Role of wnt-5a in the determination of human mesenchymal stem cells into preadipocytes

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Summary:

Increasing adipocyte size as well as numbers are important in the development of obesity and type 2 diabetes, with adipocytes being generated from mesenchymal precursor cells. This process comprises the determination of mesenchymal stem cells (MSC) into preadipocytes (PA) and the differentiation of PA into mature fat cells. While the process of determination is highly investigated, the determination in humans is poorly understood. In the present study we compared human MSC and human committed PA on a cellular and molecular level to gain further insights into regulatory mechanisms in the determination process. Both cell types showed similar morphology and expression patterns of common mesenchymal and hematopoetic surface markers. However, while MSC were able to differentiate into adipocytes and osteocytes, PA were only able to undergo adipogenesis, indicating that PA lost their multipotency during determination. Wnt-5a expression showed significant higher levels in MSC compared to PA suggesting that wnt-5a down-regulation might be important in the determination process. Indeed, incubation of human MSC in medium containing neutralizing wnt-5a antibodies abolished their ability to undergo osteogenesis while adipogenesis was still possible. An opposite effect was achieved using recombinant wnt-5a protein. On a molecular level, wnt-5a was found to promote c-Jun-N-terminal-kinase-dependent intracellular signalling in MSC. Activation of this non-canonical pathway resulted in the induction of osteopontin expression further indicating pro-osteogenic effects of wnt-5a. Our data suggest that wnt-5a is necessary to maintain osteogenic potential of MSC and that inhibition of wnt-5a signalling therefore plays a role in their determination into PA in humans.

Introduction:

Epidemiological studies suggest that obesity and related metabolic disorders, e. g. type 2 diabetes, will be increasing prevalent within the next decades [1]. Understanding the molecular mechanisms in the pathogenesis of this important disease therefore is currently a major goal in biomedical research. It has been shown in human studies that besides increasing the size of existing adipocytes the generation of mature fat cells from mesenchymal precursor cells is of importance in developing obesity [2]. This process, called adipogenesis, consists of two related steps, the determination of human mesenchymal stem cells into preadipocytes and the differentiation of preadipocytes into mature fat cells [3]. Interestingly, using fatty acid binding protein (FABP)-4 as a molecular marker, it has been shown that most precursor cells in human adult adipose tissue are committed preadipocytes rather than multipotent mesenchymal stem cells [4]. This is in agreement with a recent study in rodents which suggests that the determination of mesenchymal stem cells into preadipocytes might occur in very early stages of development, e. g. perinatal life [5]. Since the number of preadipocytes and mature fat cells has been shown to be different between lean and obese human adult subjects [6], variations in the determination process in early stages of adipose tissue development might be
important in the pathogenesis of obesity and type 2 diabetes.

Wnt molecules are secreted glycopeptides that can act in an autocrine and paracrine manner and were first discovered in Drosophila. They are able to activate two distinct signalling pathways. Most knowledge exists on the so called canonical pathway where wnt molecules bind to a receptor complex consisting of a Frizzled (Frz)-receptor and a LDL-receptor related peptide (LRP) co-receptor. Upon wnt binding to these receptors, a cytosolic protein complex containing Glycogensynthasekinase (GSK)-3β is getting inactivated which leads to stabilization of cytosolic β-catenin. The latter then translocates into the nucleus where it activates target genes by interacting with TCF transcription factors [7]. Besides this β-catenin dependent pathway at least two other so called non-canonical pathways exist. One of this is G-protein coupled whereby its activation triggers an intracellular influx of Ca^{2+} which in turn stimulates Protein kinase (PK)-C or Calmodulin dependent protein kinase (CamK)-II [8]. The other non-canonical pathway involves activation of c-Jun N-terminal kinase (JNK) upon binding of the Wnt molecules to a Frz-receptor [9] or the orphan receptor Ror-2 [10].

Wnts play an important role in many developmental processes as well as in maintaining tissue homeostasis [11]. In rodents, wnt-10b and wnt-5a are expressed in undifferentiated adipogenic precursor cells and inhibition of wnt-10b signalling in these cells has been shown to be sufficient to induce spontaneous differentiation into mature adipocytes [12]. Furthermore, in humans, induction of adipogenesis is associated with an up-regulation of Dickkopf (Dkk)-1 expression, a secreted factor known to act as an inhibitor of the canonical wnt-signalling pathway [13]. In contrast to preadipocyte differentiation, which is inhibited by wnt-signalling, osteogenesis is promoted in precursor cells in the presence of distinct wnt molecules [14].

