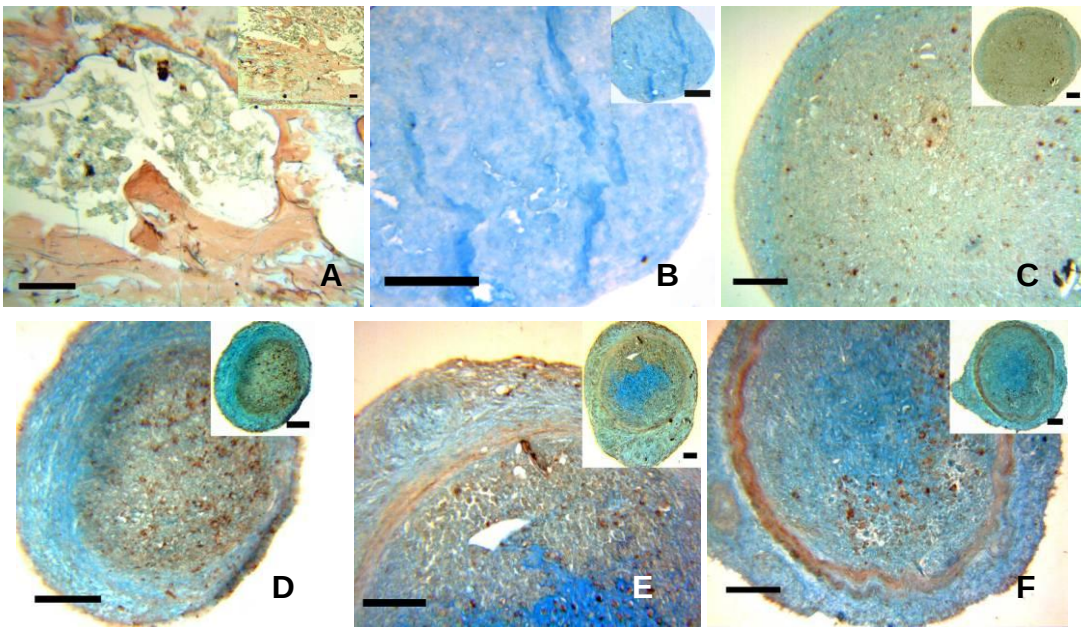


Fig. 5.10. Collagen I immuno-staining of fetal time course. A. Positive control (a section in rat femur); B. Negative control. Collagen I was found in a few cells after 7 days (C), the number of cells has markedly increased after 14 days (D). Collagen I was found in the collagenous ring on day 21 (E) and increased in intensity of the ring was evident on day 28 (F). The collagenous I staining corresponded to Sirius red staining. Scale bars = 100 μm.

The matrix was characterized by immuno-staining for the main collagenous and non-collagenous proteins in the bone matrix. Collagen I (Fig. 5.10), osteonectin (Fig. 5.11) and BSP (Fig. 5.12) displayed the same distribution. The three proteins were present in the centre of the pellet at day 7 and day 14. At day 21, the proteins appeared at the osteogenic ring. Most of the cells at the centre were still immuno-positive except in the regions where chondrogenic matrix had developed. With increasing chondrogenic matrix production on day 28, only the cells near the ring were still expressing the three proteins, while the ring acquired more intense staining.

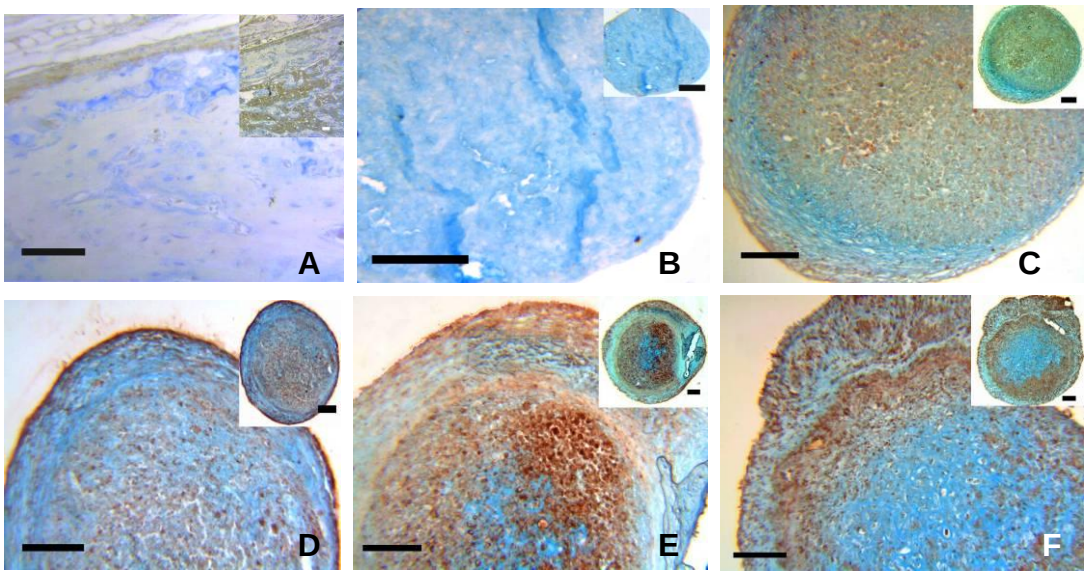


Fig. 5.11. Osteonectin immuno-staining of fetal time course. A. Positive control (a section in rat femur); B. Negative control. Osteonectin has followed the same pattern as collagen I. Osteonectin was found inside the cells after 7 days (C) and after 14 (D), while after 21 days (E) osteonectin was found in the ring while the intracellular staining became more intense. The central part of the pellet had lost the staining on day 28 (F) as the staining in the centre, was replaced by proteoglycan while osteonectin was observed to be concentrated at the ring. Scale bars = 100 μm.

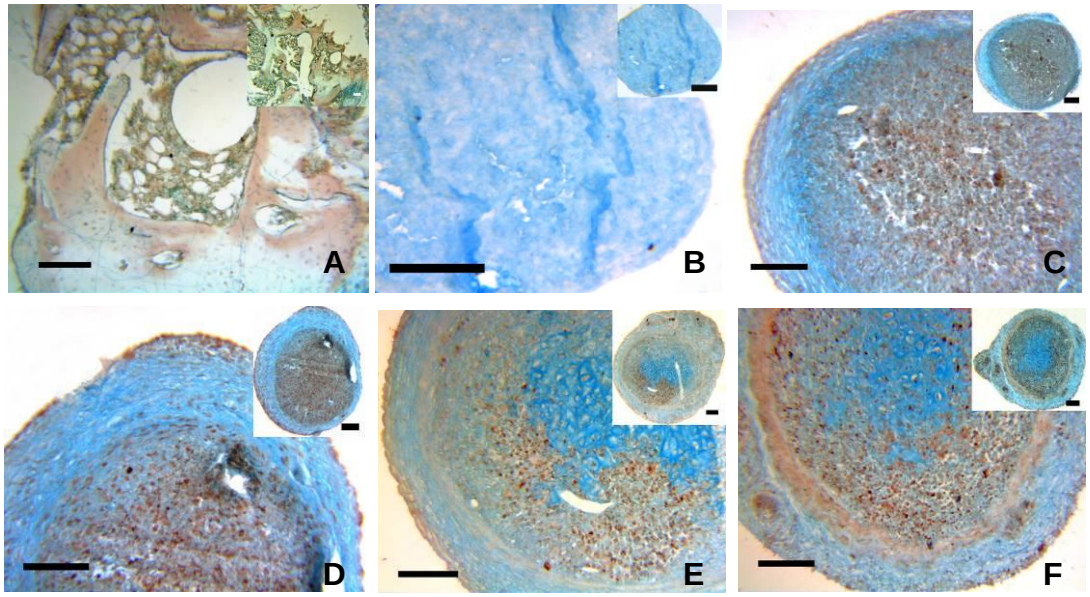


Fig. 5.12. BSP immuno-staining of fetal time course. A. Positive control (rat femur), B. Negative control. BSP has followed a similar pattern to collagen I and osteonectin, being found inside the cells after 7 days (C) and after 14 (D), while after 21 (E) BSP was found in trace amounts in the ring. The central part of the pellet had lost all staining by day 28 (F) as the staining regressed from the centre, was replaced by proteoglycan while BSP intensely stained the ring. Scale bars = 100 μm.

No immuno-staining for osteocalcin was observed at any time point (Fig.5.13).

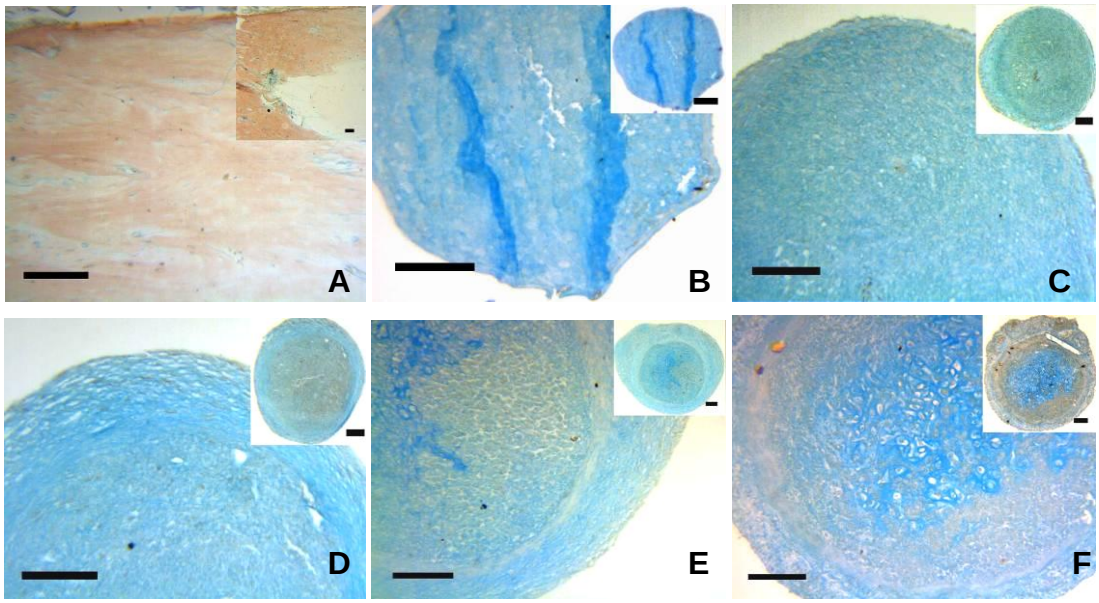


Fig. 5.13. Osteocalcin immuno-staining of fetal time course. (A) Positive control (a section in rat femur), B. Negative control. Osteocalcin was not present, either inside the cells or in the ring after 7 days (C), 14 days (D), 21 days (E) or 28 day (F). Scale bars = 100 μm.

von Willebrand factor (vWF) is a glycoprotein that is present in blood plasma and produced constitutively in the endothelium as well as in subendothelial connective tissue (Sadler, 1998). vWF has been used as a marker for angiogenesis related to osteogenesis (Kanczler et al., 2008), which is crucial for bone regeneration. vWF was found in the centre of the pellet (Fig.5.14) on days 7 and 14. The intensity of the staining was less than for collagen I, osteonectin or BSP. On day 21, very few cells were stained in the centre of the pellet and positive staining was confined to the small cell population. The ring was negative for vWF, while the tissue outside the ring displayed intense positive staining. This configuration was preserved until day 28 and was also present in the satellite rings that appeared to be budding from the main pellets.

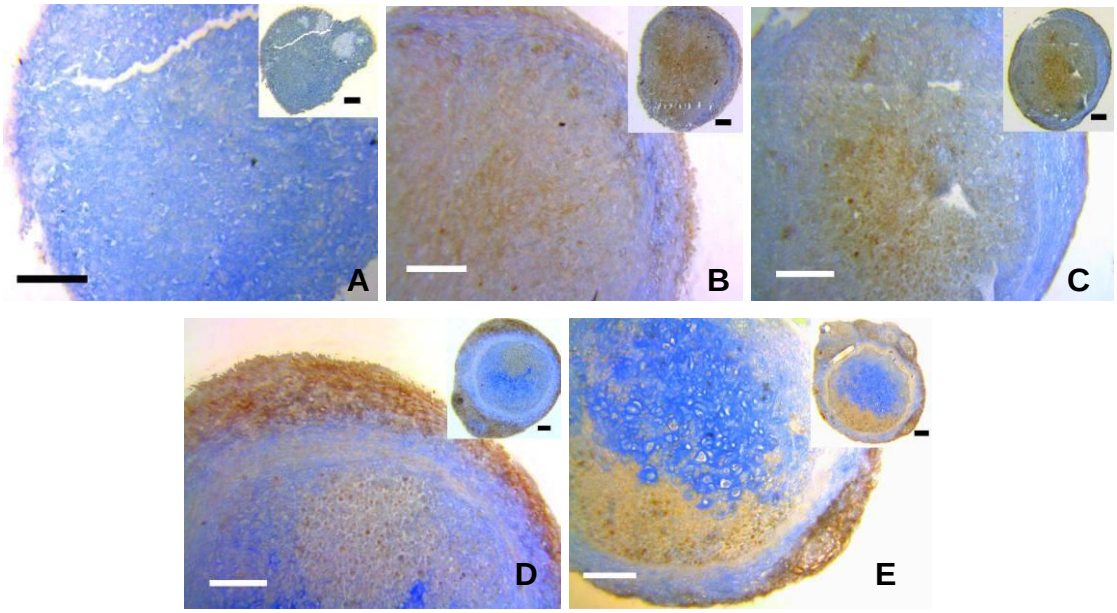


Fig. 5.14. vWF immuno-staining during the time course. A. Negative control; vWF was positive in the small cell population that was found in the centre of all sections at 7 days (B) and 14 days (C) and expression was preserved in such cell population although not the developing central cartilage at 21 days (D) and (E) when the exterior of the pellet showed positive staining. Scale bars = 100 μm.

