Graduate Program “Molecular Basis of Human Disease”

Master Thesis

Study of the NF-YA transcription factor in human bone marrow mesenchymal stem cells

Anthi Demetriadou
BSc in Biology

Supervisor: Dr. Helen A. Papadaki

Haemopoiesis Research Laboratory

Medical School
University of Crete

Heraklion 2012

Πανεπιστήμιο Κρήτης

Μεταπτυχιακό Πρόγραμμα
«Κυτταρική και Γενετική Αιτιολογία, Διαγνωστική και Θεραπευτική των Ασθενειών του Ανθρώπου»

Μεταπτυχιακή Εργασία

Μελέτη του μεταγραφικού παράγοντα NF-YA σε ανθρώπινα αρχέγονα μεσεγχυματικά κύτταρα μυελού των οστών

Ανθή Δημητριάδου
Βιολόγος

Επιβλέπων: Ελένη Α. Παπαδάκη

Ερευνητικό Εργαστήριο Αιμοποίησης

Ιατρική Σχολή
Πανεπιστήμιο Κρήτης

Ηράκλειο 2012

ΕΥΧΑΡΙΣΤΙΕΣ

Η παρούσα μεταπτυχιακή εργασία πραγματοποιήθηκε στα πλαίσια του μεταπτυχιακού προγράμματος «Κυτταρική και Γενετική Αιτιολογία, Θεραπευτική και Διαγνωστική των Ασθενειών του Ανθρώπου» στο ερευνητικό εργαστήριο Αιμοποίησης υπό την επίβλεψη της κας Ελένης Α. Παπαδάκη, καθηγήτριας Αιματολογίας της Ιατρικής Σχολής του Πανεπιστημίου Κρήτης.

Αρχικά θα ήθελα να ευχαριστήσω θερμά την κα Ελένη Παπαδάκη που μου έδωσε την ευκαιρία να γίνω και εγώ μέλος της ερευνητικής της ομάδας. Αναμφίβολα η προσφορά, το αμέριστο ενδιαφέρον της και η πολύτιμη βοήθειά της αποτέλεσαν για μένα σημαντικά εφόδια για την εκπόνηση της εργασίας αυτής. Χαίρομαι ιδιαίτερα που γνώρισα και συνεργάστηκα με αυτόν τον εξαίρετο άνθρωπο αλλά και επιστήμονα.

Στη συνέχεια θα ήθελα να ευχαριστήσω τη Χριστίνα Καστρινάκη και τον Χάρη Ποντίκογλου για τη βοήθεια, την υποστήριξη και το ενδιαφέρον τους καθ' όλη την παραμονή μου στο εργαστήριο. Η συνεισφορά τους ήταν πολύτιμη και καθοριστική για την πραγματοποίηση αυτής της εργασίας.

'Ενα τεράστιο ευχαριστώ στην Κωνσταντία Παυλάκη που με εμπιστεύτηκε και που ήταν πάντα δίπλα μου τόσο στο χώρο του εργαστηρίου όσο και σε προσωπικό επίπεδο. Η βοήθειά της ήταν πολύ σημαντική και σπουδαία για μένα και η συνεργασία μας τίποτα λιγότερο από άψογη.

Πως θα μπορούσα να ξεχάσω τη ιδιαίτερη συμβολή του Μάνου Σημαντηράκη! Πέρα από τη πολύτιμη υποστήριξή του στο πειραματικό μέρος της εργασίας αυτής, τα καθημερινά του αστεία μου έφτιαχναν πάντα τη διάθεση.

'Επειτα θα ήθελα να ευχαριστήσω την Αριστέα Μπάτσαλη, που δυστυχώς δεν μου δόθηκε η ευκαιρία να συνεργαστώ μαζί της, αλλά που υπήρξε για μένα ένα άτομο που μπορούσα να μοιράστω μαζί του νέες

ιδέες και προβληματισμούς. Οι συμβουλές της και η προσφορά της ήταν καθοριστικής σημασίας για μένα.

Η ολοκλήρωση της μεταπτυχιακής αυτής εργασίας δεν θα μπορούσε να επιτευχθεί χωρίς τη συνεισφορά και τη βοήθεια όλης της υπόλοιπης ερευνητικής ομάδας του εργαστηρίου. Θα ήθελα να ευχαριστήσω από καρδιάς την Ειρήνη Μαυρουδή, τη Σεμέλη Μαστροδήμου, τη Μαρία Βελεγράκη, την Αθηνά Δαμιανάκη, την Κλαυδία Γεμεντζή, την Έλσα Κουβίδη και την Άννα Ψαράκη που μοιράστηκαν μαζί μου τις επιστημονικές γνώσεις τους, για την υπομονή και την υποστήριξή τους. Επίσης, ευχαριστώ την Αθηνά Τρακάκη και τον Μιχάλη Κλώντζα για τη δημιουργία ενός χαρούμενου και φιλικού περιβάλλοντος, κάνοντας το καθημερινό μου έργο ευχάριστο και παραγωγικό.

Η διετής πορεία μου στο μεταπτυχιακό αυτό πρόγραμμα δεν θα ήταν ίδια χωρίς τους συμφοιτητές μου Μάνο Σημαντηράκη, Μάρω Στρατινάκη, Δημήτρη Νασσίκα και Δημήτρη Θεοφιλάτο. Μοιραστήκαμε προβληματισμούς και άγχος, συνεργαστήκαμε, διαβάσαμε και γελάσαμε μαζί. Τους ευχαριστώ για την υπόστηριξη, την κατάνοηση και τη βόηθειά τους. Επίσης, ένα μεγάλο ευχαριστώ στην κα Μαίρη Αδαμάκη που η βοήθεια και η υπομονή της υπήρξαν πολύ σημαντικά στοιχεία για την ομαλή διεξαγωγή του μεταπτυχιακού αυτού προγράμματος.

Τέλος θα ήθελα να ευχαριστήσω τον Κωνσταντίνο Παληκαρά που στάθηκε δίπλα μου με υπομονή και αφοσίωση καθ' όλη τη διάρκεια του μεταπτυχιακού μου καθώς φυσικά και τους γονείς μου, Γιάννη και Εύη, για την αμέριστη αγάπη και υποστήριξή τους και που χωρίς αυτούς η πραγματοποίηση των σπουδών μου θα ήταν αδύνατη.

Σας ευχαριστώ όλους θερμά!

CONTENTS

Page

ABSTRACT

Background

NF-Y is a CCAAT-binding transcription factor consisting of three subunits, NF-YA, NF-YB and NF-YC, which are all necessary for DNA binding. NF-YB and NF-YC form a stable dimer which acts as a binding surface for NF-YA. NF-YA is characterized as the regulatory subunit of the complex because of its differential expression during the cell cycle. NF-YA appears to have a complex role in hematopoietic stem cell (HSC) biology. So far, there are no available data on NF-Y role in mesenchymal stem cells (MSCs).

Aims

The evaluation of NF-YA expression levels during the *in vitro* expansion of human bone marrow mesenchymal stem cells (BM-MSCs) and the assessment of its possible role during the *in vitro* differentiation of BM-MSCs into the adipogenic and osteogenic lineages.

Methods

BM-MSCs were isolated from posterior iliac crest aspirates of hematologically healthy individuals undergoing orthopedic surgeries after written informed consent. BM-MSCs were *in vitro* expanded until passage-10 (P10). Total RNA was isolated from BM-MSCs at different passages (P2, P6, and P10) for the evaluation of NF-YA mRNA levels.
BM-MSCs *in vitro* differentiations into adipocytes and osteoblasts were induced using the appropriate culture media. Total RNA and proteins were isolated from differentiated BM-MSCs, and NF-YA levels, as well as lineage-specific marker genes levels were evaluated. The relative gene expression was evaluated by real-time PCR. All PCR results are expressed as $2^{-\Delta Ct}$. Protein levels of NF-YA were immunodetected by Western blot. Moreover, differentiations were verified using appropriate cytochemical stains.
In order to specifically knockdown NF-YA expression, BM-MSCs were transduced with shRNA for NF-YA (shNF-YA) lentiviral particles and adipocytic as well as osteoblastic differentiations were induced. Total RNA was isolated from transduced BM-MSCs at several time-points during differentiations.

Results

A statistically significant ($p=0.0296$) reduction in the mRNA levels of NF-YA was found in P10, as compared to P3 BM-MSC cultures ($n=9$).
During adipogenic differentiation, NF-YA mRNA and protein levels were found to significantly increase ($p=0.0478$) in day 16, as compared to the onset of differentiation induction (day 0), ($n=14$). Furthermore, NF-YA expression levels were strongly correlated

with PPARG ($r=0.74$, $p<0.0001$), C/EBPA ($r=0.76$, $p<0.0001$) and LPL ($r=0.74$, $p<0.0001$) transcription levels.

Regarding osteogenesis, in contrast with adipogenesis, no statistically significant change was detected in NF-YA mRNA levels (n=9).

In order to evaluate if NF-YA is functionally related to *in vitro* adipogenesis and osteogenesis of BM-MSCs, NF-YA knockdown was performed and adipocytic as well as osteoblastic differentiations were induced.

Throughout adipocytic differentiation process, the relative mRNA levels of NF-YA in shNF-YA-transduced BM-MSCs were reduced to 51.03% compared with control (non-transduced) cells. At day 21 of adipogenesis, a reduction of 31.22%, 70.06% and 74.3% was also observed in PPARG, C/EBPA and LPL mRNA levels, respectively. At last, Oil Red O staining indicated a lack of lipid droplets accumulation in shNF-YA-transduced BM-MSCs compared with control cells.