In the past, most experiments on the molecular regulation of adipogenesis were performed using the mouse cell line 3T3-L1. However, since these cells are preadipocytes already committed to the adipogenic lineage, data about the determination of stem cells into preadipocytes are rare. In the present study we aimed to gain further insights into the molecular regulation of this developmental process by comparing human mesenchymal stem cells and committed preadipocytes on a cellular and molecular level. Using this approach, we identified that the inhibition of JNK mediated non-canonical wnt-5a signalling is of importance in the determination of stem cells to the adipogenic versus osteogenic lineage in humans.

Experimental procedures:

Cell preparation, culture and differentiation: Primary human cell cultures: The study was approved by the local ethics committee and written informed consent was obtained from participants and in case of umbilical cord blood from both parents. Human mesenchymal stem cells (hMSC) were isolated from umbilical cord blood samples (gestational age 38-41 weeks). 20-30 ml umbilical cord blood were collected from n=350 newborns straight after delivery. RosetteSep (StemCell Technology) was added to the blood samples (50 µl RosetteSep per 1ml blood) and incubated 20min at RT. Subsequently blood was diluted 1:1 with PBS and stacked on Lymphoprep (Fresenius). Centrifugation at 2000rpm for 20 min was performed and the layer containing mononuclear cells was extracted and washed with PBS three times. Mononuclear cells were seeded on culture dishes. After 24h hMSCs were adherent and the medium containing non-adherent lymphocytes and monocytes was removed. MSC’s were grown in basalm medium (Dulbecco’s modified Eagle medium (DMEM)) containing 1g/L D-Glucose, 30% FCS and 1% penicillin/streptomycin. Human subcutaneous preadipocytes (PA) were isolated form adipose tissue biopsies from metabolically healthy subjects at the age of 18-35 years as described earlier. [15]. Blood vessels were carefully dissected from the fat biopsies. Cell lines: C3H10T1/2 cells were grown in Dulbecco’s modified Eagle medium (DMEM) containing 4,5 g/l D-Glucose, 10% NCS and 1% penicillin/streptomycin. 3T3-L1 cells were grown in Dulbecco’s modified Eagle medium containing 4,5 g/l D-Glucose, 10% NCS and 1% penicillin/streptomycin. All cells were grown in humidified atmosphere at 37°C and 5% CO_{2}. For differentiation experiments hMSC’s were transferred to 6-well plates. Two days post-confluence adipogenesis was induced by adding adipogenesis medium (DMEM, 10% FCS, 1% penicillin/streptomycin, 0.1 µmol/l...
dexamethason, 5 µg/ml Insulin, 0.5 mmol/l IBMX and 60 µmol/l indometacin). Osteogenesis was induced at 80% confluency by adding osteogenesis medium (DMEM, 10% FCS, 1% penicillin/streptomycin, 1 µmol/L dexamethason, 0.05mmol/l ascorbic acid and 10 mmol/l β-glycerophosphat). Before performing experiments the two primary cell populations were cultured for at least 3 weeks under standard conditions, since it has been shown, that even if some mononuclear or endothelial cells are present in the initial preparation, after that time most of the cells are of mesenchymal origin [16]. The cells were incubated in differentiation medium for a total of 12 days, while changing the medium every other day. 2 µg anti-wnt-5a antibodies (sc-23698, Santa Cruz), 2 µg non-specific antibodies of the same subclass (sc-2027, Santa Cruz) or 500ng/ml recombinant wnt-5a (R&D) were added per well as indicated in the figure legends.

Luciferase assay:
For Luciferase assay C3H10T1/2cells were seeded in 24-well plates and grown to 80% confluency. The cells were transfected with 1µg of TOPFLASH or pGL2-AP1 construct and 10 ng of pRL-CMV as described earlier [17]. The samples were measured in triplicates in a luminometer (Mithras LB 940, Berthold).

Immunofluorescence:
C3H10T1/2cells were transferred to 24 well plates and grown to 90% confluency. After incubation with LiCl (control) or anti-wnt-5a-antibodies as indicated in the figure legends, cells were washed with PBS and subsequently fixed with 100% methanol for 5 min. Subsequently cells were washed 3 times with PBS and blocked with 1% bovine serum albumin (BSA) in PBS for 1h. After that cells were incubated with beta-catenin antibodies for 1h and washed 3 times with 1% BSA/PBS solution. Subsequently cells were incubated 1h with appropriate secondary antibodies and washed 3 times with 1% BSA/PBS solution. After washing cells were fixed on a microscope slide using Vectorshield with DAPI (Vector Lab.).