5.3.3. Discussion

Fetal cells cultured in pellets, in osteogenic media, differentiated to a 3D configuration that was similar in some aspects to the early development of early bones. FC differentiated into osteogenic cells that produced osteoid at the external surface of the pellet, while the cells in the centre differentiated into

chondrocyte-like cells, resembling what happens during the normal development of long bones.

The constituents of the ring could only be characterized by immuno-staining from day 21 with gradual accumulation of collagen I, osteonectin and BSP. vWF could be seen by day 21 as well. vWF followed a different distribution as if it was laid on the collagenous matrix, not in between fibres.

Thus, culturing pellets of FC for 21 days was sufficient to obtain all markers and extending the culture to 28 days only succeeded in obtaining higher intensity. Hence, subsequent cultures would only be maintained for 21 days.

5.4. Cells derived from different regions of the fetal femur displayed different behaviour in culture

The fetal femur consists of two large epiphyses and a small diaphysis (Mirmalek-Sani et al., 2006b). While the epiphyses were cartilaginous in nature with a thin perichondrium, the shaft consists of a thin bone collar around a hypertrophic cartilage core (Fig 5.4.G and Fig.5.15.A). Thus, all the types of the cells found in section 5.2 and 5.3 could be found in well-defined locations in the fetal femur. Although the results obtained earlier were explained by the multipotentiality of FC, rearrangement of the osteogenic, chondrogenic and hypertrophic cartilage cells in the pellets was another possible explanation.

5.4.1. Hypothesis

Cells derived from the epiphyses display greater multipotentiality than cells from the diaphysis. Cells derived from the bone collar form predominantly bone while cells from the diaphyseal cartilaginous core form mainly cartilage, with the absence of the ring conformation.

5.4.2. Methods

Fetal femurs were obtained from an aborted fetus aged 9 weeks post-conceptional. The femurs were divided into three parts as shown in Fig 5.15.A. The first group included the two epiphyses. The second included the bone collar while the third group was the central (hypertrophic) cartilage. All the three groups were incubated with 5 mg/ml collagenase B in α-MEM at 37 ^{o}C overnight. On the next day, groups 2 and 3 produced very few cells (less than 50,000 cells) while group 1 produced three times of that number. The bone or cartilagenous structures that remained after extraction were separated from group 1, but left together with the cells in the other groups in order to give the remaining cells a chance to grow out of the explant. Cells were allowed to grow until reaching visual confluence, which varied markedly between the three groups. At this stage, the cells were counted and cell doubling was calculated at the end of passage 1. Cells in passage two were used for pellet culture and cultured for 21 days in osteogenic conditions.

5.4.3. Results

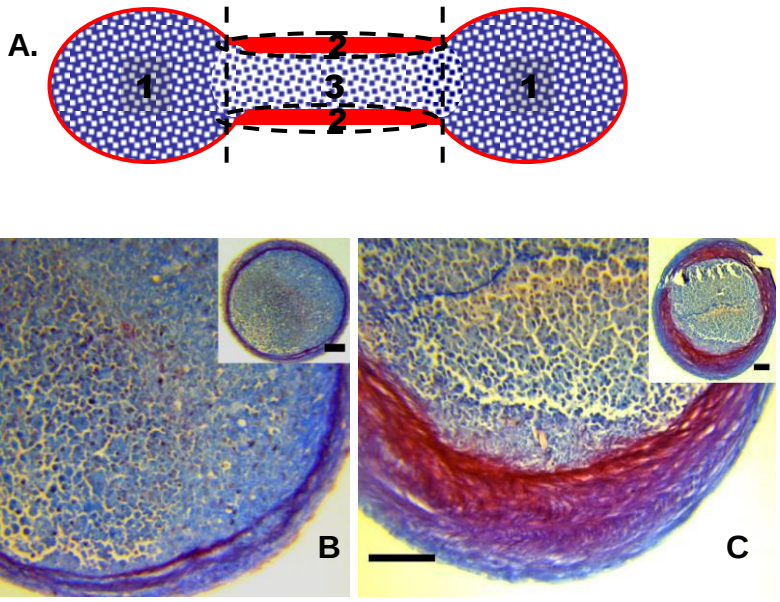


Fig.5.15. Diagrammatic representation of the fetal femur in (A), showing the three groups of the study: group 1 the epiphyses, group 2 the bone collar and group 3 the diaphyseal cartilage. Group 1 formed a well-defined ring structure with central chondrogenic area (B), which was comparable to the previous results. Group 2 formed a wide intense red ring with no chondrogenic centre (C). Scale bars = 100 μm.

The doubling time varied markedly between different study groups. Doubling time for group 2 was 122.2 hours, which was 2.4 times that of group 1 (51.7 hours). Group 3 cells had long doubling time (1248.7 hours); i.e. 24.2 times that of group 1. As the starting number of cells for the third group was very low, the final number of cells was not sufficient to form one pellet and thus this group was excluded from the study.

The typical osteoid ring formed in the pellets of both groups. Group 1 pellets had a similar structure to that derived from the whole fetal femur as shown in the previous sections; i.e. the ring stained with Sirius red and the centre stained with Alcian blue (Fig.5.15.A). Group 2 cells displayed an intense red staining ring, which was wider than the other group, and lacked the chondrogenic matrix in the centre (Fig.5.15.B).

5.4.4. Discussion

The chondrocytes of the epiphyses had the potential to develop into the osteogenic and chondrogenic lineages in a 3D configuration that was similar to the *in-vivo* cross section of the fetal femur. In contrast, the osteogenic cells of the bone collar had already differentiated down the osteogenic lineage, thus they had a longer doubling time and less ability to differentiate into different lineages. These cells proliferated and maintained their phenotype when cultured under the appropriate conditions (i.e. osteogenic conditions in 3D configuration), which could explain the presence of intense, wide collagenous ring structure with the absence of central chondrogenic matrix. The hypertrophic cartilage cells that formed the centre of the diaphysis could not proliferate efficiently as they would be in the terminal stage of differentiation.

Taking the slow rate of proliferation together with the small numbers of cells obtained from the bone shaft or hypertrophic cartilage indicates that these two regions contribute little to the cellular pool obtained from fetal femurs. Hence, the fetal cell population consisted predominantly of cells obtained from the epiphyses. In the pellets formed by epiphyses-derived cells, the intensity of

Sirius red staining at 21 days was comparable to that obtained in whole fetal-derived cells on 14 days (Fig.5.9.B).

5.5. Osteogenic ring structure was preserved up to passage 4

The production of the osteogenic ring was an interesting observation that has occurred with FC as well as cells derived from the epiphyses and bone collar. The persistence of such configuration over different cell passages would be an important in respect to regenerative medicine. With increasing number of passages, plenty of cells could be obtained from a single donor, if the cells kept their characteristics.

5.5.1. Hypothesis

The osteogenic ring structure is preserved in pellets formed using cells from higher cells passages.

5.5.2. Methods

Cells derived from the epiphyses were used at passage 2, 4 and 6 to form pellets at 0.5 X 10^6 cells/pellet. Pellets were cultured in osteogenic conditions for 21 days, followed by fixation for histology. A/S staining was carried out at the same time for all samples.

5.5.3. Results

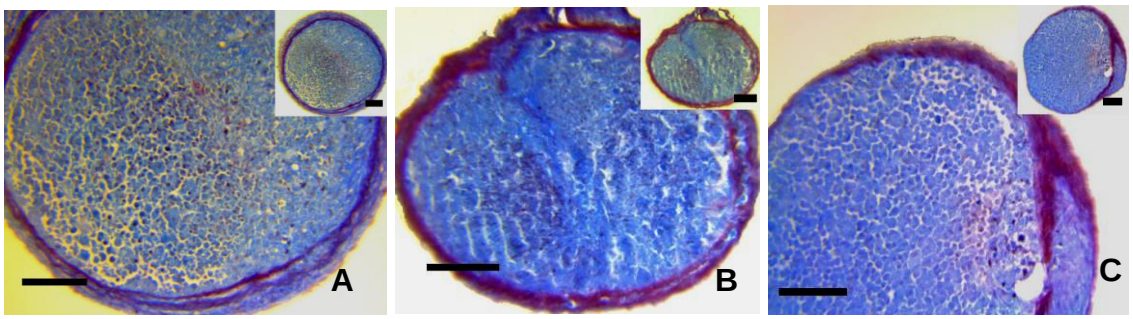


Fig. 5.16. Pellets in osteogenic conditions in passage 2 (A), 4 (B) and 6 (C). The ring structure was present in passage 2 (A) and 4 (B), while disorganized in passage 6 cultures(C). Scale bars = 100 µm.

The osteogenic matrix could be found as a ring in passage 2 and 4 cells, while the structure was lost in passage 6 cells(Fig.5.16). In pellets from

passage 6 cells, the rings were less organized and occupied only two thirds of the perimeter. The chondrogenic matrix in the centre showed a gradual decrease with the passaging. In passage 2 pellets, cartilage matrix was present in around 40% of the centre, but in less than 10 % in passage 4 and abscent in passage 6.

5.5.4. Discussion

The ability of the femur-derived cells to form the osteoid ring structure was affected by cell passage number used. This suggests a gradual loss of phenotype with increasing passage, perhaps due to the accumulation of mutations with passaging the cells, or to epigenetic silencing acquired in culture (Allegrucci et al., 2007).

5.6. The osteogenic ring structure was dependant on cell density

The osteogenic ring structure was obtained with pellets that were formed by 0.5 X 10^6 cells. The persistence of such a configuration in larger diameter pellets was studied by increasing the number of cells per pellet.

5.6.1. Hypothesis

The osteogenic ring persists until a critical diameter and pellets consisted of higher cell number do not show similar configuration

5.6.2. Methods

FC in passage two were cultured in pellets for 21 days in osteogenic conditions. The pellets were formed by 0.25 X 10^6, 0.5 X 10^6, 1 X 10^6, 2X 10^6, 4X 10^6 and 8X 10^6 cells per pellet. The pellets were fixed for histology. A/S staining was used to demonstrate the matrix pattern.

5.6.3. Results

a. Pellets maintained the ring structure up to 1 X10^6 cells/pellet

There was marked variations in the pellet size formed by different numbers of cells/pellet as illustrated in Fig 5.17.A. The ring structure was found

in pellets formed with 0.25 X 10^6 (Fig 5.17.B), 0.5 X 10^6 (Fig 5.17.C) and 1 X 10^6 (Fig 5.17.D) cells per pellet. The pellets formed with higher number of cells had not shown ring formation, although budding clusters from the pellets acquired the configuration of rings shown with 2 X 10^6 (Fig 5.17.E). In addition, patches of Sirius red staining were found with pellets formed with 4 and 8 X 10^6 (Fig 5.17.F and G).

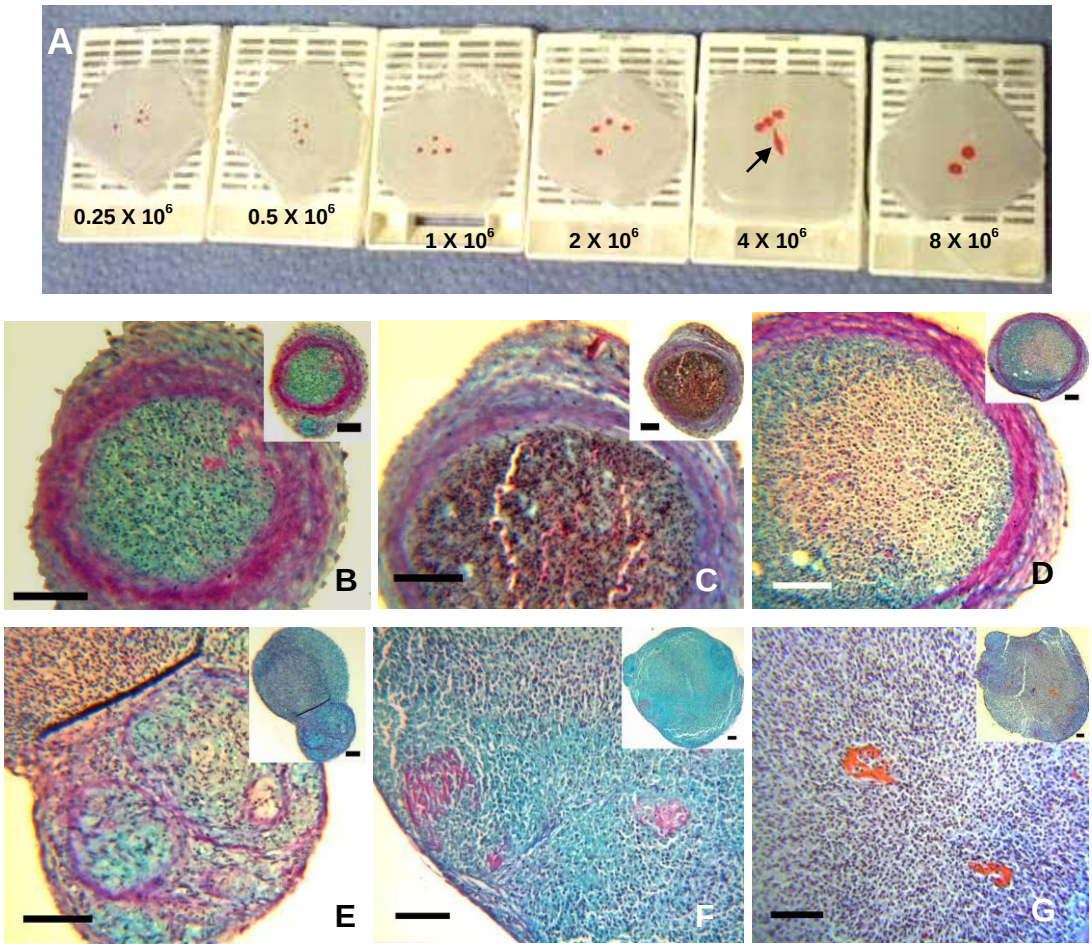


Fig. 5.17. (A) Actual pellets stained with Eosin Y and embedded in wax. Size variation was evident between different numbers of cells/ pellet. The arrow points at a special pellet that is discussed in details in the next section. The A/S staining showed the presence of the osteogenic ring configuration with pellets formed with 0.25 X 10^6 (B), 0.5 X 10^6 (C) and 1 X 10^6 (D) cells /pellet. Pellets formed with 2 X 10^6 (E) showed evidence of rings formation in budding from the pellets. Patchy Sirius red staining was found in 4 X 10^6 (F) and 8 X 10^6 (G). Scale bars = 100 µm.

b. Adherence of pellets to plastic enhanced matrix formation

One of the pellets seeded at 4 X 10^6 cells/pellet were cultured in a tube that happened to contain a plastic spicule as an extra fragment. Cells seeded in this tube formed initially as an ordinary pellet, then the pellet attached to the plastic spicule and remained attached throughout the culture period. After 21

days, the pellet was fixed with the attached plastic spicule, marked by the arrow in Fig.5.17. Cells migrated from the pellet to cover the length of the plastic spicule. The attached cells produced an intense by Sirius red stained matrix covering the whole spicule. In addition, Sirius red staining in the periphery of the pellet was slightly enhanced corresponding to the attachment to the plastic spicule. The pellet with the attached plastic spicule is shown in Fig. 5.18.A in comparison to a classical pellet of the same initial density. The staining of the pellet and different regions along the plastic spicule is shown in Fig. 5.18. B to E.

