THESIS ON NATURAL AND EXACT SCIENCES B155

Differentiation and Heterogeneity of Mesenchymal Stem Cells

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TUT PRESS

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This dissertation was accepted for the defence of the degree of Doctor of Philosophy in Gene Technology on June 19, 2013.

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Defence of the thesis: August 23, 2013

Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

/Kersti Jääger/

ISSN 1406-4723
ISBN 978-9949-23-509-4 (publication)
ISBN 978-9949-23-510-0 (PDF)

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ORIGINAL PUBLICATIONS

The current thesis is based on three original publications and one manuscript:

I. **Jääger K** and Neuman T. (2011)
Human dermal fibroblasts exhibit delayed adipogenic differentiation compared with mesenchymal stem cells.
Stem Cells and Development, Aug; 20(8):1327-36.

II. **Jääger K,** Islam S, Zajac P, Linnarsson S, Neuman T. (2012)
RNA-seq analysis reveals different dynamics of differentiation of human dermis- and adipose-derived stromal stem cells.
PLoS One, 7(6):e38833.

III. Balikova A, **Jääger K**, Viil J, Maimets T, Kadaja-Saarepuu L. (2012)
Leukocyte marker CD43 promotes cell growth in co-operation with β-catenin in non-hematopoietic cancer cells.
International Journal of Oncology, Jul; 41(1):299-309.

MANUSCRIPT

Jääger K, Fatkina A, Velts A, Orav E, Neuman T.
Variable expression of lineage regulators in differentiated stromal cells indicates distinct mechanisms of differentiation towards common cell fate.

My personal contribution:

I, II	I designed and performed the experiments, analyzed the data and wrote the paper.
III	I participated in designing and performing the experiments, and in data analysis.
Manuscript	I designed the experiments, performed immunofluorescence analysis, analyzed the data and wrote the manuscript.

INTRODUCTION

Some millions of cells in the human body die every second of our lives. Replacement of cells in adult tissues relies on stem cells' ability to self-renew and differentiate into tissue-specific cells. The processes of cell proliferation and differentiation are fundamental both in developmental biology and in regenerative medicine, and due to increased understanding of cellular differentiation over the last seven years (following the discovery of reprogramming factors by Yamanaka and colleagues in 2006), scientific interest towards these fields of biology has continued to rise. It has become evident that terminally differentiated cells in the adult body can be reversed to more primitive cells with broader developmental capacity similar to cells of embryonic stage, by a process called reprogramming. Such cellular plasticity opens up enourmous potential possibilities in cell therapy and points to our limited knowledge about molecular mechanisms that control cell fate. We still do not know why/how old skin cells are replaced by new skin cells instead of bone cells, for example.

Mesenchymal stem cells (MSCs) or stromal stem cells constitute fibroblast-like cells derived from adult tissues that can be *in vitro* expanded and differentiated into minimally three mesodermal lineages including fat, bone and cartilage. MSCs were first identified as non-hemaetopoietic multipotent bone marrow cells already in the early 1970s, but later, cells with multi-lineage differentiation potential have been isolated from virtually all mature tissues, making MSCs important players in the field of regenerative medicine. However, MSCs cannot be distinguished from other cell types by means of surface antigen expression, because multipotent cells derived from different tissues or donors have been shown to exhibit different surface characteristics, or the same surface proteins are expressed by cells that lack differentiation potential.

This thesis summarizes the studies on *in vitro* differentiation of two stromal cell populations including adipose tissue-derived MSCs (AdMSCs) and skin-derived fibroblasts (FBs). We analyzed gene expression upon differentiation of AdMSCs and FBs into adipocytes, osteoblasts and chondrocytes over time, on global scale and in cell populations derived from different donors in order to get insight into the molecular mechanisms that control cell fate decisions of stromal cells.

ABBREVIATIONS

AB – Alcian Blue
ACAN – aggrecan
AdMSC – Adipose-derived mesenchymal stem cell
ALP – alkaline phosphatase
ANOVA – analysis of variance
ARS – Alizarin Red S
BmMSC – Bone marrow-derived mesenchymal stem cell
BMP – bone morphogenetic protein
C/EBP – CCAAT/enhancer-binding protein
CD – cluster of differentiation
CREB – cAMP response element-binding protein
Dlx – distal-less homeobox
FABP4 – fatty acid-binding protein 4
FB – fibroblast
FDR – false discovery rate
GAPDH – glyceraldehyde 3-phosphate dehydrogenase
HDAC – histone deacetylase
hESC – human embryonic stem cell
IBMX – isobutyl-methyl-xanthine
iPS – induced pluripotent stem cell
MSC – mesenchymal stem cell
Msx – muscle-specific homeobox
ORO – Oil Red O
Osx – osterix
P300/CBP – p300/CREB-binding protein
PCA – principal component analysis
PFA – paraformaldehyde
PPARγ – peroxisome proliferator-activated receptor gamma
qRT-PCR – quantitative real-time polymerase chain reaction
Runx2 – runt-related transcription factor 2, also known as Cbfa-1
SHARP1 – basic helix-loop-helix transcription factor, also known as DEC2
Sox9 – sex determining region Y-box 9
SREBP – sterol regulatory element-binding protein, also known as Add1
SVF – stromal-vascular fraction
ZFP423 – zinz finger transcription factor, ZNF423 in humans
TGFβ – transforming growth factor beta

REVIEW OF THE LITERATURE

1. Mesenchymal stem cells

1.1 Identification and definition of MSCs

Stemness - self-renewal and multilineage differentiation capacity of cells - was for a long time associated only with embryonic blastocyst-derived cells, which through multiple proliferation and differentiation cascades give rise to all of the different cell types found in adult organisms. In 1968, Friedenstein and his colleagues identified spindle-shaped, clonogenic cells of non-hematopoietic origin from adult bone marrow, which were defined as colony-forming unit fibroblasts (CFU-Fs)(Friedenstein et al., 1968), and later were shown to be common predecessors of mesenchymal tissues. As a result of their supposed capacity for self-renewal and differentiation, bone marrow-derived multipotent stromal precursor cells were first considered as stem cells by Caplan who named them mesenchymal stem cells (**MSCs**) (Caplan, 1991). The first detailed description of the trilineage differentiation potential of MSCs was published 14 years ago (Pittenger et al., 1999). Nowadays, MSCs, which can alternatively be defined as multipotent stromal cells or multipotent adult progenitor cells, are generally considered a heterogeneous population of post-natally derived cells that proliferate as plastic-adherent cells, have fibroblast-like morphology, form colonies *in vitro,* and can differentiate into bone, cartilage and fat cells (Horwitz et al., 2005).

Cells that meet this criteria have been isolated from virtually any organ or tissue including bone marrow, fat, skin, lung and liver (Pittenger et al., 1999; Zuk et al., 2001; Toma et al., 2001; Sabatini et al., 2005; da Silva Meirelles et al., 2006). MSC populations lack unique biomarkers and they are typically characterized by the capacity of differentiation into three mesodermal lineages including adipocytes, osteoblasts and chondrocytes *in vitro*. There is evidence, however, that MSC preparations are heterogeneous cell cultures comprising a subset of stem cells (or different subsets of stem cells) and more differentiated (progenitor) cells.

The possible existence of MSCs as **heterogeneous populations** *in vivo* adds complexity to their study *in vitro*. It has been suggested that the *in vivo* localization of MSCs correlates with that of pericytes, cells that lie on the abluminal side of blood vessels, immediately opposed to endothelial cells (ECs)(da Silva Meirelles et al., 2008). This idea according to which MSCs are derived from blood vessel walls is consistent with the outcome that they have been isolated from nearly any organ or tissue innervated by blood vessels. However, although several studies have provided evidence that, for example, adipose-derived MSCs (AdMSCs) *in situ* reside in perivascular niche (Lin et al., 2008, 2010), the exact localization of AdMSCs in native adipose tissue is still under debate.

1.2 Molecular phenotype and characterization of MSCs

Due to the high heterogeneity of primary MSC populations (with regard to proliferation and differentiation potential), surface antigen expression profile of these cells has been extensively studied and described (Boquest et al., 2005; Mitchell et al., 2006; reviewed in Kolf et al., 2007) in hope to improve and standardize the *in vitro* propagation of MSCs. In 2006, the International Society for Cellular Therapy proposed the minimal set of surface antigens whose expression would distinguish MSCs from other cell types: they must express CD105, CD73 and CD90, and lack the expression of hematopoietic markers c-kit, CD14, CD11b, CD34, CD45, CD79α, CD19 and HLA-DR (Dominici et al., 2006). Interestingly, isolation of cells from **stromal-vascular fraction (SVF)** of different tissues based on plastic-adherence always results in populations that contain cells with positive staining of Integrin-β1/CD29, H-CAM/CD44, 5'-nucleotidase/CD73, Thy-1/CD90 and Endoglin/CD105 (Table 1). Further, all fibroblast populations analyzed express these antigens similarly with MSCs. Some studies have addressed the question whether surface marker-based cell sorting would enable purification of homogenous cell populations with higher differentiation capacity towards any specific lineage. Isolation of AdMSCs with immuno-magnetic beads coated with antibodies against either CD29, CD44, CD49d, CD73, CD90, CD105 or Stro-1 resulted in cell populations that all differentiated into osteoblasts and chondrocytes within 3 weeks similarly with the unsorted SVF cells (Rada et al., 2011). Also, both CD105-negative and CD105-positive AdMSCs exhibited adipogenic, osteogenic and chondrogenic potential (Jiang et al., 2010). Hence, these surface molecules are not sufficient to discriminate MSCs from other cell types in heterogenous tissues.

Analysis of **gene expression profiles** of different MSC populations revealed that expression of FN1 (fibronectin 1), other extracellular matrix components, and transcription factors NFIB (nuclear factor I/B), ID1 (inhibitor of DNA binding 1), and homeobox genes HOXA5 and HOXB6 were enriched but not unique for these cells (Wagner et al., 2005). Transcriptome analysis of MSCs isolated from 12 different tissues in parallel with retinal pericytes and fibroblasts showed extensive overlap in gene expression patterns of these cells (Covas et al., 2008). For example, VIM (vimentin), LGALS1 (galectin 1), ANXA2 (annexin), MMP2 (matrix metallopeptidase 2), TAGLN (transgelin), TAGLN2, SPARC (osteonectin) among others were highly expressed in all cells analyzed. The authors concluded that MSCs, pericytes and fibroblasts are related cells present at the vascular wall where they function as a source of cells for repair and maintenance of the various tissues. Proteome analysis of bone marrow-derived MSCs showed that cytoskeletal proteins, and those involved in protein folding and metabolism were most prevalent functional groups present in these cells (Wagner et al., 2006). No single marker was found to be adequate to specify MSCs.

Table 1. Surface antigen expression on MSCs isolated from different tissues.

Tissue origin of human cells	Surface antigens analyzed	Positive staining on all analyzed cells	Ratio of positive cells	Publication
Adipose tissue	HLA- ABC, HLA- DR, CD9, CD11a, CD11b, CD11c, CD10, CD13, CD14, CD18, CD29, CD31, CD34, CD44, CD45, CD49d, CD49e, CD50, CD54, CD55, CD56, CD59, CD62e, CD105, CD166	HLA-ABC, CD9, CD10, CD13, CD29, CD34, CD44, CD49e, CD54, CD55, CD59, CD105, CD166	CD29 (90 %) CD44 (60 %) CD105 (36 %)	(Gronthos et al., 2001)
Adipose tissue Bone marrow (Clonetics)	STRO-1, CD13, CD14, CD16, CD29, CD31, CD34, CD44, CD45, CD56, CD61, CD62e, CD71, CD90, CD104, CD105, CD106	STRO-1, CD13, CD29, CD44, CD71, CD90, CD105	CD44 (16.92 %) CD90 (25.9 %) CD105 (8.39 %)	(Zuk et al., 2002)
Adipose tissue Umbilical cord blood Bone marrow	HLA-ABC, HLA-DR, SSEA4, CD10, CD13, CD14, CD24, CD29, CD31, CD34, CD36, CD38, CD44, CD45, CD49d, CD73, CD90, CD105, CD106, CD117, CD133, CD166	HLA-ABC, CD13, CD29, CD44, CD73, CD90, CD105, CD166	CD29 (high) CD44 (high) CD73 (high)	(Wagner et al., 2005)
Adipose tissue	CD13, CD29, CD31, CD34, CD44, CD49a, CD63, CD73, CD90, CD105, CD144, CD146, CD166	CD13, CD29, CD44, CD49a, CD63, CD73, CD90, CD105, CD166	CD29 (87.4 %) CD44 (96.9 %) CD73 (93.9 %) CD90 (96.2 %) CD105 (68.9 %)	(Mitchell et al., 2006)
Bone marrow Skin	HLA-ABC, HLA-DR, CD13, CD14, CD26, CD29, CD34, CD44, CD45, CD49b, CD49e, CD71, CD73, CD80, CD86, CD90, CD105, CD117	HLA-ABC, CD13, CD29, CD44, CD73, CD90	CD29 (high) CD44 (high) CD73 (high) CD90 (high) CD105 (moderate)	(Lysy et al., 2007)
Adipose tissue Umbilical cord blood Bone marrow Retinal pericytes Skin Foreskin CCD27Sk fibroblasts (ATCC)	HLA-I, HLA-II, cadherin 5, glycophorin A, STRO-1, CD13, CD14, CD29, CD31, CD34, CD44, CD45, CD49e, CD73, CD90, CD146, CD166	HLA-I, STRO-1, CD13, CD29, CD44, CD49e, CD73, CD90	CD29 (78-99 %) CD44 (59-91 %) CD73 (66-97 %) CD90 (91-99 %)	(Covas et al., 2008)

Table 1. Continues

Tissue origin of human cells	Surface antigens analyzed	Positive staining on all analyzed cells	Ratio of positive cells	Publication
Bone marrow (Lonza) HNDF fibroblasts (Lonza)	caveolin 1, CD10, CD59, CD73, CD90, CD105, CD109	caveolin 1, CD10, CD59, CD73, CD90, CD105, CD109	CD73 (high) CD90 (high) CD105 (high)	(Bae et al., 2009)
Adipose tissue Bone marrow Vocal fold fibroblasts (hVFF)	CD14, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105	CD29, CD44, CD73, CD90, CD105	CD29 (97-100 %) CD44 (92.6-100 %) CD73 (97.4-100 %) CD90 (97.6-100 %) CD105 (90.4-100 %)	(Hanson et al., 2010)
Adipose tissue Skin Embryonic lung fibroblasts (WI38)	CD14, CD31, CD44, CD45, CD73, CD105	CD44, CD73, CD105	CD44 (91-98 %) CD73 (98-99.5 %) CD105 (93-99.6 %)	(Alt et al., 2011)
Adipose tissue HNDF fibroblasts (Lonza)	CD29, CD34, CD44, CD54, CD56, CD90, CD105, CD106, CD117, CD133, CD146, CD166	CD29, CD44, CD90, CD105	CD29 (91-96 %) CD44 (92-97 %) CD90 (94-97.6 %) CD105 (95-98.4 %)	(Blasi et al., 2011)
Adipose tissue Bone marrow Skin New-born skin	HLA-DR, CD13, CD14, CD29, CD34, CD44, CD45, CD73, CD90, CD105	CD13, CD29, CD44, CD73, CD90, CD105	CD29 (high) CD44 (high) CD73 (high) CD90 (high) CD105 (high)	(Al-Nbaheen et al., 2013)

1.3 Differentiation potential of MSCs

MSCs derived from different tissues including bone marrow (BmMSC) and adipose tissue (AdMSC) all differentiate into a variety of cell types: hepatocytes, myocytes, adipocytes, osteoblasts, chondrocytes and epithelial cells (Zuk et al., 2001, 2002; Gimble and Guilak 2003; Lee and Kemp 2006; Saga et al., 2005; Kitagawa et al., 2006; Lysy et al., 2007) under appropriate conditions *in vitro*. The differentiation of MSCs is usually assessed using histochemical staining of tissue-specific extracellular matrix produced by differentiated monolayer cultures *in vitro*, and/or analysis of cell type-specific gene expression either at mRNA or protein level upon long-term treatment of cells with inductive components in culture media (reviewed in Vater et al., 2011). In general, **culture supplements** required for osteogenic differentiation include dexamethasone, β-glycerol-phosphate, ascorbic acid 2-phosphate, vitamin D3, and combinations of TGF-β and BMPs. Chondrogenic medium contains dexamethasone, ascorbic acid 2-phosphate and TGF-β. Adipogenic differentiation is efficiently induced with supplements including dexamethasone, isobutylmethylxanthine (IBMX), insulin and indomethacin.

There is good evidence of the ability of MSCs to differentiate into mature adipocytes when exposed to medium containing steroids, a cAMP inducer and fatty acids (Zuk et al., 2001; Gimble and Guilak 2003). Confirmation of differentiation into adipocytes is usually performed by staining cytoplasmic lipid droplets with Oil Red O, and detection of expression of adipocyte-specific genes including PPARγ2, LPL, aP2, adiponectin (Table 2). Analysis of osteogenic differentiation of MSCs includes positive staining of calcified nodules with Alizarin Red S or von Kossa techniques. Also, increased expression and activity of alkaline phosphatase is observed in MSC-derived osteoblasts. Osteoblast-specific gene expression profile commonly includes Runx2, osteocalcin (OCN), osteopontin (OPN) and bone sialoprotein (BSP) genes. The method mostly used to demonstrate chondrogenic differentiation of MSCs is staining for increased expression of proteoglycans using Alcian blue, Toluidine blue or Safranin O. Chondrogenic differentiation is typically confirmed with expression of Sox9, collagen type 2 and aggrecan. Differentiation of MSCs is also accompanied by morphological changes from fibroblast-like shape into cuboidal, round and spherical shapes in response to osteogenic, chondrogenic and adipogenic stimulation, respectively.

These described methods are good for making general conclusions that analyzed MSC populations contain cells with differentiation potential towards defined lineages. However, staining of heterogenous cultures and detection of

Table 2. Multilineage differentiation potential of MSCs derived from different tissues.

Tissue origin of human cells	Differentiation potential	Duration of differentiation	Detection method	Marker expression	MSCs and FBs are	Publication
Bone marrow HS27 fibroblasts (ATCC) 1087Sk fibroblasts (ATCC)	Ad (+)/Os (+)/Ch (+) Ad (-)/Os (-)/Ch (-) Ad (-)/Os (-)/Ch (-)	1-3 w	ORO/AP act/ Col II	PPARγ2, LPL, aP2/ ALP/ACAN, Col II	Different	(Pittenger et al., 1999)
Bone marrow (hBM212) Fetal lung (hICIG7) Bronchial fibroblasts	Ad (+)/Os (+)/Ch (+) Ad (+)/Os (+)/Ch (+) Ad (+)/Os (+)/Ch (+)	3 w/4 w/1 w	ORO/ARS/ Col II	PPARγ/ OPN/Sox9, Col II	Similar	(Sabatini et al., 2005)
Adipose tissue Umbilical cord blood Bone marrow HS68 fibroblasts (ATCC) NHDF fibroblasts (Promocell)	Ad (+)/Os (+) Ad (+)/Os (+) Ad (+)/Os (+) Ad (-)/Os (-) Ad (-)/Os (-)	2 w/3 w	ORO/von K	NA	Different	(Wagner et al., 2005)
Skin Bone marrow	Ad (+)/Os (+)/Ch (+) NS	NS	ORO/AP act/ AB	NA	Similar	(Haniffa et al., 2007)
Bone marrow Skin	Ad (+)/Os (+) Ad (+)/Os (+)	4 w	ORO/von K, ARS	PPARγ1, PPARγ2, LPL, Adipsin/ OCN, BSP, Col I	Similar	(Lysy et al., 2007)
Adipose tissue Skin	Ad (+)/Os (+) Ad (+)/Os (+)	2 w/4 w	ORO/von K	PPARγ, LPL/ON, OCN	Similar	(Lorenz et al., 2008)
Bone marrow (Lonza) HNDF fibroblasts (Lonza)	Ad (+)/Os (+)/Ch (+) Ad (-)/Os (-)/Ch (-)	3 w/4 w 2-3 w	ORO/von K/ AB	PPARγ, aP2/ALP, BSP/ Sox9, Col X	Different	(Bae et al., 2009)
Adipose tissue Bone marrow Vocal fold fibroblasts (hVFF)	Ad (+)/Os (+)/Ch (+) Ad (+)/Os (+)/Ch (+) Ad (+)/Os (+)/Ch (+)	3 w/3 w/4 w	ORO/ARS/ Safranin-O	NA	Similar	(Hanson et al., 2010)
Adipose tissue Skin Embryonic lung fibroblasts (WI38)	Ad (+)/Os (+)/Ch (+) Ad (+)/Os (+)/Ch (+) Ad (-)/Os (-)/Ch (-)	3 w	ORO/ARS/ Col II	NA	Similar/Different	(Alt et al., 2011)
Adipose tissue HNDF fibroblasts (Lonza)	Ad (+)/Os (+) Ad (+)/Os (+)	3 w/2 w	ORO/AP act	Adiponectin	Similar	(Blasi et al., 2011)
Adipose tissue Bone marrow Skin New-born skin	Ad (+)/Os (+) Ad (+)/Os (+) Ad (+)/Os (+) Ad (+)/Os (+)	3 w	ORO/AP act	PPARγ, aP2, Adiponectin/ OPN, OCN, ALP	Similar	(Al-Nbaheen et al., 2013)

Abbreviations: AB – Alcian blue, ACAN – aggrecan, Ad – adipocytes, ALP – alkaline phosphatase, AP act – alkaline phosphatase activity, aP2 – FABP4, fatty acid-binding protein, ARS – Alizarin Red S, ATCC –American Type Culture Collection, BSP – bone sialoprotein, Ch – chondrocytes, LPL – lipoprotein lipase, NA – not analyzed, NS – not shown, OCN – osteocalcin, ON – osteonectin, OPN – osteopontin, ORO – Oil Red O, Os – osteoblasts, w – week(s), (+)- differentiation, (-)-no differentiation

confined set of marker genes represents limitations in analyzing the differentiation and molecular events that occur in individual cells. One important biological issue arising from the heterogeneity of MSC pools is whether **individual MSCs** can give rise to multiple differentiated phenotypes or whether each phenotype derives from a subset of committed progenitor cells that exist within a heterogeneous population. Clonal analysis of differentiation potential of MSCs revealed that aside from cells with monolineage (10-37%) and bilineage potential (10-47%), AdMSCs also contained cells with trilineage potential (7-49%) (Guilak et al., 2006). Similar results were obtained in a study with dermal fibroblasts: monopotent (2.1-8.5%), bipotent (6.4-12.8%) and tripotent (6.4%) cells were present in cell populations derived from skin (Chen et al., 2007). It turns out then, that both AdMSCs and fibroblasts can be regarded as truly multipotent cell types.

Such seemingly unlimited differentiation potential of MSCs represents them as exciting candidates for potential use in cellular therapies and tissue engineering strategies, which is why the focus of stem cell studies has been placed from technically and ethically-challenged embryonic stem cells on adult stem cell-based research.

1.4 *In vivo* differentiation and clinical application of MSCs

Tissue stromal cells have been reported to be responsible for regeneration of numerous organs. Besides the obvious applications of MSCs to repair or regenerate cartilage, bone, muscle or adipose tissue, the possibility of peripheral nerve regeneration, hepatic regeneration, insulin-producing islet cell regeneration, functional repair of myocardial infarction, and recovery of renal function has been shown in *in vivo* models (Petersen et al. 1999; Kopen et al. 1999; Wakitani et al. 2002; Cowan et al. 2004; Laflamme and Murry 2005; Baer and Geiger 2012).

Transplantation studies of MSCs have been carried out to analyze the ability of injected cells to repopulate adult tissues. The circulating cells have a different genotype from the solid tissue, allowing tracking their fate upon transplantation. The most common example involves transgender transplantation, for example, where Y chromosome identifies a cell as extracardiac origin in female hearts, whereas immunostaining with specific cell markers identifies the cell type. The preponderance of evidence suggests that circulating progenitors make only a very limited contribution to cardiomyocyte repopulation. Several lines of evidence indicate that direct injection of noninduced MSCs into the heart improves ventricular function postinfarction in rats and pigs. In one study, human MSCs were injected into mouse, where they located into heart, expressed cardiac genes and resembled morphologically cardiomyocytes (Toma et al., 2002). However, in another study, transplanted MSCs expressed muscle markers but retained fibroblast morphology and lacked electromechanical function (Shake et al., 2002).

The current data indicate that although bone marrow-derived MSCs were first

proposed for therapeutic purposes in regenerative medicine on the basis of their stem cell-like qualities, their therapeutic effect can result from other characteristics such as their anti-proliferative and anti-inflammatory properties. MSC secrete a variety of cytokines and growth factors that have both paracrine and autocrine activities. Indirect or **trophic effects of MSC** might explain some of the positive therapeutic effects observed with MSC without any evidence for differentiation of MSC. Such trophic effects have been proposed in treatment of stroke, myocardial infarct and meniscus repair (Caplan and Dennis 2006). Despite evidence that MSCs can transdifferentiate into multiple cell types *in vitro* and *in vivo*, the real contribution of MSCs to tissue repair through significant engraftment and differentiation into biologically and functionally relevant tissue-specific cell types, is still unclear (Uccelli et al., 2000). For example, MSC based myocardial therapy has proceeded at a rapid pace and there is sound evidence for successful cardiac regeneration or repair upon MSC treatment. However, this effect might be attributed to: 1. differentiation of the administered cells into all of the cellular constituents of the heart; 2. release of factors capable of paracrine signaling; 3. fusion of the administered cells with the existing constituents of the heart; or 4. stimulation of endogenous repair by injected cells (Wagner and Ho 2007). Current scientific evidence supports the use of MSC for tissue reconstruction through exclusive differentiation mechanisms only for bone repair (Quarto et al., 2001). Hence, the full clinical potential of MSCs awaits much deeper investigation of their fundamental biology.

1.5 Heterogeneity of MSC populations

Taken the fact that MSCs lack unique phenotype, isolation of cells with multilineage potential from SVF of different tissues based on surface markers is not possible. The only criterion for isolation is the cells' ability to adhere to tissue culture plastic and to survive and proliferate in standard culture conditions over several passages. MSCs are thus cultured and studied as heterogeneous pools of cells. Not suprisingly, the lack of standardization between research groups in defining what they mean by MSCs limits the interpretation of results and clinical progression of MSC research.

MSCs exhibit variability in their phenotypes, including proliferation capacity and expression of cell surface antigens (see Table 1), and in ability to secrete cytokines or differentiate into mesodermal lineages. Both, **inter-population** (differences between MSC populations derived from different donors) **and intra-population heterogeneities** (differences within the MSC population from an individual isolate) have been shown to account for the variation of MSC cultures (Phinney et al., 2000; Russell et al., 2010). Further, experiments have suggested that even when derived from a single cell, the progeny of MSCs can possess different properties (Ylöstalo et al., 2008). This heterogeneity could be the result of, first, alterations induced by extensive culturing, or secondly, the *in vivo* heterogeneity that represents the natural repertoire of MSCs. Although, it is

quite clear that *in vitro* culture conditions modify MSC populations, the observation that long-term cultured MSC cell strains retain their function over extensive passaging suggests that some or even most of the heterogeneity of MSCs originates *in vivo* (Pevsner-Fischer et al., 2011).

It has been argued that the *in vitro* observed tri-lineage differentiation of MSCs is not valid, and *in vivo* differentiation of MSCs upon heterotropic transplantation should be used for their characterization (Bianco et al., 2010). However, analysis of bone formation *in vivo* demonstrated that all multi-colony strains formed bone, whereas only 58.8% of single colony-derived MSCs generated bone (Kuznetsov et al., 1997), indicating that the heterogeneity is also observed *in vivo*. The point of view that stem cell is a fixed entity that follows irreversible differentiation scenario is becoming replaced by a new concept: **stemness is a transient and reversible cell state** rather than a fixed property that develops following interaction with the environment. Transitions between various differentiated states termed 'cellular plasticity', are believed to be a fundamental property of MSCs (Zipori 2006).

1.6 Heterogeneity of established cell lines

Heterogeneity is a common feature of all populations of cells since gene expression occurs and is regulated through complex mechanisms in individual cells. Stable cell lines are usually considered to be less heterogeneous compared with primary cell cultures. However, there is growing evidence that cell-to-cell variation is a common characteristic of established cell lines that could arise from **locally distinct microenvironment** generated by culture conditions, and from extensive passaging of the cells. Moreover, transformed cell lines commonly originate from tumor tissues that contain cells with heterogenous patterns of genomic aberrations that lead to distinct cellular behaviors in culture conditions (Ku et al., 2010).

Deregulated activation of β-catenin is a principal cause of colorectal cancer (Giles et al., 2003). β-catenin belongs to the cell-cell adhesion apparatus, whereas it translocates to the nucleus upon Wnt signaling, where it binds T-cell factor (TCF) and lymphocyte-enhancing factor (LEF) family transcription factors and regulates expression of genes important for proliferation and differentiation (Tetsu and McCormick 1999). Cell density has been shown to be one of the factors that affects subcellular localization and function of β-catenin (Dietrich et al., 2002). Hence, slight variations in local environment can lead to remarkable changes in gene expression and biological functions of cells in populations grown otherwise under homogenous conditions.

1.7 Identity of stromal cells: do MSCs equal fibroblasts?

The identification of self-renewing and multipotent cells has helped to explain how different types of mature cells stem from a single immature progenitor cell. However, it has also generated the understanding that stem cells are a type of cells with defined entity, and whose journey form immature state to specialized cell is unidirectional. This dogma has been challenged and now it is known that mature cell types can turn back to more primitive stage in development and give rise to a variety of different cell types (Takahashi and Yamanaka 2006).

The transition of cells between different states may relate to the phenomenon that multipotent cells can be isolated from most adult tissues. However, the identity of cells with multilineage differentiation potential isolated from SVF of different tissues has remained unclear. Tissue stromal cells or fibroblasts (FBs) exist in virtually every organ in the human body. They are defined as adherent cells, which are not endothelium, epithelium or hematopoietic origin, and which have the capacity to synthesize and remodel the extracellular matrix. Further, phenotypic and **functional properties of FBs completely overlap with those of MSCs** (see Table 1 and Table 2), including immunoregulation *in vitro* (Haniffa et al., 2007). Due to the lack of markers that would functionally discriminate different stromal cell types, it is important to recognize the common ground between the fields of fibroblast and MSC biology (Haniffa et al., 2009). Nomenclature-based differences between these cells could hamper the studies on stromal cell biology and mask true mechanisms of regulation of stromal cell behavior in response to diverse environmental stimuli.

2. *In vitro* differentiation of MSCs

2.1 Adipogenic differentiation

Several cell types contain esterified lipids but **adipocytes** are unique in the quantity of lipid that they can store, and the repertoire of secreted proteins. Among others, they produce hormones adiponectin and leptin that are important in the regulation of whole-body energy homeostasis (Lau et al., 2005). White adipose tissue, which is the predominant type of fat in adult humans, stores energy, whereas brown adipose tissue generates heat in newborns and rodents (Farmer 2008). Most studies on the mechanisms of adipogenic differentiation have been done using cell lines of mouse origin (3T3-L1 and 3T3-F442A), that are thought to recapitulate the main steps of the *in vivo* differentiation of pre-adipocytes into mature adipocytes (Green and Kehinde 1975). However, less is known about earlier steps of differentiation of pluri- or multipotent progenitor cells towards adipocyte lineage, because mesencyhmal precursor cells are heterogeneous populations and their differentiation potential changes over time in culture. The most commonly used multipotent stem cell line is C3H10T1/2 (Reznikoff et al., 1973) that can be converted by 5´-azacytidine into three

mesodermal stem cell lineages including adipocytes, chondrocytes and myoblasts (Pinney and Emerson 1989).