Electromobility Gel Shift Assay:
C3H10T1/2 cells were lysed with lysis buffer (50 mM KCl, 0.5% NP-40, 25 mM HEPES (pH 8.0), 125 µM DTT, 1x complete protease inhibitor tabs. (Roche)) for 4 min on ice. The EMSA experiment was described as reported earlier [17] using 10 µg of the nuclear protein extracts and 20 fmol 5'-biotin labeled doublestranded oligonucleotides containing the c-Jun binding site (5’-GGGCTTGATGAGTCAGCCGGACC-3’).

Fluorescent activating cell sorter (FACS)-Analysis:
4 x 10⁵ cells were used per FACS-tube. The tubes were centrifuged at 1000rpm for 5 min. After centrifugation cells were resuspended in PBS-FCS-Acid mix (2%FCS, 0.05% Na-Acid) and incubated for 15 min at 4ºC. Subsequently cells were incubated with the given antibodies (all BD Bioscience, except for CD31 (Immuno Tools)) for 30 min at 4ºC, washed twice with PBS-FCS-Acid mix and were analysed by a fluorescent activating cell sorter (Becton Dickinson FACSCalibur).

Oil red O and Alizarin red S staining:
Oil red O staining was performed as described earlier [16]. For Alizarin Staining, hMSC’s incubated in osteogenic medium were washed with PBS and fixed in 1% PBS-buffered Formaldeylyd for 10 min. The cells were then washed again with PBS and stained with 1% Alizarin red S (stock solution: 10mg/ml, Sigma) for 2 min. Excess Alizarin red S was removed by washing with H₂O.

Western blotting:
Western blotting was performed as described earlier [17]. Hypotonic cell-lysis buffer for β-catenin preparation was described previously [14]. Primary antibodies were purchased from Cell Signaling (PPAR-γ, CamKII, p-CamKII, JNK, p-JNK) and Santa Cruz (OPN, c-Jun, β-catenin) and Sigma (secondary antibodies).

Results
Human mesenchymal stem cells but not human preadipocytes exhibit multipotent differentiation capacity
In the present study we aimed to identify novel molecular regulators in the determination of mesenchymal stem cells into preadipocytes. Since there is no cell line available to investigate this developmental process in humans we used the following approach. We have isolated human mesenchymal stem cells (hMSC) from umbilical cord blood samples which have been reported to be able to develop into several mesenchymal lineages, including adipocytes, osteocytes and
myocytes and therefore exhibit truly multipotent capacity [18-21]. Besides that, mesenchymal precursor cells from the stromavascular fraction (SVF) were prepared from subcutaneous adipose tissue biopsies from human adult subjects. It has been shown in a previous report that most of these precursor cells express FABP-4, the human homologue of mouse aP2, and are therefore committed preadipocytes (PA) [4]. These two primary cell populations, hMSC and PA, were then compared on a cellular and molecular level in order to identify novel regulatory mechanisms in the determination process in humans.

Both cell populations, hMSC and PA showed similar morphology (fig. 1A). We first examined whether these cells differ in the expression of common molecular surface markers. Therefore we performed FACS analysis which revealed presence of the mesenchymal surface markers CD29, CD44 and CD73 and the absence of hematopoetic markers (CD34 and CD45) and endothelial markers (CD31) in both cell populations (fig. 1B). This result not only suggests similarity in the expression of common surface marker but also demonstrates the absence of a contamination of our primary cell culture systems with hematopoetic or endothelial cells.

Since there is no major difference in morphology and the expression of surface markers we next compared differentiation capacity of the two different cell types. As shown in figure 2A using standard induction medium without PPAR-γ agonists or bone morphogenic proteins (BMP) hMSC were able to undergo sufficient adipogenesis as well as osteogenesis. In contrast, using the same media, human subcutaneous PA were only able to undergo adipogenesis but not osteogenesis which indicates that these cells lost their multipotency during the determination process.

Wnt-5a expression differs between human mesenchymal stem cells and preadipocytes

The experiments reported so far demonstrate that hMSC are multipotent while subcutaneous PA are already committed to the adipogenic lineage. Therefore, these two cell populations serve as a suitable model to examine the determination process in humans. It is known that wnt-signalling has a strong influence on developmental processes. Of the different wnt family members, wnt-5a has been linked to osteogenesis in mice and humans [22,23] which is why we next examined wnt-5a expression in the two primary cell systems. These experiments revealed significant higher levels in hMSC compared to PA (fig. 2B+C) suggesting down-regulation of wnt-5a expression is related to the determination process.