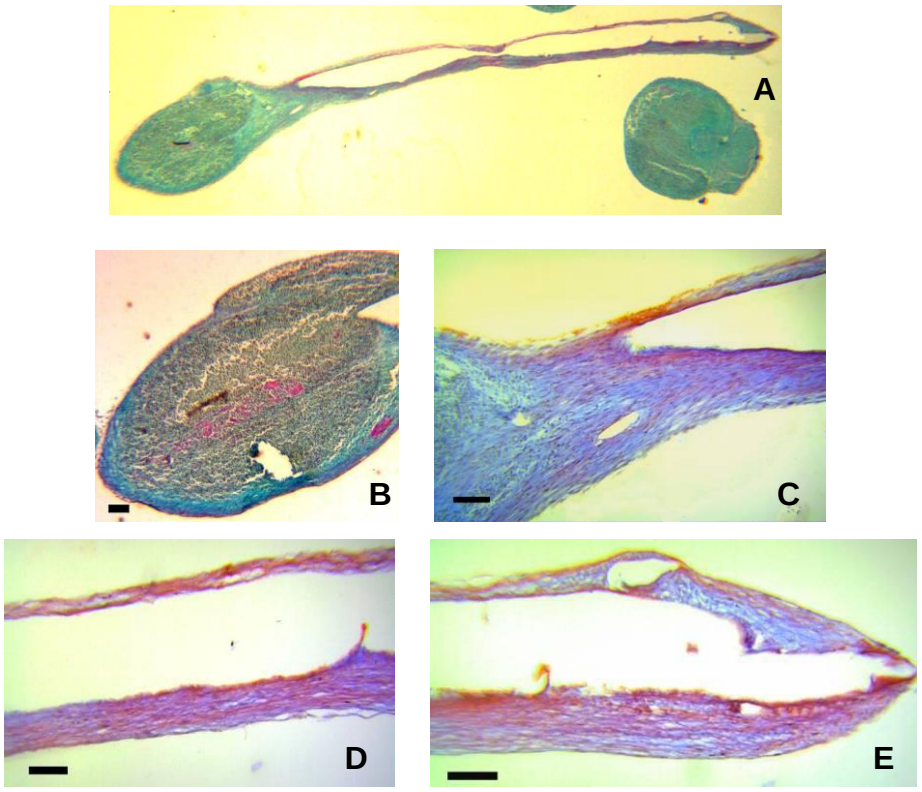


Fig.5.18. (A) On the left the pellet attached to the plastic spicule in comparison to a classical pellet formed from the same number of cells (right). Sections of the pellet itself (B) and along the proximal (C), middle (D) and distal (E) areas of the spicule covered with cells and matrix shows intense filamentous Sirius red staining of a matrix that had formed along the spicule and surrounded the pellet itself. The longitudinal patch of Sirius red staining in the pellet was in alignment with the spicule, which does not appear in this section as the spicule insertion in the pellet had 3D configuration. Scale bars = 100 µm.

Another pellet, seeded at $8X10^6$ cell /pellet, showed enhanced matrix formation by a comparable mechanism. The 8 million cells separated into two masses, the first formed a spherical pellet that was freely mobile in the medium while the second was adherent to the bottom of the tube as a mass of cells that

had the shape of inverted cone (Fig. 5.19.A). Then the mobile pellet attached to the periphery of the upper surface of the cone and increased in size gradually until covered the whole surface. At this stage, the two cells groups united and formed one big-sized pellet that lost its adherence to the plastic surface and moved freely in the medium (Fig.5.19).

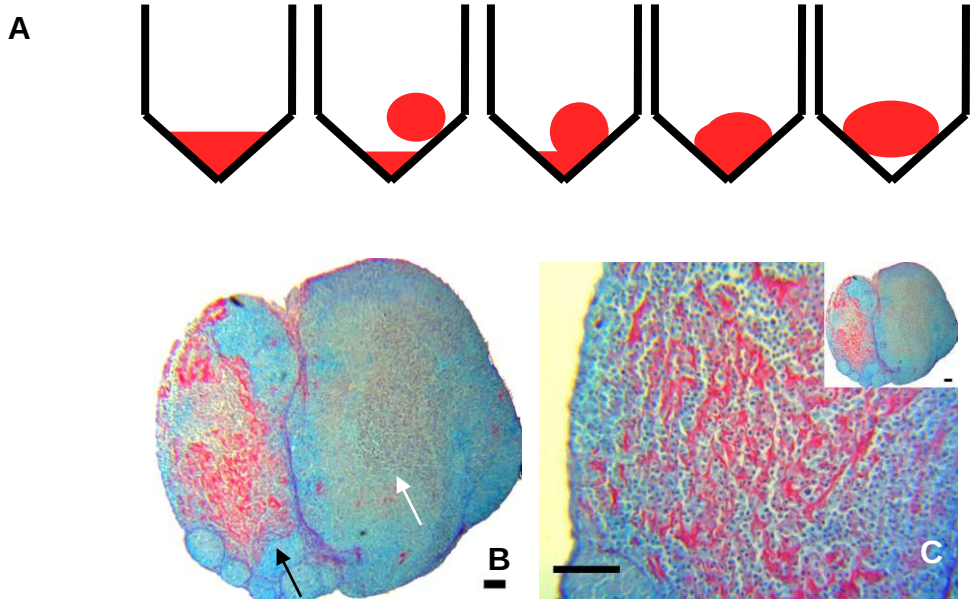


Fig. 5.19. The different stages of the pellet formation are shown in (A). After the early stage of seeding, one freely mobile pellet was formed while a smaller group of cells adhered to the tube bottom. The pellet then attached to the adherent cells, grew and united to form one big mobile pellet. A/S staining was shown in (B). The white arrow points to the larger portion of the pellet, which was very comparable to the classical pellet formed with same number of cells. This was possibly the free mobile pellet. The black arrow points at the smaller portion that had intense Sirius red stain and probably shows the attached pellet. A line between both regions forms the fusion axis between the two masses. Higher power image of the osteogenic-like portion is shown in (C). Scale bars = 100 µm.

A/S staining showed two distinct regions in that pellet. The bigger (shown by the white arrow in Fig 5.19.B) was similar to the 8 million-cell pellet (Fig.5.17.G); i.e. with few scattered patches of Sirius red staining. This region was possibly the mobile pellet. The smaller area (showed by the black arrow in Fig 5.19.B and in higher power in Fig.5.16.C) displayed intense Sirius red staining in the intracellular areas, suggestive of an osteogenic matrix. There was a well-demarcated line, possibly representing the fused edges between both regions.

5.6.4. Discussion

This study has shown two important results, the first was related to the osteogenic ring formation in the fetal pellets and the second was the enhancement of osteogenic matrix formation in relation to plastic adherence. The ring structure was preserved to a certain diameter represented by 1 X 10^6 cells per pellet. The large surface area of the bigger pellet might counteract the mechanical factors that might have enhanced the osteogenic differentiation and the ring formation in smaller pellets. Alternatively, the interaction between the cells on the surface and the centre could be lost. The presence of outgrowth from the pellets (budding) did acquire a ring-like structure, which confirmed the presence of a critical size for this configuration.

Extended serendipitous contact between the pellets and plastic surfaces enhanced formation of osteogenic matrix. The protocol for pellet culture used in all previous experiments was to move the pellet in the media by shaking the tube gently so that nutrients and oxygen would be supplied to all parts of the pellet. The adhesion to a surface might provide a platform for the cells to lay down matrix. This notion could explain the presence of matrix on certain poles of some previous pellets such as Fig. 4.8.D, 4.10.C and 4.12.A, D and E. The plastic adhesion may also provide an explanation for the ring formation in FC. As these cells were very active, attachment to the plastic between media changes might be enough to form a part of the osteogenic ring and finally the above-described configuration. However, this explanation was unlikely because the ring never showed a patchy appearance; i.e. the whole ring was homogeneous stained in every pellet for Sirius red or immuno-staining, even on day 7 of the time course (Fig. 5.9.A). In addition, there was a fibrous-like layer on the outer side of the ring, which had not acquired Sirius red staining and the ring was absent in pellets formed from cells over 1X10^6 cells. Thus, factors other than plastic adherence could be implicated in the ring formation.

The next question raised was whether this matrix formation stimulated by any surface, or a specific type of surface.

5.7. FC pellets migrated over plastic tips which enhanced osteogenic differentiation

This study was conducted to confirm and extend the previous results; i.e. the preference of the pellets to adhere to surfaces, and thereby enhance osteogenic differentiation. The principle was to increase the plastic surface area and thus hopefully enhance the differentiation process. In order to increase the plastic surface area, a sterile 20 µl micropipette tips was inserted into the plastic tubes, so that the cells had the plastic inner surface of the tube as well as the outer surface of the tips to migrate along.

5.7.1. Hypothesis

The presence of a plastic tip in the pellet of cells at day zero provides a greater surface area and enhances osteogenic differentiation.

5.7.2. Methods

FC were seeded at $2X10^6$ cell/pellet as at this concentration not much osteogenic matrix formed normally. When the cells were transferred to pellet culture and after the centrifugation step, a 20 µl micropipette tip was inserted into the tube (Fig.5.20). n = 5 constructs obtained from two fetal femurs.

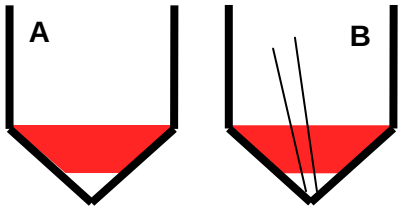


Fig.5.20. The diagrammatic representation of the study design at day zero. The control (A) was classical pellets, while in (B) a micropipette tip was inserted into the cells that would form the pellet.

The tips were left in position for the whole culture period of 21 days. The pellets attached to the tips were fixed and cut in both longitudinal and transverse sections.

5.7.3. Results

On the second day after seeding, the cells formed a pellet around the end of the tip and separated from the inner wall of the tubes (Fig.5.21). Cells

migrated upwards, against gravity to form a ring of cells. This ring has moved upwards for almost half a centimetre, which was observed on day 13 in both pellets. Only a small mass was left at the end of the tip. Afterwards the ring increased in size by growing outwards until the end of the culture.

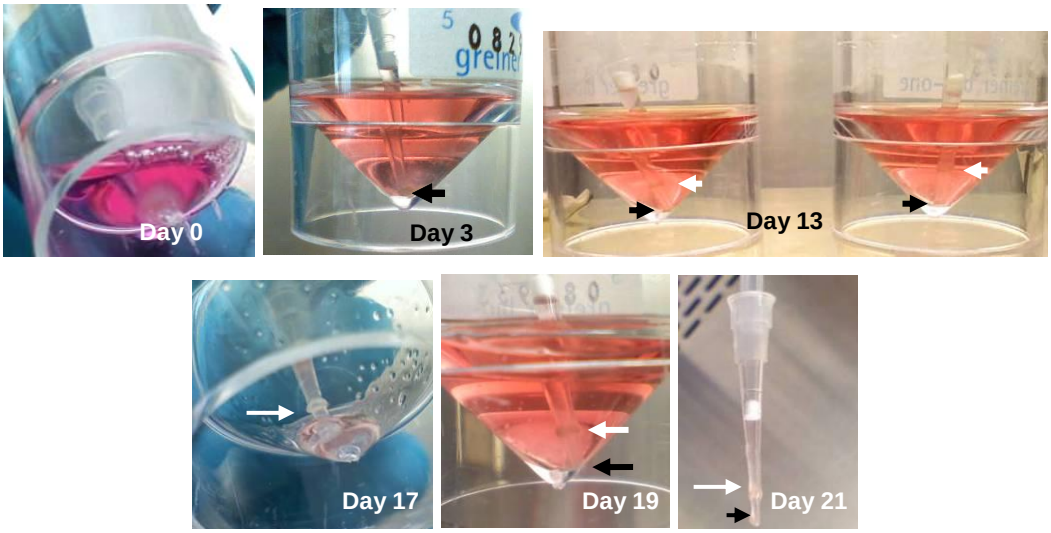


Fig. 5.21. The sequence of events started on day 0 when cells were centrifuged and a micropipette tip was inserted. After 3 days, the pellet formed around the end of the tips, shown by the black arrow. 10 days later the ring of cells had moved against gravity along the tip (white arrow), while a small pellet remained at the end of the tip. This configuration was preserved, while gradually increased in size up to the end of the culture on 21 days.