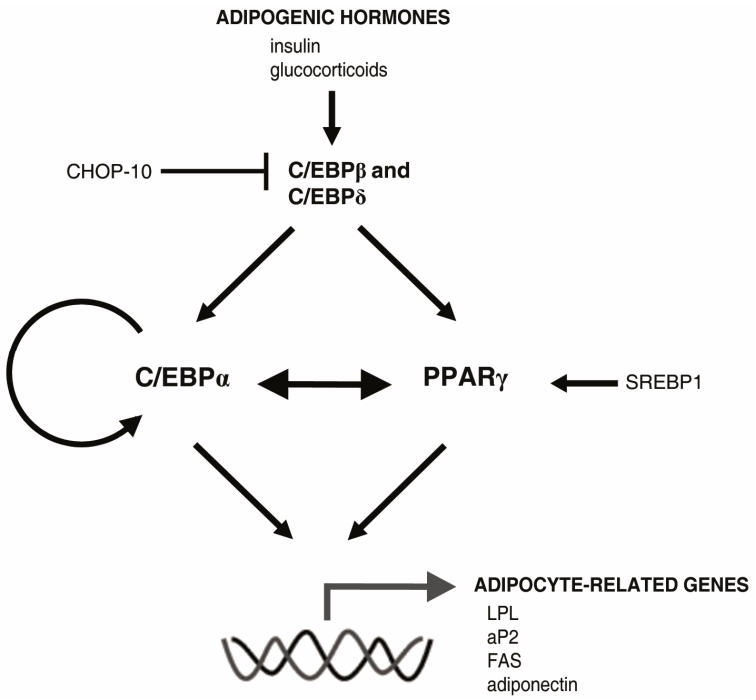

Figure 1. Transcriptional regulation of adipocyte differentiation. Adipogenic hormones induce the expression of C/EBPβ and C/EBPδ which activate C/EBPα and PPARγ. C/EBPα and PPARγ maintain the expression of each other in a positive-feedback loop, and promote the expression of adipocyte genes including lipoprotein lipase (LPL) and fatty acid synthase (FAS). Negative regulator CHOP-10 regulates early steps of the process (Frith and Genever 2008).

Transcriptional regulation of adipocyte differentiation has been reviewed by many authors (Morrison and Farmer 1992; Rosen 2005; Farmer 2006; Rosen and MacDougald 2006; Lefterova and Lazar 2009), and a cascade of expression and activity of transcription factors controlling adipogenesis is well described. Most of transcriptional regulators of adipogenesis (and other differentiation programs) operate in a feed-forward fashion, whereby they induce both other pro-adipogenic factors and then cooperate with those factors to promote downstream gene expression. Peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein (C/EBP) families of transcription factors are considered the crucial determinants of adipocyte fate. The expression of C/EBPβ and C/EBPδ is rapidly and transiently increased upon induction of differentiation, followed by the elevation of C/EBPα and PPARγ, which in turn maintain the

expression of each other, and together bind the promoters/enhancers of adipocyte genes including adipocyte fatty acid binding protein aP2 (FABP4), adoponectin and fatty acid synthetase (FAS) (Figure 1). PPARγ is the only known factor that is both necessary and sufficient to induce adipogenesis (Rosen et al., 1999), and to date no factor has been identified that can rescue adipogenesis in the absence of PPARγ.

PPARγ belongs to the nuclear receptor superfamily of ligand-activated transcription factors. It binds DNA as a heterodimer with retinoid X receptor (RXR). In the unliganded state, PPARγ associates with nuclear hormone receptor-corepressor (NCoR) or with silencing mediator of retinoid and thyroid hormone receptor (SMRT) to repress target gene expression (Farmer 2008). Commitment of MSCs to the **adipogenic lineage** involves acquisition of processes regulating production of PPARγ ligands. Interestingly, the endogenous ligand responsible for regulating PPARγ activity during adipogenesis has not been identified. *In vitro*, a mixture of dexamethasone (Dex), isobutylmethylxanthine (IBMX), indomethacin and insulin is used to promote adipogenesis. IBMX induces cAMP signaling that leads to activation of cAMP regulatory element-binding protein (CREB) and C/EBPβ expression (Zhang et al., 2004b). Induction of C/EBPδ is mediated by glucocorticoids (Dex) and C/EBPβ (Cao et al., 1991). Indomethacin contributes to the induction of C/EBPβ expression by blocking degradation of cAMP. Insulin mediates its early adipogenic effect via induction of sterol regulatory element-binding protein 1c (SREBP1c/ADD-1) that plays a role in the production of lipophilic molecules with potent PPARγ ligand activity (Kim et al., 1998). All these signals converge in generating the positive feedback loop between PPARγ and C/EBPα that together initiate the aquisition of adipocyte phenotype.

Transcription from alternative promoters of *pparγ* gene gives rise to four distinct mRNAs and two major protein isoforms **PPARγ1 and PPARγ2** (*PPARγ1,3,4* encoding PPARγ1 and *PPARγ2* encoding PPARγ2)(Zhu et al., 1995). The relative roles of these isoforms in adipogenesis are not known. PPARγ2 is expressed only in adipocytes, whereas PPARγ1 is expressed in many tissues. *In vivo* experiments with *pparγ2* knockout mice suggest that PPARγ1 can compensate for many adipogenic functions of PPARγ2, and that PPARγ2 is not absolutely required for adipocyte development *in vivo* (Zhang et al., 2004a). C/EBPα plays an important role in adipogenesis primarly by inducing the expression of PPARγ, but also by activating several adipocyte genes directly. C/EBPβ cannot induce the expression of C/EBPα in the absence of PPARγ, which is required to release histone deacetylase-1 (HDAC1) from the *c/ebpα* promoter (Zuo et al., 2006). Recently, however, it has been shown that adipogenic differentiation of NIH/3T3 cells can occur without reciprocal activation between C/EBPα and PPARγ (Shao et al., 2013).

Genome-wide analysis has revealed thousands of C/EBPα-binding sites in adipocytes and a remarkable degree of co-localization with PPARγ on DNA (Lefterova et al., 2008). C/EBPs belong to a large family of leucine zipper

transcription factors that can form homodimers and heterodimers with each other and bind to the same C/EBP consensus sequence. There is a time lag between the expression and transcriptional activity of C/EBPβ, when differentiation is induced. This is a key regulatory stage of adipogenesis and is controlled by many factors including RORα, SHARP1/DEC2 and TRB3 that block the activity of C/EBPβ. Phosphorylation of C/EBPβ by GSK3β (Park et al., 2004) releases it from interactions with inhibitory C/EBP homologous protein (CHOP-10) (Tang and Lane 2000) and enables recruitment of the chromatin remodeling complex SWI/SNF by C/EBPβ to promote transcription of *pparγ* (Salma et al., 2004). C/EBPβ activity on *c/ebpα* promoter is initially blocked by mSin3A/HDAC1 complex, but upon accumulation of PPARγ protein, it targets HDAC1 for degradation leading to the activation of C/EBPα expression (Zuo et al., 2006). Glucocorticoids induce acetylation of C/EBPβ that also results in its dissociation from HDAC1 and expression of C/EBPα (Wiper-Bergeron et al., 2003), independently from transcriptional up-regulation of *PPARγ*. Later stages of adipogenic differentiation are thus accompanied by accumulation of C/EBPα that displaces C/EBPβ from the promoters of adipogenic genes including leptin, adiponectin and *pparγ* (Salma et al., 2006).

2.2 Osteogenic differentiation

Bone is an essential mineralized tissue with critical mechanical and metabolic functions. **Osteoblasts** play a central role in the production of a characteristic extracellular matrix (ECM) and mineralization of the bone matrix. The process of differentiation of osteoblasts from mesenchymal precursor cells is tightly regulated and involves the sequential expression and activities of multiple classes of factors.

Runt-domain transcription factor **Runx2/Cbfa1** is the principal transcriptional regulator of osteoblast differentiation. Many factors function together with Runx2 to direct osteochondroprogenitor cells toward osteoblast or chondrocyte (cartilage cells) lineages. For osteoblasts, this is accomplished by the expression of **Osterix** (Osx) in preosteoblasts (Nakashima et al., 2002), followed by expression of another factor, **ATF4** that controls the transcriptional activity of mature osteoblasts (Yang and Karsenty 2004). It is believed that Osx may play a crucial role in segregating the osteoblast and chondrocyte lineages from bipotential osteochondroprogenitor cells during bone formation. Osx fulfills this function by inhibiting Sox9 expression (see below) and by fully establishing the osteoblast phenotype (Nakashima and De Crombrugghe 2003).

Alkaline phosphatase (ALP), bone sialoprotein (BSP) and collagen type I (Col1a1) are early **markers** of osteoblast differentiation, while parathyroid hormone/PTH-related peptide receptor (PPR) and osteocalcin (OCN) appear late, concomitantly with mineralization. Osteopontin (OPN) peaks twice, during proliferation and then again in later stages of differentiation (Soltanoff et al., 2009). Despite its crucial function in early differentiation, Runx2 is not essential

for the maintenance of the expression of major bone matrix proteins in mature osteoblasts (Maruyama et al., 2007). In fact, Runx2 inhibits terminal differentiation and maintains osteoblastic cells in immature stage (Liu et al., 2001). Runx2 regulatory element (also recognized by Runx1 and Runx3) is found in the promoters of several osteoblast genes including Col1a1, OPN, OCN and BSP. Although necessary for gene transcription and osteoblast development, Runx2 is not sufficient for optimal gene expression or bone formation. Not surprisingly, many of its target genes are absent in cells that express Runx2 (eg MSCs), suggesting that transcriptional activity of Runx2 is under complex regulation, and is influenced by the temporal and spatial expression of other factors.

Osteogenic differentiation is studied using MC3T3-E1 preosteoblast cells. Upon treatment with ascorbic acid (that allows cells to secrete collagenous ECM), BSP promoter becomes occupied by both Runx2 and Dlx homeodomain protein, without detectable changes in overall protein levels of either factor (Roca et al., 2005), confirming that Runx2 transcriptional activity is regulated by its ability to interact with cofactors. A number of **Runx2 binding partners** have been identified including CBFβ, ATF4, Smad proteins, Sox9, Twist, HDACs, that all modulate the activity of Runx2 and osteogenic gene expression along differentiation into osteoblasts (reviewed in Soltanoff et al., 2009). It has been shown that Runx2-induced osteoblast gene expression only occurs when expression of Twist bHLH transcription factors disappears in osteoblast precursors (Bialek et al., 2004). CBFβ, the non-DNA-binding partner of all three Runx proteins, is the most important coregulatory protein, essential for enhancement of Runx2 DNA binding (Tahirov et al., 2001). **Postranslational modification** represents the second major mechanism for controlling Runx2 activity. Ascorbic acid stimulates ECM/integrin-mediated activation of ERK/MAP kinase pathway in osteoblastic cells, that results in phosphorylation and increased transcriptional activity of Runx2 (Xiao et al., 2000). This regulation has been suggested to occur also *in vivo* (Ge et al., 2007). Also, another kinase PKCδ phosphorylates Runx2 (on a distinct site from ERK/MAPK) upon FGF2 treatment, that is required for OCN expression (Kim et al., 2003).

Osteogenesis is regulated by many of the major developmental signaling pathways including bone morphogenetic protein (BMP)/transforming growth factor β (TGFβ), Wnt and Hedgehog **signaling**. BMP2 promotes Runx2 expression in mesenchymal osteoprogenitors and Osx and Dlx5 expression in osteoblastic cells (Gori et al., 1999). TGFβ has been found to inhibit Runx2 activity *in vitro* (Kang et al., 2005), whereas it promotes bone formation *in vivo* (Ahdjoudj et al., 2002). Another factor, Indian hedgehog (Ihh) regulates osteoblast differentiation of mesenchymal cells through up-regulation of the expression and function of Runx2 (Shimoyama et al., 2007). Wnts have important modulatory function in osteogenesis. There is strong *in vivo* evidence that high levels of endogenous Wnts promote osteogenesis, whereas low levels

inhibit osteogenesis (Gaspar and Fodde 2004). Additionally, Wnt signaling stimulates osteoblast differentiation of mesenchymal precursors by suppressing C/EBPα and PPARγ (Kang et al., 2007).

Also, transcription of Runx2 is enchanced by fibroblast growth factors (FGFs), retinoic acid and hormone Dex (Kim et al., 2003; Prince et al., 2001). Short term treatment with Dex promotes osteoblast differentiation of mesenchymal cells, whereas long term treatment inhibits bone formation through inhibition of Wnt signaling and induction of osteoblast apoptosis (Smith and Frenkel 2005; Almeida et al., 2005). The cAMP pathway decreases the concentration of Runx2 protein in osteoblasts, an effect that is mediated by proteolytic degradation through ubiquitination of Runx2 and a ubiquitin-proteasome-dependent mechanism (Tintut et al., 1999).

2.3 Chondrogenic differentiation

Cartilage is avascular and non-innervated, and the primary function of its only residing cells, the **chondrocytes**, is to build, maintain and remodel the abundant ECM of the tissue. The cartilage collagen network (Col II, Col IX, Col XI) entraps a highly hydrated gel of proteoglycans and glycoproteins including aggrecan (ACN) and cartilage oligomeric protein (COMP). Chondrocytes fulfill their function in cartilage by undergoing a complex differentiation process.

The steps of sequential differentiation and maturation from chondroprogenitors to hypertrophic chondrocytes are regulated by transcription factors and growth factors including Sry-type high-mobility group box (Sox) genes, the bHLH transcription factor **Scleraxis** (Scx), the Runx genes and the TGFB superfamily (Furumatsu and Asahara 2010). **Sox9** has master roles in the onset of cartilage development, and no other transcription factor has been identified that might control early chondrogenic cell fate and differentiation upstream or in the same steps as Sox9 in all developing cartilage elements. Sox9 is turned on in chondrogenic and osteogenic mesenchymal cells prior to condensation, remains highly expressed in prechondrocytes and chondroblasts and is turned off when cells undergo prehypertrophy (Wright et al., 1995). Sox9 binding sites have been identified in the promoters or enhancers of several **cartilage-specific genes** including Col2a1, ACN and COMP. Two additional Sox family members, L-Sox5 and Sox6, which are not present in early mesenchymal condensations, but are co-expressed with Sox9 during chondrocyte differentiation (Lefebvre et al., 1998), can form homo- or heterodimers, and unlike Sox9, they contain no transcriptional activation domain. *In vitro* experiments have suggested that Sox9 and L-Sox5/Sox6 cooperate with each other to directly activate Col2a1 and ACN. However, the association between Sox9 and L-Sox5/Sox6 has not been detected. Several other transcription factors and coactivators such as Scx and p300, cooperatively modulate the Sox9-dependent transcription by interacting with Sox9.

In chondrocyte differentiation, **TGFβ stimulation** is necessary for MSC-

derived primary chondrogenesis (Pittenger et al., 1999). Several pathways following the activation of TGFβ receptors such as Smad2 and Smad3, and mitogen-activated protein kinase (MAPK) have been identified as key intracellular signals in response to TGFβ treatments (Liu 2003; Hanafusa et al., 1999). TGFβ-regulated Smad3 promotes chondrogenesis through the activation of Sox9 via p300 recruitment. It has been proposed that Sox9 may activate the transcription of its target genes in a multistep fashion, first inducing coactivator-dependent histone acetylation around Sox9-binding sites, then relaxing the chromatin structure and recruiting the Sox9-interacting activators and transcription apparatus for specific gene expression during chondrogenesis (Furumatsu et al., 2009).

In addition to its role in osteogenesis, **Runx2** also regulates chondrocyte development. It serves as a positive regulatory factor in chondrocyte maturation to the hypertrophic phenotype. The expression of Runx2 in terminal chondrocytes and mature osteoblasts suggests that these two cell types may be governed by a common or very similar set of transcription factors, also including c-Maf (MacLean et al., 2003). However, bypassing supression of Runx2 at the onset of chondrogenesis inhibits chondrocytic differentiation, indicating its importance as a modulator of the commitment of mesenchymal progenitor cells to the chondrogenic lineage. Transcriptional repressor **Bapx1/Nkx3.2** is a direct target of Sox9 for repression of Runx2 expression in chondrocytes. Bapx1/Nkx3.2 mediates transcriptional repression of target genes through interactions with BMP-responsive Smad proteins and HDAC1. It has been suggested that the molecular pathway modulated by Bapx1/Nkx3.2 links two major regulators in chondrogenesis, Sox9 and Runx2, to coordinate skeletal formation (Yamashita et al., 2009).

2.4 Cross regulation of mesenchymal cell fate

The three transcription factors PPARγ, Runx2 and Sox9 are expressed in early mesenchymal progenitors suggesting that the interactions between the **PPARγ/Runx2/Sox9 transcriptional trio** determine dynamic cell fate decisions in these mesenchymal lineages. In fact, these three transcription factors are known to interact with each other either directly or via downstream proteins. Factors of one lineage repress factors of the other lineages, thereby maintaining the undifferentiated state. Under appropriate conditions the balance is tipped leading to a cascade that promotes one cell fate while repressing the other possible fates.

Adipocyte differentiation is inhibited by Wnt signaling that blocks the expression of PPARγ and C/EBPα (Kang et al., 2007). BMP2 upregulates expression of Runx2 while simultaneously downregulating transcription of PPARγ through activation of the intermediary transcription factor TAZ (Hong et al., 2005). Sox9 inhibits adipocyte differentiation by binding to and suppressing C/EBPβ and C/EBPδ promoter activity. Further, TGFβ stimulates

chondrogenesis through Smad3 upregulation of Sox9, while simultaneously inhibiting adipogenesis by repressing C/EBP transactivation function also via Smad3 (Choy and Derynck 2003). Sox9 needs to be down-regulated for adipogenesis to occur, resulting in expression of C/EBPβ/δ as initiators of fat cell differentiation (Wang and Sul 2009). PPARγ downregulates Runx2 expression and also binds to the Runx2 protein to inhibit transactivation of osteogenic promoters (Rosen and MacDougald 2006). Sox9 binds Runx2 protein and suppresses osteogenesis (Zhou et al., 2006). Conversely, Osx has been suggested as a negative regulator of Sox9 expression (Nakashima et al., 2002).

Although each specific mesenchymal cell lineage has their own unique transcription factor signature, they also have several transcription factors in common. Specificity is achieved by context and adjustment of the functional collaboration between different transcription factor networks. For example, C/EBPβ was first identified in adipocytes as a transcriptional activator, but is evidently a regulator of growth and differentiation in chondrocytes and osteoblasts (Tominaga et al., 2008), and might function as both, repressor or activator in context dependent fashions. The change in C/EBPβ activity from repressor to activator has been suggested to involve Smad proteins and to depend on upstream signaling events (Nerlov 2008). In 3T3-L1 preadipocytes, association of Smad3 with C/EBPβ inhibited its proadipogenic activity and prevented adipocyte differentiation (Choy and Derynck 2003). In contrast, in osteogenic cells, Smad3 expression increases, binds to C/EBPβ and abrogates its inhibitory function on Runx2 transcription. In conjunction with Runx2, C/EBPβ then further drives osteoblast differentiation (Dingwall et al., 2011). However, before commitment, C/EBPβ may function as a transcriptional repressor of Runx2 and of osteoblast differentiation (Wiper-Bergeron et al., 2007). The action of Smad3 on C/EBPβ activity in adipogenesis versus osteogenesis might be part of the 'competition' of the adipogenic and osteogenic pathways, whereas in the absence of Smad signaling C/EBPβ would act as an inhibitor of mesenchymal differentiation, helping to maintain the MSC state.

To conclude, although much is known about the opposing action of several factors during lineage-specific differentiation, it is not clear how the numerous signaling networks first converge to specify lineage choice in mesenchymal stem cells.

AIMS OF THE STUDY

The potential use of MSCs for cell therapy purposes in regenerative medicine has stimulated growing interest in research on the fundamental biology of these cells. The studies were initially inspired by the observation that these cells exhibit multilineage differentiation potential, and prior to differentiation, can be expanded in culture conditions to a sufficient number for therapeutic procedures. However, the inconsistency of results between different laboratories has evoked concerns in several aspects of MSC biology including the true identity of MSCs, *in vivo* origin of MSCs, heterogeneity of MSC populations and molecular control mechanisms of MSC differentiation.

The aim of this thesis was to study the functional characteristics of MSCs derived from fat, a tissue that has been demonstrated to contain multipotent cells named adipose-derived stem cells (AdMSCs), in parallel with skin-derived cells that are classically considered fibroblasts (FBs) but that resemble AdMSCs in several essential aspects.

In order to dissect the functional identity of stromal cells in relation to potential of differentiation into diverse lineages, AdMSCs and FBs were analyzed for:

- dynamics and mechanism of adipogenic gene expression and differentiation
- transcriptome dynamics along differentiation into adipocytes, osteoblasts and chondrocytes
- heterogeneity of expression of lineage regulators across different cell populations and in single cells

MATERIALS AND METHODS

I used the following methods during the study:

- Isolation and cultivation of primary human stromal cells from adipose and skin tissue
- *In vitro* adipogenic, osteogenic and chondrogenic differentiation of cells
- Flow cytometry
- Western blotting
- Immunofluorescence analysis
- RNA isolation, cDNA synthesis and RT-PCR
- Quantitative real-time RT-PCR
- Sample preparation for multiplex-RNA sequencing on Illumina platform
- RNA-seq data analysis using Qlucore Omics Explorer
- Manipulating gene lists in web-based databases
- DNA transfection of cells
- Gene silencing using siRNA technique
- Luciferase reporter assay

RESULTS AND DISCUSSION

1. Human adipose tissue and dermis contain MSC-like cells with similar phenotype and differentiation potential (Publications I, II and Manuscript).

Self-renewal and differentiation of stem and progenitor cells determines maintenance and regeneration of adult tissues. Numerous tissues in the adult body have been found to contain MSC-like cells, including subcutaneous adipose tissue that holds a great promise for therapeutic applications as an easily accessible source of MSCs. Adipose tissue-derived MSC (AdMSC) populations express surface antigens CD73, CD90 and CD105, and differentiate into adipocytes, osteoblasts and chondrocytes under appropriate culture conditions *in vitro*. Tissue stromal cells (fibroblasts) share many characteristics of MSCs, including similar morphology and immunophenotype. However, the differentiation potential of fibroblasts (FBs) has not been uniformly demonstrated, most likely due to the different cell sources and experimental conditions used. Several studies have analyzed FB cell lines with unknown genetic backgrounds and compared them with primary AdMSCs to conclude that unlike AdMSCs, FBs do not give rise to multiple differentiated cell types. Considering all the similarities between these cell populations, we reasoned to study the differentiation potential of primary FBs and AdMSCs originating from the same donors. Surface antigen expression profiling using flow cytometry analysis revealed that AdMSCs and dermal FBs both expressed **5'nucleotidase/CD73, Thy-1/CD90, endoglin/CD105** and lacked the expression of leukocyte marker CD45, marking them as phenotypically indistinguishable cell types. To analyze the differentiation of isolated cells into adipocytes, osteoblasts and chondrocytes, FBs and AdMSCs were cultivated for 2-3 weeks in media supplemented with dexamethasone, IBMX, indomethacin and insulin for induction of adipogenesis; with dexamethasone, L-ascorbic acid 2-phosphate and glycerol 2-phosphate for induction of osteogenesis; or with L-ascorbic acid 2-phosphate, insulin and TGFβ-1 for induction of chondrogenesis. **Tissue-specific staining** of differentiated cell cultures derived from either AdMSCs or FBs under standard conditions confirmed that both starting cell populations developed into cells with positive staining for Oil Red O (ORO, stains lipid droplets characteristic to adipocytes), Alizarin Red S (ARS, stains calcified matrix of osteoblasts) and Alcian Blue (AB, stains proteoglycan-rich matrix of chondrocytes) indicating that FBs and AdMSCs are developmentally equivalent.

2. Adipogenic differentiation of fibroblasts (Publication I).

Cell differentiation is a step-wise process accompanied by the sequential expression of transcription factors and their target genes that carry out tissue-specific functions. The *in vitro* differentiation potential of MSCs and FBs is usually assessed upon long-term treatment of cells with inducive culture media, ranging from 2 to 4 weeks. However, this type of analysis neglects the possibility that the cells under study maturate at different rate. Consequently, scientific studies assessing the differentiation potential of FBs have reported contradictory results.

2.1 Dermal FBs exhibit delayed differentiation into adipocytes compared with AdMSCs.

It has been shown that the molecular changes directing cells from multipotent state to a specific differentiation pathway occur early, followed by continuous increase in expression of cell type-specific genes and aquirement of phenotype characteristic to mature cells. Hence, potential differences in gene expression in the early stages of differentiation of different starting cell populations could disappear at later stages of differentiation. We performed standard tissue-specific staining of adipo- and osteo-induced cultures of AdMSCs and FBs on days 7, 14 and 21 along differentiation in order to determine the **dynamics of differentiation** of these two stromal cell populations. We observed that despite the similar final differentiation of AdMSCs and FBs into adipocytes and osteoblasts, and similar dynamic response of these cells to osteogenic induction (based on ARS staining), there were differences in ORO staining intensities at earlier time-points of adipogenic differentiation. AdMSCs exhibited more intensive staining of ORO on day 7 and 14 upon adipogenic induction compared with FBs, whereas this difference was not detectable after 21 days of differentiation. This finding suggests that FBs exhibit delayed differentiation into adipocytes compared with AdMSCs.

We performed a more detailed analysis of gene expression of AdMSCs and FBs using qRT-PCR along multiple stages of adipogenic differentiation starting from day 1. The analysis of mRNA expression of well-known adipocyte-specific genes PPARγ2, C/EBPα and FABP4 (see overview of the literature) in AdMSCs and FBs over time revealed that the expression of these genes became elevated in AdMSCs on day 1 of adipogenic differentiation, and showed further induction on the following days in these cells, whereas in FBs their expression was induced between 3 to 7 days upon adipogenic induction. Expression of PPARγ2 and FABP4 was only slightly stimulated in FBs before day 3 upon induction and no expression of C/EBPα was detected. In fact, C/EBPα expression remained at lower level in FBs compared with AdMSCs througout the 21-day differentiation assay. It has been shown that adipogenesis can be induced by C/EBPβ and

C/EBPδ without stimulation of C/EBPα expression, probably depending on cellular context (Farmer 2006). Despite that, both AdMSC- and FB-derived adipocytes expressed similar levels of FABP4 (mRNA) and adiponectin (protein). Together, expression of adipocyte-related genes in response to adipogenic stimulation occurred much faster in AdMSCs than FBs.

2.2 The delay in adipogenesis of FBs occurs downstream of C/EBPβ expression.

The expression of PPARγ and C/EBPα in adipogenic cells is preceeded by the expression and activation of C/EBPβ that binds pparg promoter and activates its transcription (Rosen and MacDougald 2006). In turn, PPARγ stimulates the expression of C/EBPα by releasing c/ebpα promoter from histone deacetylase (HDAC1). The analysis of C/EBPβ expression in FBs and AdMSCs using western blotting showed that both cell populations exhibited transient increase in C/EBPβ protein expression on day 1 upon adipogenic induction that returned to its initial level at later time points, an expression pattern characteristic to cells undergoing adipogenic differentiation. This indicates that the observed time lag in the induction of adipogenic genes in FBs was not due to delayed expression of C/EBPβ in these cells versus AdMSCs, and further, that the early molecular step necessary for adipogenic differentiation is similarily activated in AdMSCs and FBs. Importantly, **C/EBPβ activity** is regulated by phosphorylation and several negative regulator proteins including RORα, Sharp1/Dec2 and TRB3 (Ohoka et al., 2009; Gulbagci et al., 2009; Bezy et al., 2007). We hypothized that adipogenesis could be delayed in FBs when these known inhibitors of C/EBPβ activity had higher expression in FBs compared with AdMSCs following adipogenic induction, and thereby would delay the stimulation of PPARγ2 expression by C/EBPβ. However, the expression of these genes followed similar pattern in AdMSCs and FBs during adipogenic differentiation, being first transiently down regulated upon induction and then up regulated again by day 7. These results enabled us to conclude that delayed adipogenesis of FBs is not caused by distinct regulation of C/EBPβ activity by RORα, Sharp1/Dec2 or TRB3 in these cells.

PPARγ expression is also regulated by **C/EBPβ-independent mechanisms**. As described in the overview of the literature, Wnt signaling modulates stromal cell development by directing cells towards osteoblast lineage at the expense of adipogenesis (Kang et al., 2007). A zinc finger transcription factor ZFP423 (ZNF423 in humans) is one of the few factors known to define adipogenic FBs. It regulates PPARγ expression in preadipocyte state of murine FBs (Gupta et al., 2010). We analyzed the expression of ZNF423 in AdMSCs and FBs upon adipogenic induction using qRT-PCR. Our data revealed that ZNF423 was expressed 2-3 days following adipogenic induction in AdMSCs, whereas in FBs it became detectable after 7 days of induction. These results suggest that PPARγ could be regulated by ZNF423, whose late induction can be involved in the

delayed adipogenic differentiation of FBs. Overall, our data suggest that these two stromal cell types, AdMSCs and FBs could exhibit different mechanisms for the early regulation of adipogenic differentiation, probably involving distinct regulation of PPARγ expression.

3. Global analysis of differentiation dynamics (Publication II).

Transcription factors that have been shown to have crucial functions in cell fate determination are often called 'master regulators' of certain developmental pathways. Among them are, for example, PPARγ for fat, Runx2 for bone, and Sox9 for cartilage development. Although indispensable for expression of many cell type-specific genes, the molecular interactions necessary for cell differentiation are evidently more complex and involve numerous other undescribed factors. Moreover, the known collection of factors can generate cellular outcomes in a dynamically distinct manner, as we saw for the process of adipogenesis in AdMSCs and FBs.

The improvement of high-throughput DNA sequencing methods over recent years has generated the unprecedented possibility to quantitatively detect nearly every mRNA molecule found in the cells. Moreover, the sensitivity of sequencing has come down to single-cell level, and it has been estimated that only very rare transcripts in individual cells are missed by these methods (Ramsköld et al., 2012). Combined with multiplexing sample preparation methods, tens to hundreds of different RNA samples can simultaneously be deep-sequenced and analyzed for complete transcriptome (Islam et al., 2011). We decided to use multiplex **RNA-seq technology** to study the dynamics of differentiation of AdMSCs and FBs into adipocytes, osteoblasts and chondrocytes on global scale.