Wnt-5a is important to maintain osteogenic potential of human mesenchymal stem cells

We next aimed to investigate whether this down-regulation of wnt-5a expression is of functional relevance. Since wnt-5a is a secreted glycoprotein which acts in an autocrine and paracrine manner we induced hMSC to undergo osteogenesis in the presence of neutralizing anti-wnt-5a antibodies in the culture medium. As shown in figure 3A+B, these loss-of-function experiments exhibited impaired osteogenesis as indicated by reduced staining for hydroxylapatit by alizarin as well as a reduced expression of the molecular markers osteopontin and alpine phosphatase (ALP) on protein level. To strengthen these data we also performed gain-of-function experiments by inducing osteogenesis of hMSC in the presents of recombinant wnt-5a protein in the culture medium. This treatment resulted in increased osteogenic differentiation (3C). In contrast to osteogenesis, adipogenic differentiation of hMSC was inhibited by wnt-5a as shown by increased lipid accumulation and increased expression of PPAR-γ, SREBP-1c and adiponectin in anti-wnt-5a treated cells (loss of function experiments, fig. 4A+B). Again, we also performed gain of function experiments, showing that induction of adipogenesis in the presence of recombinant wnt-5a protein resulted in reduced adipocyte formation as indicated by reduced expression of the molecular markers PPAR-γ, SREBP-1c and adiponectin (fig. 4C). Taken together, these data indicate that wnt-5a action is important to preserve osteogenic capacity of hMSC suggesting that down regulation of wnt-5a expression is a major event in the determination of these cells into preadipocytes in humans.

Human mesenchymal stem cells express canonical and non-canonical wnt-signalling pathways

In most cell systems, wnt-5a signalling is mediated by a non-canonical pathway [24,25]. However, in recent reports it has been suggested, that wnt-5a is also able to influence the beta-catenin dependent canonical signalling [26,27]. Therefore, we next aimed to investigate which of the different wnt signalling pathways are present
in primary hMSC on protein level. hMSC were lysed and expression analysis for central signalling molecules (β-catenin, CamKII, JNK) were analyzed by western blotting. As shown in fig. 5A all the signalling pathways examined are present in hMSC.

Wnt-5a signalling in human mesenchymal stem cells is mediated via the JNK-pathway

We next aimed to investigate which one of these different pathways is activated by wnt-5a. Since some transfection experiments were performed to clarify this question we decided to use the mouse mesenchymal stem cells line C3H10T1/2 first because of the much higher transfection efficiency compared to human primary cells. C3H10T1/2 cells were successfully used as a model for mesenchymal stem cell determination in previous reports [28]. The expression of the canonical and non-canonical signalling pathways is similar in these cells compared to human primary cells (data not shown). C3H10T1/2 cells were incubated in the presence or absence of neutralizing anti-wnt-5a antibodies for 24h. After that we first examined the activity of the Ca$^{2+}$ dependent CamKII non-canonical pathway. Since activation of this pathway leads to autophosphorylation of CamKII we performed western blotting for phospho-CamKII in control cells and cells treated with neutralizing anti-wnt-5a antibodies. As shown in fig. 5B no significant difference was found with respect to the phosphorylation status suggesting this pathway not to be important in wnt-5a signalling in MSC.