During osteogenesis, NF-YA mRNA levels in BM-MSCs transduced with shNF-YA were reduced to 52.37% compared to control cells. Although a reduction of 52.03% and 83.95% was observed in OSC and BSP relative mRNA levels respectively, the transcription levels of DLX5, RUNX2, OSX and ALP remained unaffected after NF-YA knockdown, in shNF-YA-transduced cells compared to control cells. Furthermore, Alizarin Red S and Von Kossa stainings did not reveal any significant difference between shNF-YA-transduced BM-MSCs compared with control cells.

Summary/Conclusion

We have found for the first time that NF-YA mRNA levels decrease after prolonged *in vitro* expansion of BM-MSCs.

We have shown for the first time that NF-YA, the regulatory subunit of the heterotrimeric transcription factor NF-Y, is implicated in the *in vitro* differentiation of BM-MSCs into the adipogenic cell-lineage. More specifically, during adipogenesis, NF-YA mRNA and protein levels were found to be elevated. Moreover NF-YA mRNA expression was positively correlated with PPARG, C/EBPA and LPL transcription levels. Furthermore, after NF-YA silencing, BM-MSCs were characterized by aberrant potential of *in vitro* adipocytic differentiation. Our results imply an emerging role of NF-Y in BM-MSCs adipogenesis.

In contrast, during induction of osteogenesis we did not observe a significant change regarding the expression levels of NF-YA. Additionally, transcription levels of most osteogenesis marker genes checked, remained unaffected, after NF-YA-silencing. Cytochemical stains, Alizarin Red S and Von Kossa, did not reveal any significant difference between shNF-YA transduced BM-MSCs and control cells.

Key words

NF-YA, bone marrow mesenchymal stem cells, adipogenesis, PPARG, C/EBPA, LPL, osteogenesis

ΠΕΡΙΛΗΨΗ

Θεωρητικό Υπόβαθρο

Ο μεταγραφικός παράγοντας NF-Y αποτελείται από τρεις υπομονάδες (NF-YA, NF-YB, NF-YC), όλες απαραίτητες για την πρόσδεσή του σε CCAAT μοτίβα σε υποκινητές γονιδίων. Οι υπομονάδες NF-YB και NF-YC δημιουργούν ένα σταθερό διμερές σύμπλοκο πάνω στο οποίο προσδένεται στη συνέχεια ο NF-YA, η ρυθμιστική υπομονάδα του συμπλόκου. Ο NF-YA φαίνεται να εμπλέκεται στη βιολογία των αρχέγονων αιμοποιητικών κυττάρων αλλά μέχρι τώρα δεν υπάρχουν δεδομένα που να περιγράφουν το ρόλο που διαδραματίζει στα μεσεγχυματικά αρχέγονα κύτταρα του μυελού των οστών.

Σκοπός

Η αξιολόγηση των επιπέδων έκφρασης του NF-YA κατά τη διάρκεια της *in vitro* ανάπτυξης ανθρώπινων μυελικών μεσεγχυματικών αρχέγονων κυττάρων (BM-MSCs), καθώς και ο προσδιορισμός του πιθανού ρόλου που μπορεί ο NF-YA να διαδραματίζει κατά την *in vitro* διαφοροποίηση των κυττάρων αυτών προς την λιποκυτταρική και οστεοβλαστική κατεύθυνση.

Μέθοδοι

Έγινε απομόνωση ανθρώπινων μεσεγχυματικών αρχέγονων κυττάρων από υγιείς δότες ύστερα από λήψη μυελού των οστών. Τα κύτταρα αυτά αναπτύχθηκαν *in vitro* μέχρι το κυτταρικό πέρασμα 10 (P10). Για τον προσδιορισμό των επιπέδων mRNA του NF-YA ακολούθησε απόμονωση ολικού RNA από διαφορετικά κυτταρικά περάσματα (P2, P6, P10). Η επαγωγή της *in vitro* διαφοροποίησης των BM-MSCs προς τη λιποκυτταρική και οστεοβλαστική κατεύθυνση έγινε με κατάλληλα θρεπτικά μέσα καλλιέργειας. Ακολούθησε απομόνωση ολικού RNA και πρωτεϊνών για τον προσδιορισμό των επιπέδων έκφρασης του NF-YA και ειδικών γονιδίων για την λιπογονική και οστεοβλαστική κυτταρική κατεύθυνση, σε διάφορα χρονικά διαστήματα κατά την κυτταρική διαφοροποίηση. Επιπλέον, η επαλήθευση των διαφοροποιήσεων έγινε με τη χρήση κυτταροχημικών χρώσεων. Για να επιτευχθεί σίγηση της έκφρασης του NF-YA, τα BM-MSCs διαμολύνθηκαν με ιϊκά σωμάτια που εκφραζουν shRNA για τον NF-YA (shNF-YA) και ακολούθησε διαφοροποίηση προς την λιποκυτταρική και την οστεοβλαστική κατεύθυνση. Απομόνωση ολικού RNA έγινε σε διάφορα χρονικά διαστήματα κατά τις κυτταρικές διαφοροποιήσεις. Η σχετική γονιδιακή έκφραση προσδιορίστηκε με τη μέθοδο της αλυσιδωτής αντίδρασης πολυμεράσης σε πραγματικό χρόνο (real-time PCR). Όλα τα αποτελέσματα από την αλυσιδωτή αντίδραση πολυμεράσης εκφράζονται

ως $2^{-\Delta Ct}$. Τα πρωτεϊνικά επίπεδα του NF-YA ανιχνεύθηκαν με τη μέθοδο του ανοσοστυπώματος Western (Western blot).

Αποτελέσματα

Κατά τη διάρκεια της *in vitro* καλλιέργειας των BM-MSCs, παρατηρήθηκε στατιστικά σημαντική (p=0.0296) μείωση των επιπέδων mRNA του NF-YA στο P10 σε σύγκριση με το P3 (n=9).

Κατά την λιπογένεση, παρατηρήθηκε στατιστικά σημαντική (p=0.0478) αύξηση στα επίπεδα mRNA και πρωτεΐνης του NF-YA την $16^{η}$ μέρα της διαφοροποίησης, σε σχέση με την ημέρα έναρξης της διαφοροποίησης (μέρα μηδέν), (n=14). Επιπλέον, τα επίπεδα έκφρασης του NF-YA βρέθηκαν να σχετίζονται θετικά με αυτά των PPARG (r=0.74, p<0.0001), C/EBPA (r=0.76, p<0.0001) και LPL, (r=0.74, p<0.0001). Όσον αφορά τα επίπεδα έκφρασης του NF-YA στην οστεογένεση, δεν παρατηρήθηκε στατιστικά σημαντική διαφορά (n=9).

Για την αξιολόγηση της πιθανής λειτουργικής σχέσης του NF-YA με τη λιπογένεση και την οστεογένεση, ακολούθησε σίγηση του NF-YA και επαγωγή της διαφοροποίησης προς τη λιποκυτταρική και την οστεοβλαστική κατεύθυνση.

Κατά τη διάρκεια της λιπογένεσης, τα μεταγραφικά επίπεδα του NF-YA βρέθηκαν μειωμένα κατά 51.03% στα διαμολυσμένα κύτταρα με το shNF-YA σε σχέση με τα μη διαμολυσμένα κύτταρα. Μείωση κατά 31.22%, 70.06% και 74.3% παρατηρήθηκε στα επίπεδα έκφρασης του PPARG, C/EBPA και LPL, αντίστοιχα. Τέλος, ύστερα από λιποκυτταρική χρώση (Oil Red O) παρατηρήθηκε έλλειψη λιπιδίων στα διαμολυσμένα κύτταρα με το shNF-YA σε σχέση με τα μη διαμολυσμένα.

Κατά τη διάρκεια της οστεογένεσης, τα μεταγραφικά επίπεδα του NF-YA, στα διαμολυσμένα κύτταρα με το shNF-YA βρέθηκαν μειωμένα κατά 52.37% σε σχέση με τα μη διαμολυσμένα κύτταρα. Τα επίπεδα mRNA των DLX5, RUNX2, OSX and ALP παρέμειναν σταθερά μετά από σίγηση του NF-YA, αν και παρατηρήθηκε μείωση κατά 52.03% and 83.95% στα επίπεδα έκφρασης της OSC και της BSP, αντίστοιχα. Επιπλέον, ύστερα από οστεοβλαστικές χρώσεις (Alizarin Red S και Von Kossa) δεν παρατηρήθηκαν διαφορές μεταξύ των διαμολυσμένων κυττάρων με το shNF-YA και των μη διαμολυσμένων.

Περίληψη/Συμπεράσματα

Δείξαμε για πρώτη φορά ότι τα μεταγραφικά επίπεδα του NF-YA ελαττώνονται κατά την παρατεταμένη *in vitro* ανάπτυξη των BM-MSCs.

Δείξαμε για πρώτη φορά ότι ο NF-YA, η ρυθμιστική υπομονάδα του ετεροτριμερούς μεταγραφικού παράγοντα NF-Y, εμπλέκεται στην διαφοροποίηση προς τη λιποκυτταρική κατεύθυνση των BM-MSCs. Πιο συγκεκριμένα, κατά τη διαφοροποίησή

τους προς τη λιποκυτταρική κατεύθυνση, τα επίπεδα έκφρασης του NF-YA αυξάνουν. Επιπλέον, τα μεταγραφικά επίπεδα του NF-YA συσχετίζονται θετικά με αυτά των PPARG, C/EBPA και LPL. Επιπρόσθετα, μετά την σίγηση του NF-YA, τα BM-MSCs χαρακτηρίζονται από διαταραγμένη ικανότητα *in vitro* λιπογονικής διαφοροποίησης. Τα αποτελέσματά μας υποδειλώνουν ότι ο NF-Y εμπλέκεται στην λιπογένεση των BM-MSCs.