A/S staining of controls showed negligible osteogenic matrix formation, which was confined to one pole of the pellet, and acquired the shape of two adjacent rings. This was very comparable to the matrix formation shown previously by pellets with same number of cells (Fig.5.17.E). The transverse section of the tip demonstrated that the matrix was arranged in a circular configuration. This may have been produced initially at the attachment at the plastic of the tip, and then possibly pushed out by growing cells. As the pellet expanded to the outer surface of the ring, many smaller Sirius red positive rings formed (Fig.5.22.B).

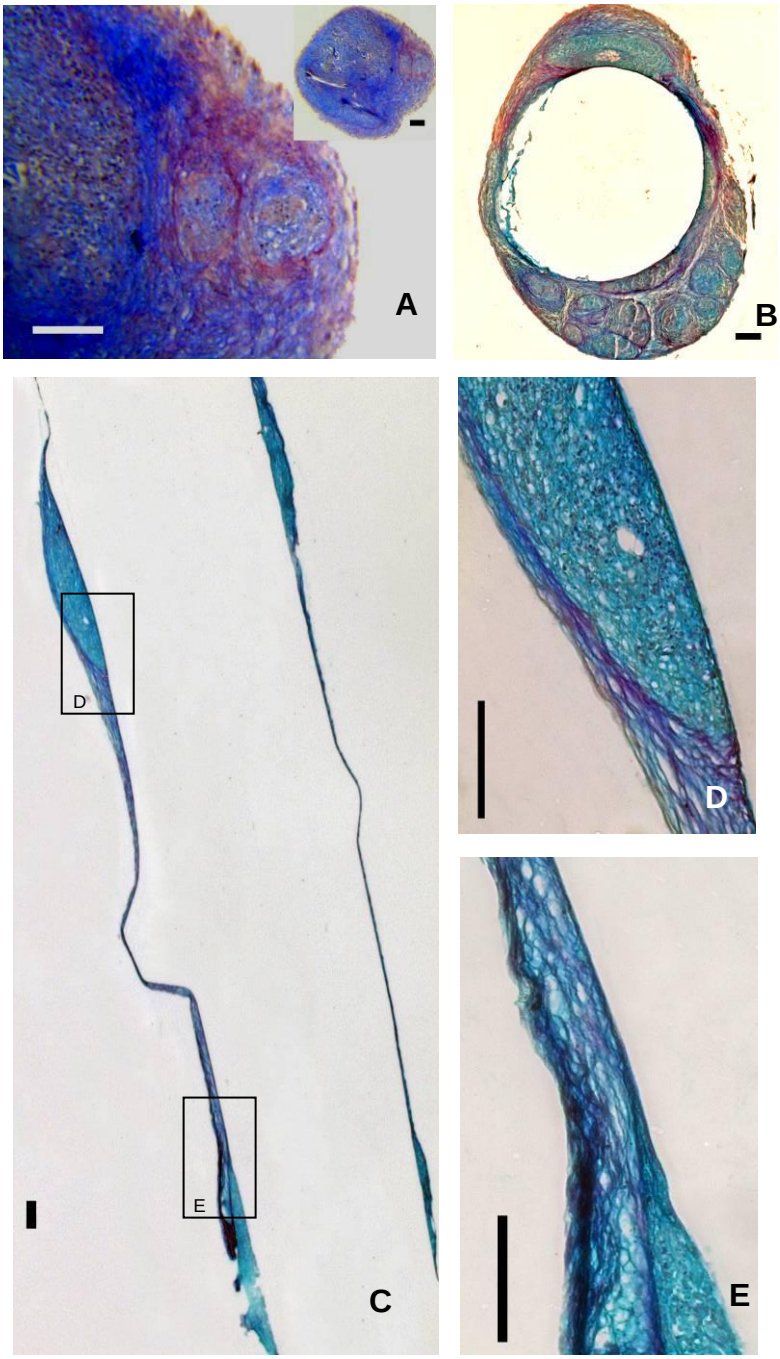


Fig. 5.22. A/S staining in control (A), being formed of $2X10^6$ cells/pellet was only present at one pole of the pellet. The cross section of the ring in the second group (B) has the configuration of a Sirius red positive ring surrounding the tip and pushed away by in-growing cell population that is shown in the longitudinal section (C) and at higher magnifications in (D) and (E) to have chondrogenic characteristics. To the outer side of the collagenous ring, there were many small new rings. The longitudinal sections also showed that the Sirius red fibres were covering the whole structure, including the distance between the tip end and the ring on the top. Scale bars = 100 µm.

On longitudinal sections (Fig. 5.22.C to E), it can be seen that Sirius red positive fibres were covering the space between the tip end, where part of the

initial pellet remained, and the upper ring. Both the ring and the pellet were covered with Sirius red positive fibres, while the thicker part of the matrix consisted of chondrogenic matrix.

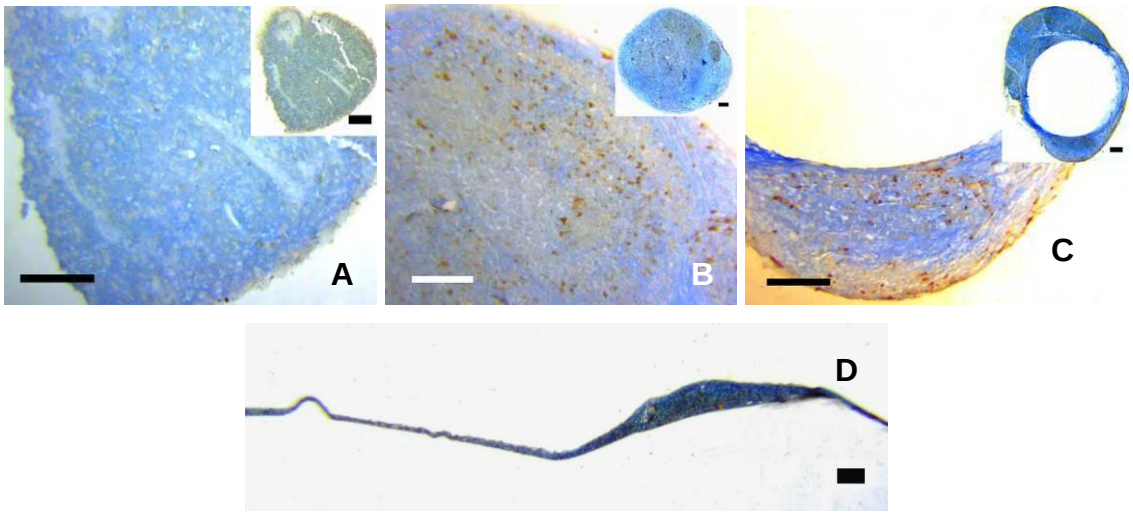


Fig. 5.23. Collagen I immuno-staining was minimal in control (B), but present at the outer perimeter of the tips (C) and along the thin layer of cells located on the surface of the tip (D). (A) The negative control. Scale bars = 100 μm.

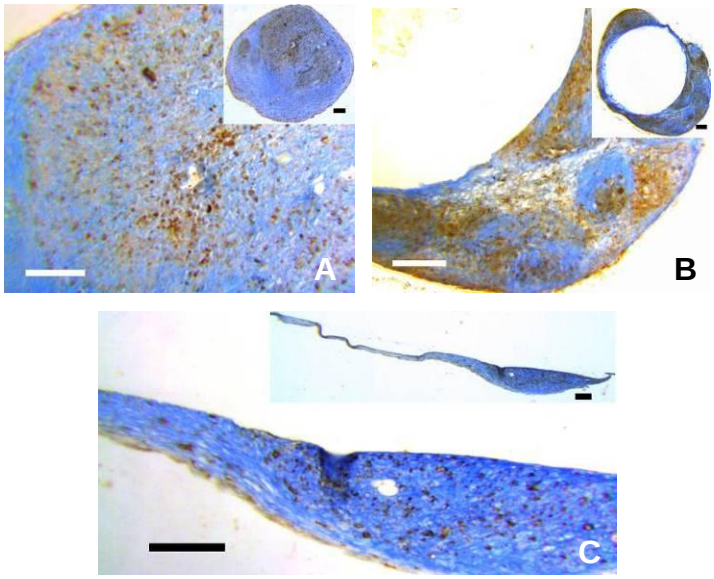


Fig. 5.24. Osteonectin immuno-staining was markedly enhanced in the matrix formed around the plastic tips (transverse section in B and longitudinal section in C) in comparison to the control (A). Scale bars = 100 μm.

To characterize the matrix, pellets were stained for the main collagenous constituent of the bone matrix, collagen I (Fig.5.23) and an important non-

collagenous constituent, osteonectin (Fig.5.24). Both proteins were enhanced in the plastic adherent system, which was more evident for osteonectin. The immuno-staining corresponded to the Sirius red staining in the matrix as well as inside the cells.

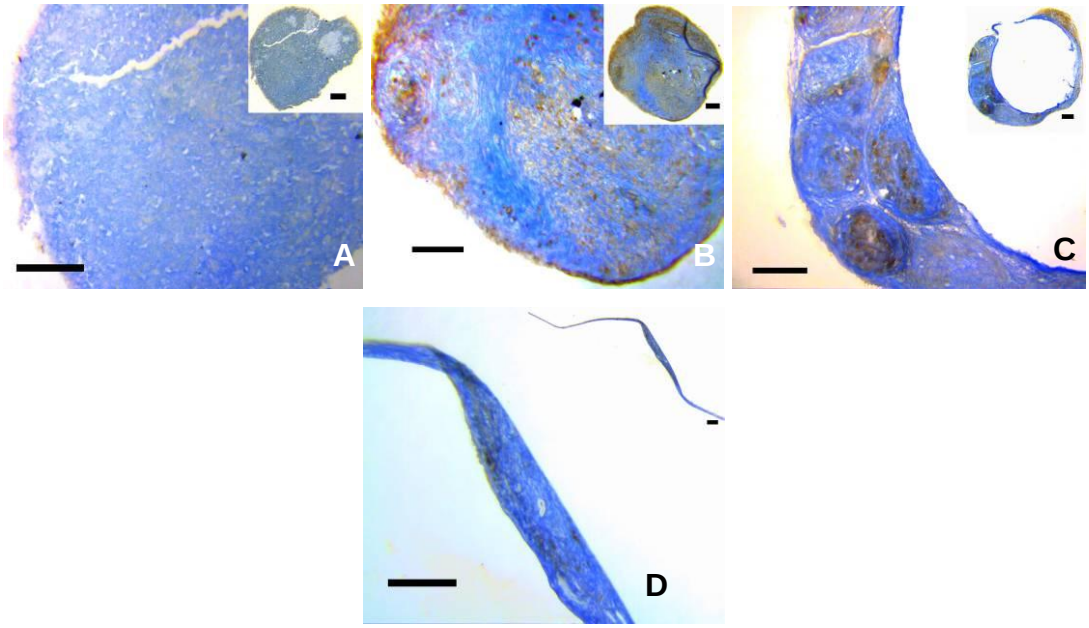


Fig. 5.25. Immuno-staining for vWF was positive in the control pellet (B), but was markedly enhanced in the plastic tips in (C) and (D). vWF immuno-staining was observed on the collagenous matrix. (A) Negative immuno-control. Scale bars = 100 µm.

vWF, the angiogenic-related marker was present in control pellets as well as the plastic adherence model. The distribution of vWF was interesting as it was laid down on the matrix rather than in between the matrix fibres.

5.7.4. Discussion

The cell pellets cultured with micropipette plastic tips showed preference to bind to the tip and migrated on the outside of the tip rather than the inner tube wall. The cells migrated against gravity to reach a certain level, where the cell layer expanded and increased in size. A small pellet remained at the end of the tip. More osteogenic matrix was present in comparison to the pellets cultured in classical conditions. Positive staining for collagen I and osteonectin confirmed that the matrix had the characteristics of osteoid along the perimeter. The bulk of the matrix was formed mainly of cartilagenous matrix that was covered by osteogenic matrix.

5.8. FC self-assembled pellets on glass surface showed enhanced osteogenic differentiation

Adherence of the pellets to the plastic had been shown to enhance the formation of an osteogenic matrix. To determine if this enhancement was due to a property of the plastic surface or just the adherence to any surface, glass tubes with flat bottom were used.

5.8.1. Hypothesis

Adherence of the pellets to any surface enhances osteogenic matrix formation especially where the pellet attaches to the surface. The difference in the shape of the tube topography does not affect the pellet formation and/or configuration.

5.8.2. Methods

FC were cultured at the density of $2X10^6$ in osteogenic conditions for 21 days. Two types of tubes were compared. They were the same shape and size, being 7-ml tubes with a flat bottom. The only difference was in their material. The first was made of polystyrene (Barloworld, cat. no. 129A) while the second was made of glass (VWR, cat. no. 212-7060). n = 5 constructs obtained from two fetal femurs.

5.8.3. Results

Differences between the two study groups were observed from day one. Cells seeded on the plastic tubes formed pellets, comparable to the standard pellets using 30 ml concave-bottomed tubes. The pellets retained their size and shape throughout the culture (Fig.5.26).

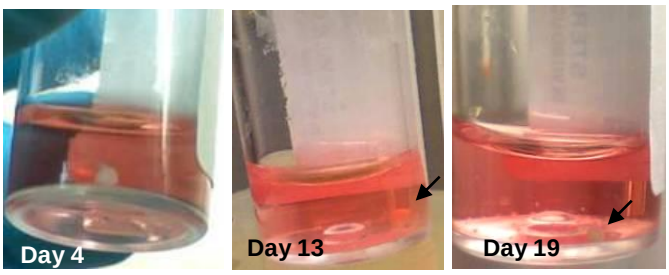


Fig. 5.26. Pellets cultured on flat-bottomed plastic tubes retain their size and configuration throughout the culture.