In order to investigate if wnt-5a signalling is mediated by the canonical pathway, first the amount of cytosolic β-catenin was compared in wnt-5a-antibody treated cells and control cells. Therefore, cells were lysed with a hypo-osmolaric lysis buffer in order to separate cytosolic from membrane bound β-catenin followed by western blotting. This experiment revealed similar amounts of cytosolic β-catenin in cells independent of the wnt-5a activity (fig. 6A). To confirm this finding, we examined wnt-5a effect on the translocation of β-catenin into the nucleus (fig. 6B). LiCl was used as positive control in this experiment due to its ability to block GSK3β activity. In contrast to LiCl treated cells, in stem cells treated with and without the neutralizing anti-wnt-5a antibody β-catenin did not enter the nucleus (fig. 6B) suggesting that wnt-5a does not strongly activate the canonical pathway. Furthermore stem cells were transiently transfected with the TOPFLASH promoter-reportergene plasmid which contains several TCF binding sites. As shown in figure 6C, treatment of transfected cells with anti-wnt-5a antibodies did not decrease activity of the TOPFLASH promoter, further demonstrating that wnt-5a does not activate the canonical pathway. Instead, in these experiments, anti-wnt-5a induced a mild but significant induction of the promoter activity. This mild effect might be recognized only in the luciferase based promoter analysis, because the sensitivity of these experiments is higher compared to western-blotting and immunocytochemistry. Taken together, the experiments reported so far suggest that wnt-5a neither activates the Ca$^{2+}$ dependent non-canonical pathway nor the β-catenin linked canonical pathway in mesenchymal stem cells. Finally we examined whether wnt-5a activates the JNK signalling pathway in MSC. Since JNK is autophosphorylated upon activation, we first compared phospho-JNK levels in cell lysates of C3H10T1/2 cells treated with neutralizing anti-wnt-5a antibodies and untreated controls. In this experiment, we observed phosphorylation of JNK to be of lesser extent in cells incubated with the anti-wnt-5a antibody compared to control cells suggesting wnt-5a transmits its signal via the JNK pathway (fig. 7A). To confirm this finding, EMSA experiments were performed using nuclear protein extracts of cells treated with anti-wnt-5a antibodies and control cells exhibiting reduced binding of c-Jun to a oligonucleotide containing an c-Jun binding site when wnt-5a signalling was blocked (fig. 7B). Furthermore, C3H10T1/2 cells were transfected with a promoter-reportergene plasmid containing the AP1 promoter which is known to be activated by c-Jun. Treatment of transfected cells with neutralizing anti-wnt-5a antibodies resulted in a significant decrease in AP1 promoter activity compared to control cells (fig. 7C). In summary, these experiments demonstrate that wnt-5a signalling in C3H10T1/2 stem cells is mediated via the JNK dependent non-canonical pathway.

In order to confirm these findings in human stem cells we performed western-blotting analysis for phospho-JNK, phospho-CamKII and β-catenin in hMSC also treated with neutralizing anti-wnt-5a antibodies and untreated control cells. As shown in figure 8A-C, in agreement with the data obtained in the mouse cell line, in primary hMSC wnt-5a activity is also mediated by the JNK non-canonical pathway.
Finally, treatment of hMSC with an unspecific antibody of the same subclass as the anti-wnt-5a antibody used for these experiments did not result in alteration of JNK signalling, indicating that the effects observed are specific for wnt-5a (fig. 8D).

**Wnt-5a signalling regulates osteopontin promoter activity in mesenchymal stem cells**

Osteopontin is one of the key regulators during osteogenesis. The proximal promoter of this gene contains a c-Jun binding site. Therefore, we cloned the cDNA of wnt-5a into an expression plasmid and performed transient co-transfection experiments together with a luciferase plasmid containing –88 to +79 of the osteopontin promoter in undifferentiated C3H10T1/2 mesenchymal stem cells (fig. 9A). Wnt-5a significantly induced osteopontin promoter activity in these experiments suggesting this pro-osteogenic factor to be a target gene of wnt-5a. This effect was abolished in the presence of the compound SP600125 (fig. 9A). Since SP600125 specifically inhibits the JNK signalling pathway (fig. 9B), this finding further indicates that wnt-5a regulates osteogenesis via the non-canonical JNK pathway.

**Discussion:**

In human adipose tissue, the number of precursor cells and mature adipocytes reflects the balance between mesenchymal stem cell determination, preadipocyte proliferation, preadipocyte differentiation as well as preadipocyte and adipocyte apoptosis [4]. Of these different biological processes, in humans least knowledge exists on the determination of multipotent stem cells to the adipogenic lineage. The data obtained in the present study suggests a role for non-canonical wnt-5a signalling in this developmental process.

A recent report on adipogenic precursor cells in the so called stroma-vascular fraction of adipose tissue in rodents revealed that most fat cells descend from a pool of proliferating progenitors that are already committed to the adipogenic lineage, either prenatally or early in postnatal life [5]. In agreement with these animal data, using FABP-4, the human homologue of mouse aP2, as a molecular marker, it has been suggested that most precursor cells in adipose tissue of human adult subjects are committed preadipocytes rather than multipotent mesenchymal stem cells [4,6]. In contrast to these studies, some authors claim that stroma cells in human adipose tissue might be used as so called “adipose tissue derived mesenchymal stem cells” and that these cells are able to differentiate into different mesenchymal lineages including adipocytes, osteocytes and myocytes [29,30]. However, osteogenic transformation of these cells often depends on the presence of certain bioactive molecules, e. g. bone morphogenic proteins (BMP), additionally to ascorbic acid and β-glycerophosphate in the standard osteogenic induction medium. According to the studies of Skillington et al. [31] this suggests that osteogenesis in this case is the result of a transdifferentiation of committed preadipocytes rather than determination of multipotent stem cells to the osteogenic lineage. This is in agreement with the results obtained in the present study, since we were not able to induce osteogenesis in adipose tissue derived precursor cells by adding standard induction medium not containing BMPs. In summary, in our opinion these data suggest that most precursor cells isolated from the stroma-vascular fraction of adult human adipose tissue biopsies are already committed to the adipogenic lineage and therefore can serve as a model for human committed preadipocytes.