3.1 Transcriptome profiles of undifferentiated AdMSCs and FBs are distinct and stay distinct upon differentiation.

We sequenced mRNAs from 96 independent bulk samples assembled into a single sequencing library using a modified method by Islam et al., 2011. For transcriptome analysis, we used multi-group ANOVA (Analysis of variance) on genes that showed significant differences between defined groups based on false discovery rate (FDR), followed by principal component analysis (PCA) to visualize differences between groups other than those used in the ANOVA. Thereby we could detect the relationships of samples (that is, differences or similarities in gene expression) with respect to tissue of origin (AdMSC or FB), patient of origin (n=2), cell type (undifferentiated, adipocytes, osteoblasts, chondrocytes) and time point (days 0-7), based on filtered differences in gene expression in the same PCA plot. First, we observed that those genes that were specifically expressed in different cell types (deliberately selected by ANOVA), were also regulated over time (visualized in PCA). Individual hESC lines and

also MSC populations have been shown to exhibit unique gene expression signature due to high genetic variability (Abeyta et al., 2004; Skottman et al., 2005; Tsai et al., 2007). In this study, however, no genes were found to be significantly differently expressed between patients, indicating that the differences between cell types overwhelmed any differences between these individuals.

Previously, comparison of gene expression profiles between AdMSCs and FBs has been carried out using cells from different donors, leading to possible variation in gene expression that is not directly related to the differences between these cells (Bae et al., 2009; Wagner et al., 2005; Kuklick et al., 2002). Importantly, here, expression of developmentally regulated genes separated undifferentiated AdMSCs and FBs derived from matching donors into **distinct populations**. Moreover, AdMSCs and FBs stayed subtly distinct in the differentiated state. When transcriptome profiles of AdMSCs and FBs in the undifferentiated state were compared using ANOVA, relatively few genes (62 genes) were found to be differently expressed between these cell populations. FBs expressed higher levels of genes that were related to cell cycle regulation, cytoskeleton stabilization and cell signaling including cell motility (S100A4) and vesicular trafficking (CAV1, DNM1). However, expression of genes associated with BMP (GREM1), VEGF (MYOF) or Wnt (ZRANB1) signaling, or biosynthetic processes, regulation of extracellular matrix organization and adhesion, was significantly higher in AdMSCs than FBs. These findings suggest that despite the similar general characteristics of AdMSCs and FBs, their gene expression profiles are initially different.

We observed from the cell type-specific PCA that unlike FBs, AdMSCs clustered together with chondrocyte population. In search for **similarities between AdMSCs and chondrocytes**, gene expression of AdMSCs, FBs and chondrocytes were simultaneously compared. Only 23 genes were found to be highly expressed in AdMSCs and AdMSC- and FB-derived chondrocytes compared with undifferentiated FBs. These included ribosomal proteins functioning in protein biosynthesis, structural components of cytoskeleton and genes that regulate ECM-mediated cell signaling and adhesion. Two genes that play important role in cartilage development (DACT1/Wnt, PDLIM7/BMP6) were enriched in AdMSCs and chondrocytes, suggesting that AdMSCs could share functional similarities with chondrocytes.

Next, we analyzed the extent of differences between AdMSCs and FBs in the differentiated state. 45, 215 and 104 genes were found to be differentially expressed between AdMSC- and FB-derived adipocytes, osteoblasts and chondrocytes, respectively, indicating that AdMSCs and FBs become more similar upon adipogenic differentiation. Further investigation into the origin of differences in gene expression in the differentiated cells revealed that some genes were differentially expressed already in the undifferentiated state, whereas others became distinctly expressed in the process of differentiation. Again, less genes remained distinctly expressed between AdMSCs and FBs upon adipogenic

induction (12 genes) compared with osteogenic (36 genes) or chondrogenic induction (22 genes). This suggests that **switch of stromal cell regulatory mechanisms** into adipocyte-specific regulation is faster than switch into osteoblast- and chondrocyte-specific regulation.

Genes that were not regulated in AdMSCs or FBs during differentiation could represent source-specific **'memory' genes**. Our data showed that expression of COL1A1, COL1A2, EFEMP1 (fibulin 3), FB1 (fibronectin 1), GGT5 (gamma-glutamyltransferase 5) and TMP2 (tropomyosin 2) were characteristic to AdMSCs and AdMSC-derived cells, whereas expression of S100A4 (fibroblast-specific protein 1) and TK1 (thymidine kinase 1) were characteristic to FBs and FB-derived cells. Future studies should confirm whether those 'memory' genes are specifically expressed in mature differentiated cell types derived from distinct tissue sources.

3.2 Changes in lineage-specific gene expession occur early in differentiation of AdMSCs and FBs.

Cell differentiation is a process of sequential induction of regulatory genes that initiate the expression of tissue-specific target genes. We had sequenced transcriptomes of AdMSCs and FBs on days 0-7 along differentiation into adipocytes, osteoblasts and chondrocytes and next, performed ANOVA between expression data of different time points to analyze gene regulation over time. In total, 213, 126 and 203 genes were found to be regulated during adipogenesis, osteogenesis and chondrogenesis, respectively, of AdMSCs and FBs. Visualization of the samples in PCA revealed that undifferentiated cells were different from all differentiated samples, except for AdMSCs that clustered together with early-stage chondrocytes (days 1-3). Hence, global changes in gene expression take place quickly upon differentiation and persist over time in both AdMSCs and FBs.

It turned out, that approximately 70% of adipocyte-related and 43% of osteoblast-related genes were down-regulated in the process of differentiation. Further, down regulation was rapid, whereas up regulation occurred slowly over the week. It has been suggested that **gene repression is a predominant early mechanism** before final cell commitment and that lineage-specific molecular processes are transcriptionally up regulated only after commitment (Scheideler et al., 2008). Our data support the idea that cells rapidly reset their original transcriptional program upon differentiation, and gradually express lineage-associated genes. Such general mechanism of gene repression is more characteristic to adipogenic than osteoblastic differentiation, again suggesting that switch into adipocyte regulation is faster than into osteoblast regulation.

3.3 AdMSCs and FBs exhibit different dynamics of chondrogenic differentiation.

Despite the similar global dynamics of adipogenesis and osteogenesis of AdMSCs and FBs, chondrogenesis-related genes showed different expression patterns in these cells. First, more genes were down regulated in AdMSCs (74%) than in FBs (62%) upon chondrogenic induction. Further, unlike in FBs, in AdMSCs smaller but bidirectional changes in gene regulation occurred throughout chondrogenesis. In contrast, FBs exhibited transient down regulation in gene expression followed by constant up regulation along chondrogenic differentiation. These trends confirm the observation that **AdMSCs and chondrocytes are more alike** and less changes in gene expression are needed in AdMSCs than in FBs to become chondrocytes. Importantly, even when AdMSCs were pre-committed to chondrocyte development, it would not affect their ability to differentiate into other cell types similarly with FBs. It has been shown that lineage-committed MSCs can transdifferentiate into other cell types in response to inducive extracellular cues (Song and Tuan 2004). Also, uncommitted adult stem cells were proposed to maintain their multipotency by expressing basal levels of genes characteristic to different lineages and that certain groups of genes are selectively suppressed upon stimulation prior to commitment to a given phenotype (Woodbury et al., 2002; Song et al., 2006). Together, our study showed that different stromal cells exhibit distinct dynamics of differentiation into mesodermal cell types under similar experimental conditions. AdMSCs and FBs exploit globally similar early mechanisms for differentiation into adipocytes and osteoblasts but show different molecular mechanisms for chondrogenic differentiation.

4. Variability of gene expression (Manuscript and Publication III).

The heterogeneity of MSC populations in terms of surface characteristics and differentiation potential imposes a challenge for development of standardized isolation and cultivation methods of these cells. This in turn, hampers the study of molecular mechanisms governing differentiation of MSCs towards diverse lineages and their efficient use in therapeutic applications. MSCs are isolated as primary cell populations from individuals with different genetic backgrounds and diseases. Also, different tissue sources of MSCs can give rise to variation in gene expression between MSC populations. To analyze the potential effect of variation of gene expression on differentiation, stromal cells were isolated from two different tissues (fat/AdMSCs and skin/FBs as described in previous sections) of **seven donors under standard conditions**, and assayed for differentiation into adipocytes, osteoblasts and chondrocytes.

4.1 AdMSC and FB populations derived from different donors exhibit multi-lineage differentiation potential.

First, we determined the potential of differentiation of isolated cell populations towards three mesenchymal lineages using cytochemical staining of cell cultures on day 14 upon induction. All seven AdMSC and FB populations differentiated into cells with positive staining for ORO (adipocytes), ARS (osteoblasts) and AB (chondrocytes) upon two-week treatment. Quantification of staining intensities revealed variation between different cell populations. However, we detected no preferential differentiation of AdMSC or FB populations into any one cell lineage, or donor-specific differentiation potential of isolated cells into all three lineages. Hence, the presence of cells at different developmental stages in these stromal cell populations must have given rise to the observed variation in staining intensities. Analysis of surface marker expression confirmed ubiquitous expression of CD73 and heterogeneous expression of CD105 on AdMSCs and FBs, whereas proliferation rates of these cell populations varied only moderately. Hence, AdMSC and FB populations exhibited **similar** growth and **tri-lineage differentiation potential** under the same *in vitro* culture conditions.

4.2 Expression of lineage-specific genes varies in AdMSC and FB populations derived from different donors.

Next, we analyzed the expression of genes that are indicative of adipogenesis (PPARγ, FABP4), osteogenesis (Runx2 and alkaline phosphatase, ALP) and chondrogenesis (Sox9 and ACAN) in AdMSC and FB populations derived from the same seven donors on day 7 upon differentiation using qRT-PCR analysis. The expression of well-known lineage-specific genes *PPARγ*, *Runx2* and *Sox9* upon adipogenic, osteogenic and chondrogenic stimulation, respectively, turned out to be **highly variable** in multipotent AdMSC and FB populations derived from different donors. Moreover, some AdMSC-derived cells did not express *PPARγ* or *Runx2* at detectable levels upon differentiation, although they expressed late marker genes *FABP4* or *ALP*, and developed into cells with positive staining of ORO or ARS, respectively. This suggests, that adipogenesis can be initiated in some AdMSCs by mechanisms that do not require elevated induction of PPARγ transcription. In fact, it has been demonstrated that glucocorticoids can stimulate adipogenesis by non-transcriptional mechanisms directly resulting in enhanced expression of C/EBPα and adipocyte-genes (Wiper-Bergeron et al., 2003). Also, it is well-known that osteoblastic cells selectively express either Runx2 or other osteoblast-genes, probably due to the regulation of Runx2 activity post-transcriptionally through binding with co-activator proteins (Schroeder et al., 2005; Franceschi et al., 2009). Interestingly, donor-specific differences in *FABP4* and *ALP* expression were overwhelmed by differences between AdMSCs and FBs: AdMSC-derived differentiated cells expressed markedly higher levels of late marker genes compared with FB-

derived differentiated cells, indicating tissue source-specific regulation of PPARγ or Runx2 activity in adipogenic or osteogenic cells, respectively.

Tissue source-specific differences in gene expression became most evident upon chondrogenic induction of AdMSCs and FBs – differentiating FB populations expressed *Sox9* and *ACAN* at higher levels compared with differentiating AdMSC populations. These results suggest that different control mechanisms of chondrogenesis are present in AdMSCs and FBs that lead to distinct dynamics of expression of marker genes upon induction of differentiation. Importantly, all cell populations analyzed exhibited similar differentiation potential, despite the donor- and tissue source-derived variations of gene expression.

4.3 Variability of gene regulation in established cell lines.

Variations of gene expression can occur between cells grown in a common environment. Therefore, analysis of gene expression across populations of cells always averages out the potential differences of gene expression between single cells, and may introduce bias to the interpretation of the results on mechanisms that regulate essential cellular functions including proliferation and differentiation. To investigate the effect of cell density on Wnt signaling pathway, known to be important in several cellular processes, localization and **activity of β-catenin in confluent and subconfluent cells** were analyzed (Publication III), using established human cell lines: COLO205 (colon adenocarcinoma), HCT116 (colon carcinoma) and H1299 (non-small lung cancer). We detected expression of β-catenin in the nucleus of subconfluent COLO205 cells, whereas in confluent cells nuclear β-catenin staining decreased, as evidenced by immunofluorescence analysis (Figure 5 in Publication III). It is widely accepted that the involvement of β-catenin in cell adhesion or transcription is strictly associated with the subcellular localization of β-catenin. To analyze the effect of cell density on β-catenin-mediated transcription, we employed the TOPflash luciferase reporter system and measured luciferase activity in transfected cells (Figure 7 in Publication III). There was a 2-fold higher transcription level of the reporter gene in subconfluent COLO205 cells compared with confluent cells. Similarly, subconfluent H1299 and HCT116 cells exhibited higher transcription from the reporter gene compared with confluent cells, though the effect was less prominent. These results indicate that in subconfluent cells transcriptionally active β-catenin accumulates in the nucleus. Along with these experiments we detected co-localization and interaction of CD43 with β-catenin in COLO205 cells and proposed that the activity of β-catenin is modulated by CD43 by both direct and indirect mechanisms. Hence, analysis of gene expression in individual cells demonstrated that cell density can affect local microenvironment in a way that changes cellular distribution, potential interactions and activity of proteins in these cells.

4.4 Variable expression of lineage regulators in individual cells.

Although it is widely acknowledged that stromal cell populations are heterogeneous mixtures of cells at different developmental stages, there is little data available on differentiation and gene expression of individual stromal cells. In order to detect the expression of specific lineage regulators PPARγ, C/EBPα and their target gene FABP4, or Sox9 and its target gene collagen type II (Col II) in individual differentiating cells, we performed immunofluorescence analysis on FBs that were differentiated towards adipocyte or chondrocyte lineages, respectively, on day 14 upon induction. We evidenced **cell-to-cell variation** of expression of adipogenic genes and unsimultaneous expression of adipocyte regulators in the same cells. It has been shown that most genes induced in adipogenic mouse 3T3-L1 cells are bound by both PPARγ and C/EBPα, while very few are PPARγ-specific (Lefterova et al., 2008). Moreover, reciprocal activation between PPARγ and C/EBPα is not necessary for adipocyte differentiation of NIH/3T3 cells (Shao et al., 2013). Our findings extend these studies towards the idea that adipocyte gene expression of individual primary stromal cells can be regulated by either PPARγ or C/EBPα alone. We have previously shown that *C/EBPα* mRNA expression remained at lower level in FB-derived than in AdMSC-derived adipogenic cells, despite similar final differentiation of both cell populations into adipocytes (Publication I). Similarly, transcriptome profiles of adipogenic AdMSCs and FBs retained source-specific patterns upon differentiation (Publication II). Together, our results suggest that individual stromal cells use alternative mechanisms to become adipocytes.

Single-cell analysis of Sox9 and Col II expression in FB-derived chondrocytes revealed that despite ubiquitous and abundant expression of Col II, Sox9 was not expressed in every cell. These results suggest that stromal cells possess Sox9-dependent and -independent mechanisms to regulate and maintain Col II expression upon chondrogenic stimulation. There are several lines of evidence from literature to support this conclusion: Sox9 exhibits cell type- or stage-specific regulatory role during differentiation (Kou and Ikegawa 2004); different proteins bind Sox9 enhancer elements within *Col2a1* gene (Zhou et al., 1998); Sox9 does not play a key role in *Col2a1* activation in human adult articular chondrocytes (Aigner et al., 2003).

Together, our findings let us conclude that the presence of distinct cell subsets in stromal cell populations gives rise to heterogenous response upon induction of differentiation and variable gene expression dynamics. Importantly, cell-to-cell variation of expression of known lineage genes indicates distinct mechanisms of differentiation to reach a common cell fate. Detailed analysis of differentiation of multipotent stromal cells towards diverse lineages could contribute to the understanding of the fundamentals of stromal cell biology and help to improve cell therapy approaches in regenerative medicine.

CONCLUSIONS

The results of the studies summarized in this thesis reveal that:

1. AdMSCs and FBs exhibit similar immunophenotype and potential to differentiate into adipocytes, osteoblasts and chondrocytes under similar culture conditions.
2. FBs show delayed induction of adipogenesis compared with AdMSCs, that occurs downstream of C/EBPβ expression probably due to distinct early regulation of PPARγ expression in these cells.
3. AdMSC and FB populations exhibit distinct global gene expression patterns in the undifferentiated state.
4. Transcriptome profiles of AdMSC- and FB-derived differentiated cells stay distinct - 'cellular memory'.
5. Lineage-specific gene regulation occurs early upon differentiation on global scale and persists over time in both AdMSCs and FBs.
6. Switch of stromal cell regulatory mechanisms into adipocyte-specific regulation occurs faster than switch into osteoblast- and chondrocyte-specific regulation.
7. AdMSCs and FBs exhibit different dynamics of chondrogenic differentiation.
8. Different stromal cell populations with similar differentiation potential exhibit donor- and tissue source-specific expression of lineage regulators.
9. Variable expression of lineage regulators in single cells indicates distinct dynamics/mechanisms of differentiation to reach a common cell fate.

It concludes that different stromal cells including AdMSCs and FBs are functionally equivalent. The differences in gene expression between these cell populations arise from dynamic transitions between different cell states of individual cells at any given time, that generates heterogeneity within single cells ('randomness' of gene expression), between cells of the same population (population-level heterogeneity), between cell populations of distinct tissue origin (tissue-source specificity), and between cells of different donors (individual variation; Figure 2). These studies confirm previous statements that cellular plasticity is a common and inherent property of stromal cells including AdMSCs and FBs which can develop into diverse cell types under the influence of appropriate extracellular stimuli.

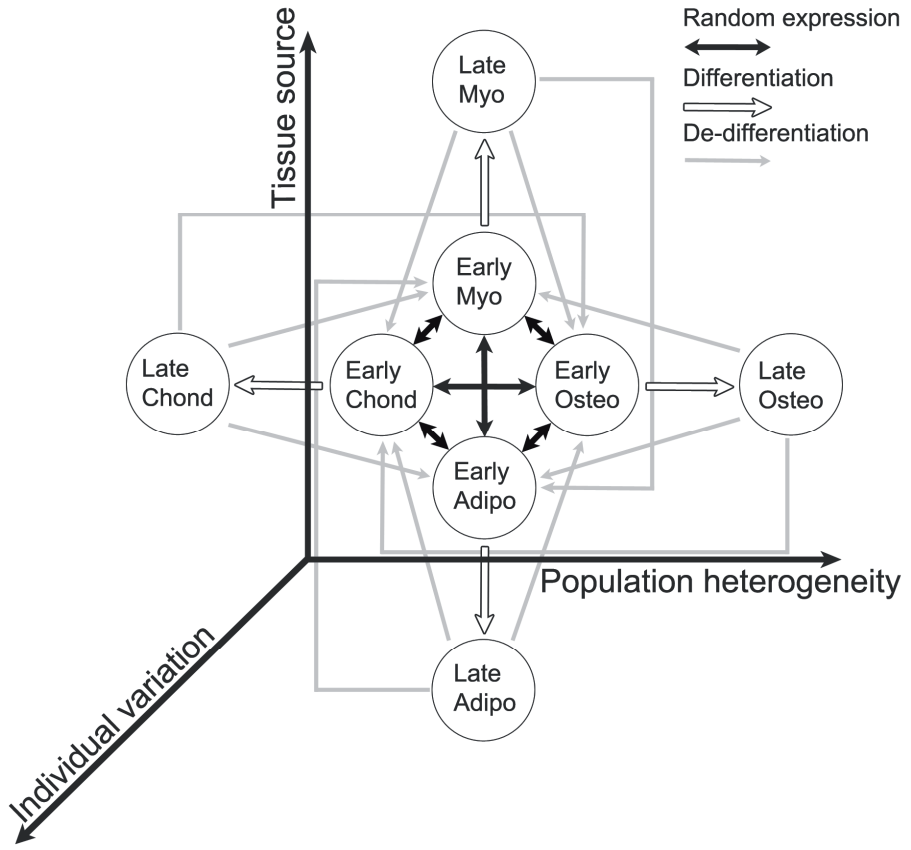

Figure 2. Cellular plasticity and origins of heterogeneity in gene expression of stromal cells. The three axes represent additional dimensions of gene expression along which each cell is 'positioned' at any given time. Abbreviations: Adipo – adipocytes, Osteo – osteoblasts, Chond – chondrocytes, Myo - myocytes (the layout of the scheme is partly borrowed from Zhu and Paul 2010).

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ACKNOWLEDGEMENTS

These studies have been financed by different projects led by different biotechnology companies in Estonia including Cellin Technologies LLC, Protobios LLC and FibroTx LLC. I am grateful to the two head figures of those companies, Toomas Neuman and Kaia Palm whose efforts have made it possible for me to work on one of the most exciting fields of biology – cell differentiation.

My biggest gratitude belongs to my mentor Toomas Neuman for his support, trust and respect for me, and for sharing with me his knowledge about cells. Tom has enabled me to carry out largely independent research and has motivated me to write this thesis.

I am grateful to Sten Linnarsson from Karolinska Institutet for inspiring collaboration and for having me in his lab to learn more about quantitative biology. I also thank Saiful Islam for his warm company in the lab, and Annalena Moliner for taking me along to the long exploratory walks outside of the lab in Stockholm.

I am thankful to all my former and present colleagues in Tallinn for creating a welcoming and friendly working environment. Special thanks to Katja Kazantseva for sharing our common efforts towards professional development. I am happy to have worked with young colleagues Anna Velts, Angelika Fatkina, Ester Orav, Mari-Liis Kauts, Laura Lambut and Vahur Luik – thank you.

I am grateful to my first supervisor Lilian Kadaja-Saarepuu at the University of Tartu for introducing me the experimental world of cell biology. I am also grateful to the head of the cell biology lab Toivo Maimets for collaboration, and to all my former labmates in Tartu for linking science with fun.

I am happy I learned to know Helena Faust during 'Otaniemi times' and Agne Velthut later in Tallinn – we have had invaluable professional and personal discussions that I am most grateful for to you. I thank my coursemates Mari Sepp, Nele Tamberg, Pirjo Spuul and Ervin Valk for encouragement and for staying close from the very first year together at the University of Tartu.

The author of this thesis has been greatly influenced by Madis whose presence and support cannot be overestimated – thank you. I also thank my mother, brother and my twin sister for unprecedented support and mind-matching discussions.

PUBLICATION I

Jääger K and Neuman T. (2011). Human dermal fibroblasts exhibit delayed adipogenic differentiation compared with mesenchymal stem cells. Stem Cells and Development, Aug; 20(8):1327-36.

STEM CELLS AND DEVELOPMENT
Volume 20, Number 8, 2011

DOI: 10.1089/scd.2010.0258

Human Dermal Fibroblasts Exhibit Delayed Adipogenic Differentiation Compared with Mesenchymal Stem Cells

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Human dermal fibroblasts (FBs) express mesenchymal stem cell (MSC)-specific cell surface markers and differentiate into several cell types under appropriate conditions. Molecular mechanisms controlling the early stages of differentiation of dermal FBs and MSCs isolated from different sources have not been well studied. Here, we have analyzed the cell type-specific changes of adipose tissue-derived mesenchymal stem cells (AdMSCs) and dermal FBs in the process of differentiation into adipocytes and osteoblasts. Analysis of gene expression in the course of adipogenic differentiation of AdMSCs and FBs isolated from the same individuals revealed a time lag in the induction of adipogenesis-related genes in FBs compared with AdMSCs, a phenomenon not previously described. Further, preliminary evidence suggests that delayed adipogenesis of FBs is related to the delayed induction of preadipocyte transcription factor ZNF423 in FBs. These findings clearly show that AdMSCs and FBs have similar developmental potential but different molecular control mechanisms of initial stages of adipogenic differentiation.

Introduction

Maintenance and regeneration of tissues in the adult body is dependent on stem and progenitor cells, which possess potential of self-renewal and differentiation into one or more cell types. Multipotent mesenchymal stem cells (MSCs) have been isolated from different tissues including bone marrow, adipose tissue, fetal and adult skin, peripheral blood, and skeletal muscle [1–5]. Tissue stromal cells have been reported to be responsible for regeneration of numerous organs including liver, bone, cartilage, brain, and heart [6–10].

Many functional characteristics and biomarkers of MSCs and stromal cells indicate that they can be considered similar or even identical [11–13]. For example, MSCs and stromal cells both express CD90, CD105, CD73, and STRO-1 [14,15]. Lorenz et al. have demonstrated that both MSCs and dermal fibroblasts (FBs) can differentiate into fat and bone cells presenting lineage-specific markers, either peroxisome proliferator-activated receptor gamma (PPARγ) and lipoprotein lipase or osteopontin and osteocalcin, respectively. Pluripotent cells from bone marrow, adipose tissue, hair papilla, and skin dermis express cell surface markers that are characteristic to MSCs (CD105, CD49d, and STRO-1) and differentiate into adipocytes and osteoblasts [16].

Adipose tissue-derived mesenchymal stem cells (AdMSCs) differentiate into a variety of cell types including osteoblasts, chondrocytes, adipocytes, tenocytes, and skeletal myocytes [2,17–19]. Gene and protein expression data show that isolated AdMSCs and dermal FBs (stromal cells) have both similarities and differences [14,20,21]. FBs derived from lamina propria have the same cell surface markers, immunophenotypic characteristics, and differentiation potential as BmMSCs and AdMSCs [22]. Dermal FBs that express vimentin, fibronectin, and collagen and AdMSCs have similar expression patterns of CD29, CD44, CD71, CD73/SH3-SH4, CD90/Thy-1, CD105/SH2, and CD166/ALCAM [12]. When cultured under appropriate conditions, both cell types differentiate along the adipogenic and osteogenic lineages. Also, adult bronchial FB-like cells revealed a similar expression pattern of antigens characteristic to BmMSCs, including CD90/Thy-1, CD73/SH3-SH4, CD105/SH2, and CD166/ALCAM, whereas STRO-1 antigen was weakly expressed in bronchial FBs [11]. Both cell types differentiated along the adipogenic, osteogenic, and chondrogenic mesenchymal pathways when cultured under appropriate conditions.

Little is known about the molecular changes that occur at the early stages of differentiation of stromal cells (FBs) and AdMSCs into osteoblasts and adipocytes. Long-term differentiation assays have demonstrated that the final differentiation of FBs and AdMSCs is nearly identical, as both express a set of lineage-specific markers. Differentiation along adipogenic and osteogenic pathways requires sequential induction of specific genes. Adipogenesis depends on the transcription factors CCAAT/enhancer binding protein alpha (C/EBPα) and PPARγ, the expression of which is facilitated by CCAAT/

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enhancer binding protein beta (C/EBPβ) and CCAAT/enhancer binding protein delta (C/EBPδ). All these transcription factors together promote adipogenic differentiation by activating the full adipose-specific gene expression program leading to adipocyte maturation [23,24]. Fatty acid binding protein 4 (FABP4), a key mediator of intracellular transport and metabolism of fatty acids in adipose tissue, is one of the adipogenic genes directly regulated by PPARγ. Recently, it was shown that zinc finger transcription factor ZFP423 robustly activates PPARγ and stimulates adipocyte differentiation [25].

Development of osteoblasts is regulated by a number of transcription factors including runt-related transcription factor 2 (RUNX2) (CBFA1), Osterix, ATF4, and β-catenin [26–29]. RUNX2 has been identified as the key transcription factor regulating cell commitment and differentiation into osteoblasts [30]; however, various regulatory factors such as TAZ, pRB, HOXA10, GRG5, and BAPX-1 control the activity of RUNX2 [31–35]. The temporal and spatial expression and activity of those regulatory factors cause RUNX2 target genes to be often absent in cells that express RUNX2 [36]. The coordinated action of different transcription factors results in expression of osteogenic genes and acquisition of the osteoblast phenotype.

Comparative studies of the multipotency of FBs and MSCs have been mostly based on long-term differentiation assays with the analysis of terminal differentiation. Also, majority of gene expression profiling studies of FBs and MSCs have been done using commercially available cells that rarely originate from the same donor, which makes the genetic background unknown and may introduce individual variation into the comparison of different cell types. The objective of this study was to investigate cell type-specific changes of AdMSCs and FBs during the course of differentiation into adipocytes and osteoblasts in donor-matched samples. Results of gene expression analysis revealed a time-lag in the induction of adipogenesis-related gene expression in FBs, compared with AdMSCs, and delayed differentiation. This delayed differentiation can be related to late induction of zinc finger protein ZNF423 in FBs, compared with AdMSCs.

Materials and Methods

Cell culture

Human subcutaneous adipose tissue and skin dermis from the same donor were used to isolate MSCs and FBs, respectively. AdMSCs were isolated according to Lin et al. and Yamamoto et al. [37,38] with slight modifications. Briefly, adipose tissue was digested with 0.1% collagenase (Gibco) in serum-free DMEM/F12 (Gibco) at 37°C for 1.5 h, followed by neutralization of enzyme activity with 20% fetal bovine serum (FBS) and 1% penicillin–streptomycin DMEM/F12 growth medium. Following centrifugation, stromal cell pellet was passed through a 100-μm nylon mesh (BD Biosciences), resuspended in growth medium, plated at a density of 10,000 cells/cm^2, and incubated at 37°C with 5% CO_2. After 48 h, medium was replaced to remove nonadherent cells. Further cultivation was performed under standard cell culture conditions. FBs were isolated from dermal skin as previously described [39]. Briefly, primary culture was established by FB outgrowth from skin explants placed onto Primaria dish (BD Falcon) containing 10% FBS and 1% penicillin–streptomycin DMEM-high glucose (Gibco) growth medium.

Immunophenotyping

About 0.5×10^6 cells were collected by trypsinization and incubated on ice for 1 h with 2.5 μg/mL antibodies in phosphate-buffered saline containing 2% bovine serum albumin. Primary antibodies against CD90 (Chemicon), CD73, and CD105 (BD Biosciences) and V5-tag (Invitrogen) as a nonreactive control were used. After incubation with primary antibodies, the cells were washed and incubated on ice for 45 min with secondary antibody conjugated with Alexa-488 (Molecular Probes), washed, and analyzed using FACS Calibur™ flow cytometer (BD).

In vitro differentiation

Passage 2 or 3 cells were plated at a density of 25,000 cells/cm^2 at 24 h prior to induction of differentiation. Growth medium containing 10% FBS and 1% penicillin–streptomycin was supplemented with 100 nM dexamethasone, 50 μM L-ascorbic acid 2-phosphate, and 10 mM glycerol 2-phosphate for osteogenic induction and 1 μM dexamethasone, 500 μM 3-isobutyl-1-methylxanthine (IBMX), 100 μM indomethacin, and 10 μg/mL insulin for adipogenic induction. Treatment media were changed twice a week. All chemicals were purchased from Sigma.

Accumulation of lipid droplets in adipocytes was determined by Oil Red O (ORO) staining at 7, 14, and 21 days postinduction. Briefly, 4% paraformalde-hyde (PFA)-fixed cells were washed with 60% isopropanol, dried for 30 min at room temperature, and incubated with 2 mg/mL ORO solution in isopropanol:water at a ratio of 3:2. Cells were washed 4 times with water and photographed. For quantitative analysis, bound ORO was eluted with 100% isopropanol and optical density was measured at 500 nm.

Osteoblasts were analyzed for the formation of calcified matrix by Alizarin Red S (ARS) staining at 7, 14, and 21 days postinduction. Briefly, 4% PFA-fixed cells were washed with water, incubated for 30 min at room temperature with 20 mg/mL ARS solution, washed 4 times with water, and photographed. For quantitative analysis, the color intensity was determined using Scion Image Analysis software (National Institutes of Health, Bethesda, MD).