Cells seeded on glass tubes formed a monolayer instead of a pellet (Fig. 5.27). The monolayer increased in density up to day 7. Then the monolayer started to peel, rolled on itself to form a sausage-like pellet by day 13. This ‘sausage’ pellet then moved along the bottom of the tube to reach the sidewall on day 15. On day 17, the pellet migrated up the wall and attached only to the sidewall several millimetres above the tube base.

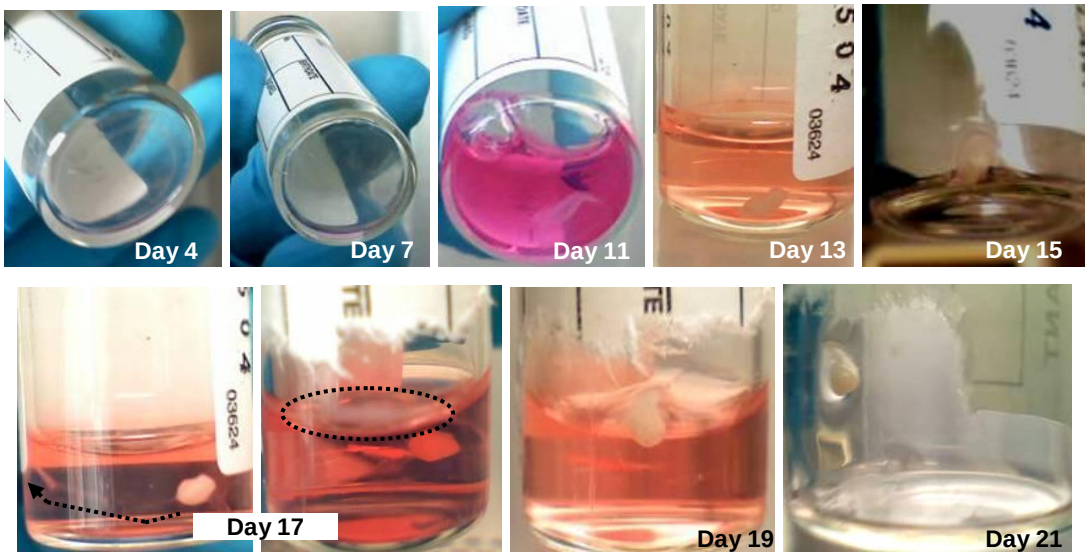


Fig. 5.27. Cells cultured on flat-bottomed glass tubes adhered to the glass surface as a monolayer for 7 days, and then the monolayer peeled and rolled over itself to form a pellet by day 13. On day 15, the pellet moved upwards on the tube sidewall and continued until day 17, when band of cells emerged from the pellet, adhered to about two thirds of the inner tube circumference to end in a thick band at the media-air interface. On day 19 the whole pellet was found at the position of the thick band, just covered with thin film of media. The culture was stopped on day 21.

A band of cells grew out of the pellet attached to the wall and extended gradually over two thirds of the inner circumference of the tube (fig.5.27. day 17) to end in a thick band of cells at the media-air interface. Two days later the whole pellet moved or rolled over the track determined by these guiding cells to the same position of the thick band. The pellet was covered by a thin film of media. The pellet remained in this position until the end of culture period. Side-view on day 21 (Fig. 5.27) demonstrated the 3D configuration of the pellet, its position relative to the tube bottom and its size relative to the tube.

The size of the pellets (Fig. 5.28) was comparable to the pellets cultured in plastic flat-bottom tube and the standard concave bottom tubes, while the self-assembled pellets cultured on glass were about 16 times larger. (Sphere

size comparison was calculated using an on-line tool http://www.csgnetwork.com/circlecalc.html).

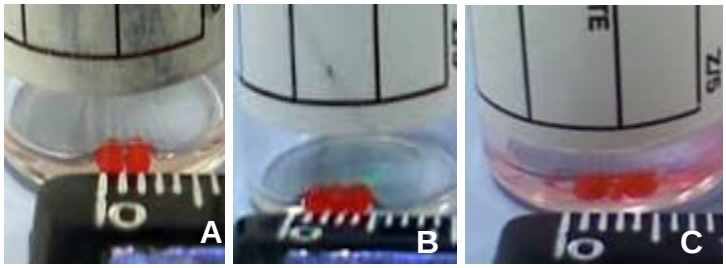


Fig. 5.28. Comparison of the size of the pellets cultured in (A) 30 ml plastic concave bottom-tubes, (B) 7 ml plastic flat-bottomed plastic tube and (C) its glass equivalent. The pellets diameter was nearly 1 mm in (A) and (B) and 2.5 mm in (C). Pellets were stained with Eosin Y.

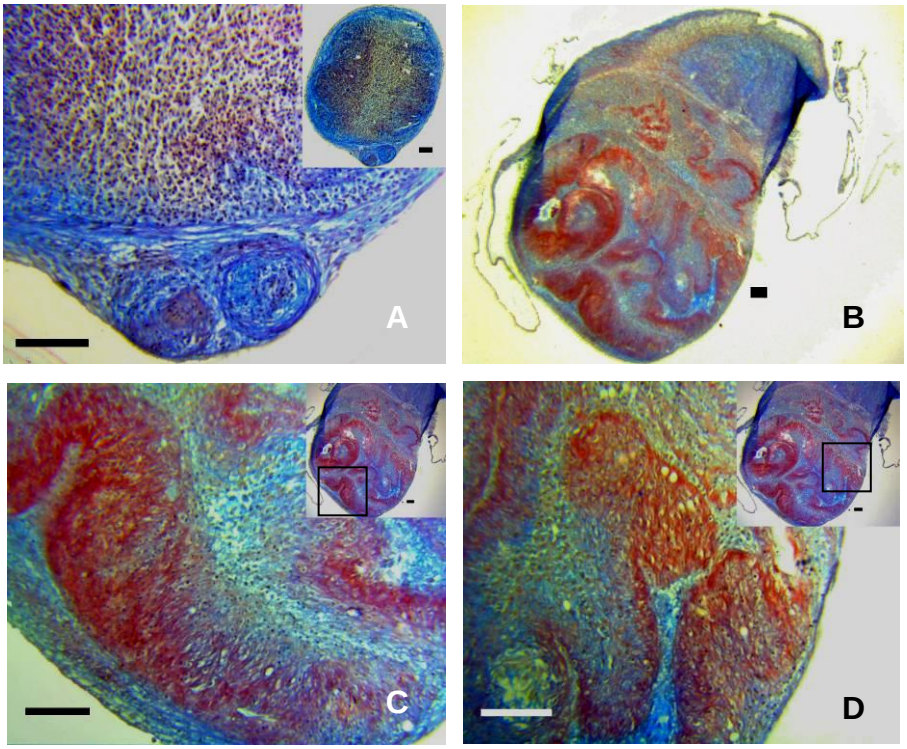


Fig. 5.29. A/S staining showed negligible matrix formation in pellets cultured on plastic surface, while marked enhancement of osteogenic matrix was obtained in self-assembled pellets cultured on glass (B-D). Scale bars = 100 μm.

A/S staining (fig. 5.29) showed that osteoid matrix had folded into sheets that may correspond to the original monolayer folding. Between the folds of Sirius red, chondrogenic matrix had formed. Further characterization of the matrix (Fig. 5.30) showed collagen I and osteonectin expression corresponding to Sirius red staining. vWF was markedly enhanced in relation to areas of osteogenic matrix.

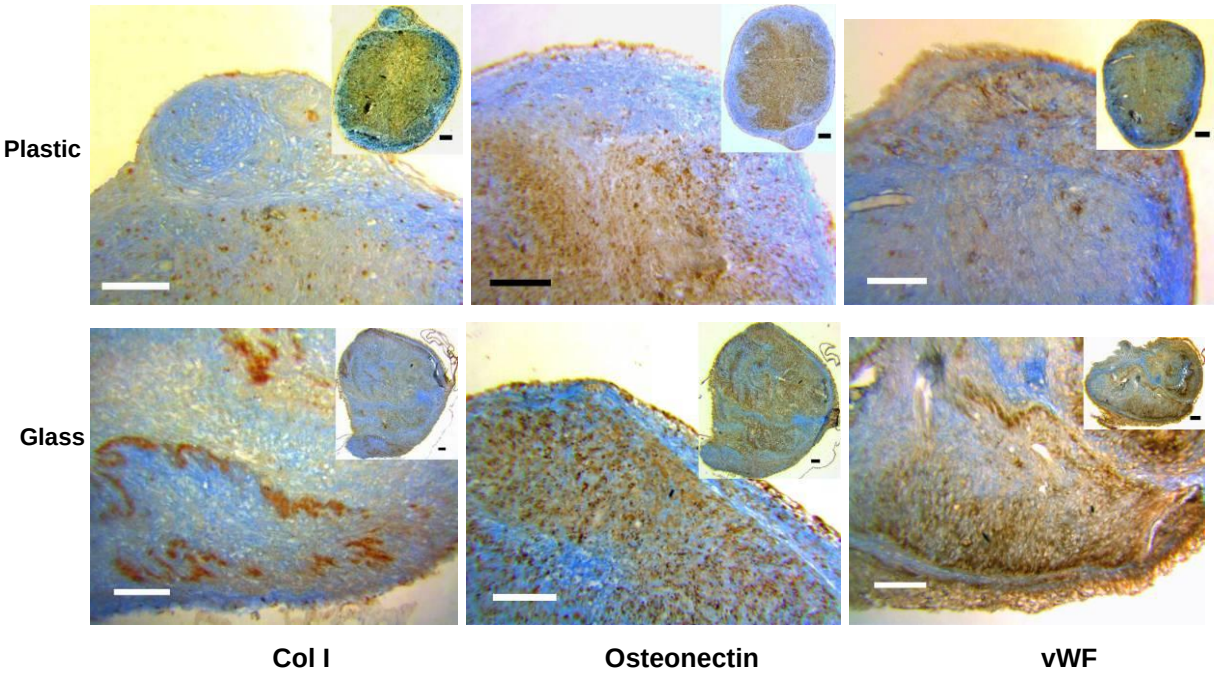


Fig. 5.30. Immuno-staining for collagen I, osteonectin and vWF for pellets cultured on plastic (upper panel) and glass surfaces (lower panel). There was enhancement for the studied markers in the pellets culture on the glass surface. Scale bars = 100 μm.

5.8.4. Discussion

Pellets cultured on glass provided a novel model to enhance osteogenesis in 3D. More bone matrix was observed to be formed and the pellets were larger than the pellets cultured on plastic. No difference could be found between the pellets cultured on the flat bottom tubes and the conical bottom tubes used in the previous experiments. This suggests that the material of the tubes was more important than the configuration of the tube itself. In other systems, evidence of enhanced osteogenic markers in monolayer was obtained by culturing chondrocytes on titanium surfaces (Boyan et al., 1996). This was explained by both the roughness of the surface as well as the adsorption of certain materials in the media that alter the microenvironment around the cells.

5.9. BMSC pre-treated with 5-Aza-dC displayed enhanced osteogenic differentiation as self-assembled pellets cultured on glass surface

The enhancement of osteoid formation that was obtained by self-assembled FC pellets raised the question about the response of BMSCs to similar culture conditions.

5.9.1. Hypothesis

Formation of an osteogenic matrix by BMSCs is enhanced when pre-treated with 5-Aza-dC and cultured on a glass surface.

5.9.2. Methods

BMSCs were obtained from a 66-year-old female and cultured on monolayer until becoming 50 % confluent, when the cells were serum-fasted for 24 hours and treated with 5-Aza-dC at 1 μM for three successive days. Treated and non-treated cells were then seeded on plastic or glass 7ml flat-bottomed tubes as 0.5 $X10^6$ cells/pellet. The pellets were cultured in osteogenic media for 28 days (2 pellets/group). The culture was extended to 28 days, because the seeding density was low.

5.9.3. Results

Pellets cultured on glass followed the sequence shown in the previous section for the FC. BMSCs formed a monolayer that detached, rolled itself to form a pellet that migrated along the base of the tube and migrated up the sidewall to reach the medium-air interface on day 19. The pellet remained at this position until day 28. At the same time, BMSCs cultured on plastic formed small mobile pellets that may have attached very briefly and weakly as a pellet to the plastic surface.