Αντιθέτως, κατά την επαγωγή της οστεογένεσης, δεν παρατηρήθηκε σημαντική αλλαγή των επιπέδων έκφρασης του NF-YA. Μετά τη σίγηση του NF-YA, τα επίπεδα των περισσοτέρων ειδικών γονιδίων της οστεογένεσης που ελέγχθηκαν, παρέμειναν σταθερά. Επιπλέον, ειδικές κυτταροχημικές χρώσεις για την οστεογένεση, δεν έδειξαν διαφορές μεταξύ των διαμολυσμένων με shNF-YA κυττάρων και των μη διαμολυσμένων.

Λέξεις-κλειδιά

NF-YA, μυελικά μεσεγχυματικά αρχέγονα κύτταρα, λιπογένεση, PPARG, C/EBPA, LPL, οστεογένεση

INTRODUCTION

NF-YA (Nuclear Factor-YA)

NF-Y is a CCAAT-binding trimeric transcription factor that was firstly identified in yeast [14, 16, 29, 32]. CCAAT-boxes are regulatory elements found in about 30% of eukaryotic promoters [26] and in more than 60% of human promoters [39]. Many types of gene promoters are characterized by the presence of CCAAT-boxes, such as developmental and housekeeping genes, tissue-specific and cell-cycle genes [25, 26]. Usually, CCAAT-boxes are located between 60 and 100 nucleotides upward of the transcriptional start site [26]. NF-Y recognizes the CCAAT consensus sequence, with a preference for specific flanking sequences [5, 12, 22].

NF-Y consists of three evolutionary conserved subunits, NF-YA, NF-YB and NF-YC, which are all necessary for DNA binding [14, 16, 29, 32, 36]. NF-YB and NF-YC have homology with each other, as well as with eukaryotic core histones H2B and H2A, respectively [1]. These two subunits form a stable dimer which acts as a binding surface for the third subunit, NF-YA, and subsequently this trimeric complex is able to bind CCAAT-boxes on gene promoters with high affinity and specificity [26, 28], (Figure 1). A possible mechanism by which NF-Y induces transcription is by interaction with nucleosomes and specifically with histones H3 and H4. When NF-Y together with other factors (co-activators like PCAF), (Figure 1) binds CCAAT-boxes on gene promoters, DNA in that region becomes more loose (euchromatin), enabling RNA polymerase II and transcription initiation factors to approach that site and induce transcription [28].

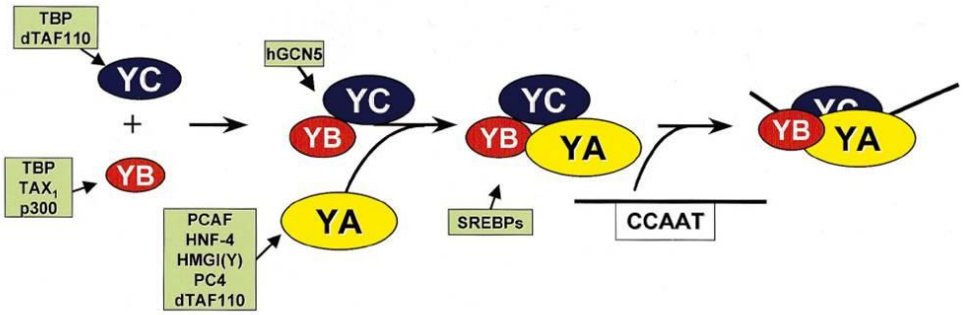

Figure 1 Schematic representation of NF-Y assembly, DNA binding and interactions with several activators [26].

All three subunits of NF-Y transcription factor include an evolutionary conserved domain from yeast to human which is indispensable for interaction with each other, with other proteins and for DNA binding [26, 20]. The conserved domain of NF-YA protein is at the C-terminal part, in contrast with NF-YB and NF-YC whose conserved domains are at the centre and at the N-terminal part, respectively [20]. During cell cycle NF-YB and NF-YC levels are stable, in contrast to NF-YA levels which vary through the

different phases of the cell cycle [7]. For that reason NF-YA is characterized as the regulatory subunit of the complex [26].

NF-YA gene is located in the short arm of chromosome 6 and consists of 9 exons. There are two NF-YA isoforms: one long and one short form. The latter lacks 28 aminoacids [21]. The alternative splicing occurs in the glutamine-rich region on exon B. The 3D structure of NF-YA is not known yet, but some biochemical data indicate that the conserved part costists of two helices, one responsible for interaction with NF-YB and NF-YC, and the other one for DNA binding [41, 24]. NF-YA has also a glutamine-rich domain and a Ser/Thr-rich domain [26], (Figure 2).

Figure 2 Schematic representation of NF-YA gene. Q: Glutamine-rich domain, BC: domain required for NF-YB and NF-YC protein interaction, DNA: domain required for DNA binding, LI: domain present only in the long isoform of NF-YA [26].

Most proteins are subjected to post-translational modifications. Specifically, NF-Y subunits have been reported to be acetylated and phosphorylated. Several histone acetylases (HATs), like KAT3B (p300) are responsible for NF-YA acetylation in lysine residues of C-terminal domain which results in the prevention of poly-ubiquitination and subsequent proteasome-mediated degradation and therefore in a prolonged half-life of the subunit [23]. Regarding phosphorylation, NF-YA is known to be phosphorylated in serines residues (Ser292, Ser298) by CDK2, emphasizing its role in cell-cycle regulation [9].

NF-YA can interact with a plethora of transcription factors as well as co-factors like C/EBPA and SP1. Usually, these interactions are synergistic and therefore transcription is activated by binding of these factors on adjacent sites (CCAAT-boxes and GC-boxes respectively) [12]. Moreover NF-YA can also repress transcription: for example, NF-YA interacts with p53 and c-Myc in order to repress transcription of G2/M genes [12, 18].

NF-Y has been studied enough in many model organisms like *S. cerevisiae*, *D. rerio*, *C. elegans*, *D. melanogaster* and mammals. NF-YA$^{-/-}$ mice (NF-YA knockout) are characterized by embryonic lethality very early in embryogenesis, due to S-phase blockade and cell apoptosis [4]. shRNA-mediated knockdown of NF-YA in human colon carcinoma cell line (HCT116) revealed a delay in S-phase progression and induced apoptosis [2].

Many genes have CCAAT-boxes in their promoters and it has been shown that NF-Y regulates the transcription of several of those genes. One example is the E2F1 gene which encodes for a transcription factor activated at G1/S phase of the cell cycle. E2F1 promoter has three CCAAT-boxes and generally is involved in the regulation of cell-cycle genes. It has been described recently that NF-YA overexpression causes apoptosis in mouse fibroblasts which is abrogated when E2F1 is missing (E2F1 knockout mouse fibroblasts) [15]. This result indicates that NF-YA indirectly causes apoptosis through E2F1 transcription hyperactivation.

NF-Y and Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) normally reside in the bone marrow (from 12 weeks post-conception in humans) and are responsible for the production of all mature blood cell types [31, 40]. HSCs apart from their differentiation ability have also the potential of self-renewal, a process in which at least one of the daughter cells retains the stem cell fate [6]. HSCs reside in the so-called bone marrow hematopoietic 'niche' which supports and controls hematopoiesis by providing the necessary signals for HSCs proliferation and differentiation, through production of several cytokines/growth factors/extracellular matrix and by cell-to-cell interactions [40].

NF-Y is implicated in HSCs regulation. Zhu and coworkers have shown that NF-Y can induce the expression of HOXB4 gene in HSCs in co-operation with USF1/2, a transcription factor which is involved in the expansion of HSCs both *in vivo* and *in vitro* [43]. Later, the same group described that overexpression of NF-YA in mice HSCs resulted in the activation of several genes involved in the proliferation and self-renewal of HSCs [44]. Furthermore, in a very recent study, Bungartz et al. made a conditional knockout mouse model which lacks NF-YA specifically in the hematopoietic system. They reported that genes implicated in HSCs proliferation, such as HOXB4, had reduced expression levels. Moreover, NF-YA deletion resulted in lower peripheral blood cell numbers and inability of HSCs to support hematopoiesis caused by bone marrow failure due to cell-cycle blockade in G2/M phase and apoptosis [8].

NF-Y and Mesenchymal Stem Cells

The bone marrow hematopoietic 'niche' includes a variety of cells like endothelial and fibroblastic cells, macrophages, adipocytes, and osteoblasts, all comprising the bone marrow microenvironment, which normally supports and controls hematopoiesis [10]. Fibroblastsic cells, adipocytes and osteoblasts are considered as mesenchymal stem cell progeny.

Mesenchymal stem cells (MSCs) are multipotent adult stem cells [33, 34] representing a rare population of stromal cells in the bone marrow. MSCs can also be isolated from a variety of other tissues, such as fat tissue and umbilical cord. MSCs and their progeny are considered to provide signals for HSC proliferation, differentiation and survival by production of cytokines, growth factors and extracellular matrix and/or by cell-to-cell interactions [10].

MSCs are characterized by the expression of a variety of cell surface markers, plastic adherence, high *in vitro* proliferative potential, and multi-lineage differentiation capacity into mainly mesenchymal lineages [34].

MSCs are able to escape immune recognition, as they do not express MHC class II, or any co-stimulatory molecules, and express MHC class I at very low levels. Another important characteristic of MSCs is their immunoregulatory ability. They inhibit the proliferation and activation of T cells. These effects are known to be mediated by soluble factors, like TGF-β1, PGE2, IDO, and by cell-to-cell interactions [30, 35]. Bone marrow derived MSCs reside in a quiescent state *in vivo.* After stimulation with a variety of physiological or pathological stimuli *in vivo* or *in vitro* [18], they can be differentiated into adipocytes, chondrocytes, or osteoblasts [33, 34, 42], (Figure 3, 4).