Reverse transcription–polymerase chain reaction analysis

Total RNA was isolated from control cells and from cells induced toward adipogenesis and osteogenesis at 1, 2, 3, 7, 14, and 21 days postinduction using a commercial RNA-aqueous kit (Ambion). RNA samples were treated with DNase I using DNA-free™ kit (Ambion) and applied to first-strand cDNA synthesis at 50°C for 60 min with SuperScript™ III Reverse Transcriptase kit (Invitrogen) using Oligo(dT)$_{20}$ primers. mRNA expression analysis was performed using semiquantitative reverse transcription (RT)–polymerase chain reaction (PCR) with FirePol Master Mix (Solis Biodyne). 7900HT Fast Real Time qPCR System (Applied Biosystems) and Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) were used for

Table 1. Primer Sets used in the Present Study

Primer name	*Orientation*	*Sequence 5′–3′*	*Transcript ID*
PPARγ2	Sense	TCCATGCTGTTATGGGTGAA	NM_015869.4
	Antisense	TCAAAGGAGTGGGAGTGGTC	
C/EBPα	Sense	AACCTTGTGCCTTGGAAATG	NM_004364.2
	Antisense	CCCTATGTTTCCACCCCTTT	
FABP4	Sense	AACCTTAGATGGGGGTGTCC	NM_001442.2
	Antisense	TGGTTGATTTTCCATCCCAT	
RUNX2	Sense	GGAGGGACTATGGCATCAAA	NM_001015051.2
	Antisense	GCTCGGATCCCAAAAGAAGT	NM_001024630.2
ALP	Sense	CACGTCTTCACATTTGGTGG	NM_000478.3
	Antisense	GCAGTGAAGGGCTTCTTGTC	
ZNF423	Sense	TCAATTTACACCTGCGATCACTG	NM_015069.2
	Antisense	GTTGTGGGTCGTCATCACCA	
SHARP1	Sense	GCATGAAACGAGACGACACCA	NM_030762.2
	Antisense	TGCTCGGTTAAGGCGGTTAAA	
GAPDH	Sense	CTCTCTGCTCCTCCTGTTCGAC	NM_002046.3
	Antisense	TGAGCGATGTGGCTCGGCT	

ALP, alkaline phosphatase; C/EBPα, CCAAT/enhancer binding protein alpha; FABP4, fatty acid binding protein 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RUNX2, runt-related transcription factor 2; SHARP1, basic helix-loop-helix transcription factor.

real-time (qRT-PCR) analysis. Primer sets used for amplification are listed in Table 1. mRNA expression values relative to GAPDH were calculated according to Applied Biosystems' Comparative Ct Method [40].

Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate) with 1×Protease Inhibitor Coctail (Roche) for protein extraction at indicated time points following initiation of differentiation. Twenty-eight micrograms of protein lysate was separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membrane using a semidry blotting system (Amersham Biosciences). The membrane was stained with Ponceau dye (Amresco) for loading control prior to blocking with 4% nonfat dry milk solution in 1×TBS–0.1% Tween 20 for 2 h at room temperature. Primary antibody incubations were performed overnight at 4°C at dilutions 1:1,000 for mouse anti-human PPARγ (Chemicon), 1:1,000 for rabbit anti-human C/EBPβ (Santa Cruz), and 1:2,000 for mouse anti-human adiponectin (Chemicon). Secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (Abcam) incubations at 1:10,000 dilutions were performed for 1 h at room temperature, followed by washing and signal visualization with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Data analysis

Differentiation assay and mRNA expression analysis were performed in triplicates using cells from 2 donors. Values for ORO or ARS staining for each time point were obtained by subtracting mean values of control cells from mean values of treated cells for each donor. The resulting values were then statistically compared (2 against 2) using Student's *t*-test ($\alpha = 0.05$), assuming equal 2-sample variance.

PPARγ2, *C/EBPα*, and *FABP4* mRNA expression values were calculated relative to their expression at day 0 time point in AdMSCs from one of the donors. Basic helix-loop-helix transcription factor (*SHARP1*) and *ZNF423* mRNA expression values were calculated relative to their expression at day 0 time point in AdMSCs. Each value for mRNA expression (from 2 donors in triplicate) was separately included in the statistical comparison using Student's *t*-test ($\alpha = 0.05$), assuming equal 2-sample variance. Single-factor analysis of variance was used to compare relative *SHARP1* and *ZNF423* mRNA expression values between different time points during differentiation.

Results

AdMSCs and FBs share similar phenotype and differentiation potential

Similarity of AdMSCs and tissue FBs (stromal cells) has been reported in several publications. Flow cytometry analysis of early passage AdMSCs and dermal FBs isolated from 2 individuals showed that both cell types express surface molecules 5′-nucleotidase/CD73, Thy1/CD90, and endoglin/CD105 that are characteristic to MSCs and lack the expression of leukocyte marker CD45 (Fig. 1). In general, we observed AdMSCs to be morphologically more heterogeneous and to exhibit lower growth rate in cell culture compared with FBs.

To compare the adipogenic and osteogenic differentiation potential of isolated AdMSCs and FBs, passage 2 or 3 cells were plated at 24 h prior to addition of differentiation media supplemented with dexamethasone, IBMX, indomethacin, and insulin for induction of adipogenesis or with dexamethasone, L-ascorbic acid 2-phosphate, and glycerol 2-phosphate for induction of osteogenesis. Cells were cultured for 21 days and the formation of lipid droplets, characteristic to mature adipocytes, and matrix mineralization, characteristic to bone cells, was determined with ORO staining or calcium phosphate staining with ARS, respectively. Induced AdMSCs and FBs developed into cells with positive staining for ORO and ARS (Fig. 2A) by day 21, indicating that FBs and AdMSCs have similar developmental potential. Quantification of lineage-specific staining was performed on days 7, 14, and 21 postinduction by measuring the optical density of eluted ORO at 500 nm or by image analysis of ARS-stained cells using

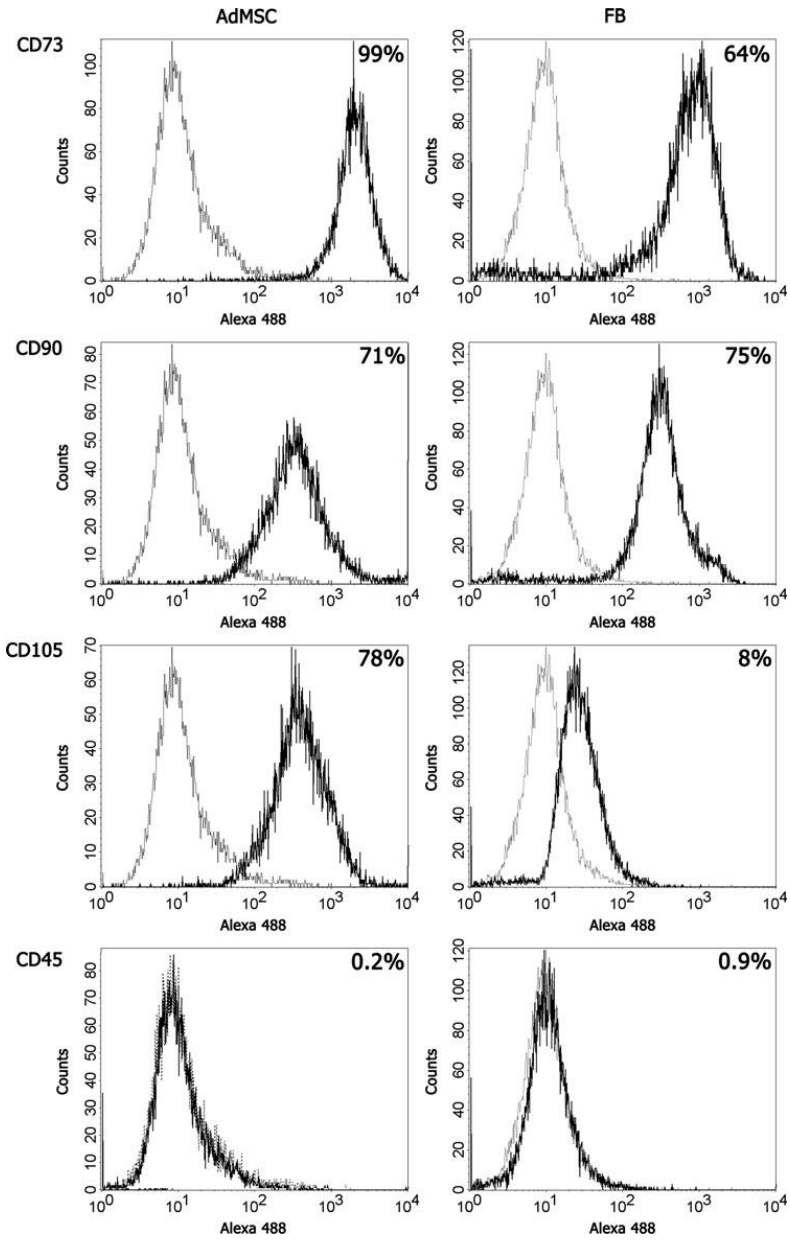

FIG. 1. AdMSCs and FBs express MSC-specific surface markers. AdMSCs (*left column*) and FBs (*right column*) were stained with antibodies against indicated surface molecules and analyzed using flow cytometry. Histograms depict positive antibody reactivity (bold line) in relation to irrelevant V5-tag antibody staining (regular line) and are representative of cells from 2 donors. AdMSCs, adipose tissue-derived mesenchymal stem cells; FBs, fibroblasts.

Scion Image software. ORO staining demonstrated relatively high lipid level produced by AdMSCs on day 7, which remained unchanged throughout the 21 days (Fig. 2B). In contrast, FBs showed weak staining for ORO on days 7 and 14, but gradually reached the level similar to that of AdMSCs by day 21, suggesting that FBs exhibit a delayed response to adipogenic induction compared with AdMSCs, but it does not affect their final differentiation. AdMSCs and FBs showed similar ARS staining intensity along osteogenic differentiation, which increased in time and became slightly higher in FBs by day 21 (Fig. 2C), indicating that both cell types have similar response to osteogenic induction. The formation of adipocytes and osteoblasts from AdMSCs and FBs was further confirmed by demonstration of induction of *PPARγ2* and *C/EBPα* (adipocytes) or *RUNX2* and alkaline phosphatase (*ALP*) (osteoblasts) using RT-PCR (Fig. 2D).

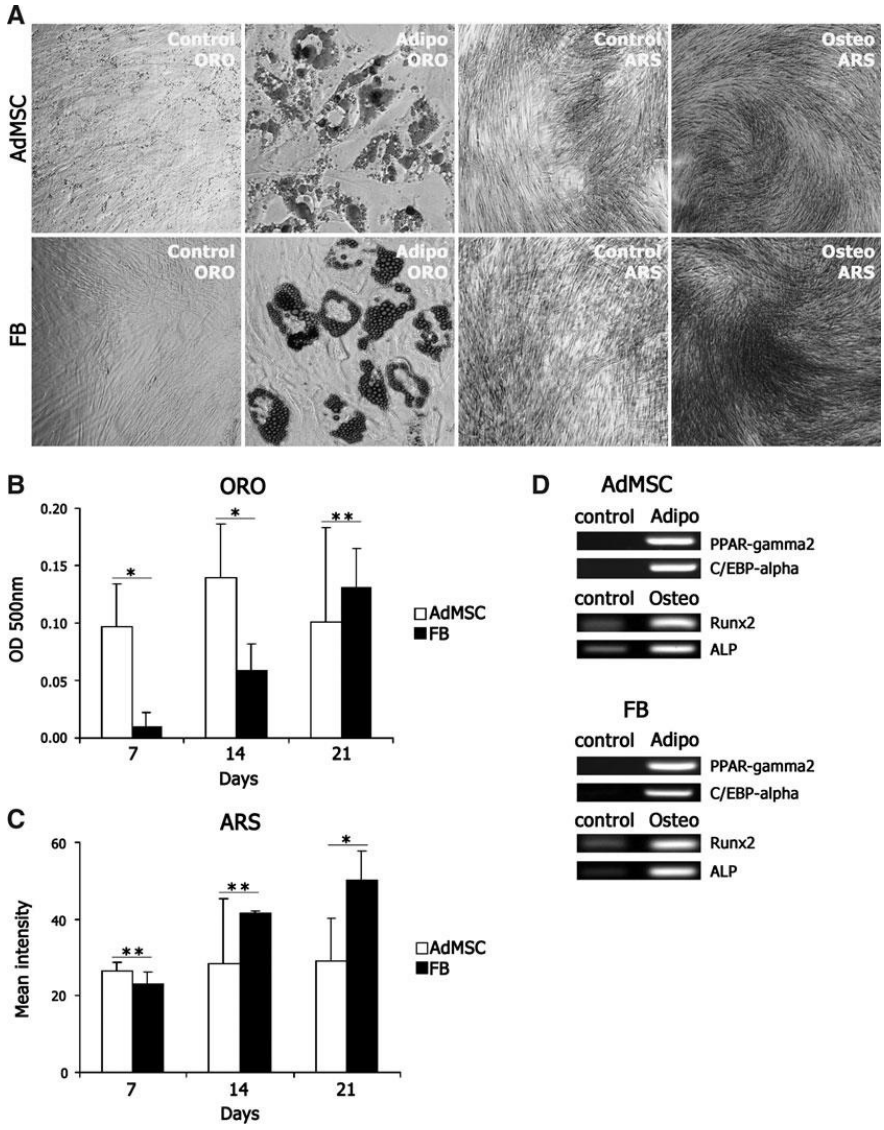

FIG. 2. AdMSCs and FBs differentiate into adipocytes and osteoblasts with a delay in adipogenic differentiation of FBs. ORO and ARS staining of unstimulated cultures (control) and adipogenic or osteogenic cultures, respectively, following 21 days of differentiation. **(A)** *Upper panel*, AdMSCs; *lower panel*, FBs. Quantification of **(B)** ORO staining at indicated time points during adipogenic differentiation by measuring optical density of eluted dye at 500 nm or of **(C)** ARS staining during osteogenic differentiation by analyzing mean color intensity per culture well using Scion Image software. Error bars represent mean $\pm$ SD of 2 donors. Statistical significance (Student's *t*-test): $^{*}P < 0.05$, $^{**}P > 0.05$. *White columns*, AdMSCs; *black columns*, FBs. mRNA expression profile of the adipogenic markers peroxisome proliferator-activated receptor gamma (*PPARγ2*) and CCAAT/enhancer binding protein alpha (*C/EBPα*) and the osteogenic markers runt-related transcription factor 2 (*RUNX2*) and alkaline phosphatase (*ALP*) in AdMSCs (*upper panels*) and FBs (*lower panels*) of unstimulated cultures (control) and 14-day adipogenic or osteogenic cultures, respectively **(D)**. ARS, Alizarin Red S; ORO, Oil Red O.

Adipogenesis of dermal FBs is delayed compared with AdMSCs

To explore the differences in adipogenic induction of AdMSCs and FBs, the expression of well-characterized adipogenic transcription factors was studied in both cell types using western blot analysis. Cells were plated at a density of 10,000 cells/cm^2 at 3 days prior to adipogenic induction and protein lysates were prepared from both cell types on days 0, 1, 3, 5, and 7 postinduction. Western blot analysis of C/EBPβ, PPARγ, and its target gene adiponectin confirms the onset of differentiation in both AdMSCs and FBs (Fig. 3A). Nearly identical induction of C/EBPβ protein was detected during differentiation of both cell types. In contrast, induction of PPARγ was different in FBs compared with AdMSCs. PPARγ expression was induced within 24 h following the start of differentiation in AdMSCs, whereas induction of PPARγ in FBs was delayed 3 days. The lag in PPARγ protein expres-

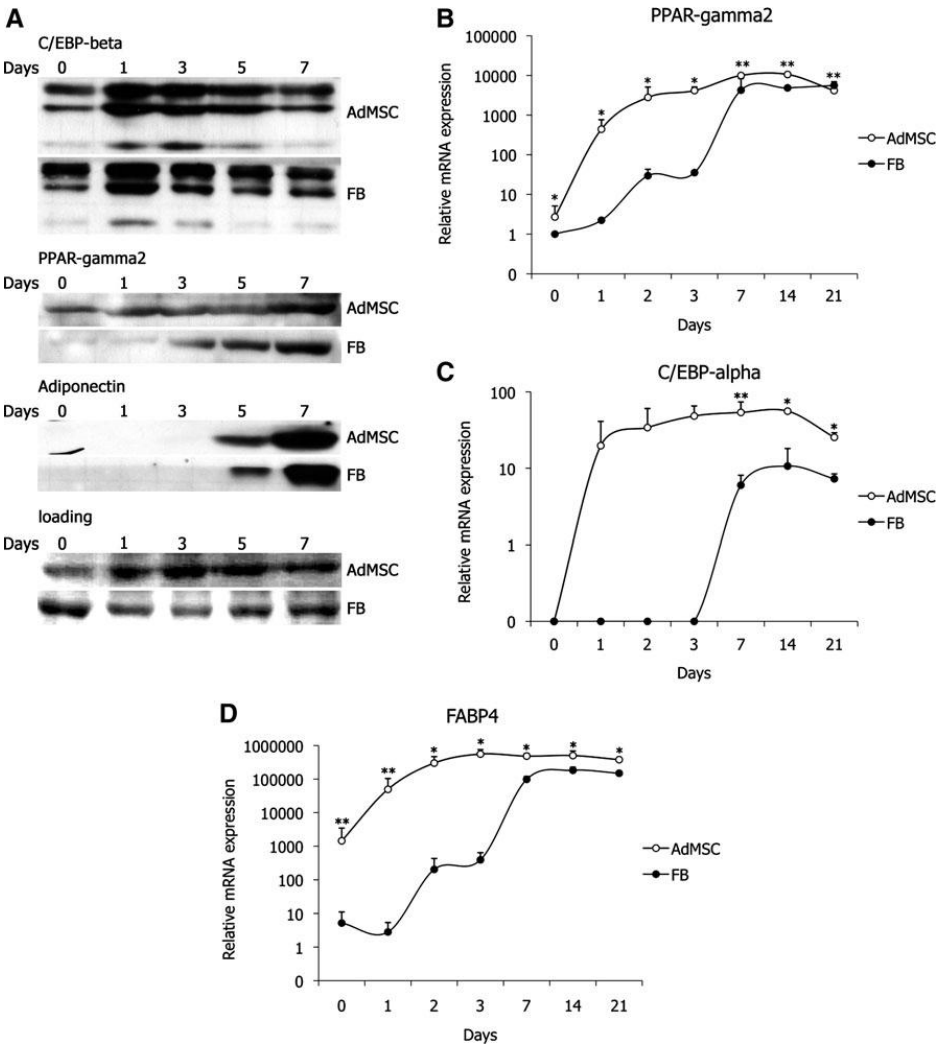

FIG. 3. AdMSCs and FBs express adipocyte markers, but the induction of marker expression is delayed in FBs compared with AdMSCs. Western blot analysis of adipogenic proteins at indicated time points in AdMSCs (*upper rows*) and FBs (*lower rows*) stimulated with adipogenic medium. Ponceau-stained membrane serves as a loading control **(A)**. Relative mRNA expression levels of the adipogenic markers *PPARγ2* **(B)**, *C/EBPα* **(C)**, and *FABP4* **(D)** at indicated time points upon adipogenic induction determined by real-time (qRT)–polymerase chain reaction. Values on *Y*-axis are presented in logarithmic scale. Error bars represent mean ± SD of 2 donors. Statistical significance (Student's *t*-test): $*P < 0.05$, $**P > 0.05$. *Unfilled circles*, AdMSCs; *filled circles*, FBs. FABP4, fatty acid binding protein 4.

sion early in adipogenic induction strongly correlates with the delayed formation of lipid vacuoles in FBs during adipogenic differentiation (Fig. 2B).

To further elucidate the induction of lineage-specific genes during adipogenesis in AdMSCs and FBs, expression of *PPARγ2*, *C/EBPα*, and *FABP4* was analyzed using qRT-PCR on days 0, 1, 2, 3, 7, 14, and 21 following induction of differentiation. Rapid induction of mRNA expression of all 3 genes within 1 day upon induction of adipogenesis with further elevation by day 3 (Fig. 3B–D) was observed in AdMSCs. Interestingly, expression of *PPARγ2* and *FABP4* was only slightly stimulated in FBs (Fig. 3B, D), and no expression of *C/EBPα* mRNA was detected (Fig. 3C). The expression of *PPARγ2*, *C/EBPα*, and *FABP4* was induced between 3 and 7 days of adipogenic induction; however, the mRNA level of *C/EBPα* in FBs never reached the level of that in AdMSCs during the 21-day differentiation assay. Taken together, the mRNA expression of adipocyte-related transcription factors and their target genes in response to adipogenic stimulation occurs much faster in AdMSCs compared with FBs, with a lag period between 3 and 7 days in FBs.

Delayed adipogenesis is not due to inhibition of C/EBPβ activity in FBs

As a master regulator of adipogenesis, C/EBPβ activity is regulated by numerous transcription factors by protein/protein interactions or DNA binding. It has been shown that the transcription factors RORα, SHARP1, and pseudokinase TRB3 suppress the activity of C/EBPβ in several cell types [41–43]. We studied the expression of *RORα*, *SHARP1*, and *TRB3* using qRT-PCR in AdMSCs and dermal FBs following adipogenic induction. *SHARP1* expression was transiently downregulated following adipogenic induction and upregulated again by day 7 in both AdMSCs and FBs (Fig. 4A). This is consistent with previously published data [42]. *RORα* and *TRB3* showed similar expression patterns (data not shown). Together, our results demonstrate that the expression of the inhibitors of C/EBPβ activity is equally suppressed in AdMSCs and FB upon adipogenic induction, suggesting that the delayed adipogenesis of FBs compared with AdMSCs cannot be explained by the inhibition of C/EBPβ activity by RORα, SHARP1, or TRB3 in FBs.

ZNF423, a regulator of PPARγ expression, shows delayed induction in FBs

It has been shown that PPARγ expression can be regulated independently from the C/EBPβ activity. Recently, a zinc-finger protein ZFP423 (ZNF423 in humans) was identified as a critical regulator of PPARγ expression in the preadipocyte state of murine FBs [25]. We analyzed the expression of *ZNF423* using qRT-PCR in AdMSCs and FBs following adipogenic induction. Expression of *ZNF423* was induced on day 2 postinduction and was further induced by day 3 (Fig. 4B) in treated AdMSCs. In contrast, no induction of *ZNF423* expression was observed in FBs until day 7 of adipogenic induction. The results indicate that ZNF423 could regulate PPARγ expression in AdMSCs and FBs, and therefore, its late induction can be involved in the delayed adipogenic differentiation of FBs. The specific differences in the molecular mechanisms of early stages of induced adipogenesis of AdMSCs and FBs remain to be elucidated.

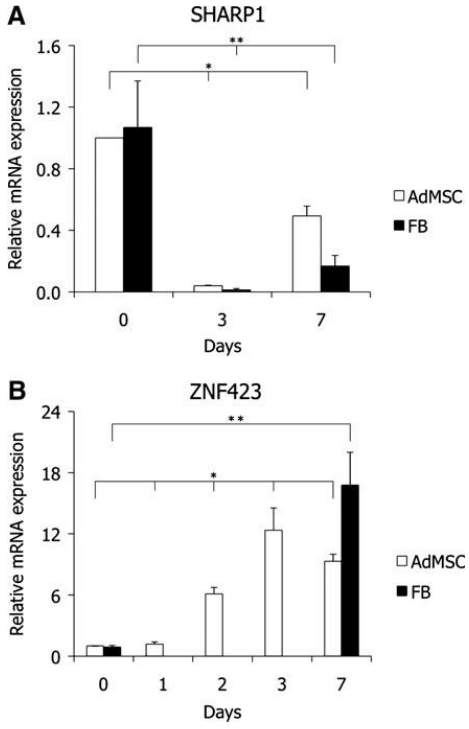

FIG. 4. (*SHARP1*) and *ZNF423* expression in AdMSCs and FBs during adipogenic differentiation. Results of qRT–polymerase chain reaction analysis show transient downregulation of *SHARP1* mRNA levels in both AdMSCs and FBs **(A)**, whereas *ZNF423* mRNA levels are induced on day 3 in AdMSCs and on day 7 in FBs **(B)**. Error bars represent mean ± SD of 2 donors. Statistical significance (analysis of variance): $*P < 0.01$, $**P < 0.05$. *White columns*, AdMSCs; *black columns*, FBs.

Discussion

Adult MSCs possess stem cell-like properties such as self-renewal and differentiation into a variety of cell types of mesodermal origin. In vitro studies have led to the use of a limited collection of surface molecules expressed by MSCs and differentiation of cells toward mesenchymal lineages such as adipocytes, osteoblasts, and chondrocytes to confirm the MSC-like nature of isolated cells. Several adult tissues have been found to contain MSCs, including subcutaneous adipose tissue that holds a great promise for therapeutic applications as an easily accessible source of MSCs.

FBs are present in many tissues including adipose tissue, bone marrow, skin, synovium, and cord blood [44,45] and they have many characteristics of MSCs. There are no MSC-specific markers or profiles of markers available that clearly distinguish MSCs from FBs (stromal cells). The minimal criteria proposed by the Mesenchymal and Tissue Stem Cell

Committee of the International Society for Cellular Therapy, to distinguish MSCs from other cell types, has proven not to be sufficient because all the characteristics described for MSCs are equally possessed by FBs [46]. In this study, we further demonstrate that dermal FBs and AdMSCs express the same set of surface antigens, marking FBs and AdMSCs as phenotypically indistinguishable cell types. Gene expression profiling has uncovered slight differences between MSCs and FBs [21]; however, similar differences also exist between different populations of MSCs [14]. Translation of the variation in gene expression into functional characteristics of these cells, for example, multipotent differentiation capacity, has not been achieved until today, leaving the question about the different identities of MSCs and FBs unanswered.

Assessment of in vitro mesenchymal differentiation is commonly performed by detection of lineage-specific marker expression or cytochemical staining of terminally differentiated cells grown under stimulating conditions for 2–4 weeks. In accordance with previous reports, our work shows that by the end of the long-term differentiation assay, both AdMSCs and FBs have differentiated into adipocytes and osteoblasts, indicating that these cells are developmentally equivalent.

The process of differentiation of multipotent cells into functionally distinct mature cell types requires a sequential expression of genes that determine the phenotype of the differentiated cells. FBs and AdMSCs have similar dynamics of osteogenic differentiation based on the analysis of calcium phosphate deposits using staining with ARS. In contrast, analysis of adipogenic differentiation of AdMSCs and FBs using ORO staining showed significant differences in the dynamics of differentiation between MSCs and FBs. AdMSCs and FBs exhibit different degrees of adipocyte-specific staining early during adipogenic differentiation, whereas this difference was not detectable after 3 weeks of differentiation.

One of the earliest events in adipogenesis is the induction of transcription factor C/EBPβ, which together with C/EBPδ activates the expression of transcription factors PPARγ and C/EBPα which, in turn, orchestrate the expression of full adipogenic differentiation program. Analysis of expression of C/EBPβ transcription factor showed no difference in AdMSCs and FBs during adipogenic differentiation. On the contrary to the induction of C/EBPβ expression, induction of PPARγ expression was significantly delayed in FBs compared with AdMSCs, raising the question of different regulatory mechanisms controlling the early stages of adipogenic differentiation of MSCs and FBs. Analysis of *PPARγ*, *C/EBPα*, and *FABP4* expression clearly demonstrated delayed induction of these genes in FBs compared with MSCs following induction of adipogenesis. Interestingly, in FBs, the mRNA level of *C/EBPα*, the second most important transcription factor in adipogenesis, never reached the level of that in AdMSCs. Despite these differences, known markers for adipocytes such as *FABP4* and adiponectin showed similar expression levels in FB- and AdMSC-derived adipocytes. This could be explained by previous observations that C/EBPβ and C/EBPδ induce adipogenesis with no stimulation of C/EBPα expression in FBs [24]. Further, it is noteworthy that the mRNA of *C/EBPα* is undetected until day 7 when *PPARγ* mRNA level peaks in FBs. This finding is in correlation with the fact that C/EBPβ cannot induce the expression of C/EBPα without PPARγ, which is required to release histone deacetylase 1 (HDAC1) from C/EBPα promoter [47,48].

As C/EBPβ protein was equally induced in AdMSCs and FBs during adipogenic stimulation and the induction of PPARγ expression was delayed in FBs, we analyzed several potential inhibitors of C/EBPβ activity in FBs and AdMSCs following adipogenic induction. The expression of PPARγ depends on the activity of C/EBPβ, which binds *PPARγ* promoter to activate its transcription. We analyzed whether C/EBPβ activity could be suppressed in FBs by known negative regulators and therefore result in the delayed induction of PPARγ. Analysis of expression of well-known inhibitors of C/EBPβ activity, such as transcription factors *SHARP1* and *RORα* as well as a pseudokinase *TRB3*, did not reveal any difference in the expression in AdMSCs and FBs following adipogenic induction. Transient downregulation of the expression of these regulators upon adipogenic induction was occurring similarly in both cell types. We concluded that the activity of C/EBPβ protein induced upon adipogenic stimulation in AdMSCs and FBs was not inhibited in FBs for an extended period of time to cause the delayed induction of PPARγ and adipogenic differentiation in these cells. PPARγ expression and adipogenesis are also regulated by Wnt and cell cycle-related regulatory proteins that function independently of C/EBPβ. For example, WNT10b shifts stromal cell development from adipogenesis toward osteoblast differentiation by suppressing the expression of PPARγ and C/EBPα [49,50]. pRB has been reported to associate with PPARγ, together with HDAC3, leading to the inhibition of adipogenesis [51]. The analysis of C/EBPβ-independent factors as potential regulators of delayed adipogenesis of FBs was beyond the scope of the present work.

One of the few factors known to define the adipose-lineage–committed FBs is a zinc finger transcription factor ZFP423, which controls preadipocyte determination of murine FBs [25] by regulating PPARγ expression. Our data show that the expression of *ZNF423* was detected 2–3 days following adipogenic induction in AdMSCs, whereas in FBs it became detectable after 7 days of induction. These results indicate that ZNF423 could regulate PPARγ expression in AdMSCs and FBs upon adipogenic induction, and therefore, its late induction can be involved in the delayed adipogenic differentiation of FBs.

Presented data suggest that AdMSCs and FBs may possess different mechanisms for the regulation of the initial stages of adipocyte differentiation that involves distinct regulation of PPARγ expression.

Acknowledgment

The authors thank Sten Linnarsson for useful comments on the manuscript.

Author Disclosure Statement

None of the authors has any disclosure to declare. No competing financial interests exist.

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Received for publication June 30, 2010
Accepted after revision December 8, 2010
Prepublished on Liebert Instant Online December 8, 2010

PUBLICATION II

Jääger K, Islam S, Zajac P, Linnarsson S, Neuman T. (2012). RNA-seq analysis reveals different dynamics of differentiation of human dermis- and adipose-derived stromal stem cells. PLoS One, 7(6):e38833.