During the last decades, advances in biotechnology rendered the possibility to isolate human mesenchymal stem cells from umbilical cord blood. It has been shown in many reports that these cells are truly multipotent [18-21]. This is confirmed by our data, since we were able to induce adipogenesis and osteogenesis in these cells under standard conditions without the use of PPAR-γ agonists or BMPs. Therefore, in order to gain insights into the molecular regulation of the adipogenic determination process in humans in the present study we compared human multipotent umbilical cord blood mesenchymal stem cells with committed preadipocytes isolated from adipose tissue biopsies from adult human subjects.

On a cellular level we found similar morphology and expression of common mesenchymal surface markers (fig. 1). We then examined the expression of wnt-5a in both primary cell populations because this molecule has been related to osteogenesis in previous reports [22,23]. Interestingly, we found that expression
of this signalling molecule was significantly higher in stem cells compared to preadipocytes (fig. 2). Furthermore, by using neutralizing anti-wnt-5a antibodies as an experimental tool to inhibit wnt-5a signalling we showed that wnt-5a is important to maintain osteogenic potential of mesenchymal stem cells. These data are in agreement with previous reports showing that wnt-5a enhances osteogenesis of bone marrow mesenchymal stem cells ex vivo [32]. From our point of view, these data suggest that inhibition of wnt-5a signalling is an important molecular mechanism in the determination of human mesenchymal stem cells into preadipocytes.

In contrast to osteogenesis, the effects of wnt-5a on adipogenesis are discussed controversingly in the literature. In one report it has been purposed that in 3T3-L1 cells wnt-5a promotes adipogenesis despite the fact that expression of wnt-5a is down-regulated during 3T3-L1 preadipocyte differentiation into mature fat cells [33]. In contrast, it has been shown on a molecular level that wnt-5a signalling inactivates the function of PPAR-γ, the master regulator of adipogenesis [34]. The data from the second study support our findings that wnt-5a inhibits adipogenesis of human mesenchymal stem cells.

In the present study we found that wnt-5a activates JNK non-canonical signalling in human mesenchymal stem cells as well as in the mouse C3H10T1/2 cell line. Interestingly, it has been shown, that JNK signalling is essential in the regulation of bone formation and that inactivation of JNK signalling impairs osteogenesis while adipogenesis in promoted [35]. Furthermore, on a molecular level it is known, that the proximal osteopontin promoter contains a c-Jun binding site [36]. Therefore, in the present study we examined the effect of ectopically overexpressed wnt-5a on osteopontin promoter activity and found an increase compared to control cells. Since osteopontin is known to be an important pro-osteogenic factor, this might suggest a molecular mechanism by which wnt-5a promotes osteogenesis.

We also noticed a weak but significant inhibitory effect of wnt-5a on the β-catenin dependent canonical pathway in luciferase promoter reporterene experiments using the TOPFLASH plasmid. This is of interest since in a recent report a crosslink between the non-canonical and the canonical pathway was postulated whereby wnt-5a induces an inactivation of β-catenin [26,27]. In many developmental processes the canonical signalling is thought to maintain proliferation capacity of cells [37]. Thus, inhibition of proliferation of human mesenchymal stem cells via influencing β-catenin might be a second important function of wnt-5a besides promotion of osteogenic differentiation via the JNK signalling pathway.

The data obtained in the present study support the theory that most precursor cells in human adult adipose tissue are committed preadipocytes rather than multipotent stem cells. This raises the question on the time point when the determination of stem cells into preadipocytes occurs during human development. As described earlier, a recent report in rodents suggests that progenitor cells in adipose tissue are committed either prenatally or early in postnatal life [5]. This is of interest, since many epidemiological data support the notion that the perinatal period is a sensitive part in human development in respect to the pathogenesis of obesity and type 2 diabetes [38,39]. Since the number of preadipocytes and mature fat cells differs between lean and obese human adults, further studies will be necessary to clarify if abnormalities in the determination of mesenchymal stem cells into preadipocytes in the early childhood might contribute to the so called “perinatal priming” of metabolic diseases of adult human subjects.