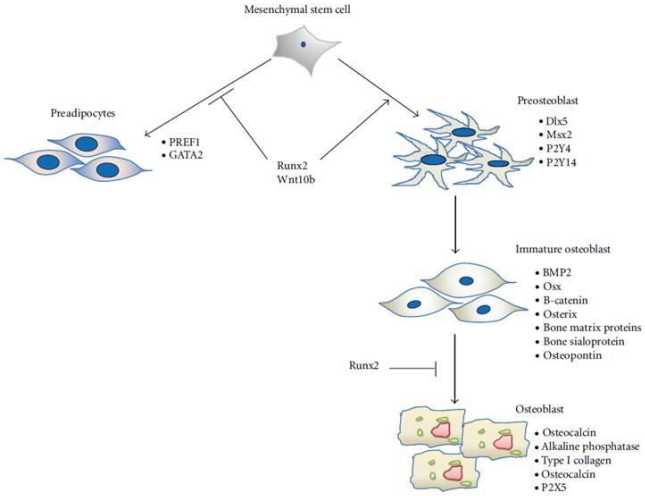

Figure 3 Differentiation of MSCs into osteoblasts [42]

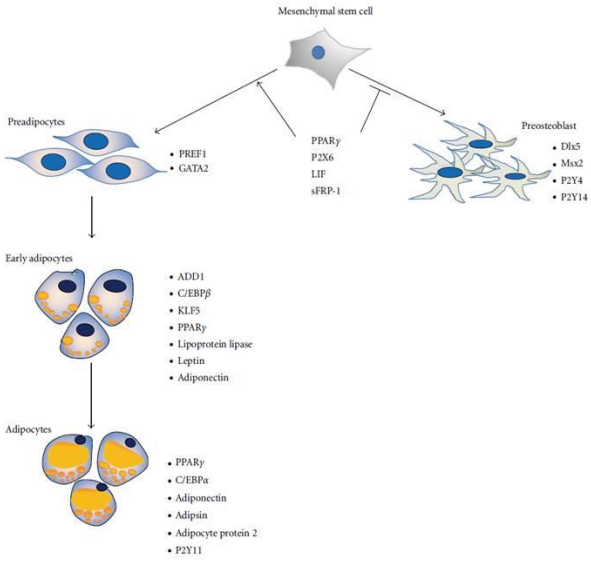

Figure 4 Differentiation of MSCs into adipocytes [42]

However, several authors indicated that they can be differentiated also into vascular smooth muscle, sarcomeric muscle (skeletal and cardiac), endothelial, neural and hepatocytic lineages, having therefore a wider differentiation capacity [34]. MSCs are positive for several non-specific cell surface markers, such as CD29 (integrin b1 chain), CD44 (hyaluronan receptor), CD71 (Transferrin receptor protein 1), CD73 (ecto-5-

nucleotidase), CD90 (thy-1), CD105 (TGF-β receptor III), CD106 (vascular cell adhesion molecule 1, VCAM), CD120a (TNFR1), CD124 (IL4R), CD146 (melanoma cell adhesion molecule, MCAM or cell surface glycoprotein, MUC 18) and many other surface proteins, but they do not express the hematopoietic markers CD11b, ITGAM (integrin αM), CD14, CD45 (Pan-leukocyte antigen) and CD34 [19, 33, 34].

Until now there are no data regarding the possible role of NF-Y in the regulation of proliferation and differentiation of BM-MSCs.

Aim of the study

The aim of this study is to describe the possible role of NF-YA, the regulatory subunit of NF-Y transcription factor, during the *in vitro* expansion of human bone marrow mesenchymal stem cells (BM-MSCs), as well as during BM-MSCs differentiation towards the adipogenic and osteogenic cell lineages.

MATERIALS & METHODS

1. Samples

Human bone marrow derived mesenchymal stem cells (BM-MSCs) were isolated from posterior iliac crest aspirates of 14 hematologically healthy individuals undergoing orthopedic surgeries after informed consent.

2. Isolation and *in vitro* expansion of BM-MSCs

Bone marrow aspirates were overlaid on Lymphodex (Inno-train Diagnostics GmbH) to obtain the bone marrow mononuclear cells (BMMCs), after density centrifugation. BMMCs were isolated, counted in Neubauer hemocytometer using 0,4% Trypan Blue (Sigma-Aldrich) vital dye.
BMMCs were plated at a concentration of $1x10^6$ BMMCs/cm^2 in culture flasks at 37^oC/5% CO_2 fully humidified atmosphere. After 24hr, culture medium was completely replaced with fresh medium [DMEM Low Glucose Glutamax™ (Gibco), 10% fetal bovine serum (FBS) heat inactivated (Gibco), 1% Penicillin/Streptomycin (Gibco)], removing the non-adherent hematopoietic cells. The remaining BM-MSCs were further expanded as previously described [11]. Upon 70% confluency, BM-MSCs were detached using 0,1% Trypsin-EDTA (Gibco) and re-seeded for a total of ten passages (P).

3. *In vitro* differentiation of BM-MSCs into adipocytes and osteoblasts

Differentiation of BM-MSCs into adipocytes and osteoblasts was induced using different culture media. The adipocytic differentiation medium consisted of DMEM Low Glucose Glutamax™ (Gibco) supplemented with 20% FBS (Gibco) and 1% Penicillin/Streptomycin (Gibco), 0,5mM IBMX (Sigma-Aldrich), 1μM dexamethasone (GAP), 0,2μM indomethacin (Sigma-Aldrich) and 10μg/ml insulin (Sigma-Aldrich). The osteoblastic differentiation medium consisted of DMEM Low Glucose Glutamax™ (Gibco) supplemented with 10% fetal bovine serum heat inactivated (Gibco), 1% Penicillin/Streptomycin (Gibco) and 0,1μM dexamethasone (GAP).
Differentiated MSCs were detached using 0,1% Trypsin-EDTA (Gibco) at different time points for total RNA and proteins isolation.

4. shRNA-mediated knockdown of NF-YA in BM-MSCs

BM-MSCs were transduced with either NF-YA shRNA Lentiviral Particles or either cop GFP Control Lentiviral Particles (Santa Cruz Biotechnology, INC) according to the manufacturer instructions. Non-transduced cells were used as control cells.

5. RNA isolation from BM-MSCs

Total RNA was isolated using the TRIzol reagent (Invitrogen) following the manufacturer instructions. RNA concentration was measured with NanoDrop Spectophotometer.

6. Reverse Transcription

1μg of total RNA was reverse transcribed to cDNA with the Superscript II Reverse Transcriptase kit (Invitrogen) using random hexamer primers, according to the manufacturer instructions.

7. Real-time PCR

Gene expression was evaluated by real-time RT-PCR using KAPA SYBR FAST qPCR Master Mix 2x (KAPA BIOSYSTEMS). The reactions were performed on a Rotor-Gene 6000 (Corbett, QIAGEN). Every single reaction had a final volume of 20μl and it contained 25ng cDNA, 400nM of the respective pair of primers and KAPA SYBR FAST qPCR Master Mix 1x. Products were normalized according to GAPDH expression. Thermocycling conditions were 95^{o}C for 3 min, 95^{o}C for 3 sec and 60^{o}C for 30 sec for 40 cycles. All PCR results were expressed as $2^{-\Delta Ct}$. All primer sequences are listed in Table 1.

	NF-YA	forward primer	5'-TCA ATT CAG GAG GGA TGG TC-3'
		reverse primer	5'-ACG GTG GTA TTG TTT GGC AT-3'
adipo-specific genes	PPARG	forward primer	5'-TCA GGT TTG GGC GGA TGC-3'
		reverse primer	5'-TCA GCG GGA AGG ACT TTA TGT ATG-3'
	C/EBPA	forward primer	5'-AAG AAG TCG GTG GAC AAG AAC AG-3'
		reverse primer	5'-AAC GCG ATG TTG TTG CG-3'
	LPL	forward primer	5'-TGG ACG GTA ACA GGA ATG TAT-3'
		reverse primer	5'-GTC CAG AGG GTA GTT AAA CTC-3'
osteo-specific genes	RUNX2	forward primer	5'-GGC CCA CAA ATC TCA GAT CGT T-3'
		reverse primer	5'-CAC TGG CGC TGC AAC AAG AC-3'
	DLX5	forward primer	5'-GCC ACC AAC CAG CCA GAG AA-3'
		reverse primer	5'-GCG AGG TAC TGA GTC TTC TGA AAC C-3'
	OSC	forward primer	5'-GAG GGC AGC GAG GTA GTG AAG A-3'
		reverse primer	5'-CGA TGT GGT CAG CCA ACT CG-3'
	OSX	forward primer	5'-GCC AGA AGC TGT GAA ACC TC-3'
		reverse primer	5'-TAA CCT GAT GGG GTC ATG GT-3'
	BSP	forward primer	5'-GGG CAG TAG TGA CTC ATC CGA AG-3'
		reverse primer	5'-CTC CAT AGC CCA GTG TTG TAG CAG-3'
	ALP	forward primer	5'-CCT GGA GCT TCA GAA GCT CAA-3'
		reverse primer	5'-ACT GTG GAG ACA CCC ATC CC-3'
housekeeping gene	GAPDH	forward primer	5'-CAT GTT CCA ATA TGA TTC CAC C-3'
		reverse primer	5'- GAT GGG ATT TCC ATT GAT GAC-3'

Table 1. Sequences of primers used in Real-time PCR

8. Protein Isolation from BM-MSCs

Whole proteins were isolated using RIPA buffer (10% glycerol, 10% NP-40, 5% Na-deoxycholate, 50mM Tris-HCl pH 7,5, 0,15M NaCl, 1mM EDTA) following the lab protocol. Bradford protein assay was performed for protein concentration measurement.