OPEN ACCESS Freely available online

PLoS one

RNA-Seq Analysis Reveals Different Dynamics of Differentiation of Human Dermis- and Adipose-Derived Stromal Stem Cells

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Abstract

Background: Tissue regeneration and recovery in the adult body depends on self-renewal and differentiation of stem and progenitor cells. Mesenchymal stem cells (MSCs) that have the ability to differentiate into various cell types, have been isolated from the stromal fraction of virtually all tissues. However, little is known about the true identity of MSCs. MSC populations exhibit great tissue-, location- and patient-specific variation in gene expression and are heterogeneous in cell composition.

Methodology/Principal Findings: Our aim was to analyze the dynamics of differentiation of two closely related stromal cell types, adipose tissue-derived MSCs (AdMSCs) and dermal fibroblasts (FBs) along adipogenic, osteogenic and chondrogenic lineages using multiplex RNA-seq technology. We found that undifferentiated donor-matched AdMSCs and FBs are distinct populations that stay different upon differentiation into adipocytes, osteoblasts and chondrocytes. The changes in lineage-specific gene expression occur early in differentiation and persist over time in both AdMSCs and FBs. Further, AdMSCs and FBs exhibit similar dynamics of adipogenic and osteogenic differentiation but different dynamics of chondrogenic differentiation.

Conclusions/Significance: Our findings suggest that stromal stem cells including AdMSCs and dermal FBs exploit different molecular mechanisms of differentiation to reach a common cell fate. The early mechanisms of differentiation are lineage-specific and are similar for adipogenic and osteogenic differentiation but are distinct for chondrogenic differentiation between AdMSCs and FBs.

Citation: Jääger K, Islam S, Zajac P, Linnarsson S, Neuman T (2012) RNA-Seq Analysis Reveals Different Dynamics of Differentiation of Human Dermis- and Adipose-Derived Stromal Stem Cells. PLoS ONE 7(6): e38833. doi:10.1371/journal.pone.0038833

Editor: Wolfgang Wagner, RWTH Aachen University Medical School, Germany

Received January 24, 2012; **Accepted** May 15, 2012; **Published** June 19, 2012

Funding: The authors have no support or funding to report.

Competing Interests: The authors have read the journal's policy and have the following conflicts: Kersti Jääger: Paid employment at Cellin Technologies LLC. Status - compensated. Toomas Neuman: Ownership of stocks or shares at Protobios LLC. Status - compensated. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

Tissue regeneration is dependent on progenitor cells that self-renew and differentiate into different cell types with specialized functions. Mesenchymal stem cells (MSCs) have been isolated from many different adult organs and tissues including skin, lung, liver and fat [1–4]. *In vitro* studies have demonstrated that MSCs can be expanded in culture and differentiated into several cell types under appropriate conditions. In addition to fat, bone and cartilage cells, MSCs have been demonstrated to give rise to muscle and nerve cells *in vitro* [4–7].

In contrast, differentiation of dermal fibroblasts (FBs) into various mesodermal cell types under similar conditions has produced contradictory results. In some experimental settings FBs were shown to lack multilineage differentiation potential [8,9], whereas other reports show that FBs and MSCs can be equally differentiated into several types of mesodermal cells [10–13]. Also, we have previously shown that dermal FBs and adipose tissue-derived MSCs (AdMSCs) originating from the same donors both differentiate into osteoblasts and adipocytes [14]. The immunophenotypes of MSCs and FBs are similar based on numerous surface markers currently used to identify MSCs. Both cell types express cell surface antigens CD73, CD90 and CD105 [9,10,13].

The molecular characterization of MSCs is hampered by the lack of biomarkers that would allow their selective isolation from different tissue sources with heterogeneity of cell populations. MSCs are currently isolated as plastic-adherent cells with fibroblast-like morphology that can be differentiated into several mesodermal cell types [15]. These parameters are not sufficient to discriminate MSCs from FBs and do not aid in the understanding of the identity of these cell types. Another problem is comparison of different types of stromal cells including dermal FBs and AdMSCs isolated from individuals with different genetic backgrounds. This could lead to differences in gene expression patterns and cellular functions that cannot directly be associated with distinct cell identities.

Here we aimed to analyze the transcriptome profiles of several differentiated cells starting from AdMSCs and dermal FBs

obtained from two matching donors and differentiated under similar experimental conditions towards adipocytes, osteoblasts and chondrocytes (Figure 1A). RNA-seq-derived gene expression data was compared by a multi-group ANOVA, and differences between groups other than those used in the ANOVA were then visualized using principal component analysis (PCA). To our knowledge, this is the first study to compare the dynamics of differentiation of AdMSCs and FBs into three mesodermal cell types on global scale.

Results

Transcriptome Profiles of Multipotent AdMSCs and FBs

Both AdMSCs and FBs exhibit adipo-, osteo- and chondrogenic developmental potential. Prior to the analysis of the global gene expression profiles of differentiating AdMSCs and FBs in more detail, we aimed to verify that both of these cell populations exhibit multipotency. Cells derived from two donors were plated at 72 h prior to addition of differentiation media and cultivated for 14 days until analysis (see Materials and Methods). *In vitro* differentiation of AdMSCs and FBs was confirmed by detection of formation of lipid droplets with Oil Red O staining (ORO, adipocytes), matrix mineralization with Alizarin Red S staining (ARS, osteoblasts) or formation of proteoglycan-rich matrix with Alcian Blue staining (AB, chondrocytes). Induced AdMSCs and FBs (from both donors) differentiated into cells with positive staining for ORO, ARS and AB confirming the similar developmental capacity of these cell types (Figure 1B). Quantification of lineage-specific staining showed that the differentiation potential of FBs and AdMSCs is indeed comparable (Figure 1B, lower panel shows staining intensities of FBs relative to AdMSCs). This analysis together with previous reports [10,13,14] confirms that multipotency is not solely restricted to AdMSCs but is also characteristic to fibroblasts. Immunophenotyping showed that AdMSCs and FBs from both donors expressed cell surface antigens CD73 and CD105 (data not shown).

Global transcriptome profiling reveals AdMSC- and FB-specific gene expression patterns. For transcriptome analysis, cells were treated as described in Materials and Methods section and RNA was isolated every 24 h on days 0–7 upon adipogenic, osteogenic and chondrogenic differentiation. Single sequencing library was then generated from the resulting 96 RNA samples (Table S1) using a method by Islam *et al*, 2011 [16] with slight modifications (see Materials and Methods). Deep sequencing yielded 45 million mapped reads and 60% of those mapped to known transcripts in the human genome. 9000 most highly expressed features with normalized hit values ranging from 6.25 to 23 437.5 transcripts per million (t.p.m), that cover 99% of the transcripts and include both the most highly expressed genes as well as rare transcripts, were used in gene expression analysis. Five samples were removed from the analysis (Table S1) due to unsatisfactory RNA quality (total read number was below 0.01% of all samples). Each time point in the assay was represented by a single replicate except for day 0 that was sequenced in triplicate (three different RNAs). Each sample from the total of 91 was annotated according to its tissue of origin (AdMSC or FB), patient of origin, cell type and time point.

First, we analyzed how different samples are connected to each other using principal component analysis (PCA) on complete gene expression data without prior statistical filtering (Figure 2). The circles in Figure 2 represent individual samples and are visualized according to cell type (undifferentiated cells, adipocytes, osteoblasts and chondrocytes). PCA shows that samples belonging to the same cell group cluster together, except for a few adipocyte-samples and one osteoblast-sample that stay apart from the clusters. Interestingly, undifferentiated cells make up two distinct clusters. The analysis shows that the RNA-seq-derived transriptome profiles are characteristic to different cell types.

The analysis above was performed with unfiltered data. However, PCA can be used to visualize filtered data. We used multi-group ANOVA to compare gene expression between defined groups and then used the ANOVA-filtered data in subsequent PCAs to visualize differences between other groups (not between those used in the ANOVA). The genes were selected for ANOVA based on false discovery rate (FDR) to control the effects for multiple testing. The step-wise filtering and vizualisation of the data was performed with Qlucore Omics Explorer.

Next, we analyzed how different cell types (undifferentiated cells, adipocytes, osteoblasts and chondrocytes) are related to each other based on filtered differences in gene expression. A multi-group ANOVA with a FDR of 0.1% recovered 792 differentially expressed genes between different cell types. PCA was then used to visualize the relationship of the individual samples (Figure 3). The

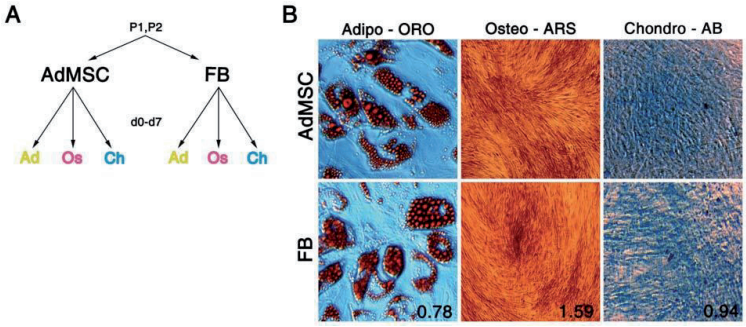

Figure 1. Cell differentiation. A) AdMSCs and FBs were isolated from two patients (P1, P2) and differentiated towards adipocytes, osteoblasts and chondrocytes. RNA was isolated on days 0–7 during differentiation and the resulting 96 RNA samples were used to generate single sequencing library for gene expression analysis. B) *In vitro* differentiation of AdMSCs (upper panel) and FBs (lower panel) was confirmed by ORO staining of adipocyte, ARS staining of osteoblast and AB staining of chondrocyte cultures on day 14 upon induction of differentiation. The quantified stainings of FBs are represented relative to AdMSCs (lower panel; AdMSC = 1). Abbreviations: P, patient; AdMSC, adipose-derived mesenchymal stem cell; FB, fibroblast; Ad, adipocyte; Os, osteoblast; Ch, chondrocyte; d, day; ORO, Oil Red O; ARS, Alizarin Red S; AB, Alcian Blue.
doi:10.1371/journal.pone.0038833.g001

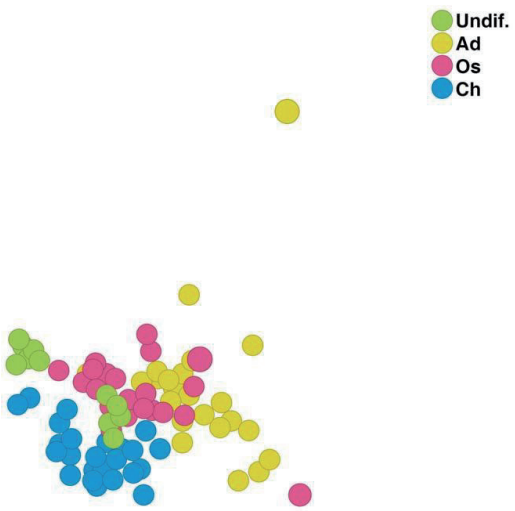

Figure 2. Principal component analysis (PCA) of non-filtered data. 9000 most highly expressed genes were visualized by PCA based on cell type (undifferentiated, adipocytes, osteoblasts and chondrocytes) without prior statistical filtering. Different cell types cluster together upon PCA. Abbreviations: Undif., undifferentiated; Ad, adipocyte; Os, osteoblast; Ch, chondrocyte.
doi:10.1371/journal.pone.0038833.g002

edges in Figure 3 connect each sample with the four other most closely related samples (in A and B). The same PCA plot was used to visualize the samples based on different annotations such as cell type, AdMSC or FB, time group ('early' including days 1–3 and 'late' including days 4–6) and patient of origin. Cell type-based visualization (Figure 3A) shows that 792 genes clearly generate clusters from samples belonging to the same cell group. This is unsurprising, since the ANOVA selected for genes that distinguish between cell types. However, the samples also show a clear separation by time group, demonstrating that those genes that distinguish cell types were also differentially regulated over time. Importantly, undifferentiated cells make up two distinct clusters. One of them (FB) locates separately with no connections to other clusters, whereas the other (AdMSC) is closely connected to chondrocytes (Figure 3B). Upon differentiation, clusters of AdMSCs and FBs become close already on day 1 and stay close in all time groups (Figure 3C). Interestingly, despite the loss of initial differences between AdMSCs and FBs upon differentiation, AdMSC- and FB- specific sub-clusters still remain apparent within adipocytes, osteoblasts and chondrocytes. The samples originating from two patients were intermingled, verifying the reproducible and patient-independent formation of cell type-specific clusters (Figure 3D). ANOVA between the two patients over the total expression data (9000 genes) identified no genes that were significantly (FDR of 1%) differently expressed between the individuals. Hence, the differences between cell types overwhelm any differences between these donors. Since different media was used to cultivate undifferentiated AdMSCs and FBs at optimal conditions (media was chosen so that AdMSCs and FBs exhibited similar growth rate), it cannot be excluded that some of the differences in gene expression between AdMSCs and FBs arise from the different media compositions. Taken together, these data show that AdMSCs and FBs represent initially distinct populations with regard to the expression of developmentally regulated genes, and they also stay subtly distinct in the differentiated state. The *in vitro* development of mature cell types usually takes 2–4 weeks. It is thus possible that the differences between AdMSCs and FBs that are evident after one week of differentiation may disappear after longer differentiation.

Undifferentiated AdMSCs and FBs are Different

AdMSCs and FBs exhibit different gene expression patterns in the undifferentiated state. The observation that undifferentiated AdMSCs and FBs clustered separately based on the expression of 792 lineage-specific genes raised the question how different are AdMSCs and FBs before differentiation. Heat map-view of differentially expressed genes (including 9000 genes) was generated using all replicate samples (5 of AdMSCs and 6 of FBs). The scale in Figure 4A shows the up (red) or down regulation (blue) in standard deviations from the mean expression for each gene. Altogether 62 genes were found to have significantly (FDR of 1%) different expression between AdMSCs and FBs, 38 with higher and 24 with lower expression in FBs than in AdMSCs. ANOVA with five times higher false discovery rate (5%) resulted in 116 more genes (Figure S1). The relatively small number of differentially expressed genes between AdMSCs and FBs could be explained by their common mesodermal origin that probably determines the general transcription profile of the cells. Also, in cell culture, AdMSCs grow as fibroblast-like cells and exhibit morphology similar to FBs, so that the substantial overlap in gene expression patterns between the cells can be expected.

Genes with Various Functions are Distinctly Expressed between AdMSCs and FBs

We then asked whether 62 differentially expressed genes represent functional differences between AdMSCs and FBs. These genes were grouped according to their known function that resulted in six predominant classes (Table 1). 20 genes out of 38 with higher expression in undifferentiated FBs than AdMSCs are related to cell cycle regulation, more specifically to G2/M phase of the cell cycle. Also, the majority of genes involved in the regulation of cytoskeleton stability and in cellular signaling pathways (cell motility - *S100A4*, vesicular trafficking - *CAV1*, *DNM1*) had higher expression in FBs compared to AdMSCs. However, expression of genes associated with either BMP (*GREM1*), VEGF (*MYOF*) or Wnt signaling (*ZRANB1*) was significantly higher in AdMSCs compared to FBs. Most of the genes that participate in the biosynthetic processes or in the regulation of extracellular matrix organization and adhesion had higher expression in AdMSCs than FBs. Interestingly, we identified high expression of developmentally important gene chromobox homolog 8 (*CBX8*) in AdMSCs but not in FBs (Table 1). CBX8 is an essential component of the Polycomb group (PcG) multiprotein PRC1 complex that is required to maintain transcriptionally repressive state of many genes, including Hox genes, throughout development [17]. Whether CBX8 has any functional role in determining the differences between AdMSCs and FBs remains to be elucidated in future studies. Together, our results suggest that despite the similar general characteristics of AdMSCs and FBs, the gene expression profiles are distinct due to differences in expression of genes involved in the regulation of cell cycle and developmental processes and also in the structural organization of the cell.

AdMSCs are more similar to chondrocytes than FBs. The observation that AdMSCs are closely connected to chondrocytes (Figure 3A and B, PCA of developmentally regulated genes) reveals important aspects of differences between AdMSCs and FBs. In search for similarities between AdMSCs and

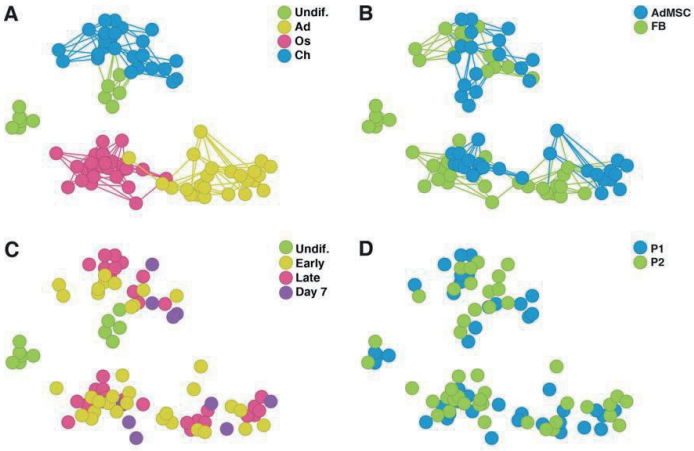

Figure 3. PCA of cell type-specific gene expression. 9000 most highly expressed genes were analyzed by multi-group ANOVA to find differentially expressed genes between cell types: undifferentiated AdMSCs and FBs, and AdMSC- and FB-derived adipocytes, osteoblasts and chondrocytes using false discovery rate (FDR) of 0.1%. PCA of the resulting 792 genes was used to visualize the relationship of the samples based on annotations such as A) cell type, B) cell origin (AdMSC or FB), C) time groups of differentiation and D) patient. Each circle represents one sample, and is connected by edges to four other most closely related samples in A and B. The same genes that separate different cell types, also separate undifferentiated AdMSCs and FBs and are regulated over time with no differences between patients. However, AdMSCs and FBs retain characteristic gene expression even in the differentiated state. Abbreviations: Undif., undifferentiated; Ad, adipocyte; Os, osteoblast; Ch, chondrocyte; AdMSC, adipose-derived mesenchymal stem cell; FB, fibroblast; P, patient.
doi:10.1371/journal.pone.0038833.g003

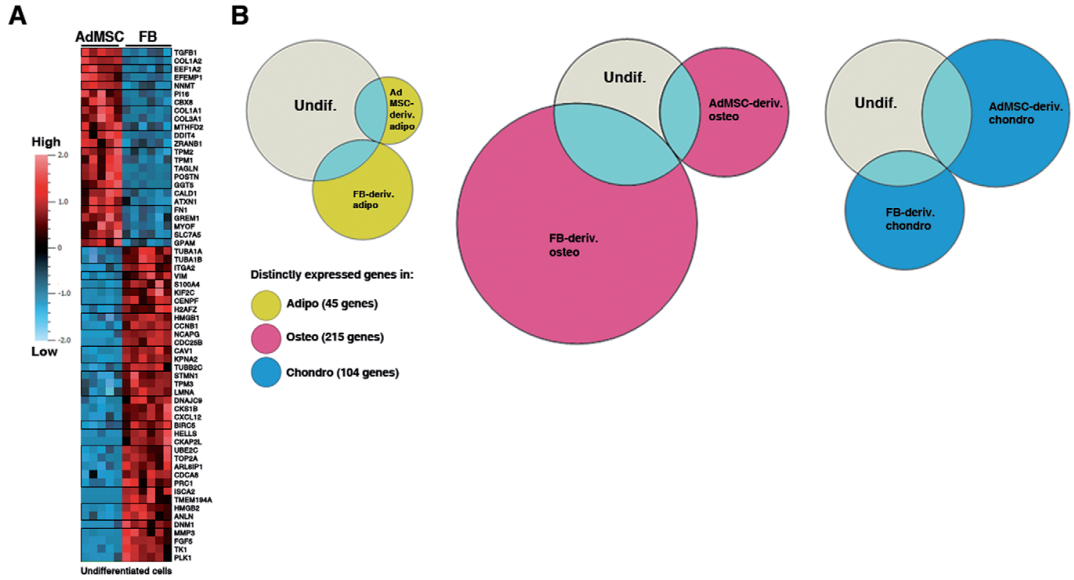

Figure 4. Differences in gene expression of AdMSCs and FBs. A) ANOVA with FDR of 1% between undifferentiated AdMSCs (5 replicates) and FBs (6 replicates) recovered 62 differentially expressed genes, 24 with higher and 38 with lower expression in AdMSCs than FBs. The scale shows the up (light red) or down regulation (light blue) in standard deviations from the mean expression for each gene. B) Comparison of differentially expressed genes between AdMSCs and FBs in the undifferentiated state (light grey) and in AdMSC- and FB-derived adipocytes, osteoblasts and chondrocytes using Venn diagram. Many genes remain (light blue) and many differentiation-related genes become (yellow, pink or blue) differentially expressed in AdMSC- and FB-derived differentiated cells. Abbreviations: AdMSC, adipose-derived mesenchymal stem cell; FB, fibroblast; Undif., undifferentiated; Adipo, adipocyte; Osteo, osteoblast; Chondro, chondrocyte; deriv., derived.
doi:10.1371/journal.pone.0038833.g004

Table 1. Distinctly expressed genes between undifferentiated AdMSCs and FBs (based on ANOVA with FDR of 1%).

	Genes with higher expression in:	
	FBs	**AdMSCs**
Cell cycle	ANLN, BIRC5, CCNB1, CDC25B, CDCA8, CENPF, CKS1B, H2AFZ, HELLS, HMGB1, HMGB2, KIF2C, KPNA2, LMNA, NCAPG, PLK1, PRC1, TK1, TOP2A, UBE2C	
Cytoskeleton	CKAP2L, STMN1, TPM3, TUBA1A, TUBA1B, TUBB2C, VIM	CALD1, TAGLN, TPM1, TPM2
Extracellular matrix and adhesion	ITGA2, MMP3	COL1A1, COL1A2, COL1A3, EFEMP1, FN1, POSTN, TGFBI
Biosynthesis	ISCA2	EEF1A1, GGT5, GPAM, MTHFD2, NNMT, PI16, SLC7A5
Signal transduction	CAV1, CXCL12, DNM1, FGF5, S100A4	GREM1, MYOF, ZRANB1
Development		CBX8

doi:10.1371/journal.pone.0038833.t001

chondrocytes, 792 differentially expressed genes were analyzed to identify genes that are highly expressed in AdMSCs and chondrocytes but not in FBs. We compared the expression of genes in undifferentiated cells and in day 1 AdMSC- and FB-derived chondrocytes, since gene expression patterns become similar at later time points of chondrogenic differentiation. As few as 23 genes were found to have higher expression in AdMSCs and AdMSC- and FB-derived chondrocytes compared with undifferentiated FBs (Table 2). The genes were grouped into five functional classes including cytoskeleton, extracellular matrix and adhesion, processes of biosynthesis, signal transduction and development. The majority of genes that were enriched in AdMSCs and chondrocytes encode ribosomal proteins and function in protein biosynthesis. Also, structural components of the cytoskeleton and genes that regulate ECM-mediated cell signaling and adhesion showed higher expression in AdMSCs and chondrocytes compared to FBs. Two genes, *DACT1* (Wnt signaling) and *PDLIM7* (BMP6 signaling) involved in developmental processes were common to AdMSCs and chondrocytes. Both of these pathways play important role in cartilage development [18,19]. Our data show that different cell types have different expression of lineage-specific genes (Figure 3A) and suggests that unlike FBs, undifferentiated AdMSCs may share functional similarities with chondrocytes.

AdMSCs and FBs Exhibit Cellular 'Memory'

AdMSCs and FBs become more similar upon induction of differentiation. Gene expression patterns of AdMSCs and FBs become more similar upon differentiation, but they still remain distinguishable within differentiated cell clusters indicating that cells 'remember' their origin (Figure 3A and B). We asked the question how different are gene expression patterns of AdMSC- and FB-derived cell lineages, and whether the differences vary according to cell lineages. Undifferentiated cells together with lineage-specific samples were included in the ANOVA to find differentially expressed genes (FDR of 1%) between AdMSC- and FB-derived adipocytes, osteoblasts and chondrocytes. 45 genes were found to be differentially expressed between AdMSC- and FB-derived adipocytes (Figure 4B). For AdMSC- and FB-derived osteoblasts or chondrocytes the number of differentially expressed genes was 215 and 104, respectively. This result first confirms that differences between different cell types (792 genes) are greater than differences between AdMSC- and FB-derived cells. Secondly, the fact that more genes were differentially expressed between AdMSC- and FB-derived osteoblasts and chondrocytes than between AdMSC- and FB-derived adipocytes, indicates that AdMSCs and FBs became more similar upon adipogenic differentiation. It suggests that switch of stromal cell regulatory mechanisms into adipocyte-specific regulation requires less time than switch into osteoblast- and chondrocyte-specific regulation.

AdMSC- and FB-derived cells exhibit distinct gene expression. To answer the question whether genes that are initially distinctly expressed in AdMSCs and FBs also remain differentially expressed in differentiated cells, the comparison of genes differentially expressed in undifferentiated and differentiated AdMSCs and FBs was done and the extent of overlap was determined for each AdMSC- and FB-derived differentiated cell type. Results of the analysis were visualized using Venn diagram, where the size of a circle is proportional to the number of genes it represents (Figure 4B). A fraction of distinctly expressed genes between undifferentiated AdMSCs and FBs (light grey) stay distinct in the differentiated cells (light blue), but also many differentiation-related genes become differently expressed in the AdMSC- and FB-derived cells (yellow, pink or blue) as shown in Figure 4B. Interestingly, the number of genes that become different in adipocytes (33 genes) is smaller than in other differentiated cells (179 genes in osteoblasts; 82 genes in

Table 2. The list of genes that are highly expressed in AdMSCs and chondrocytes but not in FBs.

Cytoskeleton	**FRMD6, TPM1, TTN**
Extracellular matrix and adhesion	COL5A1, FN1, SPARC
Biosynthesis	BOP1, EEF1A1, ENPP7, FKBP7, RPL23, RPL39, RPLP1, RPLP2, RPS16, RPS25, SERPINE1
Signal transduction	C5orf13, IQCG, IQSEC1, TSNAX
Development	DACT1, PDLIM7

doi:10.1371/journal.pone.0038833.t002

chondrocytes). Also, equally small number of genes remains distinctly expressed between AdMSCs and FBs upon adipogenic induction (12 genes), which is less evident upon osteogenic (36 genes) and chondrogenic (22 genes) induction. This result confirms that AdMSC- and FB-derived adipocytes are more alike than other AdMSC- and FB-derived cells. Further, osteogenic differentiation has the smallest effect on the regulation of genes that are initially differently expressed between undifferentiated AdMSCs and FBs compared with adipogenic and chondrogenic differentiation. Taken together, the differences between AdMSC- and FB-derived differentiated cells originate from both the initially distinct gene expression patterns and gene expression acquired in the process of differentiation.

AdMSCs and FBs express cellular 'memory' genes. The fact that several genes that are differently expressed in AdMSCs and FBs remain differently expressed in AdMSC- and FB-derived differentiated cells raises the possibility that the cells express so called 'source'-specific cell 'memory' genes that are not regulated during the differentiation. Our data show that high expression of *COL1A1*, *COL1A2*, *EFEMP1 (fibulin 3)*, *FN1 (fibronectin 1)*, *GGT5 (gamma-glutamyltransferase 5)* and *TPM2 (tropomyosin 2)* is characteristic for AdMSCs and AdMSC-derived cells. On the other hand, expression of *S100A4 (fibroblast-specific protein 1)* and *TK1 (thymidine kinase 1)* is characteristic for FBs and FB-derived cell types. It would be of interest to learn whether after longer period of differentiation the differential expression of those 'memory' genes in AdMSC- and FB-derived mature adipocytes, osteoblasts and chondrocytes will remain present or disappear.

AdMSCs and FBs Exhibit Similar Dynamics of Adipogenic and Osteogenic Differentiation but Distinct Dynamics of Chondrogenic Differentiation

Lineage-specific gene regulation occurs early in differentiation and persists over time. It is well known that cell differentiation is a process of sequential induction of regulatory genes that in turn initiate the expression of a pile of tissue-specific target genes. Still, each developmental process requires the activation of a specific transcriptional program. Our data show that global changes in cell type-specific gene expression take place quickly upon differentiation of AdMSCs and FBs (Figure 3C). Next we performed more detailed analysis of dynamics of differentiation of AdMSCs and FBs into adipocytes, osteoblasts and chondrocytes.

To visualize transcriptome profiles of differentiating AdMSCs and FBs along adipogenic, osteogenic and chondrogenic lineages over time, the daily time points except day 0 as 'undif.' and 'day 7', were assembled into the following groups: 'early' including days 1–3 and 'late' including days 4–6. Gene expression at different time points was compared using ANOVA (FDR of 1%) and significantly differentially expressed genes were used to visualize the linkage of different samples in the PCA plot based on time group, and AdMSC or FB annotations (Figure 5). In total, 213 lineage-specific genes were found to be regulated over time during adipogenesis, 126 during osteogenesis and 203 during chondrogenesis. The genes are listed in Table S2. AdMSCs and FBs differentiated into adipocytes, osteoblasts and chondrocytes cluster together or are connected with each other through edges with little effect of time. In contrast, the genes that were regulated over time clearly placed undifferentiated cells into separate clusters that have no edge-connections with differentiated samples, except for undifferentiated AdMSCs that were related to 'early' chondrocytes. Hence, it reveals that major changes in lineage-specific gene expression occur early in differentiation and persist over time.

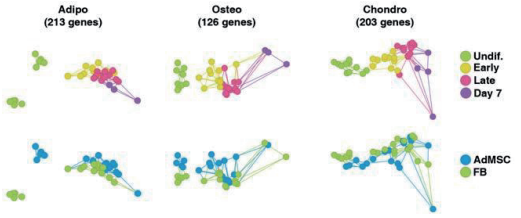

Figure 5. PCA of lineage-regulated gene expression. ANOVA with FDR of 1% between different time points recovered 213 genes in adipogenesis, 126 genes in osteogenesis and 203 genes in chondrogenesis that were regulated over time. These genes were used to visualize the samples in a PCA plot. Major changes in gene expression occur early in differentiation and persist over time. Abbreviations: Undif., undifferentiated; Adipo, adipocyte; Osteo, osteoblast; Chondro, chondrocyte; AdMSC, adipose-derived mesenchymal stem cell; FB, fibroblast.
doi:10.1371/journal.pone.0038833.g005

Gene expression dynamics upon chondrogenic differentiation is different between AdMSCs and FBs. The analysis of above described gene expression data shows that approximately 70% of adipogenesis-related and 43% of osteogenesis-related genes are down regulated in the process of differentiation of both AdMSCs and FBs (Table S3). These results show that gene repression is the major mechanism of differentiation of adipocytes, whereas osteogenic differentiation is accompanied by smaller changes in global gene expression with slightly more genes up regulated (57%) than down regulated during differentiation. Chondrogenesis-related genes show different expression patterns in AdMSCs and FBs (Table S3). More genes were down regulated in AdMSCs (74%) upon chondrogenic differentiation than in FBs (62%). Next we analyzed whether the up and down regulation of gene expression occured similarly over time. The scale in line plots (Figure 6) shows gene regulation in standard deviations from the mean expression for each gene. Down regulation in gene expression was quick but up regulation occurred slowly over the week upon adipogenic and osteogenic differentiation of AdMSCs and FBs (Figure 6A and B). Interestingly, AdMSCs and FBs exhibited distinct gene expression dynamics upon chondrogenesis. Smaller but bidirectional changes in gene regulation occurred in AdMSCs throughout chondrogenesis, whereas in FBs a transient down-regulation in gene expression was followed by constant up-regulation along chondrogenic differentiation. This observation confirms that the transcriptome profiles of AdMSCs and chondrocytes are more alike and less changes in gene expression need to occur in AdMSCs than in FBs to become chondrocytes.