In summary, in the present study we compared human mesenchymal stem cells and committed preadipocytes on a cellular and molecular level. Using this approach, we identified that JNK dependent non-canonical wnt-5a signalling is important to maintain the potential of multipotent stem cells to undergo osteogenesis and that inhibition of this pathway is a major event in the determination of these cells into preadipocytes in humans.

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Figure legends:

Fig. 1: Comparison of human mesenchymal stem cells and preadipocytes with respect to morphology and expression of common cell surface markers: (A) Morphology of hMSC and PA, light microscopy, 200x. (B) Expression of surface markers: preconfluent human MSC and PA were analyzed by FACS analysis for expression of the mesenchymal markers CD29, CD44, CD73, the hematopoietic markers CD34, CD45 and the endothelial marker CD31.

Fig. 2: (A) Induction of adipogenesis and osteogenesis in human mesenchymal stem cells and preadipocytes: MSC and PA were incubated in induction medium for adipogenesis or osteogenesis for 7-10 days. Upper panel: adipogenesis in MSC and PA, light microscopy (100x), lower panel: osteogenesis in MSC and PA, Alizarin staining and light microscopy (100x) (B) and (C) Comparison of wnt-5a expression in human mesenchymal stem cells and preadipocytes: Preconfluent human MSC and PA were lysed by RIPA buffer and western blotting was performed for wnt-5a. (B) representative western blot of MSC and PA from three independent human individuals, each. (C) statistical analysis of western blotting experiments of MSC or PA from n=5 independent individuals, each. Wnt-5a expression was normalized to the amount of total protein. Mean ±SEM. Student’s t-test, *=p<0.05.

Fig. 3: Wnt-5a effect on osteogenesis of human mesenchymal stem cells: Human MSC were induced to undergo osteogenesis in the presence or absence of neutralizing anti-wnt-5a antibodies or recombinant wnt-5a protein within the tissue culture medium. (A) Alizarin staining. (B+C) Western blotting for osteopontin (OPN) and alkaline phosphatase (ALP), two molecular markers for osteogenesis. β-actin was used to normalize for total protein content. rec. =recombinant. Shown is one example of n=3 independent experiments using different cells from independent human individuals.

Fig. 4: Wnt-5a effect on adipogenesis of human mesenchymal stem cells: Human MSC were induced to undergo adipogenesis in the presence or absence of neutralizing anti-wnt-5a antibodies or recombinant wnt-5a protein within the tissue culture medium. (A) Oil Red O lipid staining, microscopically image (200x). (B+C) Western blotting of molecular markers for adipogenesis (PPAR-γ, SREBP-1c) and the adipokine adiponectin. β-actin was used to normalize for total protein content. rec. =recombinant. Shown is one example of n=3 independent experiments using different cells from independent human individuals.

Fig. 5: (A) Expression of intracellular signalling molecules of the canonical and non-canonical pathway in human mesenchymal stem cells. Western blotting of whole cell lysates of MSC from three independent human individuals. (B) Wnt-5a does not activate the CamKII-dependent non-canonical pathway in C3H10T1/2 mesenchymal stem cells. C3H10 cells were incubated in medium containing neutralizing anti-wnt-5a antibodies or control medium for 24 hours. Afterwards cells were lysed using RIPA buffer containing phosphatase inhibitors and western blotting was performed for Phospho-CamKII (upper panel) and whole CamKII (loading control, lower panel). Shown is one example of n=3 independent experiments.