9. Western Blot

30μg of whole protein extracts were used for protein separation with SDS-PAGE which was performed on 10% (10ml) running gel and 5% (3ml) stacking gel. Protein extracts were heated to 95^{o}C for 5 minutes for protein denaturation. After the completion of electrophoresis the proteins were transferred into a nitrocellulose membrane. Next, blocking of non-specific binding was achieved by incubation of the nitrocellulose membrane in a non-fat dry milk in Tris-Buffered Saline (TBS) 0,1%

Tween for 1 hour at room temperature. After that, membrane was incubated overnight at 4^{o}C with the first antibody for NF-YA (rabbit, Santa-Cruz Biotechnology, INC) in 5% non-fat dry milk in TBS 0,1% Tween, at a concentration of 400ng/ml (1:500 ratio). After the overnight incubation, membrane was rinsed with TBS 0,1% Tween to remove the unbound antibody and then was incubated with the second antibody (Goat anti-Rabbit IgG HRP-conjugated), (Millipore) in 5% non-fat dry milk in TBS 0,1% Tween, at a concentration of 500ng/ml (1:2000 ratio), for 1 hour in room temperature. Then, the membrane was rinsed with TBS 0,1% Tween to remove the unbound antibody. The bound antibodies were visualized with ECL Western Blotting Detection Reagents (GE Healthcare) and recorded in x-ray films.
Next, membrane was incubated with the first antibody for α-tubulin (mouse, Sigma-Aldrich) in 5% non-fat dry milk in TBS 0,1% Tween, at 1:2000 ratio, for 1 hour at room temperature. After that membrane was rinsed with TBS 0,1% Tween to remove the unbound antibody and then was incubated with the second antibody (Goat anti-Mouse IgG HRP-conjugated), (Millipore) in 5% non-fat dry milk in TBS 0,1% Tween, at a concentration of 500ng/ml (1:2000 ratio), for 1 hour at room temperature. The same procedure as above was followed for signal detection.
The solution components for preparing the gels, the components for the buffers as well as the whole procedure were all obtained from "Molecular Cloning, A Laboratory Manual", 2nd Edition, Sambrook, Fritsch, Maniatis.

10. Cell staining of adipocytes and osteoblasts

Differentiation of BM-MSCs into adipocytes and osteoblasts was induced using different cultures mediums. For the adipo-differentiation medium was used DMEM Low Glucose Glutamax^{TM} (Gibco) supplemented with 20% fetal bovine serum heat inactivated (Gibco) and 1% Penicillin/Streptomycin (Gibco), 0,5mM IBMX (Sigma-Aldrich), 1μM dexamethasone (GAP), 0,2μM indomethacin (Sigma-Aldrich) and 10μg/ml insulin (Sigma-Aldrich). For the osteo-differentiation medium was used DMEM Low Glucose Glutamax^{TM} (Gibco) supplemented with 10% fetal bovine serum heat inactivated (Gibco), 1% Penicillin/Streptomycin (Gibco), 0,1μM dexamethasone (GAP), 0,15mM L-ascorbic-2-phosphate (Sigma-Aldrich) and 3mM NaH_2PO_4. Twenty one (21) days after the onset of differentiation induction, cells were stained using appropriate cell stains. For adipocytes, Oil Red O (Sigma-Aldrich) staining (quantification of lipid droplets) was used while for the osteoblasts were used Alizarin Red S (Sigma-Aldrich) (determination of the presence of calcium deposition by cells of an osteogenic lineage which is an early stage marker of matrix mineralization) and Von Kossa (Sigma-Aldrich) stainings (quantification of cell mineralization).

RESULTS

1. **NF-YA expression levels during *in vitro* expansion of BM-MSCs**

The mRNA levels of NF-YA were measured with real-time PCR. Passages (P)-2, -6, -10 BM-MSCs from 9 healthy individuals were used for RNA isolation. A statistically significant (p=0.0296) reduction in the mRNA levels of NF-YA was found in P10, as compared to P3 BM-MSCs cultures (One way ANOVA test), (Figure 5).

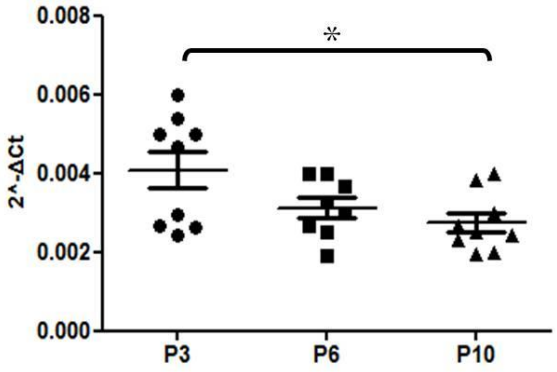

Figure 5 NF-YA relative gene expression during *in vitro* expansion of BM-MSCs (p=0,0296)

2. **NF-YA expression levels during *in vitro* differentiation of BM-MSCs into the adipogenic and osteogenic lineages**

P3 BM-MSCs were *in vitro* induced to adipogenic and osteogenic differentiation, from 14 and 9 normal subjects, respectively, using the appropriate differentiation media. RNA and proteins were isolated at different time points of the adipogenic and osteoblastic differentiations. NF-YA mRNA and protein levels were evaluated with real-time PCR and Western blot, respectively. Differentiations were verified by evaluating gene expression of lineage-associated markers for both adipogenesis and osteogenesis, as well as by appropriate cytochemical stains. Peroxisome proliferator-activated receptor gamma (PPARG), CCAAT/enhancer-binding protein alpha (C/EBPA) and lipoprotein lipase (LPL) expression levels were evaluated for adipogenesis, and Distal-less homeobox 5 (DLX5), Runt-related transcription factor 2 (RUNX2), osteocalcin (OSC), osterix (OSX), bone sialoprotein (BSP) and alkaline phosphatase (ALP) expression levels were evaluated for osteogenesis. Oil Red O staining was used for the evaluation of adipocytic differentiation and Alizarin Red S as well as Von Kossa stainings were used for osteoblastic differentiation. During adipogenic differentiation, NF-YA mRNA and protein levels were found to

significantly (p=0.0478) increase in day 16 as compared to the onset of differentiation induction (day 0), (Mann Whitney U test), (Figures 6, 7).

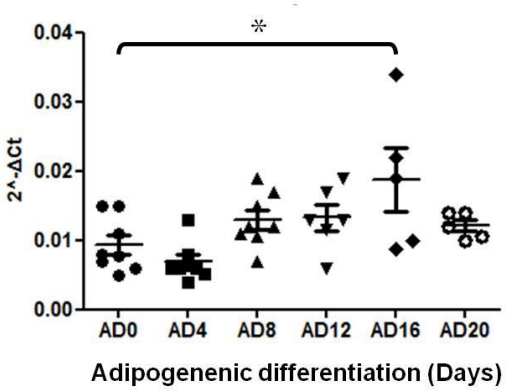

Figure 6 NF-YA relative gene expression during *in vitro* adipogenic differentiation of BM-MSCs

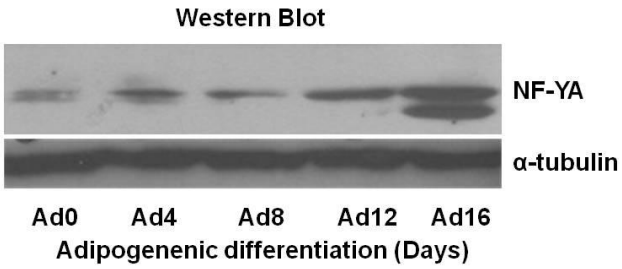

Figure 7 NF-YA protein levels during *in vitro* adipogenic differentiation of BM-MSCs. a-tubulin was used as a loading control.

Furthermore, NF-YA expression levels were strongly correlated with PPARG (r=0.74, p<0.0001), C/EBPA (r=0.76, p<0.0001) and LPL (r=0.74, p<0.0001) transcription levels (Figures 8, 9, 10).

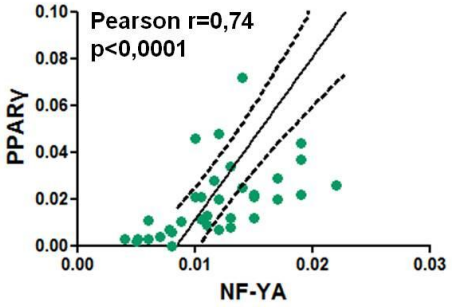

Figure 8 Correlation of NF-YA expression with PPARγ expression levels during *in vitro* adipogenic differentiation of BM-MSCs

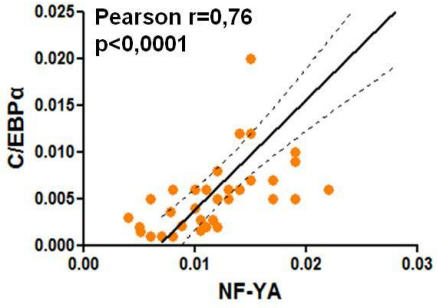

Figure 9 Correlation of NF-YA expression with C/EBPα expression levels during *in vitro* adipogenic differentiation of BM-MSCs

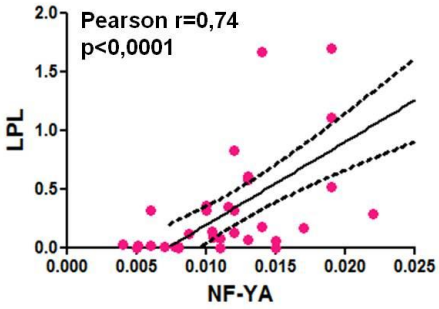

Figure 10 Correlation of NF-YA expression with LPL expression levels during *in vitro* adipogenic differentiation of BM-MSCs

Oil Red O staining was used for the identification of lipid droplet accumulation in adipocytes (Figure 11).