Discussion

Stem cells are promising tools to study mechanisms of development and regeneration. Molecular characterization of MSCs is held back by the lack of marker genes that would distinguish them from other cell types in different tissues. MSCs are similar to FBs in growth properties, morphology, surface marker expression and developmental potential as well as origin. The global gene expression analysis of AdMSCs and FBs, both in the undifferentiated state and in the process of differentiation along adipogenic, osteogenic and chondrogenic lineages using cells from the same donors, allowed the identification of cell type-specific gene expression dynamics of two closely related stromal stem cells.

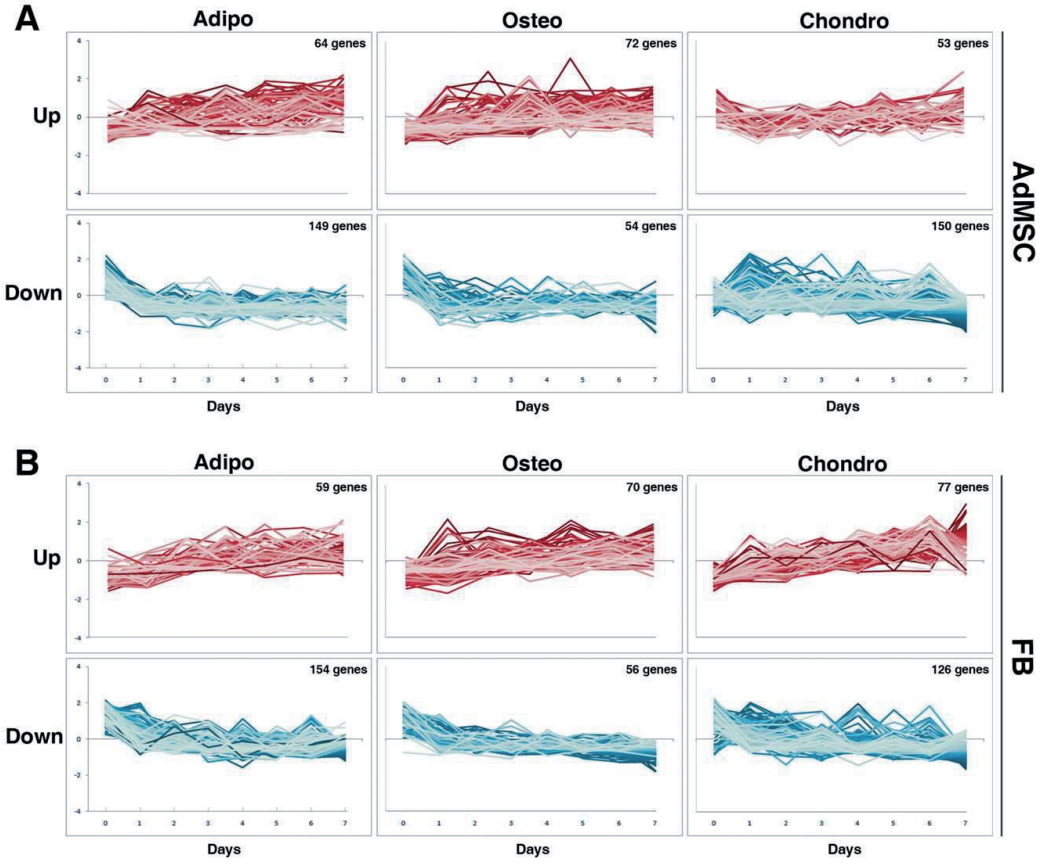

Figure 6. Gene expression dynamics. The expression dynamics of lineage-regulated genes in A) AdMSCs and B) FBs was visualized using line plots. The scale on y-axis shows the up or down regulation in standard deviations from the mean expression for each gene. AdMSCs and FBs share similar gene expression dynamics - quick down regulation (lower panels, blue) but slow up regulation (upper panels, red) in gene expression along adipogenesis and osteogenesis. However, the dynamics of chondrogenesis differs between AdMSCs and FBs. Abbreviations: Adipo, adipocyte; Osteo, osteoblast; Chondro, chondrocyte; AdMSC, adipose-derived mesenchymal stem cell; FB, fibroblast.
doi:10.1371/journal.pone.0038833.g006

First, our study reveals that the transcriptome profiles of undifferentiated AdMSCs and FBs are distinct and stay distinct upon differentiation despite the similar general characteristics of the cells. In previous studies the comparison of gene expression profiles between AdMSCs and FBs has been carried out using cells from different donors, body locations and developmental stage (eg fetal or adult tissues), leading to possible variation in gene expression that is not directly related to the differences between these cell types [8,9,20]. Independently-derived hESC lines were identified to exhibit unique gene expression signature due to high genetic variability [21,22]. Moreover, different MSC populations have been shown to exhibit a unique genomic signature [23]. We found, that the global gene expression patterns differ between AdMSCs and FBs derived from matching donors. Differences between AdMSCs and FBs did not disappear completely upon one week of differentiation probably due to the slow proccess of transition of the original cell to another cell type. In fact, we noticed many new differentially expressed genes to be present in AdMSC- and FB-derived differentiated cells compared with undifferentiated cells. Little attention has been paid to the comparison of gene expression profiles of differentiated cells that are derived from different progenitors but under similar differentiation conditions. Our data also indicate, that cells retain the expression of some 'memory' genes that trace back to the tissue origin of the cells. Similar phenomenon of cellular memory has been described for induced pluripotent stem cells (iPS). The gene expression analysis of iPS cells generated from different mature tissue types has revealed that iPS cells recall their original tissue type, although they all share similar morphology and expression of pluripotency genes [24]. However, it has been proposed that reprogramming of cells is a slow process and the memory of the cells' origin will be erased over time [25]. It is possible then that the differences in gene expression profiles of AdMSC- and FB-derived adipocytes, osteoblasts and chondrocytes will disappear after longer differentiation.

Secondly, the analysis of gene expression profiles over time shows that the differences in lineage-specific gene expression occur early in differentiation of both AdMSCs and FBs. Interestingly,

changes in the gene expression of AdMSCs and FBs upon induction are related initially to rapid down-regulation of gene expression, whereas up regulation occurs slowly over the week. It has been suggested that gene repression is a predominant early mechanism before final cell commitment and that lineage-specific molecular processes are transcriptionally up regulated only after commitment [26]. The results of our analysis support the idea that cells respond to induction of differentiation by rapidly resetting their original transcriptional program and gradually expressing lineage-associated genes. Although such general mechanism is shared by AdMSCs and FBs along differentiation into adipocytes and osteoblasts, the extent of gene repression is higher upon adipogenic induction. Notably, our findings suggest that the switch from stromal regulation to adipogenic regulation is faster than the switch to osteoblast and chondrocytes regulation.

Thirdly, dynamics of chondrogenic differentiation is different in AdMSCs and FBs. Unlike in FBs, in AdMSCs several genes that become up regulated along chondrogenesis are initially down-regulated and *vice versa*, many of those genes that become down regulated over the week, are initially up regulated upon differentiation. The distinct pattern of gene regulation upon chondrogenesis in AdMSCs could be related to the observation that AdMSCs are more similar to chondrocytes in the undifferentiated state than FBs. It is intruiging to speculate that AdMSCs are pre-committed to chondrocyte development and initiation of differentiation does not involve global transcriptional reprogramming. Such pre-commitment of AdMSCs seems not to affect their ability to differentiate into other cell types similarly with FBs. It has been shown that lineage-committed MSCs can transdifferentiate into other cell types in response to inducive extracellular cues [27]. Also, it has been proposed that uncommitted adult stem cells maintain their multipotency by expressing basal levels of genes characteristic to different lineages and that certain groups of genes are selectively suppressed upon stimulation prior to commitment to a given characteristic phenotype [28,29]. It turns out then that AdMSCs and FBs use globally similar early mechanisms of differentiation into adipocytes and osteoblasts but exhibit distinct mechanisms of chondrogenic differentiation.

Together, our study shows that stromal stem cells including adipose-derived AdMSCs and dermal FBs exhibit distinct dynamics of differentiation into mesodermal cell types under similar experimental conditions. AdMSCs and FBs exploit similar early mechanisms for differentiation into adipocytes and osteoblasts but show different molecular mechanisms for chondrogenic differentiation. Further finding suggests that the switch from stromal regulation to adipocyte regulation is faster than the switch to osteoblast and chondrocyte regulation. The results of the global study provide relevant insight to the molecular mechanisms of differentiation of stromal stem cells that can be used in further studies.

Materials and Methods

Ethics Statement

Experiments with human tissues were approved by National Institute for Health Development and Ethics Committee in Estonia (Approval No 2234 from Dec 09, 2010).

Cell Isolation and Cultivation

AdMSCs were isolated from human subcutaneous adipose tissue according to Lin et al. and Yamamoto et al. [30,31] with slight modifications. Briefly, adipose tissue was digested with 0.1% collagenase (Gibco) in serum-free alphaMEM (a modification of Minimum Essential Medium (MEM), contains sodium pyruvate, lipoic acid, vitamin B_{12}, biotin, and ascorbic acid, Gibco 32571) at 37°C for 1.5 h, followed by neutralization of enzyme activity with 20% fetal bovine serum (FBS) and 1% penicillin-streptomycin alphaMEM growth medium. Following centrifugation, stromal cell pellet was passed through a 100 μm nylon mesh (BD Biosciences) and resuspended in 10% FBS growth medium, plated at a density of 10 000 cells/cm^2 and incubated at 37°C with 5% CO_2. After 48 h medium was replaced to remove non-adherent cells. Further cultivation was performed under standard cell culture conditions. Fibroblasts were isolated from dermal skin of the same donors as AdMSCs, using a method described before [32]. Briefly, primary culture was established by fibroblast outgrowth from skin explants placed onto Primaria dish (BD Falcon) in 10% FBS and 1% penicillin-streptomycin DMEM-High Glucose (a modification of Eagle's Minimal Essential Medium, contains sodium pyruvate, higher glucose levels, Gibco 10569) growth medium.

In vitro Differentiation

Passage three or four cells were plated at density of 15 000 cells/cm^2 72 hours prior to induction of differentiation. 10% FBS and 1% penicillin-streptomycin containing growth medium was supplemented with:

- 1 μM dexamethasone, 500 μM IBMX (3-isobutyl-1methyl-xanthine), 100 μM indomethacin and 10 μg/ml insulin for adipogenic induction,
- 100 nM dexamethasone, 50 μM L-ascorbic acid 2-phosphate and 10 mM glycerol 2-phosphate for osteogenic induction,
- 50 μM L-ascorbic acid 2-phosphate, 6,25 μg/ml insulin and 10 ng/ml TGFbeta-1 (Peprotech) for chondrogenic induction.

Treatment media was changed once (on day 3) during the 7-day differentiation assay or twice a week during a long-term differentiation assay. All chemicals, if not specified differently, were purchased from Sigma. Accumulation of lipid droplets in adipocytes was determined by Oil Red O (ORO) staining as previously described [14]. For quantitative analysis, optical density of eluted ORO was measured at 510 nm. Osteoblasts were analyzed for the formation of calcified matrix by Alizarin Red S (ARS) staining as described in [14]. For quantitative analysis, ARS-stained cell monolayers were scraped off the dish in 10% acetic acid and optical density of the supernatant was measured at 405 nm. Chondrocyte differentiation was determined by Alcian Blue (AB) staining of proteoglycan-rich matrix. Briefly, 4% PFA-fixed cells were washed with water, incubated for 30 min at RT with 10 mg/ml AB solution in 5% acetic acid, washed 4 times with water, and photographed. For quantitative analysis, AB-stained cell monolayers were scraped off the dish in 6 M guanidine HCl and optical density of the supernatant was measured at 600 nm.

RNA Isolation

Cells were lyzed at day 0, 1, 2, 3, 4, 5, 6 and 7 of adipogenic, osteogenic and chondrogenic differentiation for total RNA extraction using Trizol reagent (Invitrogen). Following a phenol/chloroform extraction and isopropanol precipitation, RNA samples were treated with DNase I using DNA-free ™ kit (Ambion). The resulting 96 RNA samples were applied to sample preparation for deep sequencing.

Multiplex RNA-seq and Data Analysis

Gene expression analysis was performed as previously described for single-cells [16]. Multiplex mRNA-seq was performed using the same approach, but starting with 10 ng

of total RNA instead of single cells, and using only 10 cycles of PCR for the cDNA amplification. Statistical analysis (ANOVA), hierarchical clustering and PCA were performed using the Qlucore Omics Explorer (Qlucore AB, Lund, Sweden). Selection of genes for ANOVA (Analysis of variation) was based on the false-discovery rate (FDR = q) to control for multiple testing. FDR was used as a measure of significance of the observed effects. PCA was used on ANOVA-filtered data (except Figure 2) to visualize differences between groups other than those used in the ANOVA, or within the groups used in the ANOVA (See Results section for specifications). Raw sequencing data is publically available at NCBI (GEO accession number GSE37521).

Supporting Information

Figure S1 Differences in gene expression of AdMSCs and FBs. ANOVA (with FDR of 5%) between undifferentiated AdMSCs and FBs resulted in 178 differentially expressed genes, 59 with higher and 119 with lower expression in AdMSCs than in FBs. The scale shows the up (light red) or down regulation (light blue) in standard deviations from the mean expression for each gene.
(TIF)

Table S1 The list of samples used in the study.
(DOCX)

Table S2 The list of lineage-specific genes.
(XLSX)

Table S3 Up and down regulation of genes during differentiation.
(DOCX)

Acknowledgments

We thank Indranil Sinha for introduction into Qlucore Omics Explorer; Ats Metsis, Annalena Moliner and the members of our laboratories for helpful discussions.

Author Contributions

Conceived and designed the experiments: KJ SL. Performed the experiments: KJ SI PZ. Analyzed the data: KJ SL. Contributed reagents/materials/analysis tools: SL TN. Wrote the paper: KJ. Contributed to manuscript writing: SL TN.

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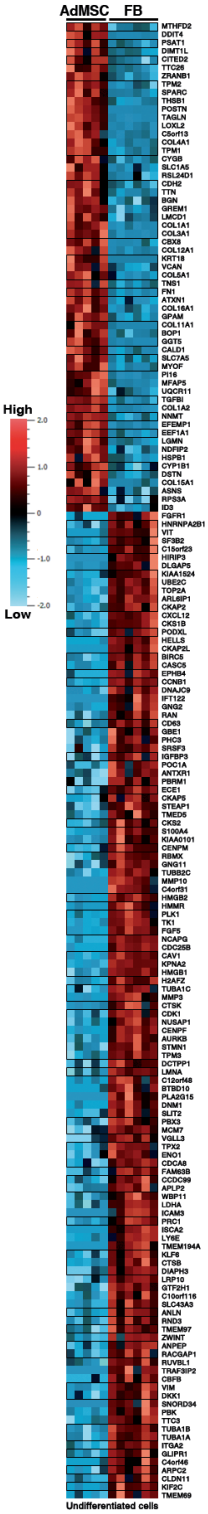

Figure S1. Differences in gene expression of AdMSCs and FBs. ANOVA (with FDR of 5%) between undifferentiated AdMSCs and FBs resulted in 178 differentially expressed genes, 59 with higher and 119 with lower expression in AdMSCs than in FBs. The scale shows the up (light red) or down regulation (light blue) in standard deviations from the mean expression for each gene.

Table S1. The list of samples used in the study.

Sample No	Cell type	Donor	Lineage	Time point - day	Sample No	Cell type	Donor	Lineage	Time point - day
1	AdMSC	1	Undifferentiated	0	**49**	FB	1	Undifferentiated	0
2	AdMSC	1	Adipogenesis	1	**50**	FB	1	Adipogenesis	1
3	AdMSC	1	Adipogenesis	2	**51**	FB	1	Adipogenesis	2
4	AdMSC	1	Adipogenesis	3	**52**	FB	1	Adipogenesis	3
5	AdMSC	1	Adipogenesis	4	**53**	FB	1	Adipogenesis	4
6	AdMSC	1	Adipogenesis	5	**54**	FB	1	Adipogenesis	5
7	AdMSC	1	Adipogenesis	6	**55**	FB	1	Adipogenesis	6
8	AdMSC	1	Adipogenesis	7	**56**	FB	1	Adipogenesis	7
9	AdMSC	2	Undifferentiated	0	**57**	FB	2	Undifferentiated	0
10	AdMSC	2	Adipogenesis	1	**58**	FB	2	Adipogenesis	1
11	AdMSC	2	Adipogenesis	2	**59**	FB	2	Adipogenesis	2
12	AdMSC	2	Adipogenesis	3	**60**	FB	2	Adipogenesis	3
13	AdMSC	2	Adipogenesis	4	**61**	FB	2	Adipogenesis	4
14	AdMSC	2	Adipogenesis	5	**62**	FB	2	Adipogenesis	5
15	AdMSC	2	Adipogenesis	6	**63**	FB	2	Adipogenesis	6
16	AdMSC	2	Adipogenesis	7	**64**	FB	2	Adipogenesis	7
17*	AdMSC	1	Undifferentiated	0	**65**	FB	1	Undifferentiated	0
18*	AdMSC	1	Osteogenesis	1	**66**	FB	1	Osteogenesis	1
19	AdMSC	1	Osteogenesis	2	**67**	FB	1	Osteogenesis	2
20	AdMSC	1	Osteogenesis	3	**68**	FB	1	Osteogenesis	3
21	AdMSC	1	Osteogenesis	4	**69**	FB	1	Osteogenesis	4
22	AdMSC	1	Osteogenesis	5	**70**	FB	1	Osteogenesis	5
23	AdMSC	1	Osteogenesis	6	**71**	FB	1	Osteogenesis	6
24*	AdMSC	1	Osteogenesis	7	**72**	FB	1	Osteogenesis	7

Table S1. Continues

Sample No	Cell type	Donor	Lineage	Time point - day	Sample No	Cell type	Donor	Lineage	Time point - day
25	AdMSC	2	Undifferentiated	0	**73**	FB	2	Undifferentiated	0
26	AdMSC	2	Osteogenesis	1	**74**	FB	2	Osteogenesis	1
27	AdMSC	2	Osteogenesis	2	**75**	FB	2	Osteogenesis	2
28*	AdMSC	2	Osteogenesis	3	**76**	FB	2	Osteogenesis	3
29	AdMSC	2	Osteogenesis	4	**77**	FB	2	Osteogenesis	4
30	AdMSC	2	Osteogenesis	5	**78**	FB	2	Osteogenesis	5
31	AdMSC	2	Osteogenesis	6	**79**	FB	2	Osteogenesis	6
32	AdMSC	2	Osteogenesis	7	**80**	FB	2	Osteogenesis	7
33	AdMSC	1	Undifferentiated	0	**81**	FB	1	Undifferentiated	0
34	AdMSC	1	Chondrogenesis	1	**82**	FB	1	Chondrogenesis	1
35	AdMSC	1	Chondrogenesis	2	**83**	FB	1	Chondrogenesis	2
36	AdMSC	1	Chondrogenesis	3	**84**	FB	1	Chondrogenesis	3
37	AdMSC	1	Chondrogenesis	4	**85**	FB	1	Chondrogenesis	4
38	AdMSC	1	Chondrogenesis	5	**86**	FB	1	Chondrogenesis	5
39	AdMSC	1	Chondrogenesis	6	**87**	FB	1	Chondrogenesis	6
40*	AdMSC	1	Chondrogenesis	7	**88**	FB	1	Chondrogenesis	7
41	AdMSC	2	Undifferentiated	0	**89**	FB	2	Undifferentiated	0
42	AdMSC	2	Chondrogenesis	1	**90**	FB	2	Chondrogenesis	1
43	AdMSC	2	Chondrogenesis	2	**91**	FB	2	Chondrogenesis	2
44	AdMSC	2	Chondrogenesis	3	**92**	FB	2	Chondrogenesis	3
45	AdMSC	2	Chondrogenesis	4	**93**	FB	2	Chondrogenesis	4
46	AdMSC	2	Chondrogenesis	5	**94**	FB	2	Chondrogenesis	5
47	AdMSC	2	Chondrogenesis	6	**95**	FB	2	Chondrogenesis	6
48	AdMSC	2	Chondrogenesis	7	**96**	FB	2	Chondrogenesis	7

*Samples that were removed from gene expression analysis

Table S2. The list of lineage-specific genes

Adipo-specific genes				Osteo-specific genes				Chondro-specific genes			
FB		**AdMSC**		**FB**		**AdMSC**		**FB**		**AdMSC**	
Up	**Down**	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**
ABCC1	ACTB	ABCC1	ACTB	CEBPD	ADAM12	GLIPR1	ADAM12	CNPY2	ACMSD	PGK1	ACMSD
ACACB	ACTC1	ACACB	ACTC1	H2AFJ	ADAMTS6	GLUL	ADAMTS6	COPS6	ACTA2	PPIB	ACTA2
AKR1C1	ACTG1	AKR1C1	ACTG1	GLIPR1	ADM	GREM1	ADM	COX7A2	ADD3	PRPF8	ADD3
AKR1C2	ACTN1	AKR1C2	ACTN1	GLUL	ALPK2	HLA-E	ALPK2	DKK1	AHNAK	RAC1	AHNAK
AKR1C3	ACTR3	AKR1C3	ACTR3	GREM1	ANXA5	IGFBP3	ANXA5	EIF1	ARF4	RASA4	ARF4
AOX1	ADAM9	AOX1	ADAM9	HLA-E	APOD	IGFBP5	APOD	EIF3C	ARPC3	RBBP7	ARPC3
CBX5	ADAMTS6	CBX5	ADAMTS6	IGFBP3	BLOC1S1	IL1R1	BLOC1S1	ENPP2	ASF1B	RBM3	ASF1B
CNOT3	AEBP1	CNOT3	AEBP1	IGFBP5	BTG1	ITGA10	BTG1	FKBP1A	ATOH8	RBMX	ATOH8
COMP	ALPK2	COMP	ALPK2	IL1R1	C10orf10	LOC730755	C10orf10	FKBP7	ATP2B4	RHOG	ATP2B4
CRISPLD2	ANGPTL4	CRISPLD2	ANGPTL4	ITGA10	C1QTNF1	MALL	C1QTNF1	FXC1	ATP5B	RPL26	ATP5B
ECH1	ANLN	ECH1	ANLN	LOC730755	C1R	MARCKS	C1R	HSPB7	ATP5E	RPL32	ATP5E
EEF1A1	ANP32E	EEF1A1	ANP32E	MALL	C1S	MARS	C1S	JAK1	ATP5I	RPL36	ATP5I
EEF1G	ANXA1	EEF1G	ANXA1	MARCKS	CABC1	MEGF10	CABC1	KIAA1199	ATP5L	RPL39	ATP5L
EVC	AP2M1	EVC	AP2M1	MARS	CALU	MT1E	CALU	LILRA3	AXL	RPS25	AXL
FADS1	ARPC5	FADS1	ARPC5	MEGF10	CBLB	MT1L	CBLB	LMNA	BMPER	RPS27L	BMPER
FAM133B	ASF1B	FAM133B	ASF1B	MT1E	CD5	MT2A	CD5	LTA4H	C11orf31	RPS6	C11orf31
FKBP7	ATP2B4	FKBP7	ATP2B4	MT1L	CFD	MTRNR2L2	CFD	LUM	C15orf63	SASH1	C15orf63
FZD6	AXL	FZD6	AXL	MT2A	CKAP4	MYOF	CKAP4	MCM7	C19orf53	SCOC	C19orf53
GLUL	C12orf75	FTH1	C12orf75	MTRNR2L2	CNR1	NAP1L4	CNR1	MEG3	C1QTNF3	SEC61B	C1QTNF3
HTRA1	C1R	GLIPR1	C1R	MYOF	CORIN	NID1	CORIN	MYOF	CALU	SERF2	CALU
IDH1	CALM2	GLUL	CALM2	NAP1L4	CPM	NNMT	CPM	NAV1	CAT	SH2D4A	CAT
IQCG	CALU	HTRA1	CALU	NID1	CRABP2	NTM	CRABP2	NDUFA4	CAV1	SH3BGRL	CAV1
JMJD6	CCDC21	IDH1	CCDC21	NNMT	CYB5A	PDE4DIP	CYB5A	NPC2	CCDC72	SH3BGRL3	CCDC72
KIR3DL1	CCDC80	IQCG	CCDC80	NTM	DAB2	PDGFRA	DAB2	NR3C1	CCDC80	SHFM1	CCDC80
LAMB1	CCNB1	JMJD6	CCNB1	PDE4DIP	DCAF12	PDLIM7	DCAF12	OXSR1	CCDC94	SLC7A1	CCDC94
LOC728066	CCND1	KIR3DL1	CCND1	PDGFRA	DCN	PFKP	DCN	PABPC4	CD151	SNRPF	CD151
LYRM2	CD63	LAMB1	CD63	PDLIM7	DPT	PKM2	DPT	PKM2	CITED2	SSR1	CITED2
MAOA	CDCA8	LOC728066	CDCA8	PFKP	DPY30	PLIN2	DPY30	PLIN2	CLIC4	STC2	CLIC4
MAP1A	CENPK	LOC730755	CENPK	PKM2	ECH1	PODXL	ECH1	PMM2	CLU	ZNF185	CLU
MTRNR2L2	CENPM	LYRM2	CENPM	PLIN2	EPRS	PPP1R3C	EPRS	PODXL	COL11A1	ZNF75D	CNPY2
MTRNR2L8	CFL1	MAOA	CFL1	PODXL	FBLN1	PRELP	FBLN1	PPP1CA	COL8A1	TBCA	COL11A1
NNMT	CHMP2A	MAP1A	CHMP2A	PPP1R3C	FBLN5	PRPS1	FBLN5	PPP2R1A	COMMD6	TFPI	COL8A1
NR1H3	CKS2	MGLL	CKS2	PRELP	FBN2	PSAT1	FBN2	PRKDC	COPE	THRAP3	COMMD6
PAK1IP1	COPG	MTRNR2L2	COPG	PRPS1	FBXL17	PTGFR	FBXL17	PTGIS	CORO1C	TIMP3	COPE
PSMC3IP	CORIN	MTRNR2L8	CORIN	PSAT1	FGF2	RAB3B	FGF2	PTPN21	CRIM1	TLN1	COPS6
RAB11FIP1	CORO1C	NNMT	CORO1C	PTGFR	FLNC	RANBP3	FLNC	RAB1B	CSRP2	TMBIM6	CORO1C
REXO2	CRABP2	NR1H3	CRABP2	RAB3B	FTH1	RASSF4	FTH1	RAB3B	CTGF	TMEM165	COX7A2
RNF24	CTGF	PAK1IP1	CTGF	RANBP3	FTL	RDBP	FTL	RANBP3	CYGB	TMEM43	CRIM1
RPL12	DAB2	PSMC3IP	DAB2	RASSF4	FXYD5	REV3L	FXYD5	RASA4	DCN	TMEM47	CSRP2

Table S2. Continues

Adipo-specific genes				Osteo-specific genes				Chondro-specific genes			
FB		**AdMSC**		**FB**		**AdMSC**		**FB**		**AdMSC**	
Up	**Down**	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**
RPL29	DIAPH1	RAB11FIP1	DIAPH1	RDBP	GAPDH	SAA2	GAPDH	RBBP7	DDAH1	TMEM50A	CTGF
RPL3	DPT	REXO2	DPT	REV3L	GLRX	SAMD11	GLRX	RHOA	DDR2	TNFRSF11B	CYGB
RPL39	DSP	RNF24	DSP	SAA2	GPX3	SAMHD1	GPX3	RHOC	DDX21	TRPC4	DCN
RPL6	DUSP3	RPL12	DUSP3	SAMD11	GREM2	SARS	GREM2	RHOG	DIAPH1	TSEN54	DDAH1
RPS16	DYNC1I2	RPL29	DYNC1I2	SAMHD1	H3F3B	SERPINF1	H3F3B	RNASEK	ECE1	TSR1	DDR2
RPS3	ECM1	RPL3	ECM1	SARS	HNMT	SERTAD2	HNMT	RPL26	EHBP1	TXNDC17	DDX21
RPS3A	EFEMP2	RPL39	EFEMP2	SERPINF1	HSPB7	SH2D4A	HSPB7	RPL30	EIF3CL	TXNRD1	DIAPH1
RPS5	EMP3	RPL6	EMP3	SERTAD2	IER3IP1	SLC7A1	IER3IP1	RPL36AL	ELL2	UQCR11	DKK1
S100A11	EXOSC9	RPS16	EXOSC9	SH2D4A	IFI30	SORBS1	IFI30	RPL39	ENG	UQCRH	ECE1
SERPINB6	EXT1	RPS3	EXT1	SLC7A1	IFITM2	STARD13	IFITM2	RPS16	ENO1	UQCRQ	EHBP1
SERPINF1	FAM129A	RPS3A	FAM129A	SORBS1	IL10RB	STMN2	IL10RB	RPS27L	ENPP7	VAMP3	EIF1
SOD2	FKBP5	RPS5	FKBP5	STARD13	ITGBL1	TCEAL4	ITGBL1	SASH1	ERLIN1	WASF2	EIF3C
STMN1	FLNC	RRM1	FLNC	STMN2	KIR3DL1	TFPI2	KIR3DL1	SCOC	EXT1	WHAMM	EIF3CL
ZADH2	FRMD6	S100A11	FRMD6	TCEAL4	MAP1A	TIMP3	MAP1A	SEC61B	F3	YWHAH	ELL2
TIMP3	FTH1	SERPINB6	FTHL3	TFPI2	MAP1B	TMEM119	MAP1B	SERF2	FAT4		ENG
TNFRSF11B	FTHL3	SERPINF1	G2E3	TIMP3	CEBPD	TMEM126A		SERINC1	FBXO16		ENO1
TRIM59	G2E3	SOD2	GREM1	TMEM119	H2AFJ	TMEM59		SH2D4A	FGF2		ENPP2
TRPC4	GLIPR1	STMN1	GREM2	TMEM126A		TMTC1		SH3BGRL	FKBP8		ENPP7
VAT1	GREM1	ZADH2	GTF2F1	TMEM59		TNRC6C		SH3BGRL3	GAPVD1		ERLIN1
VIM	GREM2	TIMP3	HEG1	TMTC1		TPI1		SNRPF	GGCT		EXT1
	GTF2F1	TNFRSF11B	HJURP	TNRC6C		TPM1		SSR1	GNA14		F3
	HEG1	TRIM59	HMGB1	TPI1		TRPC4		ZNF185	GREM2		FAT4
	HJURP	TRPC4	HSD3B7	TPM1		TUBA1A		ZNF75D	GTF2F1		FBXO16
	HMGB1	VAT1	HSPB7	TRPC4		TXNIP		TBCA	HEXA		FGF2
	HSD3B7	VIM	IGFBP3	TUBA1A		UCHL1		TFPI	HGS		FKBP1A
	HSPB7		IGFBP4	TXNIP		UPK2		THRAP3	HNRNPF		FKBP7
	IGFBP3		IL32	UCHL1		VIM		TIMP3	IFI27L2		FKBP8
	IGFBP4		ISLR	UPK2		WARS		TLN1	IFI30		FXC1
	IL32		ITGB1	VIM		WISP1		TMBIM6	IFITM1		GAPVD1
	ISLR		KCNK2	WARS		ZFP36L2		TMEM165	IFITM3		GGCT
	ITGB1		KDELR3	WISP1		ZNF451		TMEM43	IQCG		GNA14
	KCNK2		KIAA1199	ZFP36L2				TMEM47	ISLR		GREM2
	KDELR3		KIAA1949	ZNF451				TNFRSF11B	ITGA10		GTF2F1
	KIAA1199		KIF20A					TSEN54	LAMA4		HEXA
	KIAA1949		KIF23					TXNDC17	LAMC1		HGS
	KIF20A		KIRREL3					VAMP3	LARP1		HNRNPF
	KIF23		LMNA					WASF2	LASP1		HSPB7
	KIRREL3		LMO7					YWHAH	LDHB		IFI27L2
	LMNA		LOC541471						LOC541471		IFI30