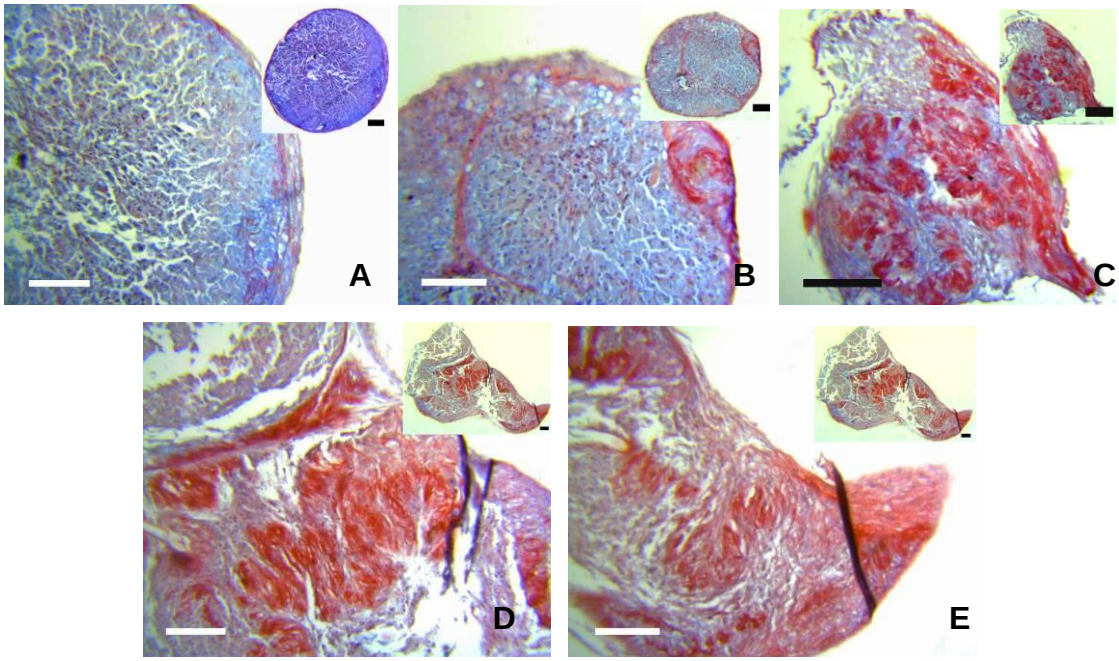


Fig. 5.31. A/S staining of the four groups of the study. 5-Aza-dC pre-treated cells showed enhanced osteogenic formation on plastic (B) in comparison to non-treated cells (A) as well as those cultured on glass (D and E) in comparison to non-treated cells (C). In addition, pellets cultured on glass had enhanced matrix formation in comparison to those cultured on plastic. Scale bars = 100 μm.

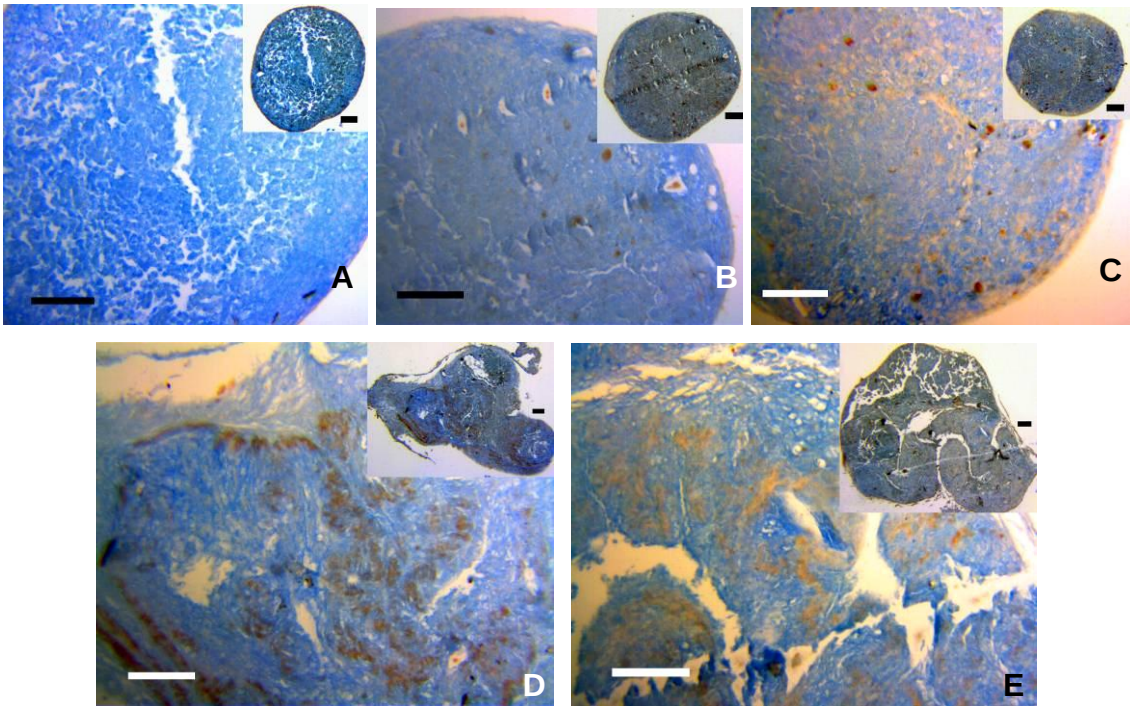


Fig. 5.32. Immuno-staining for collagen I showed minimal staining in non-treated cells cultured on plastic (B) while 5-Aza-dC pre-treated cells has shown enhanced expression. Cells cultured on glass without 5-Aza-dC pre-treatment (D) and with pre-treatment (E) showed enhanced expression of collagen I in comparison to those cultured in plastic, being more intense in cells without initial treatment (D). Negative control is shown in (A). Scale bars = 100 μm.

A/S staining of the pellets showed enhanced osteogenic matrix formation for cells pre-treated with 5-Aza-dC cultured either on plastic (Fig.5.31.B compared to control Fig.5.31.A) or on glass (Fig.5.31.D and E compared to controls Fig.5.31.C). The amount of Sirius-red positive osteoid matrix induced by glass with or without initial 5-Aza-dC treatment was considerably greater than that of pellets cultured on plastic. The combination of 5-Aza-dC and glass produced slightly more osteoid than glass alone.

Further characterization showed enhancement of collagen I staining (Fig.5.32) in pellets cultured on plastic in osteogenic conditions with 5-Aza-dC pre-treatment in comparison to non-treated cells. Collagen I immuno-staining was enhanced in pellets cultured on glass, being more intense in the absence of 5-Aza-dC initial treatment.

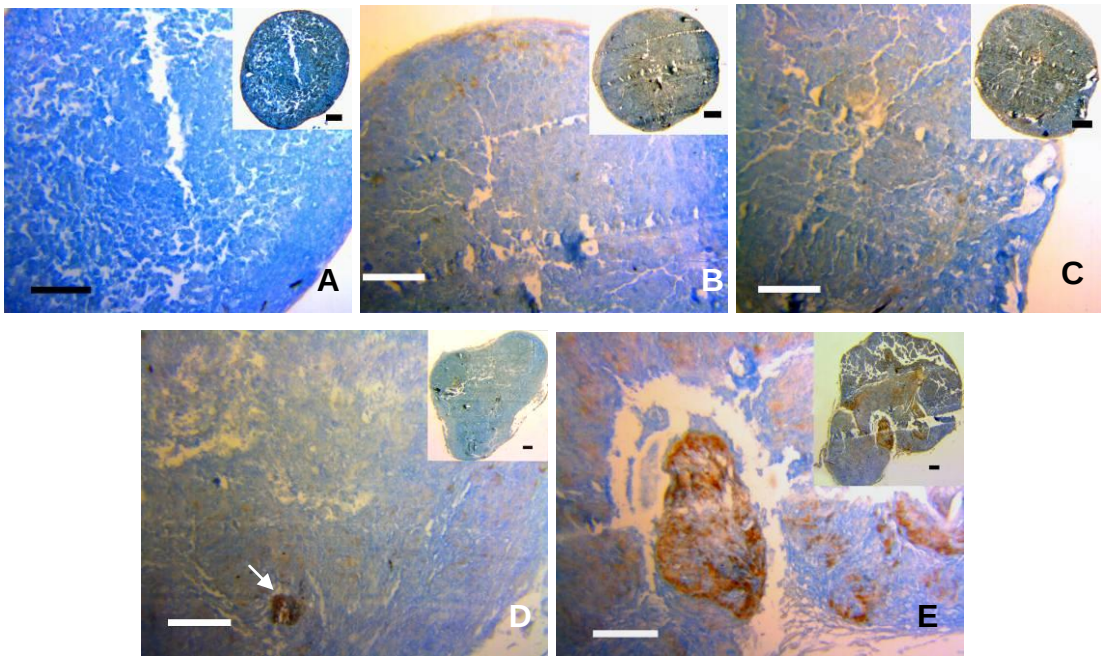


Fig. 5.33. Immuno-staining for collagen vWF showed minimal staining in non-treated cells cultured on plastic mainly on the periphery (B) while 5-Aza-dC pre-treated cells showed enhanced expression. Cells cultured on glass without 5-Aza-dC pre-treatment (D) had comparable induction to the previous group; in addition, there were wider areas of localized intense staining, marked by the arrow. 5-Aza-dC pre-treated cells cultured on glass (E) showed marked vWF expression throughout the pellet, with some areas of localized intense staining. Negative control is shown in (A). Scale bars = 100 µm.

Minimal immuno-staining for vWF (Fig.5.33) was present in the controls pellets cultured on plastic without 5-Aza-dC (Fig. 5.33.B), but some staining was

present after 5-Aza-dC treatment (Fig. 5.33.C). By contrast, vWF was induced in pellets cultured on glass even without 5-Aza-dC treatment (Fig.5.D). Some areas displayed a distinct staining of a circular arrangement (marked by the arrow in Fig.5.33.D). However, 5-Aza-dC increased both the intensity and distribution of vWF immuno-staining. Cells pre-treated with 5-Aza-dC and cultured on glass (fig.5.33.E) showed widespread intense staining that occupied most of the pellets. Some areas showed particular intense staining.

5.9.4. Discussion

This study has confirmed the induction of osteogenesis in BMSCs pre-treated with 5-Aza-dC as previously shown in chapter 4. In addition, culturing BMSCs on glass followed the same sequence of events shown by FC (section 5.8), until the formation of osteogenic matrix. However, the amount of osteogenic matrix obtained by culturing non-treated cells on glass was superior to that obtained with 5-Aza-dC pre-treated cells and cultured on plastic. Adding the two factors together, i.e. cells pre-treated with 5-Aza-dC and cultured on glass, only resulted in a slight enhancement of Sirius red staining. Further characterization showed more collagen I staining in the non-treated pellet cultured on glass in comparison to treated counterparts. In addition, the presence of vWF with such intensity may raise the question of the nature of the construct obtained in these conditions and if it is related to vascular components. It is tempting to speculate that either 5-Aza-dC enhanced the production of vWF in an attempt to attract endothelial cells and consequently vascularisation, which is not possible *in vitro* or alternatively 5-Aza-dC has enhanced the trans-differentiation of BMSCs into the endothelial lineage in this system.

The PCR array results had shown up-regulation of PDGF and EGF, which could stimulate vascularisation (section 3.6). Thus, the adhesion of the BMSCs to glass can induce osteogenic genes and consequently the osteogenic development. In such system, there was no need for the 5-Aza-dC pre-treatment. These constructs are expected to be safer than the epigenetically

modified pellets in terms of undesirable gene activation with possible side effects and thus could be better candidates for tissue regeneration purposes.

5.10. Conclusions

FC were cultured in four variants of the pellet culture system. 1) The role of TSA in enhancing chondrogenesis was confirmed in the standard pellet culture system, while the cells respond to the osteogenic media without the need for 5-Aza-dC, which was required for enhanced osteogenesis in BMSCs. 2) FC showed their ability to differentiate into a sphere that resembled the structure of a cross section of fetal bone. 3) FC have high capacity to adhere to plastic surfaces. This capacity was maintained, even when the plastic surface was vertical and the cells had to move against gravity to acquire as much adherence as possible. Such adherence enhanced the formation of an osteoid matrix. 4) Self-assembled pellets on glass surfaces produced larger constructs that contained a large amount of osteoid matrix formation. This model combined the advantages of cellular contact to the surface, contact of cells to each other and with the developing matrix as well as the high oxygen tension at the air-media interface. In addition, the cells were free to migrate, form 3D construct or approach the level of higher oxygen tension. This final model was useful for BMSCs differentiation, which showed enhanced osteogenic matrix formation in bigger constructs.

DISCUSSION, LIMITIATIONS OF STUDY AND FUTURE PROSPECTUS

6.1. Discussion

Bone marrow stroma is one of the richest sources of mesenchymal progenitor cells. These cells have the ability to differentiate into many lineages *in vitro* (reviewed in (Tare et al., 2008; Abdallah & Kassem, 2009)). The current approach for investigating the differentiation potential of the cells is to combine cells with growth factors and an appropriate scaffold (Luyten et al., 2001). The international editorial board of the journal of 'Tissue Engineering' aimed for clinical application of tissue engineering by the year 2021 (Johnson et al., 2007). However, it is still difficult to obtain a pure cell population of the desired lineages.

Murine BMSCs were shown to differentiate into neural-like cells when the cells were treated with 5-Aza-dC (DNA demethylating agent), TSA (histone deacetylation inhibitor) or their combination and cultured in neuronal induction media (Alexanian, 2007). Thus, the epigenetic modifiers induced the differentiation of neural cells, originally derived from the ectoderm, from BMSCs that had a mesodermal origin. The author suggested a potential role for epigenetics in governing stemness, commitment, differentiation, and maintenance of these states. The concept behind Alexanian's study is in agreement with the results obtained in this thesis as the differentiation potential of BMSCs was enhanced when the cells were pre-treated with the epigenetic modifiers, 5-Aza-dC and TSA and cultured in specialized media. In the Alexanian study, 5-Aza-dC and TSA had similar effects. In addition, there was a synergistic effect of the combined treatment, which was in agreement with a study performed on the Oct-4 gene (Hattori et al., 2004) in which the authors showed activation of the Oct-4 gene in primary murine trophoplastic cells by one or both agents. In the murine embryonic fibroblast cell line (NIH/3T3), which is known not to express Oct-4, only the combination of treatments could up-regulate the gene. The authors demonstrated variations in the level of DNA methylation and histones H3 and H4 acetylation in the gene promoter between the trophoplastic cells and the murine embryonic stem cells, which express Oct-4. The authors concluded that the epigenetic silencing of this gene involved DNA methylation and histone de-acetylation. In addition, the individual effects of

each agent on Oct-4 expression may indicate an overlap mode of action. The authors confirmed the role of MECP-2 in maintaining gene silencing, which had been shown earlier in mice (Nan et al., 1998b).