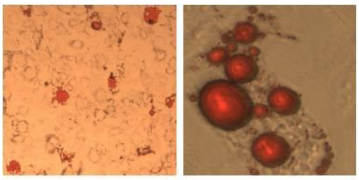

Figure 11 Oil Red O staining was used for the identification of lipid droplets in adipocytes

Alizarin Red S and Von Kossa stainings were used for the identification of calcium deposition and for mineralization, respectively (Figure 12, 13). During osteogenesis no statistically significant change was detected in NF-YA mRNA or protein levels (Mann Whitney U test), (Figure 14).

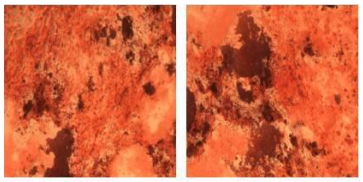

Figure 12 Alizarin Red S staining was used for the identification of calcium deposition by osteoblasts' extracellular matrix

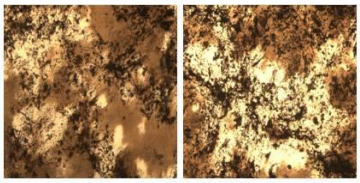

Figure 13 Von Kossa staining was used for the quantification of extracellular matrix mineralization of osteoblasts

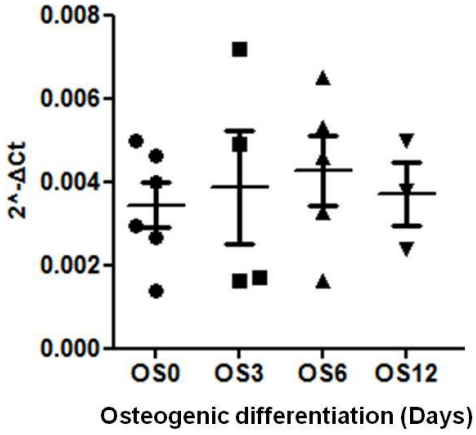

Figure 14 NF-YA relative gene expression during *in vitro* osteogenic differentiation of BM-MSCs

3. shRNA-mediated knockdown of NF-YA during *in vitro* adipogenesis of BM-MSCs

In order to evaluate if NF-YA is functionally related to adipogenesis of BM-MSCs, lentiviral particles expressing NF-YA shRNA were used to specifically knockdown NF-YA expression during induction of *in vitro* adipogenesis. BM-MSCs were transduced with either NF-YA shRNA lentiviral particles (shNF-YA) or GFP lentiviral particles (transduction control). Non-transduced cells were also used as

control cells. The mRNA levels of NF-YA as well as of PPARG, C/EBPA and LPL were evaluated with real-time PCR. Moreover Western blot was performed for the evaluation of NF-YA protein levels. In addition, Oil Red O stain was used for the identification of lipid droplets accumulation, in day 21 of adipogenesis.

A constant reduction of the relative mRNA levels of NF-YA was demonstrated for shNF-YA transduced cells compared to control (non-transduced) cells, from day 0 (51,36%) until day 14 (46,78%) of differentiation (Figure 15). At day 14 of adipogenesis, a reduction of 31.22%, 70.06% and 74.3% was also observed in PPARG, C/EBPA and LPL mRNA levels, respectively (Figure 15).

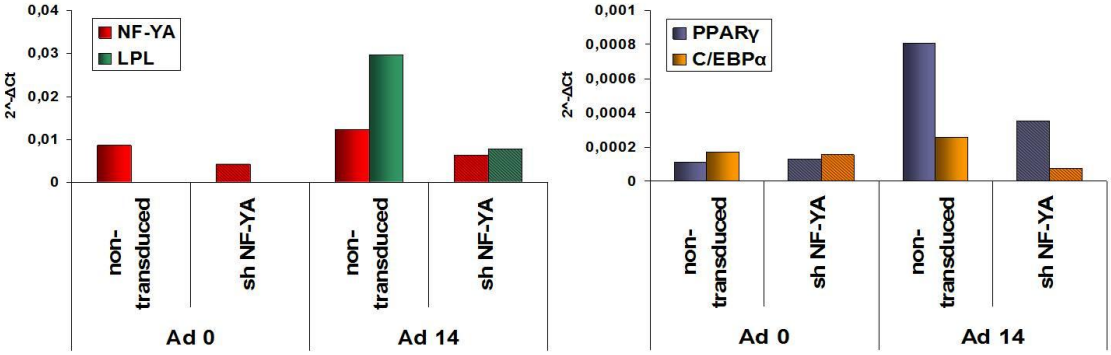

Figure 15 NF-YA, LPL, PPARγ and C/EBPα relative gene expression during *in vitro* adipogenic differentiation of control (non-transduced) and shNF-YA-transduced BM-MSCs

Regarding NF-YA protein levels in BM-MSCs, they were also reduced compared with those of control cells (Figure 16).

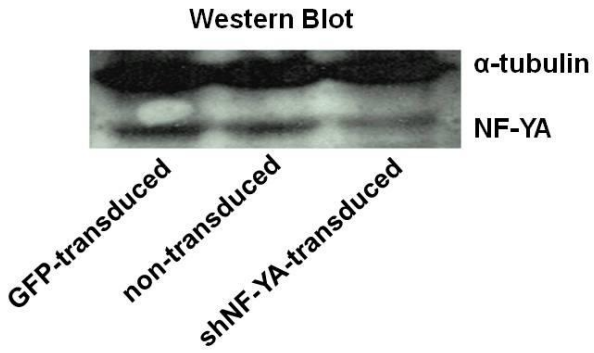

Figure 16 NF-YA protein levels after transduction of BM-MSCs with GFP lentiviral particles (GFP-transduced), nothing (non-transduced) and shRNA for NF-YA lentiviral particles (shNF-YA transduced). a-tubulin was used as a loading control.

Moreover Oil Red O staining indicated a lack of lipid droplets accumulation in shNF-YA- transduced BM-MSCs compared to control cells (Figure 17).

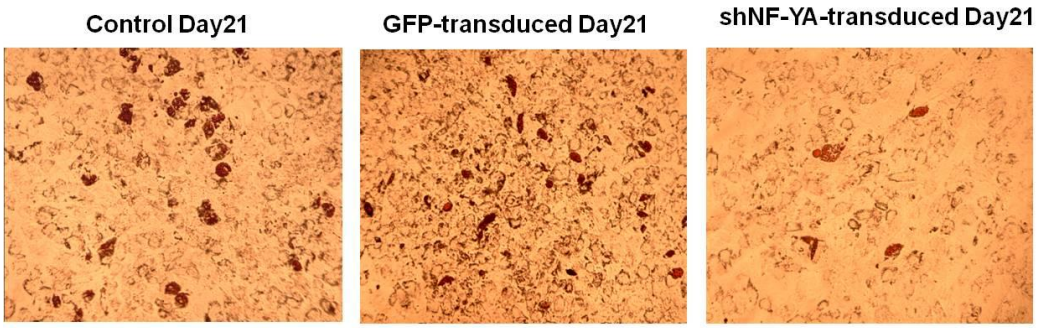

Figure 17 Oil Red O staining of lipid droplets in control (non-transduced) cells, in GFP-transduced cells and in shNF-YA-transduced cells at Day21 of adipogenesis induction

4. shRNA-mediated knockdown of NF-YA during *in vitro* osteogenesis of BM-MSCs

In order to evaluate if NF-YA is functionally related to *in vitro* osteogenesis of BM-MSCs, cells transduced with shNF-YA lentiviral particles, or GFP lentiviral particles, or non-transduced cells (control cells) were induced to the standard osteogenic differentiation protocol. The mRNA levels of NF-YA as well as of the osteogenesis markers DLX5, RUNX2, OSC, OSX, ALP, BSP were evaluated with real-time PCR. Moreover Western blot was performed for the evaluation of NF-YA protein levels. In addition, Alizarin Red S and Von Kossa stains were performed in day 21 of osteogenesis for the identification of calcium deposition and mineralization, respectively.

A mean reduction of 52.37% in the relative NF-YA expression was observed during the whole osteogenic differentiation procedure for shNF-YA-transduced cells. At day 21 of osteogenesis, NF-YA mRNA levels in shNF-YA-transduced BM-MSCs were reduced to 74.12% compared to control cells (Figure 18). Additionally NF-YA protein levels in shNF-YA transduced BM-MSCs, were reduced compared to control cells, as expected (Figure 16). Although a reduction of 52.03% and 83.95% was observed in OSC and BSP relative mRNA levels respectively, the transcription levels of DLX5, RUNX2, OSX and ALP remained unaffected after NF-YA knockdown (Figure 18).

Cytochemical stains, Alizarin Red S and Von Kossa, did not reveal any significant difference between shNF-YA-transduced BM-MSCs and control cells (Figure 19).