Table S2. Continues

Adipo-specific genes				Osteo-specific genes				Chondro-specific genes			
FB		AdMSC		FB		AdMSC		FB		AdMSC	
Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
	LMO7		LRRC2						LOC730755		IFITM1
	LOC541471		LRRC59						LPCAT3		IFITM3
	LOC730755		MAD2L1						MALL		IQCG
	LRRC2		MALL						MAP1B		ISLR
	LRRC59		MCM4						MCM4		ITGA10
	MAD2L1		MCM5						MGLL		JAK1
	MALL		MCM7						MSN		KIAA1199
	MCM4		MED14						MYADM		LAMA4
	MCM5		MED15						NADSYN1		LAMC1
	MCM7		MELK						NAP1L4		LARP1
	MED14		MSN						NCRNA00152		LASP1
	MED15		MT1E						NDUFA1		LDHB
	MELK		MXRA5						NDUFA13		LILRA3
	MGLL		MYH9						NDUFA2		LMNA
	MSN		MYL12B						NDUFB2		LOC541471
	MT1E		MYOF						NDUFB4		LOC730755
	MXRA5		NCAPG2						NDUFB9		LPCAT3
	MYH9		NCRNA00152						NEDD8		LTA4H
	MYL12B		NID1						NME1-NME2		LUM
	MYOF		NTM						NRP1		MALL
	NCAPG2		NUSAP1						NUPR1		MAP1B
	NCRNA00152		PAMR1						OST4		MCM4
	NID1		PBX3						PABPC1		MCM7
	NTM		PDLIM7						PFDN5		MEG3
	NUSAP1		PKM2						PGK1		MGLL
	PAMR1		PLIN3						PHPT1		MSN
	PBX3		PLP2						PPAP2B		MYADM
	PDLIM7		PODXL						PPIB		MYOF
	PKM2		PON3						PRPF8		NADSYN1
	PLIN3		PPP2R1A						RAC1		NAP1L4
	PLP2		PRPS1						RBM3		NAV1
	PODXL		PRR11						RBMX		NCRNA00152
	PON3		PSG5						RPL32		NDUFA1
	PPP2R1A		PTRF						RPL36		NDUFA13
	PRPS1		RAB3B						RPS25		NDUFA2
	PRR11		RANBP3						RPS6		NDUFA4
	PSG5		RHOA						SEPT7P2		NDUFB2
	PTRF		RHOC						SHFM1		NDUFB4
	RAB3B		S100A16						SLC7A1		NDUFB9

Table S2. Continues

Adipo-specific genes				Osteo-specific genes				Chondro-specific genes			
FB		**AdMSC**		**FB**		**AdMSC**		**FB**		**AdMSC**	
Up	**Down**	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**
	RANBP3		SAE1						STC2		NEDD8
	RHOA		SBDS						TMEM50A		NME1-NME2
	RHOC		SERPINE1						TRPC4		NPC2
	RRM1		SERTAD2						TSR1		NR3C1
	S100A16		SH2D4A						TXNRD1		NRP1
	SAE1		SH3BGRL3						UQCR11		NUPR1
	SBDS		SHROOM3						UQCRH		OST4
	SERPINE1		SLC44A1						UQCRQ		OXSR1
	SERTAD2		SLC7A1						WHAMM		PABPC1
	SH2D4A		SMS								PABPC4
	SH3BGRL3		SRPX								PFDN5
	SHROOM3		SSX2IP								PHPT1
	SLC44A1		TAF15								PKM2
	SLC7A1		TAGLN2								PLIN2
	SMS		TMEM173								PMM2
	SRPX		TNRC6C								PODXL
	SSX2IP		TOE1								PPAP2B
	TAF15		TOMM22								PPP1CA
	TAGLN2		TPM1								PPP2R1A
	TMEM173		TPM3								PRKDC
	TNRC6C		TPX2								PTGIS
	TOE1		TRMU								PTPN21
	TOMM22		TTLL12								RAB1B
	TPM1		TUBA1A								RAB3B
	TPM3		TUBA1B								RANBP3
	TPX2		TUBA1C								RHOA
	TRMU		TUBB6								RHOC
	TTLL12		UCHL1								RNASEK
	TUBA1A		USF2								RPL30
	TUBA1B		WHSC1								RPL36AL
	TUBA1C		YIPF5								RPS16
	TUBB6		YWHAZ								SEPT7P2
	UCHL1										SERINC1
	USF2										
	WHSC1										
	YIPF5										
	YWHAZ										

Table S3. Up and down regulation of genes during differentiation.

Gene regulation	**Adipo**		**Osteo**		**Chondro**	
	AdMSC	FB	AdMSC	FB	AdMSC	FB
Up	30%	28%	57%	56%	26%	38%
Down	70%	72%	43%	44%	74%	62%
Total	**213**		**126**		**203**	

PUBLICATION III

Balikova A, **Jääger K**, Viil J, Maimets T, Kadaja-Saarepuu L. (2012).
Leukocyte marker CD43 promotes cell growth in co-operation
with β-catenin in non-hematopoietic cancer cells.
International Journal of Oncology, Jul; 41(1):299-309.

Leukocyte marker CD43 promotes cell growth in co-operation with β-catenin in non-hematopoietic cancer cells

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Received January 3, 2012; Accepted March 19, 2012

DOI: 10.3892/ijo.2012.1440

Abstract. The Wnt/β-catenin pathway regulates key cellular processes such as differentiation, proliferation, apoptosis; and its activation promotes development of several cancer types. Expression of CD43 (leukosialin), the predominant leukocyte transmembrane sialoglycoprotein, has been detected in many tumors of non-hematopoietic origin. CD43 participates in cell adhesion and regulates intracellular signal transduction pathways involved in cell proliferation and survival. The cytoplasmic domain of CD43 has been reported to translocate to the nucleus, interact with β-catenin and affect its target gene expression, but the impact of this action on cell fate is still unknown. We demonstrate, here, by colony formation assay and siRNA-mediated gene silencing that CD43 and β-catenin co-operate in promoting cell growth. Moreover, in cells with down-regulated β-catenin expression the activation of p53 in response to CD43 overexpression is significantly impaired. In addition, the presence of both CD43 and β-catenin is required for the TCF/LEF-mediated transcription. Presumably, the full-length CD43 participates in this transcriptional regulation. We show that the mature CD43 localizes to the nucleus, where it binds chromatin, co-localizes and co-immunoprecipitates with β-catenin, and enhances the reporter gene expression regulated by β-catenin. These observations provide clear evidence linking CD43 to the Wnt/APC/β-catenin signaling pathway and supporting our hypothesis according to which CD43 plays a role in tumor development.

Introduction

CD43 (also known as leukosialin, sialophorin) is one of the most prevalent leukocyte transmembrane sialoglycoproteins expressed on cells of hematopoietic lineage including hematopoietic stem cells (1) but excluding mature erythrocytes and B-cell subsets (2,3). CD43 has a highly conserved cytoplasmic domain and mucin-like extracellular domain which is extensively *O*-glycosylated (4). The cytoplasmic domain of CD43 interacts with cytoskeletal proteins and regulates a variety of intracellular signal transduction pathways involved in cell activation, proliferation, and survival (5). Notably, the cross-linking of CD43 induces apoptosis of human hematopoietic progenitor cells but not stem cells (6). Whereas, in T-cells and monocytes the cross-linking of CD43 stimulates proliferation via protein kinase C (PKC) pathway (7). The high level of glycosylation and negative charge of the extracellular part of CD43 explain anti-adhesive properties of the CD43 molecule (8). CD43 also transmits signals enabling other ligand-receptor interactions to promote cell adhesion (9,10) and may function as a ligand for ICAM-1 and E-selectin on endothelial cells (11,12). Early expression of CD43 on hESC-derived hematopoietic progenitors may also indicate a possible role of CD43 in hematopoietic development, including acquisition of anti-adhesive properties by emerging hematopoietic cells (13).

Most of the work investigating CD43-mediated signaling has been done in the context of hematopoietic cells because CD43 expression has long been considered specific exclusively for immune cells. However, a number of studies demonstrate CD43 expression in different tumors of non-hematopoietic origin, including lung, breast and colon, but not in normal tissues (14-16). CD43 expression is also detected in several cancer cell lines (17-19). Aberrant expression and glycosylation of CD43 have been proposed to contribute to cancer progression (15,20) and are associated with immune deficiency (21,22). While in blood cells CD43 is expressed on the cell membrane, in non-hematopoietic cancer cells, in contrast, CD43 is located primarily intracellularly and even nuclearly (23). The cytoplasmic domain of CD43 contains a functional NLS (nuclear localization signal) and interacts with the nuclear transporter protein Ran which offers an explanation for the nuclear localization of CD43 (24). The translocation of the cytoplasmic part of CD43 to the nucleus is possible due to its γ-secretase-dependent proteolytic removal from the membrane. Human CD43 is processed by γ-secretase in several carcinoma cell lines (25). The resulting CD43 cytoplasmic tail has been shown to localize into a subnuclear structure, known as PML leukaemia nuclear body, and is involved in the regulation of apoptosis (26). Also, the cleavage in the extracellular domain of CD43 is well described (27,28), and the

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Key words: leukosialin, sialophorin, CD43, β-catenin, cell growth, cell density, colon cancer

soluble CD43 ectodomain fragment has been detected in human plasma (29). Therefore, CD43 might be involved in the regulated intramembrane proteolysis (RIP) signaling pathway similarly to Notch-1 (30), ErbB-4 (31), CD44 (32). In RIP signaling the intramembrane cleavage does not take place until the bulk of the protein on the extracytosolic (luminal or extracellular) face has been removed by a primary cleavage. This primary cleavage can occur in the lumen of the ER, in a post-ER compartment, or at the cell surface. The secondary intramembrane cleavage of type I transmembrane proteins, among which belongs CD43, requires presenilin-1 that cleaves off the cytoplasmic tail a few amino acids into the membrane. The released cytoplasmic fragment has in some cases been shown to translocate into the nucleus where it affects gene activation (33).

In our previous studies on the function of CD43 in non-hematopoietic cancer cells we have identified CD43 as a potential oncogene. Its overexpression causes accumulation of the tumor suppressor protein p53 and induction of apoptosis which is an ARF-dependent process (34). Recently, we have shown that CD43 increases the colony formation ability of both human and mouse cells due to increased growth rate but only in the absence of either p53 or ARF in the cells. Moreover, we found that cells overexpressing CD43 are more resistant to Fas-mediated apoptosis, the mechanism possibly giving these cells the growth advantage (35). In addition, CD43 has been suggested to have a role in Wnt/β-catenin signaling as the cytoplasmic tail of CD43 interacts with β-catenin and promotes its target gene expression (24).

β-catenin, a potent proto-oncogene, is a multifunctional protein which is involved in embryonic development and renewal of adult tissue and which aberrant activation has been shown to play a critical role in the development of different cancers including colon (36,37). β-catenin belongs to the cell-cell adhesion apparatus, and the signaling pool of β-catenin is a key component of the complex network of proteins acting in the Wnt signaling pathway. Upon Wnt signaling β-catenin translocates to the nucleus where it interacts with T-cell factor (TCF) and lymphocyte-enhancing factor (LEF) family transcription factors and with other transcriptional co-factors to form complexes that regulate genes important for proliferation, differentiation, and apoptosis (36,38). The functioning of β-catenin is controlled by a large number of binding partners that affect the stability and the localization of β-catenin. Thereby, β-catenin is able to participate both in cell adhesion and in gene expression processes.

In this study our aim was to investigate the involvement of CD43 in tumorigenesis focusing on the cross-talk between CD43- and β-catenin-mediated signaling. Based on published evidence by our group and others and our preliminary results, we hypothesize that CD43 promotes cell growth in co-operation with β-catenin and this in turn leads to the induction of ARF/p53-dependent apoptosis which is the main mechanism against malignant growth.

We used colon carcinoma cell line HCT116 with an increased Wnt/β-catenin activity, a non-small lung cancer cell line H1299 where Wnt/β-catenin signaling is very low, and a colon adenocarcinoma cell line COLO205 which expresses endogenous CD43 at a high level (17). We used siRNA-mediated gene silencing to knock down β-catenin or CD43 expression and examine the role of either protein by different experimental methods. We also applied subcellular fractionation and subsequent immunoprecipitation of CD43 and β-catenin in order to provide more detailed information about the nature of CD43 and β-catenin interaction.

The data gathered so far support our hypothesis according to which CD43- and β-catenin-mediated pathways interact to regulate cell growth towards survival and/or proliferation. The interaction between CD43 and β-catenin links CD43 to Wnt/APC/β-catenin signaling pathway and provides additional evidence on the involvement of CD43 in tumor development.

Materials and methods

Cell culture and transfections. The H1299 (p53-null human non-small cell lung carcinoma), COLO205 (mutant p53 expressing colon adenocarcinoma) and HCT116 (human ARF-null and wild-type p53 expressing colon carcinoma) cell lines (originally obtained from the American Type Culture Collection) were cultured in 5% CO_2 at 37˚C in Iscove's modified DMEM (PAA) supplemented with 10% fetal bovine serum and penicillin/streptomycin (both 100 U/ml) (PAA).

Plasmid transfections were performed using the electroporation method as described earlier (39) or with ExGen 500 *in vitro* Transfection Reagent according to the manufacturer's instructions (Fermentas). Human wild-type *TP53* in pCG vector and human *SPN* in pcDNA vector have been previously described (34,39). Human *CTNNB1* in pCI-neo vector encoding mutated β-catenin (S33Y) was obtained from Dr B. Vogelstein. The pEGFP-F vector expressing farnesylated EGFP was purchased from Clontech. The sequences of the human *MDM2* and *CDKN1A* promoters were cloned into the promoterless luciferase reporter plasmid pGL-3-basic (Promega). The TOPflash reporter plasmid containing three optimal TCF-binding motifs upstream of a minimal *FOS* promoter (40) and FOPflash plasmid containing mutant TCF-binding sites, both driving luciferase expression, were purchased from Upstate (Millipore). *Renilla* luciferase control reporter vector pRL-TK containing the herpes simplex virus-thymidine kinase (HSV-TK) promoter was purchased from Promega.

siRNA transfections were performed using HiPerFect Transfection Reagent according to the manufacturer's instructions (Qiagen). Cells were first transfected with siRNA and 24 h later with expression vectors. Cells were analyzed 48 h after siRNA treatment. siRNA oligomers targeting CD43 and β-catenin (Ambion) were as follows: 5'-GCAAACUCUCUAGGAUCCCtt-3' (sense) and 5'-GGGAUCCUAGAGAGUUUGCtg-3' (anti-sense) for *SPN* (targeting second exon); 5'-GGUGGUGGUUAAUAAGGCUtt-3' (sense) and 5'-AGCCUUAUUAACCACCACCtg-3' (anti-sense) for *CTNNB1* (targeting fifth exon). A non-specific siRNA was used as a negative control (Ambion).

Colony formation assay. The colony formation ability of cells was estimated as described earlier (35).

Reporter assay. Luciferase reporter gene expression was determined with the dual-luciferase reporter assay system (Promega). The luciferase activity was normalized to *Renilla* luciferase activity from co-transfected internal control plasmid pRL-TK.

Western blot analysis and antibodies. Western blot analysis was performed as previously described (34). The primary antibodies used were: mAb anti-CD43-4D2 (23), mAb anti-β-catenin (BD Biosciences), pAb anti-phospho p53Ser15 (Cell Signaling), mAb anti-β-actin (AC-15, Abcam), pAb anti-GRP78/BiP (Abcam), mAb anti-RNA polymerase II (4H8, Abcam) and pAb anti-histone H3 (Abcam). The secondary antibodies used were: biotinylated goat anti-mouse (DakoCytomation), biotinylated goat anti-rabbit (DakoCytomation), horseradish peroxidase (HRP) conjugated with streptavidin (DakoCytomation) and HRP-conjugated goat anti-mouse (Pierce).

Fractionation and immunoprecipitation. Subcellular protein fractionation kit (Pierce) was used for preparing fractions from COLO205 cells. Protein concentration in fractions was estimated by BCA (bicinchoninic acid) protein assay (Pierce). Equal amounts of protein from each fraction were used for further applications. Immunoprecipitations were performed using sheep anti-mouse IgG coated Dynabeads M-280 (Dynal) [1 μg mAb anti-CD43-4D2, mAb anti-CD43-1G10 (BD Biosciences) or mAb to negative control for Mouse IgG1 (Abcam) per 10^7 Dynabeads] or a co-immunoprecipitation kit (Pierce) (20 μg mAb anti-CD43-4D2 or mAb to negative control for Mouse IgG1 per 50 μl Agarose Resin slurry) following the manufacturer's instructions.

Immunofluorescence using confocal microscopy. Cells were grown on cover slides at high or low density, fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.2% Triton X-100 for 10 min on ice. Slides were blocked with normal goat serum for 1 h at room temperature. Target proteins were stained with anti-β-catenin pAb (Cell Signaling) and anti-CD43-4D2 mAb (23) or anti-CD43-L-10 mAb (Caltag) followed by incubation with the secondary antibodies Alexa Fluor 564-conjugated goat anti-mouse and Alexa Fluor 488-conjugated goat anti-rabbit (Molecular Probes). Slides were mounted with glycerol/1 M Tris pH 9.5 1:1 containing DAPI. Images were captured with a confocal laser scanning microscope (Olympus FluoView FV1000) using a 100x oil immersion objective and analyzed by Olympus FV1000 software FV10-ASW version 1.6a.

Results

CD43 and β-catenin co-operate in promoting colony formation. Aberrant activation of β-catenin-dependent signaling plays an important role in colon cancer development. It has been shown that inhibition of β-catenin expression reduces growth of colon cancer cells (41). We have shown previously, that CD43 overexpression promotes cell growth and down-regulation of its expression results in reduced cell growth (35).

We exploited siRNA-mediated gene silencing to explore whether CD43 and β-catenin co-operate in promoting colony formation ability of the cells. Using human cell lines, H1299 and HCT116, we knocked down β-catenin expression in the cells overexpressing CD43 and contrariwise, inhibited CD43 expression in β-catenin overexpressing cells, and estimated the colony formation efficiency. Results are shown in Fig. 1. In the cells treated with control siRNA there were more colonies formed in β-catenin and CD43 overexpressing cells which

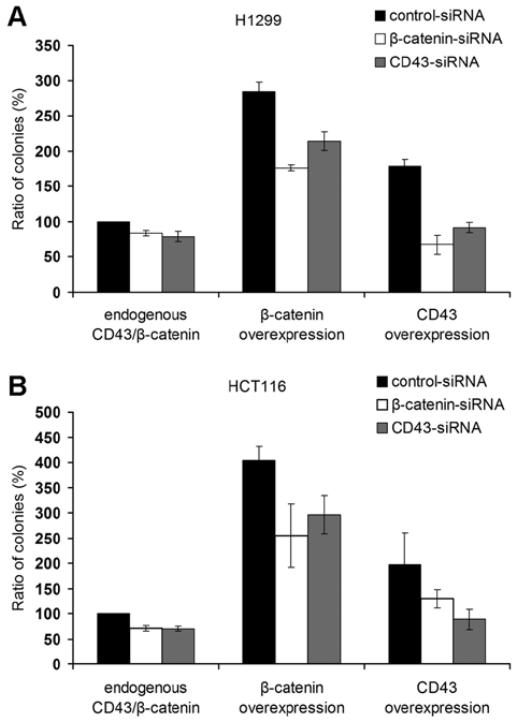

Figure 1. CD43 and β-catenin jointly promote colony formation. H1299 (A) and HCT116 (B) cells were transfected with indicated siRNAs followed by the transfection of plasmids expressing CD43 or β-catenin. Data from three independent experiments are presented relative to the cells transfected with control siRNA.

is consistent with our previous results. Down-regulation of β-catenin expression resulted in the reduction of colony formation both in β-catenin and CD43 overexpressing cells. Also, the cells overexpressing CD43 or β-catenin formed fewer colonies when CD43 expression was inhibited.

These results suggest a role for CD43 in promoting cell growth in co-operation with β-catenin-mediated signaling because the cell growth-promoting property of CD43 overexpression was abolished by the down-regulation of β-catenin expression. Therefore, CD43 has a potential role in Wnt/β-catenin signaling pathway, which is often de-regulated in human cancers.

CD43 requires β-catenin to induce p53-dependent response. We have previously shown that CD43 overexpression induces the accumulation of transcriptionally active p53, and this occurs due to the stimulatory effect of CD43 on cell growth (34,35). As CD43 seems to co-operate with β-catenin in promoting colony formation, we tested whether it is also the case in p53 activation using luciferase reporter gene assay with p53-dependent *MDM2* and *CDKN1A* promoters. The p53-null H1299 cells were treated with β-catenin siRNA or CD43 siRNA (as a control) followed by the co-transfection of expression plasmids and reporter constructs.

In the cells with decreased β-catenin expression the induction of the transcriptional activity of p53 in response to CD43 overexpression was inhibited (Fig. 2A and B). One can see that

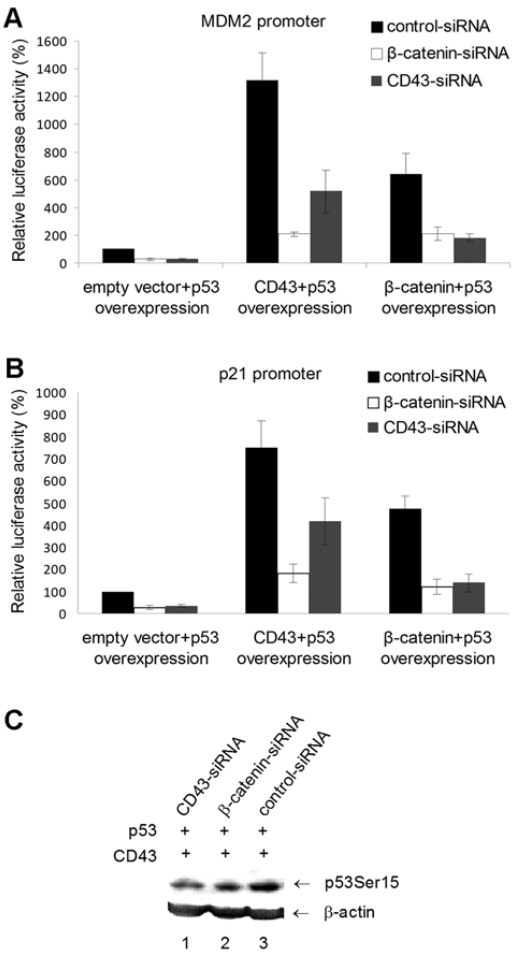

Figure 2. Activation of p53 in response to CD43 overexpression requires the presence of β-catenin. H1299 cells were transfected with indicated siRNAs followed by the transfection of p53 expressing plasmid together with plasmids expressing CD43 or β-catenin or empty vector control and with a reporter plasmid, containing either *MDM2* (A) or *CDKN1A* (B) promoter. The results of three independent experiments are presented relative to the cells transfected with control siRNA, p53 expression plasmid, empty vector control and the reporter plasmid. (C), p53 protein expression levels and phosphorylation at serine 15 were analyzed by Western blot analysis in cells treated with different siRNAs and followed by co-expression of CD43 and p53. β-actin serves as an internal loading control.

even the basal activity of p53 is lower in the cells treated with siRNAs against β-catenin or CD43. The same cells were examined by Western blot analysis to determine p53 protein levels and phosphorylation at serine 15. The latter indicates stabilization and activation of p53 probably due to the inhibition of MDM2-mediated degradation of p53 (42). Phosphorylation of serine 15 on p53 protein is induced in response to CD43 overexpression as well (34). Fig. 2C shows less phosphorylation at serine 15 when the expression of β-catenin or CD43 is down-regulated in the cells overexpressing CD43. Our results show that CD43 requires β-catenin to induce p53-dependent response.

Full-length CD43 enhances the reporter gene expression regulated by β-catenin. β-catenin is a transcriptional co-activator; it binds to the members of the TCF/LEF family of DNA-binding proteins forming a transcriptionally active complex (43). The activation of β-catenin/TCF/LEF-mediated transcription is the main down-stream consequence of Wnt signaling.

We studied the possible impact of CD43 on the transcriptional activity of β-catenin by a reporter assay in H1299 and HCT116 cells. We transfected cells with TOPflash luciferase reporter vector which reflects activation of LEF/TCF-sensitive transcription (40) together with EGFP expressing vector (negative control), β-catenin (positive control) or CD43 expressing vectors and measured luciferase activity. The overexpression of CD43 increased the transcription level of the reporter gene by several times compared to the control cells but not as much as the overexpression of β-catenin (Fig. 3A). The co-expression of both exogenous CD43 and β-catenin showed more pronounced luciferase activity relative to exogenous β-catenin alone (Fig. 3A).

To verify the role of CD43 in β-catenin-mediated transcriptional activation, we also assessed luciferase activity in case where either β-catenin or CD43 were down-regulated. The cells were treated with siRNA oligos followed by the transfection of expression vectors as described above. Indeed, the down-regulation of CD43 expression resulted in lower luciferase activity both in H1299 and HCT116 cells (Fig. 3B and C). All reporter assays were repeated using FOPflash reporter vector, which contains mutant TCF-binding sites, as a negative control. Luciferase activities in the control assays were very low as expected (data not shown). These results demonstrate that in our experimental system the presence of both CD43 and β-catenin is required for the TCF/LEF-mediated transcription.

Full-length CD43 localizes to the nucleus, binds chromatin and interacts with β-catenin. Different CD43 molecules have been described in the cell: the mature full-length glycosylated CD43, the non-glycosylated CD43 precursor, the CD43-specific cytoplasmic tail fragment (denoted as the CD43-CTF), and the CD43 cytoplasmic tail (designated here as CD43ct). The CD43-CTF which is suggested to be formed by the proteolytic removal of the ectodomain includes a small part of the extracellular domain, the intact transmembrane and intracellular domains. The CD43ct is released as a result of the intramembrane γ-secretase cleavage that follows the cleavage in the extracellular domain (25).

Subcellular localization of a protein indicates its function to some extent. Therefore we investigated which CD43 molecules are present in different subcellular fractions. We used COLO205 cell line that expresses high levels of endogenous CD43. CD43 protein was visualized with the mAb anti-CD43-4D2 which reacts with all CD43 molecules of interest because the epitope is located near the C-terminal end of CD43 intracellular domain (aa 337-343). Surprisingly, the full-length glycosylated CD43 was detected in the soluble nuclear fraction and even at a more significant level in the chromatin-bound nuclear fraction (Fig. 4A). The precursor CD43 was found in the both nuclear fractions as well. The membrane fraction contained the highest amount of the precursor CD43 and the mature CD43. The precursor CD43 was absent from the soluble cytoplasmic frac-

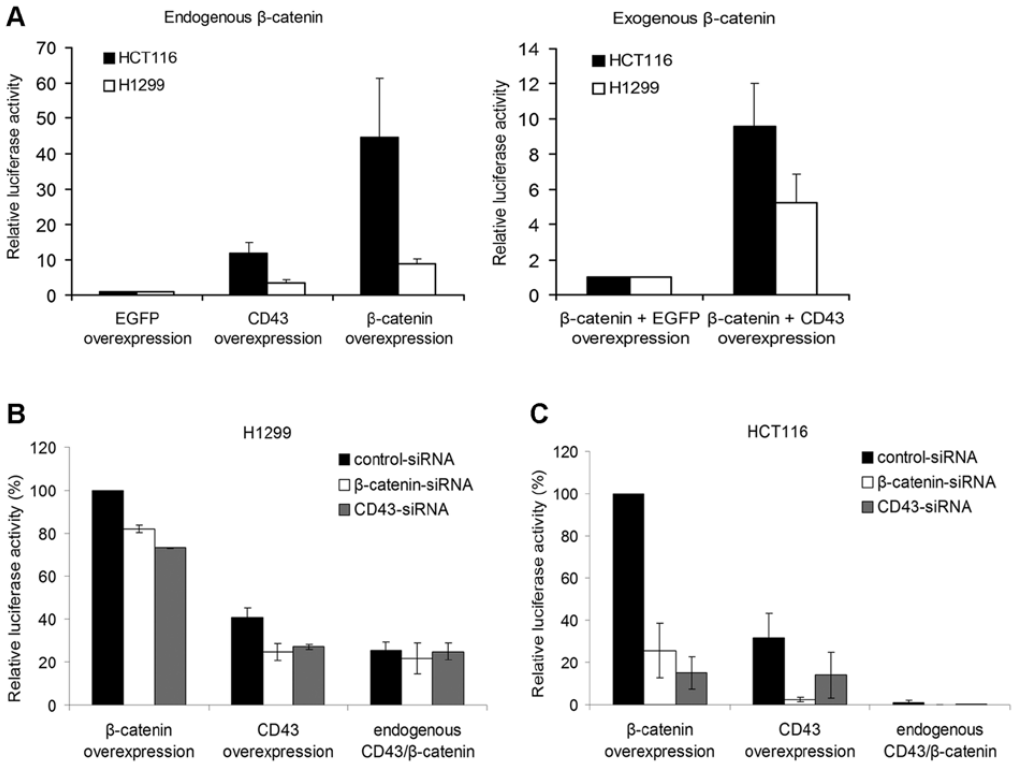

Figure 3. CD43 and β-catenin cooperate in the activation of TCF/LEF-dependent promoter. (A), H1299 and HCT116 cells were co-transfected with indicated expression vectors and TOPflash reporter plasmid. The results of three independent experiments are presented relative to the cells transfected with EGFP expression vector. (B), H1299 and (C), HCT116 cells were transfected with indicated siRNAs followed by the co-transfection of CD43 or β-catenin expression vectors and TOPflash reporter plasmid. The results of three independent experiments are presented relative to the cells transfected with control siRNA and β-catenin expression vector.