A recent study (Steele et al., 2009) showed that combined treatment of 5-Aza-dC with the histone deacetylase inhibitor PXD101 was more effective in the induction and expression of two tumour suppressor genes, both in vitro and in vivo (cells injected subcutaneously as xenograft in nude mice). Those genes were silenced by DNA methylation in the cell line used in their study, although the authors did not investigate possible changes of histone modifications.

In our system, Sox-9 expression was increased by TSA and the TSA + 5-Aza-dC combined treatment in comparison to the 5-Aza-dC only treatment. At the same time, there was a decrease in the DNA methylation level of the Sox-9 promoter. This contradicts the findings of another study (Sng et al., 2005) as the authors injected mice intra-peritoneally with TSA and examined the expression of *c-jun* and *c-fos* in the hippocampus neurons. In addition, the authors cultured the hippocampus neurons *in vitro* and added TSA following the same protocol used in the *in vivo* study. In both studies, there was immediate induction of both genes and the expression returned to the basal level after 12 hours. Although epigenetic changes usually take longer to be produced, these findings suggested that other mechanisms might cause the up-regulation of those genes.

Previous studies had shown that the differentiation potential of skeletal cells in pellets was enhanced due to cell-cell and cell-matrix contact (Tare et al., 2005). In our system, the combination of epigenetic modifiers with the pellet culture system enhanced the differentiation potential of BMSCs. The effect of epigenetic modifiers was investigated some time after the application of the treatment. BMSCs were treated with the epigenetic modifiers when the cells were 50 % confluent in monolayer and the cells were kept in monolayer until confluence. The cells were transferred to the pellet culture upon reaching confluency and then cultured in specialized media for 3-4 weeks with no further

addition of epigenetic modifiers. 5-Aza-dC affects only the dividing cells as the altered base requires to be integrated into the newly synthesized DNA to influence methylation. When the cells divide, each daughter cell acquires one original DNA strand but if the newly synthesized strand has 5-Aza-dC incorporated, it cannot be methylated. In addition, the 5-Aza-dC traps the DNA methyltransferases DNMT1. It has yet to be determined if the incorporation of 5-Aza-dC in a few locations is sufficient to trap the enzyme and have a wider effect or if 5-Aza-dC has to be incorporated into the promoter of a particular gene for the gene to be activated. TSA may affect the dividing cells in a similar way, as daughter cells would inherit half of the histones that were affected by TSA. In addition, TSA affects non-dividing cells. In these experiments, thefore, the effects of the modifiers were transmitted to daughter cells, consistent with long-term epigenetic changes (Wolffe & Matzke, 1999), in contrast to the transient up-regulation in the Sng et al study (2005).

TSA induced both osteogenic and chondrogenic lineages, as shown by the dose response studies. TSA as well as Valporic acid (another HDAC inhibitor) was shown to induce osteogenesis (Cho et al., 2005). In addition to the skeletal lineages, TSA has been shown to induce the differentiation of murine BMSCs into pancreatic islet-like structures upon culturing in corresponding conditions (Tayaramma et al., 2006). Thus, TSA induces a wide range of differentiation including, in our system, the chondrogenic lineage in both BMSCs and FC. In addition, TSA treated pellets displayed an enhanced physiological cellular and matrix architecture, as shown *in vitro* and *in vivo* with BMSCs as well as FC.

ALP activity, as a marker of osteogenesis, was enhanced more markedly by 5-Aza-dC than by BMP2, the most potent osteogenic inducer. These results were in agreement with the Zuscik et al study in which chondrocytes derived from chick bones were treated with 5-Aza-dC and the cells cultured in D-MEM with 5% FCS and ascorbate for 5 and 10 days (Zuscik et al., 2004). 5-Aza-dC enhanced ALP activity more than BMP2 in articular chondrocytes. However, a discrepancy between the ALP activity and transcription was found; i.e.

transcription was reduced and activity increased by 5-Aza-dC. This was also found in a different system using human BMSCs in which ALP activity was induced by ascorbate more efficiently than by BMP2, 4 or 7 (Diefenderfer et al., 2003). The osteogenic media protocol applied in our system included 50 µM ascorbate. Thus, the decrease of the ALP transcription in the PCR arrays could be, at least in part, in agreement with the Diefenderfer et al (2003) study. Brown et al (1990) studied the induction of ALP activity by cAMP and showed an increase of 80-fold upon stimulation, while the transcription was only up-regulated 10 folds, which illustrates that transcription does not always correlate with the activity of ALP. The most interesting result was the rapid drop of ALP level as soon as cAMP was withdrawn from the culture, which could indicate the induction of a gene rather than the differentiation of cells (Brown et al., 1990).

Based on our PCR array results, the differentiation of the BMSCs along the osteogenic lineage with 5-Aza-dC may have been induced by the up-regulation of the master osteogenic induction pathways, including TGF-β pathway and BMP6. The up-regulation of TGF-β1, TGF-β2 and Smad3 might suggest a role of the TGF-β pathway of the induction of osteogenesis. This assumption was re-enforced by the up-regulation of FGF-1, FGF-2 and PDGF, which are induced via the TGF-β pathway (Leof et al., 1986; Matsuyama et al., 2003). In addition, FGF-1 and FGF-2 enhance osteogenesis through the up-regulation of TGF-β (Nakamura et al., 1995). The PCR array study showed a marked downregulation of the TGF-β receptor 2, which is constitutively active, while receptor 1 was not significantly changed. This pattern of downregulation would not exclude a role of such a pathway, as the receptors already present could be sufficient to conduct the signal. The downregulation of the TGF-β receptor 2 has also been observed in non-transformed intestinal epithelial cells as a consequence of chronic exposure of the cells to TGF-β1 and was associated with cell transformation (Sheng et al., 1999).

In contrast, BMP 6, which can induce osteogenesis through the BMP pathway, was up-regulated. EGF is also capable of inducing BMP6, in addition to BMP receptors, which were not included in the arrays (Elabd et al., 2007).

One of the key differences between the TGF-β and BMP pathways is their effect on ALP transcription. While BMP6 enhances ALP activity, TGF-β decreases transcription as well as activity (Gruber et al., 2003; Mehlhorn et al., 2007). Thus, in our culture system, we had indications of both pathways, although involvement of other pathways could be neither excluded nor predicted, as the PCR arrays study was conducted in comparison to cells cultured in osteogenic conditions and showed a degree of osteogenic differentiation shown by the ALP activity.

Cho et al studied fracture healing as a post-natal developmental process and related that to the stages of bone development (Cho et al., 2002). In the fracture healing process, both BMP and TGF pathways were active. BMP2 reached a peak on day 1, TGF-β2 and TGF-β3 on day 7, while TGF-β1, BMP6 and GDF-10 were expressed from day 3 to day 21. In our system, in addition to TGF-β1 and BMP6 up-regulation, GDF-10 was up-regulated 1.94 folds in 5-Aza-dC pre-treated BMSCs. In agreement with our results, Cho et al concluded that different members of TGF-β superfamily had distinctive role in the differentiation process and the effects of different members were integrated towards osteogenic differentiation (Cho et al., 2002). These results were in agreement with our findings of BMSCs pre-treated with 5-Aza-dC in osteogenic media, in terms of the activation of multiple growth factors. Thus, pre-treatment of BMSCs with 5-Aza-dC and culture of cells in osteogenic conditions may mimic the osteogenic development that takes place during the fracture repair process.

The formation of osteogenic matrix in pellets formed by 5-Aza-dC pre-treated cells and cultured in chondrogenic conditions indicated the powerful induction of selected genes that were related to the osteogenic lineage. Similarly, Akiyama et al cultured bovine periosteal cells with ascorbate for 5 weeks in monolayer then transferred the cells to pellets at high density ($2X10^7$ cell/pellet) which were then implanted subcutaneously into nude mice. Six weeks later, at sacrifice, the pellets were intact, in position and had formed

osteogenic matrix in a process comparable to the intra-membranous bone development (Akiyama et al., 2006).

Bone develops in the embryo from mesenchymal cells by intra-membranous and endochondral ossification. The former takes place in flat bones when a group of cells, surrounded by high vasculature differentiate directly into pre-osteoblasts and then osteoblasts. The main characteristics of intra-membranous ossification are the absence of preferential orientation of the collagen fibres, the delay in calcification and the rich blood vasculature (Baron, 1999). Endochondral ossification depends on the formation of a cartilaginous 'anlage' of the developing bone, with chondrocytes in lacunae within a cartilage matrix. A ring of bone formed by intra-membranous ossification is then formed at the mid-shaft, where initial periosteum is formed, followed by vascular penetration of the cartilage to form the haematopoietic bone marrow. The chondrocytes enlarge to become hypertrophic chondrocytes and undergo apoptosis. Blood vessels penetrate into the empty lacunae. Transverse septa are resorbed while longitudinal septa provide the scaffold onto which marrow-derived osteoblasts deposit bone (Baron, 1999). From these criteria and in agreement with Akiyama et al., it could be assumed that BMSCs pellets cultured in osteogenic conditions mimicked an intra-membranous mode of development while the FC pellets formed from one million cells or less and cultured in osteogenic conditions may resemble the very early stages of endochondral bone development.

FC, derived mainly from the active chondrogenic epiphyses of the fetal femur, showed a natural propensity for osteogenic development when the cells were cultured in pellet form under osteogenic conditions. The configuration of the matrix formed mimicked that of a fetal bone, i.e. a chondrogenic centre with some early progenitors, a peripheral osteoid-rich matrix surrounded by a periosteum. Chan et al also found that FC formed osteoid as well as chondrogenic matrix when the cells were suspended in Matrigel and implanted below the renal capsule in mice (Chan et al., 2009).

In our system, the sphere configuration of the pellet may have created stretch forces on the exterior of the pellets by the out-growing chondrogenic matrix and the surface tension of the media, in addition to the force of fluid flow during media change. Ku et al showed enhanced synthesis of type III collagen when stretch force was applied to BMSCs in monolayer (Ku et al., 2006). The authors used a method that was similar to that used by Nakatani et al (2002), which depended on using a vacuum unit to apply stretch forces on human cells cultured on flexible polystyrene well bottom. The authors demonstrated increased transcription of collagen III and V after 48 hours of application of the stretch forces to human ligamentum flavum derived cells. This induction was secondary to increased production of TGF-β1 (Nakatani et al., 2002).

In addition, Datta et al showed that murine BMSCs enhanced osteogenesis when cultured on a titanium mesh and exposed to fluid shear stresses. In addition, the authors demonstrated that culturing the BMSCs on the titanium mesh covered with extracellular matrix markedly enhanced osteogenesis in comparison to non-covered mesh or to mesh covered with denatured extracellular matrix. The authors concluded that the matrix is important in the osteogenic differentiation and showed the variability in the differentiation potential on different surfaces (Datta et al., 2006).

The above-mentioned results were in agreement with the observations of culturing the pellets on different surfaces. In our studies, pellets cultured on plastic surfaces were smaller and less osteogenic in comparison to their counterpart cultured on glass. In addition, the cells rapidly formed pellets on plastic, whilst remaining adhered to the glass as monolayer for 7-10 days. Based on Datta's et al study, it could be anticipated that BMSCs displayed preferential adherence to the glass, and hence formed a monolayer and osteogenic matrix. When the monolayer reached confluence, it contracted, peeled from one side and rolled onto itself. When viewed, after histological preparation, intense matrix staining was present at the folding line more than at other regions of the pellets.

In another interesting study, Kieswetter et al (1996) cultured the osteosarcoma cell line MG63 on titanium surfaces of different roughness as well as on plastic. The authors found that the level of TGF-β1 in culture media increased three to five times when was cultured on rough titanium in comparison to cells cultured on plastic, while cell proliferation had an inverse relationship with the roughness of the surface (Kieswetter et al., 1996).

Primary bovine osteoblasts showed enhanced attachment and adhesion to titanium, while proliferation increased on zirconium surfaces (Depprich et al., 2008). A further explanation was provided by Keselowsky et al. (2005) The authors used surfaces coated with fibronectin as well as chemically modified fibronectin; i.e. two different ligands for the cell surface integrins (Keselowsky et al., 2005). When the ligand terminal contained –OH or $–NH_2$, there was enhanced ALP activity, up regulation of osteogenic genes and matrix mineralization in comparison to the terminals with –COOH or $–CH_3$. Integrin blocking antibody inhibited the mineralization with –OH or $–NH_2$, while functioning antibody stimulated the matrix mineralization with –COOH and $–CH_3$. Thus, the surface effect on the cells could be transmitted through the cell surface integrins. In another study, rat-derived BMSCs displayed enhanced ALP activity when cultured over discs made of a mixture of glass and ceramic in comparison to titanium discs (Ozawa & Kasugai, 1996).

Self-assembled pellets have been described by Hu and Athanasiou (2006). The authors cultured bovine chondrocytes at high density on agarose ($5.5 X10^6$ cells in 300 µl media). Cells remain detached from the plastic surface and form one cohesive nodule after 24 hours (Hu & Athanasiou, 2006). Along the course of the culture (12 weeks), the nodule appeared smooth and flat while the comparable construct cultured on plastic was contorted into folds. In addition, the agarose-cultured nodule was significantly higher in collagen and proteoglycan content at different time points. The authors argued that the self-assembling constructs would avoid the drawbacks of scaffolds such as the toxic products of degradation, loss of phenotype associated with solid scaffolds, inhibition of cell migration and cell-to-cell communication, obstruction of cell

growth and stress shield of cells from mechano-transduction. In addition, in our system the adhesion of cells to the glass for the longest possible period enhanced matrix formation especially at the folding levels. Self-assembled pellets had greater size in comparison to classical pellets. In classical pellets, some cells did not integrate into the pellets. Similarly, the seeding of scaffolds is usually a critical step that is associated with attachment of some cells into the scaffold while others drop through the scaffold. In the self-assembled pellets, all seeded cells formed a monolayer and then folded into a 3D configuration, and thus there was no significant loss of cells.