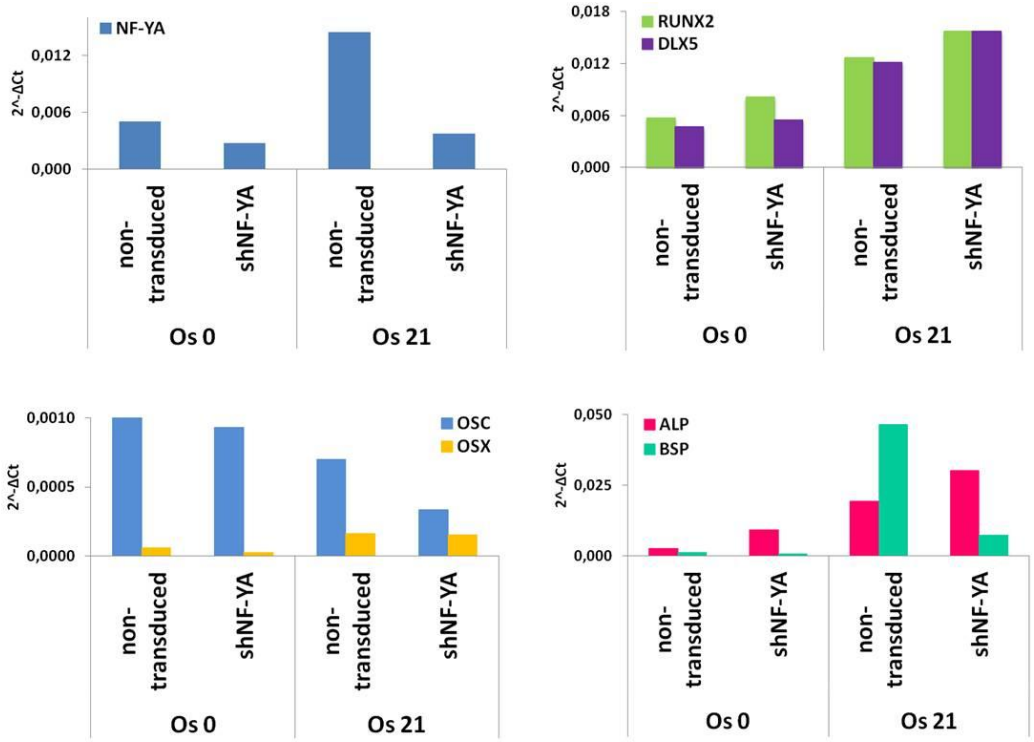

Figure 18 NF-YA, RUNX2, DLX5, OSC, OSX, ALP, BSP relative gene expression during *in vitro* osteogenic differentiation of control (non-transduced) and shNF-YA-transduced BM-MSCs

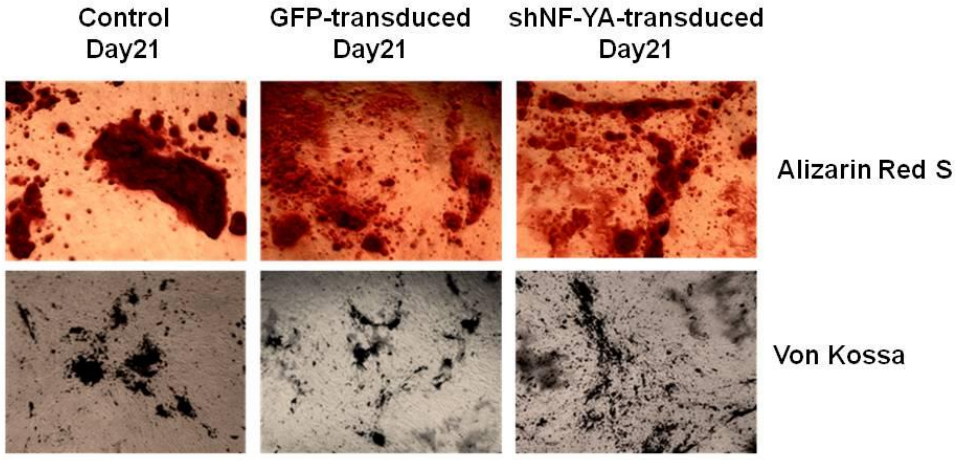

Figure 19 Alizarin Red S and Von Kossa stainings for the identification of calcium deposition and mineralization, respectively in control (non-transduced) cells, in GFP-transduced cells and in shNF-YA-transduced cells at Day21 of osteogenesis induction

DISCUSSION

This is the first study of NF-YA in bone marrow mesenchymal stem cells. We have shown for the first time that the relative expression levels of NF-YA, the regulatory subunit of the heterotrimeric transcription factor NF-Y, are decreased, at senescent (P10) BM-MSCs, compared to P3 BM-MSCs. Moreover we have shown for the first time, that both protein and mRNA expression levels of NF-YA, increase during *in vitro* differentiation of BM-MSCs into the adipogenic cell-lineage. Furthermore we have shown that after specifically knocking-down NF-YA, BM-MSCs possess aberrant *in vitro* adipogenic differentiation capacity. In contrast, during *in vitro* osteogenesis of BM-MSCs we did not observe a significant change regarding the expression levels of NF-YA. In accordance, after knocking-down NF-YA in BM-MSCs, their osteoblastic capacity was not considerably affected.

We have observed a significant reduction in the mRNA levels of NF-YA in P10, as compared to P3 BM-MSC cultures. P10 BM-MSCs have been extensively *in vitro* expanded, and it is known that MSCs prolonged *in vitro* proliferation results in cell replicative senescence [48]. Our results showing that NF-YA expression levels decrease in senescent (P10) BM-MSCs are in accordance with previously reported data on senescent cells. Cellular senescence is enforced by the p53/p21 and/or p16/Rb tumor suppressor pathways. Senescence is accompanied by the induction of expression and/or activity of p16 and p53 [45, 46] and p21, resulting in CDK (Cyclin-dependent kinase) inhibition and subsequent E2F inhibition [47], leading to senescence-related cell cycle arrest. NF-Y is known to be implicated in the regulation of cell cycle regulatory genes. More specifically, NF-Y regulates a group of cell cycle genes that are involved in G2/M phase of the cell cycle (such as CDK1, Cyclin B genes [7, 17]), or in G1/S phase (E2F1 [15, 22]). It has been reported that p53 suppress NF-Y function resulting in the prevention of transcription of NF-Y target genes, such as E2F1, a transcription factor implicated in the regulation of the cell cycle. As a result the cell cycle is blocked [28].

During *in vitro* adipogenic differentiation of BM-MSCs, NF-YA mRNA and protein levels were found significantly elevated in day 16 of differentiation, compared to the onset of differentiation induction (day 0). Moreover, the expression levels of adipogenesis marker genes (PPARG, C/EBPA, LPL) positively correlated with NF-YA expression levels, implying a possible implication of NF-YA to MSCs adipogenic differentiation.

In order to assess whether NF-YA is functionally related to BM-MSCs adipogenesis, we performed specific shRNA-mediated knockdown of NF-YA and proceeded with standard differentiation protocol. shNF-YA-transduced cells demonstrated a constant reduction of NF-YA mRNA levels, compared to control (non-transduced) cells, during the differentiation procedure. Two weeks after the onset of differentiation, shNF-YA-transduced BM-MSCs demonstrated a reduction of 46.78% in

NF-YA mRNA levels, and additionally a decrease of 31.22%, 70.06% and 74.3% in PPARG, C/EBPA and LPL mRNA levels, respectively. The decrease of adipogenic related gene expression implies a defect in the differentiation capacity of shNF-YA-transduced cells. Moreover Oil Red O staining indicated a lack of lipid droplets accumulation in shNF-YA-transduced BM-MSCs compared to control cells. All the above findings are indicative of a functional implication of NF-YA in MSCs adipogenesis. Our results show for the first time that NF-YA knock-down in BM-MSCs results in an aberrant adipocytic differentiation capacity. Our results are indicative of an essential role of NF-YA in adipogenesis of BM-MSCs.

Regarding the osteoblastic differentiation potential of BM-MSCs, our results demonstrated that during *in vitro* differentiation of BM-MSCs, the relative NF-YA expression levels remained relatively constant. Moreover shNF-YA-transduced BM-MSCs were subjected to *in vitro* osteoblastic differentiation, in order to evaluate whether NF-YA is essential for MSCs osteogenesis. The mean reduction of the relative NF-YA mRNA levels in shNF-YA-transduced BM-MSCs was 52.37% compared to control (non-transduced) cells, confirming NF-YA knockdown. At differentiation day 21, the transcription levels of DLX5, RUNX2, OSX and ALP remained unaffected after NF-YA knockdown, while a reduction of 52.03% and 83.95% was observed in OSC and BSP relative mRNA levels, respectively. The above results indicate that NF-YA is not strongly implicated in the induction of most osteogenic genes tested, although a contribution to the regulation of certain osteogenic genes (OSC, BSP) cannot be excluded. The cytochemical stains, Alizarin Red S and Von Kossa, did not reveal any significant difference between shNF-YA-transduced BM-MSCs and control cells, further suggesting that NF-YA is not essential for MSCs osteoblastic differentiation.

Several studies indicate a possible role of NF-YA in cellular differentiation. For example, it has been suggested that terminally differentiated myotubes lack NF-YA, but not NF-YB and NF-YC expression [13]. NF-YA has a role in monocyte to macrophage differentiation: monocytes found in circulation are characterized by NF-YA absence, while in differentiated macrophages NF-YA is elevated [27]. In contrast, another study has shown that NF-YA increases during differentiation of CaCo-2 cell line and also that it is responsible for the transcriptional induction of mature enterocyte markers [3]. Furthermore, a role of NF-Y has been reported in osteoblasts. NF-Y was shown to transcriptionally induce Fibroblast Growth Factor Receptor 2, a positive regulator of ossification [38]. Moreover NF-Y was found to be implicated in myeloid differentiation by positively regulating the expression of JunB as well as other myeloid differentiation primary response (MyD) genes having therefore an indispensable role in differentiation of myeloid progenitors [37].

In summary, we have shown that NF-YA has an indispensable role in adipogenesis of MSCs. During *in vitro* adipocytic differentiation of BM-MSCs, NF-YA mRNA and protein levels increased and also, the expression levels of adipogenesis marker genes are positively correlated with the mRNA levels of NF-YA. This central role of NF-YA in adipogenesis of MSCs is further supported by an aberrant adipocytic differentiation potential of MSCs, after NF-YA knockdown. Regarding, the *in vitro* osteoblastic differentiation of BM-MSCs, the transcription levels of NF-YA remained relatively constant. Moreover NF-YA silencing in BM-MSCs, revealed that their osteoblastic differentiation capacity was not affected, suggesting that NF-YA is not necessary for MSCs osteoblastic differentiation. Additionally, we observed a decrease in NF-YA mRNA levels after long *in vitro* expansion of BM-MSCs (senescent cells, P10).
This novel role of NF-YA in terms of adipogenic differentiation of BM-MSCs could further be investigated, by evaluating, the direct (or indirect) regulation of adipogenesis marker genes (PPARG, C/EBPA, LPL) by NF-YA.