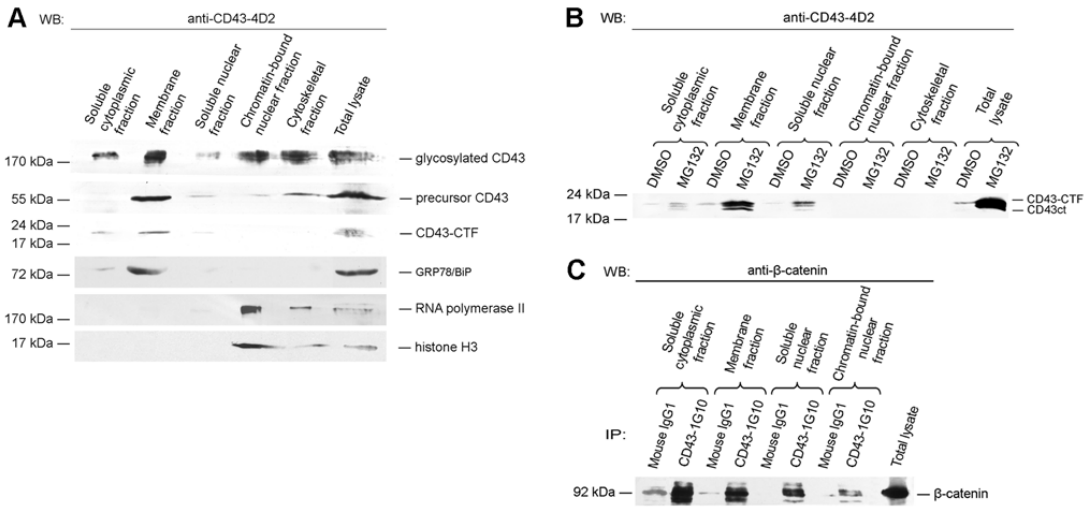

Figure 4. Full-length CD43 binds chromatin and interacts with β-catenin in the nucleus. (A), COLO205 cells were fractionated and examined by Western blot analysis using anti-CD43-4D2 mAb that recognizes all CD43 molecules in the cell. GRP78/BiP, a molecular chaperone of ER, RNA polymerase II and histone H3 were used to show fraction purity. (B), COLO205 cells were treated with the proteasome inhibitor MG132 (20 μM) for 12 h, fractionated and examined by Western blot analysis using anti-CD43-4D2 mAb. CD43 cytoplasmic fragments produced by intracellular cleavage are presented. DMSO was used as a negative control for MG132-treatment. (C), β-catenin co-immunoprecipitation with CD43 in subcellular fractions from COLO205 cells is shown by a precipitation with anti-CD43-1G10 mAb that recognizes the mature CD43 followed by a Western blot analysis probed with the mAb anti-β-catenin. The negative control for immunoprecipitation was Mouse IgG1 and the total lysate shows β-catenin migration.

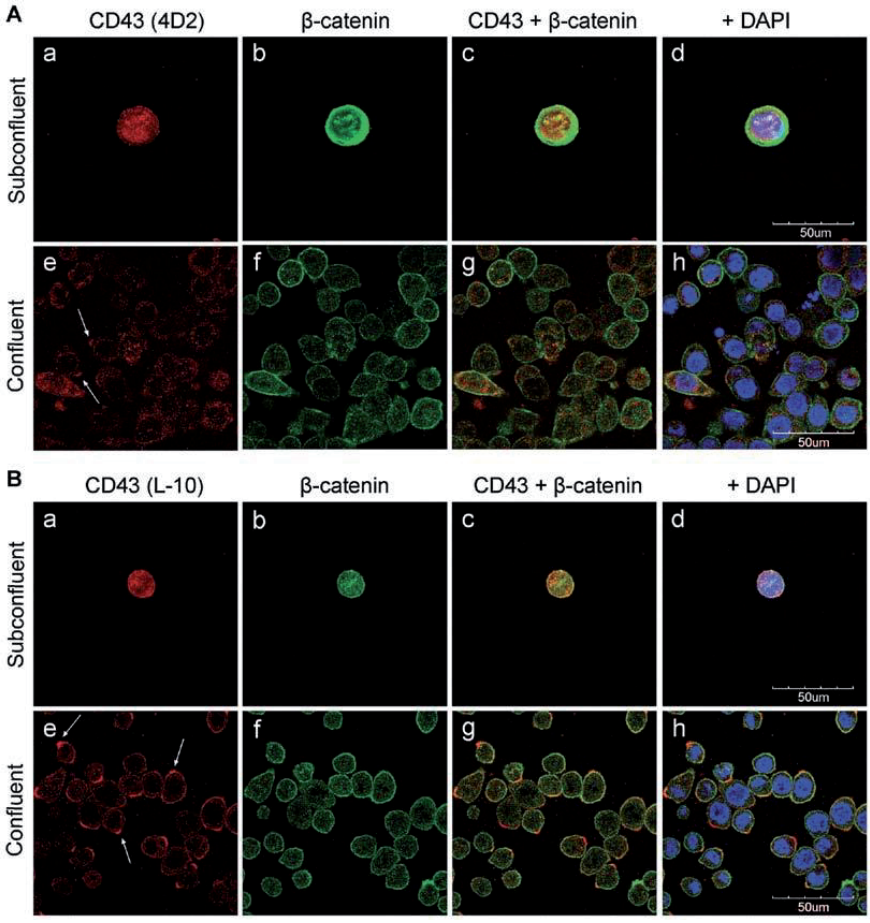

Figure 5. Confocal microscopy images of confluent and subconfluent cells showing partial co-localization between CD43 and β-catenin. (A), COLO205 cells at low density (a-d) and high density (e-h). The mAb anti-CD43-4D2 that recognizes all CD43 molecules in the cell was used to stain for CD43 (red). (B), COLO205 cells at low density (a-d) and high density (e-h). CD43 (red) was stained using the mAb anti-CD43-L-10 which recognizes only the full-length glycosylated CD43 protein. Green coloration represents β-catenin staining. 4',6-Diamidino-2-phenylindole (DAPI) was used to visualize nuclei (blue). The white arrows indicate CD43 focuses.

tion, which indicates that the precursor is compartmentalized in the cell apparently being incorporated into the endoplasmic reticulum (ER) and the Golgi apparatus for glycosylation. A considerable amount of the precursor and the mature CD43 proteins was detected in the cytoskeletal protein fraction. This is consistent with the previous findings showing that the cytoplasmic domain of CD43 associates with the cytoskeleton via ERM family adapter proteins (44). The smaller CD43 fragment, supposedly the CD43-CTF that has been shown to migrate at an apparent mass of approximately 20 kDa (in COLO205 cell line slightly higher) (25), was observed in the soluble cytoplasmic fraction, in the membrane fraction and very poorly in the soluble nuclear fraction.

When COLO205 cells were incubated with the proteasome inhibitor MG132, the amount of the CD43-CTF increased as previously described (25), and even smaller CD43 fragments could be detected (Fig. 4B). We believe that the lower band corresponds to the cytoplasmic tail of CD43, because the expected difference in molecular weight between the CD43-CTF and the CD43ct is approximately 2.5 kDa [calculated using Protein Molecular Weight Calculator at http://www.sciencegateway.org and potential cleavage sites described in (29) and (25)]. The CD43ct has been previously detected by immunoblotting only as a recombinant protein probably due to the rapid degradation of the native form (25). Upon addition of the proteasome inhibitor none of these CD43 molecules translocate to the chromatin-bound nuclear fraction; the CD43 fragments appeared only in the fractions where CD43-CTF was present. These results indicate that the mature CD43 might have a role in regulating gene expression in the nucleus. This is consistent with our previous finding indicating that the cells overexpressing the full-length CD43, and not the CD43ct, are more resistant to Fas-mediated apoptosis (35).

In order to investigate whether the mature CD43 binds to β-catenin and in which part of a cell the interaction might take place, we performed a co-immunoprecipitation from the different subcellular fractions of COLO205 cells. CD43 was immunoprecipitated using the mAb anti-CD43-1G10 which

recognizes only the full-length glycosylated CD43 protein. The immunoprecipitation was followed by a Western blot analysis using the mAb anti-β-catenin. Mouse IgG1 was used as a negative control. β-catenin was found to be co-immunoprecipitated with CD43 from all fractions isolated (Fig. 4C). The interaction between the mature CD43 and β-catenin in the chromatin-bound nuclear fraction suggests that the full-length CD43 might modulate β-catenin/TCF/LEF-mediated transcription and contribute to impaired Wnt signaling in colon cancer.

Cell density-dependent interaction between CD43 and β-catenin. CD43 and β-catenin are both involved in cell adhesion. Moreover, cell density-dependent subcellular localization of CD43 (24) and β-catenin (45) has been reported before. We studied CD43 and β-catenin co-localization by confocal microscopy in confluent and subconfluent COLO205 cells. CD43 was detected with the mAb anti-CD43-4D2 that reacts with all CD43 molecules in the cell (Fig. 5A) or with the glycosylation specific mAb anti-CD43-L-10 that recognizes only the full-length CD43 because its epitope is located in the extracellular part of CD43 (aa 1-78) (Fig. 5B). Our results demonstrate that CD43 and β-catenin partly co-localize and the co-localization pattern depends on cell density (Fig. 5A, panels c and g; Fig. 5B, panels c and g). A considerable portion of CD43 staining was intracellular as previously described for CD43 in cancer cells (23). In subconfluent populations CD43 was found throughout the cells, significantly concentrating into the nucleus (Fig. 5A, panels a and d; Fig. 5B, panels a and d). In confluent populations CD43 formed focuses (Fig. 5A, panel e; Fig. 5B, panel e). Previous reports also show granular localization of CD43 in cancer cells (23). Similarly to CD43 β-catenin was detected in the nucleus in subconfluent cells (Fig. 5A, panel b; Fig. 5B, panel b), whereas in confluent cells nuclear β-catenin staining decreased (Fig. 5A, panels f and h; Fig. 5B, panels f and h). CD43 and β-catenin co-localization in subconfluent cells was clearly seen in the nucleus as yellow areas (Fig. 5A, panels c and d; Fig. 5B, panels c and d). In confluent cells CD43 and β-catenin co-localized more in the cytoplasm and membrane (Fig. 5A, panel g; Fig. 5B, panel g). Remarkably, the mature CD43 protein accumulated in the nucleus and co-localized with β-catenin in subconfluent cell populations. This is consistent with our hypothesis that the full-length glycosylated CD43 co-operates with β-catenin in the promotion of a better survival of cancer cells.

To address whether the intensity of interaction between CD43 and β-catenin depends on cell density, we performed a co-immunoprecipitation in confluent and subconfluent COLO205 cells. CD43 was immunoprecipitated using the mAb anti-CD43-4D2 followed by a Western blot analysis using the mAb anti-β-catenin. Mouse IgG1 was used as a negative control (data not shown). In confluent cells the level of β-catenin co-precipitated with CD43 was slightly increased (Fig. 6A). To further investigate the impact of cell density on the interaction between CD43 and β-catenin, we repeated the co-immunoprecipitation in subcellular fractions from confluent and subconfluent COLO205 cells. CD43 and β-catenin were found to interact in all fractions regardless of cell density (as previously shown with anti-CD43-1G10 mAb in Fig. 4C). Interestingly, CD43 binding to β-catenin in the soluble nuclear fraction and in the chromatin-bound fraction

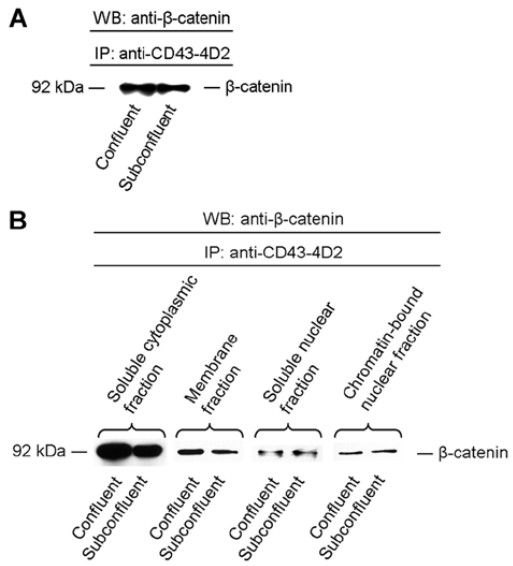

Figure 6. The influence of cell density on the interaction between CD43 and β-catenin. (A), CD43 and β-catenin co-precipitation from the total lysate of confluent and subconfluent COLO205 cells. (B), CD43 and β-catenin co-precipitation from the subcellular fractions of confluent and subconfluent COLO205 cells. CD43 was immunoprecipitated using the mAb anti-CD43-4D2 followed by a Western blot analysis using the anti-β-catenin mAb. A negative control for a precipitation from each subcellular fraction was done using Mouse IgG1 (data not shown).

was equal in confluent and subconfluent cells (Fig. 6B). The difference between confluent and subconfluent cells appeared in the cytoplasmic fraction where CD43 binding to β-catenin was increased in confluent cells as compared to subconfluent cells (Fig. 6B). The discrepancy between co-localization and co-immunoprecipitation patterns might be conditioned by the fact that co-localization indicates only the potential to interact, which is verified here by co-IP.

β-catenin-mediated transcription depends on cell density. Colon cancer formation is commonly caused by the up-regulation of β-catenin/TCF/LEF-mediated transcription leading to the activation of genes that stimulate cell growth. Although we did not see any cell density-dependent changes in the interaction between CD43 and β-catenin in the nucleus, we studied the possible influence of cell density on β-catenin-mediated transcription. We transfected confluent and subconfluent COLO205, H1299 and HCT116 cells overexpressing CD43 with TOPflash luciferase reporter vector and measured luciferase activity. In COLO205 cell line there was a 2-fold higher transcription level of the reporter gene in subconfluent cells compared to confluent cells. In H1299 and HCT116 cells the effect was not so prominent (Fig. 7).

All reporter assays were repeated using FOPflash reporter vector as a negative control. Luciferase activities in the control assays were very low as expected (data not shown). These results indicate that in subconfluent cells transcriptionally active β-catenin accumulates in the nucleus. Thereby subconfluent cells might gain growth advantage over confluent cells.

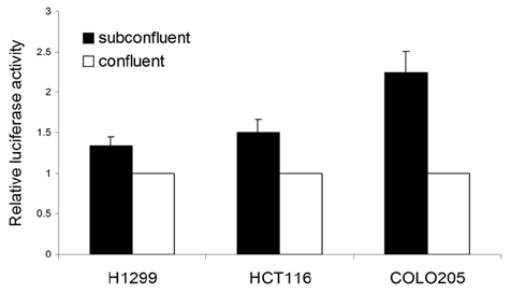

Figure 7. Transcriptional activity of β-catenin depends on cell density. TOPflash luciferase reporter vector was transfected into H1299, HCT116 or COLO205 confluent and subconfluent cells overexpressing CD43. The results of three independent experiments are presented relative to the confluent cells.

Discussion

The concept of deregulated activation of β-catenin as a principal cause of colorectal cancer is well accepted. Expression of the leukocyte marker CD43 in colon tumors but not in normal tissues has been described by different research groups. The contribution of CD43 to carcinogenesis is largely unknown, although some insights have been reported by our group and by others. Similarly to well-studied oncogenes (e.g., β-catenin) CD43 overexpression activates ARF/p53 tumorsuppressor pathway resulting in apoptosis. When either ARF or p53 is absent, CD43 overexpressing cells gain growth advantage due to the better survival of these cells. In addition, cytoplasmic tail of CD43 has been shown to interact with β-catenin and induce expression of its target genes (24). This suggests that CD43 is involved in colon tumor development via Wnt signaling pathway.

In the present study we provide new evidence for the interaction between CD43- and β-catenin-dependent signaling pathways. We address the possibility of the interaction between these two pathways by colony formation assay using siRNA against CD43 and β-catenin. We show that in both CD43- and β-catenin-overexpressing cells the depletion of either protein causes a reduction in the number of colonies. Besides, inhibition of β-catenin abrogates the growth-promoting effect of CD43 overexpression. The results confirm that both proteins are required for cell growth and CD43 is indeed involved in Wnt signaling.

Moreover, CD43 requires β-catenin to induce p53-dependent response. We have previously shown that the overexpression of the full-length CD43, but not the CD43ct-EGFP, causes the accumulation of p53 and apoptosis in the cells (34). β-catenin is able to activate p53 as well (46). Here we demonstrate that silencing β-catenin expression by siRNA inhibits the induction of the transcriptional activity of p53 in response to CD43 overexpression. Also, if expression of β-catenin or CD43 is down-regulated in the cells overexpressing CD43, the serine 15 on p53 protein is less phosphorylated, revealing the destabilization and deactivation of p53. This further confirms our hypothesis according to which CD43, as a potential oncogene, participates in signal transduction via Wnt-pathway.

The additional evidence supporting the role of CD43 in β-catenin-mediated signaling comes from our next finding employing the reporter assay. We show that the overexpression of CD43 enhances the reporter gene expression regulated by β-catenin. On the other hand, in the case where CD43 expression is silenced, the overexpression of β-catenin does not enhance the activation of its reporter gene expression, indicating important cross-talk between the CD43- and β-catenin-dependent pathways.

Applying fractionation and co-immunoprecipitation we have seen that the full-length glycosylated CD43 is able to localize into the nucleus where it binds chromatin and interacts with β-catenin. When cells were treated with the proteasome inhibitor MG132, two cytoplasmic fragments of CD43 became detectable in the soluble cytoplasmic fraction, in the membrane fraction and in the soluble nuclear fraction. We presume that the smaller fragment is the cytoplasmic tail of CD43, because it is an expected position for the CD43ct relative to the CD43-CTF (25,29). Both of the fragments are produced from the cytoplasmic part of CD43 because the epitope of the anti-CD43-4D2 mAb used for detection is located in the distal C-terminus of CD43. In our experiments with MG132 proteasome inhibitor we have noticed that the intracellular domain of CD43 is cleaved in multiple sites (data not shown), which explains the appearance of the other fragment, but the biological meaning of this phenomenon is still unclear. The effect of the proteasome inhibitor implies that these CD43 molecules have a short protein half-life and are quickly degraded in the proteasome pathway. Besides, it has previously been proposed that CD43ct is quickly metabolized (25). Upon addition of the proteasome inhibitor none of these CD43 fragments translocate to the chromatin-bound nuclear fraction, the fragments appeared only in the fractions where CD43-CTF accumulated. This is somewhat contradictory to the published data indicating that CD43ct binds β-catenin to activate the expression of β-catenin target genes *MYC* and cyclin D1 (24). However, the presence of the mature CD43 in the chromatin-bound nuclear fraction and the failure of the CD43 cytoplasmic fragments to accumulate in this fraction correlate with our results showing that the overexpression of the full-length CD43, and not the CD43ct, helps the cells with defective ARF-p53 signaling to evade Fas-mediated apoptosis and promotes cell growth (35). β-catenin is also known to inhibit Fas expression on the cell surface (47). This supports the idea of the mature CD43 acting as a pro-survival factor via Wnt-pathway.

We propose that the CD43ct and the mature CD43 may have distinct functionality in the nucleus. The CD43ct, being SUMOylated and recruited into PML nuclear bodies (26), might participate in homeostasis and apoptosis primarily by interacting with PML-associated proteins (e.g., DAXX, p53, HIPK2) and the mature CD43, being capable of binding chromatin, regulates gene expression. However, the role of CD43 in β-catenin-mediated transcriptional activation is not fully understood. Still, we do not know whether CD43 acts as nothing but a chaperone/stabilizer for β-catenin and this way contributes to its transcriptional activity, or CD43 belongs to the protein complex that binds to the promoter regions of β-catenin target genes. It is tempting to speculate that the CD43ct stabilizes β-catenin and this way causes the activation of *MYC* and cyclin D1 expression while the mature CD43 binds DNA and interacts with TCF/LEF/β-catenin complex at promoter regions of β-catenin target genes. Besides, Seo and Ziltener demonstrated that the CD43ct is recruited into

PML by confocal microscopy. However, they detected the full-length glycosylated CD43 by Western blot analysis using the same antibody. In addition, they observe that the mature CD43 may be SUMOylated as well. Hence, it cannot be excluded that only the mature CD43, or the both molecules localize in PML nuclear bodies.

The mechanism for the translocation of the full-length glycosylated CD43 into the nucleus can be based on the presence of the NLS in the cytoplasmic domain of the protein (24) or on the reversible attachment of *O*-linked N-acetylglucosamine (*O*-GlcNAc) which is known to play an important role in the modulation of the biological activity of intracellular proteins. The involvement of *O*-GlcNAc in the NLS-independent nuclear transport of cytosolic proteins was first sustained by studies on bovine serum albumin showing that the protein could be actively carried to the nucleus when it was modified with sugars (48). One of the mechanisms through which *O*-GlcNAc might act as a nuclear localisation signal is by counteracting the function of phosphorylation (48,49). Phosphorylation has been shown to affect nuclear translocation of cytosolic proteins (49,50). The localization of CD43 might be regulated by the same mechanism because CD43 is phosphorylated and extensively *O*-glycosylated carrying core 2 *O*-glycan structures which contain GlcNAc (51).

Moreover, the *O*-glycan structures of CD43 might account for oncogenic properties of the protein. It has been reported that abnormal expression level of certain *O*-glycan structures as well as occurrence of truncated forms, precursors, or novel structures of *O*-glycans may affect ligand-receptor interactions (e.g., modulating the binding to alternative ligands) and thus interfere with regulation of signal transduction (52). Indeed, aberrant glycosylation of proteins, including CD43, may contribute to cancer progression by modifying cell signaling, adhesion, migration and survival (20,53,54). Tumor-specific glycoforms of CD43 are expressed in different carcinomas, but not in normal tissues from the same patients (55). Also, altered expression of distinct glycoforms of CD43 has been associated with neoplastic transformation (14,15,23). Aberrant signaling of these CD43 glycoforms may promote tumorigenesis by activating β-catenin, NF-κB, NFAT and AP-1, which are pro-survival transcription factors and promote a tumor phenotype when deregulated (25,56). In addition, proteolytic processing of certain proteins demonstrates a requirement for *O*-glycans at specific sites in order to prevent proteolytic cleavage which eliminates biological activity or prevents continued residence/activity of the intact protein at its designated subcellular location (51). Thus, abnormal glycosylation could modulate the proteolytic processing of CD43 and therefore interfere with CD43 functionality.

Another factor which is able to modulate CD43 and β-catenin localization and function is cell density. Cell density-dependent subcellular localization of β-catenin and CD43 has been described before (24,45). Meanwhile, it is widely accepted that the involvement of β-catenin in cell adhesion or transcription is strictly associated with the subcellular localization of β-catenin. Here, we demonstrate that in subconfluent cells CD43 and β-catenin co-localize in the nucleus where, based on our hypothesis, CD43 could contribute to tumor formation in co-operation with β-catenin. We had supposed that the amount of CD43 bound to β-catenin increases in subconfluent cells, but it was not confirmed by the co-immunoprecipitation experiment. Moreover, the co-immunoprecipitation from different subcellular fractions revealed equal intensity of CD43 and β-catenin interaction in the nucleus of confluent and subconfluent cells. Apparently, the interaction outside the nucleus correlates with cell density (probably due to the involvement of both CD43 and β-catenin in cell adhesion), whereas the interaction in the nucleus presumably serves the purpose of transcriptional activation of β-catenin/TCF target genes which might participate in the inhibition of apoptosis. Hence, the interaction between CD43 and β-catenin in the chromatin-bound fraction and the co-operation in transactivation offer a possible explanation for the mechanism of the previously described phenomenon. Namely, CD43 overexpression increased the growth rate of cells making them more resistant to Fas-mediated apoptosis (35). In spite of the co-immunoprecipitation results, we found, employing the reporter assay, that the ability of β-catenin to activate reporter gene transcription is increased in subconfluent cells compared to confluent cells suggesting that the abnormal activation of β-catenin in cancer cells is not conditioned exclusively by CD43 or that CD43 might influence β-catenin-mediated transcription indirectly.

Furthermore, in colorectal carcinomas nuclear accumulation of β-catenin occurs in cells found at the invasive front and in disseminated cells at the metastatic site. The expression of cytoplasmic and membranous β-catenin is seen in the central areas of the metastases and primary tumors (57). We believe that the conditions in a subconfluent cell population mimic the conditions of the disseminated cells during metastasis. Interestingly, the tumor cells at the invasive front and the disseminated cells at the metastatic site have lost their proliferative activity (57). This suggests that the contribution of β-catenin to cancerogenesis is implemented by a better survival of the cells with nuclear β-catenin and not by an enhanced proliferation of these cells. The ability of β-catenin to interact with CD43 which conveys a survival advantage and helps to evade apoptotic responses (26,35), also the co-operation between β-catenin and CD43 in enhancing cell growth is consistent with this hypothesis.

In conclusion, we have established a relation between CD43- and β-catenin-mediated signaling pathways which supports the idea that CD43 could have a role in tumor development.

Acknowledgements

This work was partly supported by Estonian Science Foundation grants nos. 7545 and 7578, European Commission grant no. 205419 (ECOGENE) to Estonian Biocentre, and European Social Fund grant no. 1.2.0401.09-0073 to the University of Tartu (Graduate School of Biomedicine and Biotechnology). We thank Dr G. Hansson and Ch. Andersson for the CD43 expression vector and Dr B. Vogelstein for β-catenin (S33Y) expression vector.

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MANUSCRIPT

Jääger K, Fatkina A, Velts A, Orav E, Neuman T. Variable expression of lineage regulators in differentiated stromal cells indicates distinct mechanisms of differentiation towards common cell fate.

ABSTRACT

Mesenchymal stem cells (MSCs) are cells residing in adult tissues that can proliferate and differentiate into minimally three specialized mesodermal cell types including adipocytes, osteoblasts and chondrocytes *in vitro*. These qualities make MSCs highly potential candidates for cell therapy in regenerative medicine. MSCs have been isolated from the stromal fraction of virtually all tissues. However, the molecular characteristics of these cells have remained poorly known. MSCs express surface antigens CD105, CD90 and CD73 and exhibit multipotency. However, the same properties are shared by fibroblasts (FBs). Studies on the differentiation potential of FBs have reported controversial results. Some FBs have been shown to exhibit differentiation potential into fat, bone and cartilage cells under similar conditions with MSCs, whereas others have not. Conflicting data may arise from different cell sources and experimental conditions used.

The aim of this thesis was to study the functional properties of different stromal cells including adipose-derived MSCs (AdMSCs) and dermal FBs in terms of multilineage differentiation potential under standard conditions, in attempt to gain insight to the regulation of cell fate choices. We analyzed the dynamics of gene expression, global transcriptome patterns and variations of gene expression between AdMSCs and FBs upon stimulation towards adipocyte, osteoblast and chondrocyte pathways. The results of the described work allow to affirm that AdMSCs and FBs are phenotypically indistinguishable cells and exhibit similar potential of differentiation. Differences between these cells arise from distinct gene expression dynamics – AdMSCs exhibit faster adipogenic differentiation compared with FBs. Transcriptome profiles of AdMSCs and FBs are distinct and retain some differences upon differentiation – a phenomenon referred to as cell 'memory'. Global gene expression dynamics is similar along adipogenesis and osteogenesis between AdMSCs and FBs, but different for chondrogenic lineage. Heterogeneous expression of lineage-specific genes in AdMSC and FB populations arises from variations of gene expression between single differentiating cells. Single-cell analysis indicates distinct molecular mechanisms of differentiation of stromal cells towards common cell fate.

It concludes that the heterogeneity and distinct differentiation of AdMSCs and FBs arise from the dynamics of gene expression, that causes variation in expression patterns at single cell, cell population, tissue-source and donor level. Despite that, AdMSCs and FBs are functionally equivalent and can differentiate into diverse cell types upon appropriate extracellular stimuli.

KOKKUVÕTE

Mesenhümaalsed tüvirakud (MSC-d) on täiskasvanud kudedes olevad rakud, mis omavad võimet paljuneda ja diferentseeruda vähemalt kolmeks spetsialiseerunud rakutüübiks nagu adipotsüüdid, osteoblastid ja kondrotsüüdid *in vitro*. Need omadused teevad MSC-dest kõrge potentsiaaliga rakuteraapiakandidaadid regeneratiivses meditsiinis. Neid rakke on eraldatud peaaegu kõikide kudede stroomast ent nende molekulaarne kirjeldus on senini puudulik. Nad ekspresseerivad pinnamolekule CD105, CD90, CD73 ja on multipotentsed, ent samasugused omadused on ka koefibroblastidel. Fibroblastid (FB-d) on klassikalised sidekoerakud, mis tagavad kudede toestuse. FB-de diferentseerumisvõime kohta leidub kirjanduses vastakaid andmeid. Osad tööd näitavad, et MSC-d ja FB-d diferentseeruvad ühtemoodi rasva-, luu-, ja kõhrerakuks, kuid on töid, mis väidavad, et just diferentseerumisvõime eristab MSC-sid FB-st. Vastuolulised andmed võivad tuleneda sellest, et uuritud rakud pärinevad erinevatest kudedest ja on kasvatatud erinevates tingimustes.

Käesoleva doktoritöö eesmärgiks oli uurida erinevate stroomarakkude sealhulgas MSC ja FB-de mitmesuunalist diferentseerumist ühtlustatud tingimustes, et selgitada välja, kuidas raku jagunemise ja diferentseerumise valikuid reguleeritakse. Me analüüsisime geeniekspressiooni dünaamikat, ülegenoomseid ekspressioonimustreid ja geeniekspressiooni varieeruvust rasvkoest eraldatud MSC-des (AdMSC) ja nahast eraldatud FB-des, mida stimuleeriti diferentseeruma rasva-, luu- ja kõhrearengu suunas. Kirjeldatud töö tulemused lubavad väita, et AdMSC-d ja FB-d on immuunofenotüübilt eristamatud ja ühesuguse diferentseerumise võimega rakud. Erinevused nende rakkude vahel tulenevad geeniekspressiooni dünaamikast – AdMSC-d diferentseeruvad kiiremini rasvarakuks kui FB-d. Transkriptoomi-profiilid on neil rakkudel erinevad, ent erinevused vähenevad diferentseerumise käigus. Osaline ekspressiooniprofiili säilumine AdMSC-del ja FB-del peale diferentseerumise signaalide käivitamist viitab raku ’mälule’. Globaalne geeniekspressiooni dünaamika sarnaneb AdMSC-del ja FB-del rasva- ja luuarenguradades, ent erineb kõhrerajas. Arengusuuna-spetsiifiliste regulaatorite ja sihtmärkgeenide ekspressiooni varieeruvus AdMSC ja FB populatsioonides tuleneb geeniekspressiooni heterogeensusest üksikute diferentseeruvate rakkude vahel. Ühe-raku analüüs viitab sellele, et strooma rakud kasutavad samas arengusuunas diferentseerumisel erinevaid molekulaarseid mehhanisme.

Nendest tulemustest järeldub, et AdMSC ja FB-de heterogeensus ja erinevused diferentseerumises tulenevad geeniekspressiooni dünaamikast, mis põhjustab varieeruva ekspressioonimustri ühe raku, rakupopulatsiooni, koe ja indiviidi tasandil. Sellest sõltumata on AdMSC-d ja FB-d funktsionaalselt samasugused rakud, mis suudavad kindla rakuvälise stiimuli mõjul diferentseeruda erinevateks rakutüüpideks.

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Publikatsioonid:

Jääger K, Islam S, Zajac P, Linnarsson S, Neuman T. (2012). RNA-Seq Analysis Reveals Different Dynamics of Differentiation of Human Dermis- and Adipose-derived Stromal Stem Cells. PLoS One, 7(6):e38833

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