The migration of the pellets against gravity towards the air-medium interface has not been described before, to the best of our knowledge. Bryant et al described the migration of leucocytes against gravity on glass slides (Bryant et al., 1966). This process was preceded by centrifugation of the whole blood and mounting on glass slides, where the migration process continued for 12 hours. The authors found that leukocyte migration was not affected by changes of the plasma pH, electrolytes or glucose level. Alternatively, the migration was enhanced by increasing the temperature from 25^{o}C to 40 ^{o}C and both the adhesion to glass and the migration were markedly inhibited by heating the cell-free plasma at 56^{o}C for 30 minutes. The authors conclude that certain heat labile factors are important in the adhesion and migration of the cells. Annabi et al studied the effect of hypoxia on murine BMSCs on Matrigel. The cells migrated in the Matrigel to form a capillary-like network after 4 hours of culturing the cells in 1% oxygen (Annabi et al., 2003). The membrane-type 1 metalloproteinase was markedly induced in those cells and blocking the catalytic domain of this enzyme diminished the migration ability of the cells.

In our system, we assume that when BMSCs and FC form self-assembled constructs, the constructs remain attached at a certain point to the glass from which some cells migrated against gravity, guided by the increasing oxygen concentration to the air-media interface, pulling the construct in a similar mechanism to the self-assembly process; i.e. folding of the construct over the upwards moving cell sheet. In addition, FC preferentially adhered to the plastic

tips rather than the plastic wall of the tube. This adherence could be enhanced by the fact that the cells were still in contact with each other as well as with the plastic tip. The cells migrated towards higher oxygen levels, but did not reach the air-media interface, which may be caused by the increasing diameter of the tip.

6.2. Limitations of current studies and future prospectus

These studies were performed on primary cells, which required significant cell culture from scarce bone marrow samples, extraction of the cells, and expansion to a level that could be used in subsequent studies. In addition, these cells are very refractory to transfection studies. Blocking of single genes would be helpful in mapping the molecular mechanisms underlying the enhancement of differentiation in different models. The use of an appropriate cell line in future studies would be advantageous, although results obtained in cell lines are not necessary applicable to the primary cells. In addition, there are, at present, no commercially available BMSCs-like cell lines. FC have the advantage of rapidly growing cells, which allow, for example the study of cell culture on glass. The results could be transferred to BMSCs. However, FC are not expected to be a good candidate for transfection studies as they are primary cells.

The BMSCs treated with 5-Aza-dC and cultured either in pellets or self-assembled constructs as well as BMSCs and FC cultured in self assembled constructs without initial treatment under osteogenic conditions may in the future be used to replace bone loss or for application to bone defects. In addition, BMSCs and FC pre-treated with TSA in chondrogenic conditions may be helpful in cartilage defects. The safety and the spectrum of gene expression of 5-Aza-dC and TSA treated cells has not been yet defined. Microarray studies for gene expression will be necessary for pre-treated versus non-treated cells. In addition, further molecular characterization such as for example, PCR arrays for BMSCs in basal conditions versus osteogenic conditions with and without 5-Aza-dC pre-treatment, needs to be addressed. Such a study could help in determining the genes affected by the 5-Aza-dC without the influence of the

media, the effect of the media on the persistence and the level of activation of those genes as well as unmasking the genes that have already been activated in osteogenic conditions without initial treatment. The promoters of the key transcription factors, such as TGF-β1 should be mapped using the bisulphite sequencing method in order to determine whether the effects include permanent changes in the methylation status of the gene promoter. Histone changes were not examined in this thesis. From the results of the chondrogenic pellet culture work, it could be anticipated that histone acetylation of the promoters of key chondrogenic genes, such as Sox-9, may be crucial for chondrogenesis. To study this, chromatin immuno-precipitation studies could be designed.

The effect of the epigenetic modifiers could be improved by achieving better synchronization of the cell cycle before the initial application of the modifiers. A powerful agent such as mimosine could be used for such purpose. Mimosine is a plant amino acid that arrests the eukaryotic cells in the S phase (Krude, 1999). The effect of such chemical on the differentiation process needs to be evaluated.

Doubling time were calculated using an equation that depends on counting cells at two time points. A more sensitive method using multipoint calculation could be undertaken in the future studies.

The study of the differentiation of BMSCs pellets over different time points may help in mapping matrix formation. In addition, the mechanical properties of the pellets and constructs will need to be evaluated in comparison to equivalent primary explants. In addition, all the different pellets and constructs need to be studied *in vivo* for stability and further differentiation. Animal studies will be necessary for such purpose. A bone gap or cartilage defect has to be created, filled with pellets/constructs and followed up. The self-assembled pellets cultured on glass would be particularly interesting to investigate in the bone defect model due to the larger size and the expression of vWF, which could facilitate the vascularisation of the construct. The extensive

expression of vWF in BMSCs cultured on glass as self-assembled constructs requires further characterization, both *in-vitro* and *in-vivo*, using other endothelial markers such as CD 31.

Additionally, the local application and/or systemic administration of 5-Aza-dC may help to enhance the differentiation of BMSCs that are present in the callus at the site of defect. Similarly, intracapsular injection of TSA may help in the chondrogenic differentiation and/or chondrogenic matrix formation, which would be helpful in cartilage defects as well as in cartilage degenerative diseases. TSA has a wide spectrum of effects including the passive acetylation of histones, transcription factors and other cellular proteins as well as the DNA demethylation (Selker, 1998; Cervoni & Szyf, 2001; Huang et al., 2005). Chromatin immuno-precipitations assays for acetylated histones in relation to the promoters of key chondrogenic genes may help in revealing the underlying mechanism of TSA-induced chondrogenesis. Similar assays could be applied to nuclear extracts to investigate the acetylation of key transcription factors, such as Sox-9, in our system.

Cells treated with SSA have been found to have specific characteristics that were not comparable to TSA. Significant cell death was observed whilst those that survived, replenished cell numbers to a considerable extent. In addition, these cells in pellets showed negligible matrix and continued to express nucleostemin, a stem cell marker. As SSA inhibits mainly class I HDACs, this may have as yet undetermined effects. Further characterization including fluorescent sorting techniques of these cells may help to identify a population with early progenitor or stem cell charactersistics.

The differentiation of FC in pellets cultured under osteogenic conditions into the ring configuration is important from a developmental perspective and could have a role in the future, as an alternative model to animal studies in pharmaceutical testing. Longer culture times *in vitro* as well as *in vivo* (without diffusion chambers) would provide further characterization of these spherical bone-like constructs. In addition, FC lost this configuration on passage. It would

be interesting to investigate if treatment of passage 6 cells with 5-Aza-dC would restore the capacity to form the rings again, which may provide evidence that DNA methylation had been acquired over long-term culture.

In addition, the antigenicity of BMSCs and FC are still debatable. Detailed studies for the compatibility of the epigenetically modified constructs between donors and recipient will be of extreme importance.

The studies included in this thesis demonstrate evidence of the role of epigenetic modifiers on the differentiation of BMSCs into the osteogenic and chondrogenic lineages. However, the details of how the modifiers exert this role require further investigation and detailed understanding before applying these treatments into regenerative medicine programs. In addition, the different models of 3D osteogenic constructs may form foundations for future studies and possibly for tissue engineering strategies to replace bone defects. Thus, we hope that these studies will contribute a small step forward in this era of tissue regeneration.

Appendix

Ct values and fold regulation for the PCR array study

	AVG Ct		Standard Deviation		Fold Regulation
	5-Aza-dC	Control	5-Aza-dC	Control	
AHSG	35	35	0	0	1.101
ALPL	24.66	23.52	2.821356	1.562706	-2.0017
AMBN	35	35	0	0	1.101
AMELY	34.22	34.19	1.103087	1.145513	1.0783
ANXA5	20.91	20.2	2.467803	2.206173	-1.4807
BGLAP	28.81	28.35	2.206173	1.682914	-1.2494
BGN	21.3	21	1.647559	1.1243	-1.1183
BMP1	23.5	23.76	0.049497	0.947523	1.3229
BMP2	31.17	30.95	0.565685	1.315219	-1.0579
BMP3	34.57	35	0.615183	0	1.4884
BMP4	27.43	26.95	0.629325	1.378858	-1.2669
BMP5	30.47	30.28	0.79196	0.961665	-1.0362
BMP6	28.48	30.34	1.513209	0.39598	3.9965
CALCR	35	35	0	0	1.101
CD36	31.25	31.71	1.491995	0.59397	1.5197
CDH11	21.7	21.22	2.114249	1.534422	-1.2669
COL10A1	29.88	29.09	0.848528	2.467803	-1.576
COL11A1	21.73	21.83	1.110158	1.979899	1.1841
COL12A1	18.2	19.56	0.028284	1.279863	2.8162
COL14A1	23.75	25.16	0.473762	0.763675	2.9358
COL15A1	26.2	26.66	1.081873	0.615183	1.5144
COL1A1	17.69	17.96	0.629325	0.982878	1.3275
COL1A2	16	16.62	0.424264	0.671751	1.6862
COL2A1	35	35	0	0	1.101
COL3A1	19.16	20.79	1.117229	0.06364	3.3958
COL4A3	32.67	33.21	1.739483	1.301076	1.6008
COL5A1	21.66	22.59	0.72832	0.615183	2.0976
COMP	20.99	21.21	0.841457	1.491995	1.2823
CSF2	35	35	0	0	1.101
CSF3	35	35	0	0	1.101
CTSK	19.77	20.5	0.233345	1.796051	1.8324
DMP1	33.14	33.82	1.477853	0.417193	1.7639
DSPP	34.72	35	0.39598	0	1.3368
EGF	32.42	33.5	1.202082	2.12132	2.3274
EGFR	24.21	24.47	0.374767	1.421285	1.3184
ENAM	35	35	0	0	1.101
FGF1	28.44	30.55	0.509117	1.286934	4.7527
FGF2	26.06	26.92	0.452548	1.202082	1.9983
FGF3	35	35	0	0	1.101
FGFR1	26.82	27.09	0.685894	1.442498	1.3321
FGFR2	26.22	26.13	1.025305	1.45664	1.038
FLT1	35	34.21	0	1.1243	-1.576
FN1	16.81	16.63	0.353553	1.902117	-1.0326
GDF-10	34.19	35	1.152584	0	1.9369
ICAM1	26.1	26.08	1.442498	0.226274	1.0858
IGF1	25.85	25.71	0.282843	1.442498	-1.0009

IGF1R	25.64	25.17	0.799031	1.59099	-1.2581
IGF2	24.36	25.11	0.360624	1.301076	1.858
ITGA1	25.23	27.62	0.19799	1.011163	5.7507
ITGA2	30.29	31.56	0.33234	0.403051	2.6551
ITGA3	26.28	26.95	0.028284	0.077782	1.7456
ITGAM	31.31	32.15	0.155563	0.176777	1.9639
ITGB1	20.34	20.3	0.353553	1.216224	1.0708
MINPP1	26.09	26.05	1.067731	1.244508	1.0746
MMP10	33.92	33.91	1.364716	1.541493	1.0971
MMP2	19.5	19.98	0.692965	0.042426	1.5355
MMP8	34.65	35	0.494975	0	1.4032
MMP9	31.78	31.09	0.721249	0.961665	-1.4654
MSX1	29.28	28.94	1.491995	1.937473	-1.1457
NFKB1	23.9	24.44	0.509117	1.491995	1.5952
PDGFA	27.49	30.24	0.049497	2.071823	7.4063
PHEX	31.94	33.24	0.13435	0.360624	2.7109
RUNX2	24.29	25.56	0.091924	0.127279	2.6643
SCARB1	30.12	29.35	0.424264	1.251579	-1.5543
SERPINH1	19.12	18.83	0.141421	0.714178	-1.1144
SMAD1	25.76	25.27	0.940452	1.534422	-1.2757
SMAD2	25.16	25.51	0.282843	2.566798	1.3984
SMAD3	23.49	24.81	0.289914	0.014142	2.7582
SMAD4	23.98	24.03	0.438406	0.820244	1.1398
Sox9	28.41	27.86	0.473762	1.442498	-1.3252
STATH	35	33.7	0	1.845549	-2.2443
TFIP11	25.45	24.6	0.339411	1.718269	-1.6429
TGFB1	25.06	26.02	1.697056	1.343503	2.1417
TGFB2	26.9	28.41	0.997021	0.33234	3.1356
TGFB3	25.93	26.33	0.282843	0.353553	1.4527
TGFBR1	26.35	26.13	0.46669	0.070711	-1.0579
TGFBR2	31.74	28.2	0.049497	1.774838	-10.5652
TNF	32.56	32.27	0.756604	1.32229	-1.1105
TUFT1	27.21	26.67	0.296985	2.107178	-1.3207
TWIST1	22.71	22.82	0	0.516188	1.1841
VCAM1	18.98	18.32	0.487904	1.449569	-1.4352
VDR	26.02	25.14	0.678823	1.166726	-1.6774
VEGFA	25.55	25.38	0.473762	1.131371	-1.0184
VEGFB	21.97	22.09	0.601041	1.237437	1.1964
B2M	18.29	18.37	0.240416	1.294005	1.1597
HPRT1	25.49	25.54	0.360624	1.506137	1.1398
RPL13A	19.89	19.69	0.502046	1.209153	-1.0434
GAPDH	19.29	19.01	0.742462	1.407142	-1.1029
ACTB	17.7	17.55	0.502046	1.19501	-1.0078
HGDC	35	35	0	0	1.101
RTC	22.54	22.25	0.692965	0.360624	-1.1144
RTC	22.52	22.23	0.707107	0.014142	-1.1105
RTC	22.58	22.24	0.735391	0.077782	-1.1537
PPC	19.16	19.1	1.364716	1.385929	1.0598
PPC	19.41	19.01	1.527351	1.294005	-1.2027
PPC	19.44	19.05	1.343503	1.180868	-1.1944

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