REFERENCES

1. Baxevanis AD, G. Arents et al (1995). “A variety of DNA-binding and multimeric proteins contain the histone fold motif.” *Nucleic Acids Res* **23**:2685–2691.

2. Benatti P., D. Dolfini et al (2011). “Specific inhibition of NF-Y subunits triggers different cell proliferation defects.” *Nucleic Acids Res.* **39**(13):5356-68.

3. Bevilacqua MA, MC Faniello et al (2002). “Transcription factor NF-Y regulates differentiation of CaCo-2 cells.” *Arch Biochem Biophys* **407**:39–44.

4. Bhattacharya A., JM Deng et al (2003). “The B subunit of the CCAAT box binding transcription factor complex (CBF/NF-Y) is essential for early mouse development and cell proliferation.” *Cancer Res* **63**:8167–8172.

5. Bi W., L. Wu et al (1997). “DNA binding specificity of the CCAAT-binding factor CBF/NF-Y.” *J Biol Chem*. **272**(42):26562-72.

6. Blank U., G. Karlsson et al (2008). “Signaling pathways governing stem-cell fate.” *Blood* **111**(2):492-503.

7. Bolognese F., M. Wasner et al (1999). “The cyclin B2 promoter depends on NF-Y, a trimer whose CCAAT-binding activity is cell-cycle regulated.” *Oncogene* **18**:1845–1853.

8. Bungartz G., H. Land et al (2012). “NF-Y is necessary for hematopoietic stem cell proliferation and survival.” *Blood* **119**(6):1380-9.

9. Chae HD, J. Yun et al (2004). “Cdk2-dependent phosphorylation of the NF-Y transcription factor is essential for the expression of the cell cycle-regulatory genes and cell cycle G1/S and G2/M transitions.” *Oncogene* **23**:4084–4088.

10. Dazzi F., R. Ramasamy et al (2006). “The role of mesenchymal stem cells in haematopoiesis.” *Blood Rev* **20**(3):161–71.

11. Delorme B. and P. Charbord (2007). “Culture and characterization of human bone marrow mesenchymal stem cells.” *Methods Mol Med.* **140**:67-81.

12. Dolfini D., R. Gatta et al. (2012). “NF-Y and the transcriptional activation of CCAAT promoters.” *Crit Rev Biochem Mol Biol.* **47**(1):29-49.

13. Farina A., I.Manni et al (1999). “Down-regulation of cyclin B1 gene transcription in terminally differentiated skeletal muscle cells is associated with loss of functional CCAAT-binding NF-Y complex.” *Oncogene* **18**:2818–2827.

14. Forsburg SL and L. Guarente (1989). “Identification and characterization of HAP4: A third component of the CCAAT-bound HAP2/HAP3 heteromer.” *Genes Dev* **3**:1166–1178.

15. Gurtner A., P. Fuschi et al (2010). “Transcription factor NF-Y induces apoptosis in cells expressing wild-type p53 through E2F1 upregulation and p53 activation.” *Cancer Res* **70**:9711–9720.

16. Hahn S., J. Pinkham et al (1988). “The HAP3 regulatory locus of *Saccharomyces cerevisiae* encodes divergent overlapping transcripts.” *Mol Cell Biol* **8**:655–663.

17. Hu Q., JF. Lu et al (2006). “Inhibition of CBF/NF-Y mediated transcription activation arrests cells at G2/M phase and suppresses expression of genes activated at G2/M phase of the cell cycle.” *Nucleic Acids Res.* **34**(21):6272-85.

18. Imbriano C., A. Gurtner et al (2005). “Direct p53 transcriptional repression: *In vivo* analysis of CCAATcontaining G2/M promoters.” *Mol Cell Biol* **25**:3737–3751.

19. Kastrinaki MC, I. Andreakou et al (2008). “Isolation of human bone marrow mesenchymal stem cells using different membrane markers: comparison of colony/cloning efficiency, differentiation potential, and molecular profile.” *Tissue Eng Part C Methods* **13**:333-9.

20. Li XY., R. Mantovani et al (1992). “Evolutionary variation of the CCAAT-binding transcription factor NF-Y.” *Nucleic Acids Res.* **20**:1087–1091.

21. Li XY., R. Hooft van Huijsduijnen et al (1992). “Intron-exon organization of the NF-Y genes. Tissue-specific splicing modifies an activation domain.” *J Biol Chem.* **267**(13):8984-90.

22. Maity SN and B. de Crombrugghe (1998). “Role of the CCAAT-binding protein CBF/NF-Y in transcription.” *Trends Biochem Sci.* **23**(5):174-8.

23. Manni I., G. Caretti et al (2008). “Posttranslational regulation of NF-YA modulates NF-Y transcriptional activity.” *Mol Biol Cell* **19**:5203–5213.

24. Mantovani R., XY Li et al (1994). “Dominant negative analogs of NF-YA.” *J Biol Chem* **269**:20340–20346.

25. Mantovani R. (1998). “A survey of 178 NF-Y binding CCAAT boxes.” *Nucleic Acids Res*. **26**:1135–1143.

26. Mantovani R. (1999). “The molecular biology of the CCAAT-binding factor NF-Y.” *Gene* **239**(1):15-27.

27. Marziali G., E. Perrotti et al (1999). “The activity of the CCAAT-box binding factor NF-Y is modulated through the regulated expression of its A subunit during monocyte to macrophage differentiation: Regulation of tissue-specific genes through a ubiquitous transcription factor.” *Blood* **93**:519–526.

28. Matuoka K. and KY. Chen (2002). “Transcriptional regulation of cellular ageing by the CCAAT box-binding factor CBF/NF-Y.” *Ageing Res Rev*. **1**(4):639-51.

29. McNabb DS, Y. Xing et al (1995). “Cloning of yeast HAP5: A novel subunit of a heterotrimeric complex required for CCAAT binding.” *Genes Dev* **9**:47–58.

30. Nauta AJ and WE. Fibbe (2007). “Immunomodulatory properties of mesenchymal stromal cells.” *Blood* **110**: 3499-506.

31. Ogawa M. (1993). “Differentiation and proliferation of hematopoietic stem cells.” *Blood* **81**(11):2844-2853.

32. Pinkham JL, JT Olesen et al (1987). “Sequence and nuclear localization of the *Saccharomyces cerevisiae* HAP2 protein, a transcriptional activator.” *Mol Cell Biol* **7**:578–585.

33. Pittenger MF, AM. Mackay, et al. (1999). "Multilineage potential of adult human mesenchymal stem cells." *Science* **284**(5411): 143-7

34. Pontikoglou, C., F. Deschaseaux et al (2011). “Bone Marrow Mesenchymal Stem Cells: Biological Properties and Their Role in Hematopoiesis and Hematopoietic Stem Cell Transplantation.” *Stem Cell Rev* **7**(3):569-89.

35. Ren G., L. Zhang et al (2008). *“*Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide.” *Cell Stem Cell* **2**: 131-50.

36. Sinha S., SN. Maity et al (1995). “Recombinant rat CBF-C, the third subunit of CBF/NFY, allows formation of a protein-DNA complex with CBF-A and CBF-B and with yeast HAP2 and HAP3.” *Proc. Natl. Acad. Sci.* **92**:1624–1628.

37. Sjin RM, K. Krishnaraju et al (2002). “Transcriptional regulation of myeloid differentiation primary response (MyD) genes during myeloid differentiation is mediated by nuclear factor Y.” *Blood* **100**(1):80-8.

38. Sun F., Q. Xie et al (2009). “Nuclear Factor Y is required for basal activation and chromatin accessibility of Fibroblast Growth Factor Receptor 2 promoter in osteoblast-like cells.” *J Biol Chem* **284**(5):3136-3147.

39. Suzuki Y., T. Tsunoda et al (2001). “Identification and characterization of the potential promoter regions of 1031 kinds of human genes.” *Genome Res.***11**:677–684.

40. Wang LD, AJ. Wagers (2011). “Dynamic niches in the origination and differentiation of haematopoietic stem cells.” *Nat Rev Mol Cell Biol*. **12**(10):643-55.

41. Xing Y., S. Zhang et al (1994). “Subunit interaction in the CCAAT-binding heteromeric complex is mediated by a very short alpha-helix in HAP2.” *Proc Natl Acad Sci* **91**:3009–3013.

42. Zhang Y., D.Khan et al (2012). “Mechanisms underlying the osteo- and adipo-differentiation of human mesenchymal stem cells.” *Scientific World Journal* **2012**:793823.

43. Zhu J., DM Giannola et al (2003). “NF-Y cooperates with USF1/2 to induce the hematopoietic expression of HOXB4.” *Blood* **102**:2420-2427.

44. Zhu J., Y. Zhang et al (2005). “NF-YA activates multiple haematopoietic stem cell (HSC) regulatory genes and promotes HSC self-renewal.” *PNAS* **102**:11728-11733.

45. Serrano M., A.W. Lin et al (1997). “Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a.” *Cell* **88**(5):593-602.

46. Beausejour CM., A. Krtolica et al (2003). “Reversal of human cellular senescence: roles of the p53 and p16 pathways.” *EMBO J.* **22**(16):4212-22.

47. Sherr CJ. and F. McCormick (2002). “The RB and p53 pathways in cancer.” *Cancer Cell* **2**(2):103-12.

48. Wagner W., P. Horn et al (2008). ”Replicative senescence of mesenchymal stem cells: a continuous and organized process.” *PLoS One* **3**(5):e2213.