Fig. 6: Wnt-5a does not activate the canonical signalling pathway in C3H10T1/2 mesenchymal stem cells: (A) Comparison of the amount of cytosolic β-catenin in stem cells treated with neutralizing anti-wnt-5a antibodies for 24h or control cells. In this experiment cells were lysed using a hypotonic lysis buffer to separate cytosolic from membrane bound β-catenin. β-actin was used to normalize for total protein content. Shown is one example of n=3 independent experiments. (B) Immunofluorescence for β-catenin in stem cells treated with neutralizing anti-wnt-5a antibodies (+AB) or control cells (-AB). Incubation of cells in LiCl was used as a positive control for translocation of β-catenin into the nucleus. Shown is one example of n=3 independent experiments. (C) Promoter-Reportergene analysis using the TOPFLASH plasmid in cells treated with neutralizing anti-wnt-5a antibodies (+AB) and controls (-AB). pRL-CMV was used for normalization of transfection efficiency and unspecific promoter effects in this experiment. Mean±SEM of n=5 independent experiments. Student’s t-test, *=p<0.05.
Fig. 7: Wnt-5a activates the JNK-dependent non-canonical signalling pathway in C3H10T1/2 mesenchymal stem cells. (A) Comparison of the amount of phosphorylated JNK in control cells and C3H10T1/2 cells incubated in medium containing neutralizing anti-wnt-5a antibodies (upper panel). Amount of whole (phosphorylated and non-phosphorylated) JNK was used as a loading control (lower panel). Shown is one example of n=3 independent experiments. (B) Electromobility Gel Shift Assay (EMSA) for c-Jun binding to an oligonucleotide containing a c-Jun binding site. + = nuclear proteins from cells treated with neutralizing anti-wnt-5a antibodies, - = nuclear proteins from control cells. Competitive reaction was performed under identical conditions adding 200 fold molar ratio of unlabeled oligonucleotides. Samples were separated by 5% PAGE and final analysis was performed according to the LightShift® Chemiluminescent EMSA Kit (Pierce). Shown is one example of n=3 independent experiments. (C) Promoter-Reporter gene analysis using a pGL plasmid containing the AP1 promoter in C3H10T1/2 cells treated with neutralizing anti-wnt-5a antibodies (+AB) and control cells (-AB). The AP1 promoter is a known target of c-Jun. pRL-CMV was used for normalization of transfection efficiency and unspecific promoter effects. Mean±SEM of n=5 independent experiments. Student’s t-test, *=p<0.05.

Fig. 8: Wnt-5a specifically activates the JNK-dependent non-canonical signalling pathway in human mesenchymal stem cells. (A-C) Comparison of the amount of phospho-JNK, β-Catenin and phospho-CamKII in control cells and human mesenchymal stem cells incubated in medium containing neutralizing anti-wnt-5a antibodies. Shown is one example of n=3 independent experiments. (D) In order to exclude unspecific effects due to the neutralizing antibody human mesenchymal stem cells were either untreated or treated with an unspecific control antibody (unspec. AB) of the same subclass or a specific antibody for wnt-5a (anit-wnt-5a AB) for 24h. Afterwards cells were lysed using RIPA buffer containing phosphatase inhibitors and western blotting was performed for phospho-JNK (upper panel) as well as total JNK (lower panel). Shown is one example of n=4 independent experiments.

Fig. 9: Wnt-5a induces osteopontin promoter activity in C3H10T1/2 mesenchymal stem cells via JNK signalling pathway: (A) C3H10T1/2 cells were transiently co-transfected with 500ng of an expression plasmid containing the coding sequence of human wnt-5a (pcDNA3.1-wnt-5a) and 200ng of a luciferase construct (pXP2-OPN) with –88 to + 79 bp of the proximal osteopontin promoter containing a c-Jun-binding site (TGAGCCA) kindly provided by David T. Denhardt [36]. 10ng pRL-CMV was co-transfected for normalization of transfection efficiency and unspecific promoter effects. Cells were lysed after 24-48h and luciferase acivity was measured by Dual Luciferase Reporter gene Assay (Promega). In order to test that the effect of wnt-5a on osteopontin promoter activity is mediated via the JNK signalling pathway, luciferase experiments were performed in the presence of the pharmacological JNK inhibitor SP600125 as indicated in the figure. ***=p<0.001 by student’s t-test of n=8 independent experiments. Mean±SEM. ns=non significant. (B) Western-blot for phospho-JNK and total JNK as well as phospho-ERK and total ERK in pre-confluent mesenchymal stem cells treated with the JNK inhibitor SP600125 and anti-wnt-5a (AB) antibodies for 24 hours in order to demonstrate that SP600125 is specific for the JNK signalling pathway in these cells. Shown is one example of n=3 independent experiments.

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fig. 1

A

MSC

PA

B

MSC

PA

CD29

CD44

CD73

CD34

CD45

CD31
fig. 2
fig. 3

A

+ anti-wnt-5a

- anti-wnt-5a

B

anti-wnt-5a

+ -

osteopontin

ALP

β-actin

C

rec. wnt-5a

+ -

osteopontin

ALP

β-actin
fig. 4

A

B

C

|       | anti-wnt-5a | +   | -   |
|-------|-------------|-----|-----|
| PPAR-γ|             |     |     |
| SREBP-1c|           |     |     |
| Adiponectin|       |     |     |
| β-actin|             |     |     |

|       | rec. wnt-5a | +   | -   |
|-------|-------------|-----|-----|
| PPAR-γ|             |     |     |
| SREBP-1c|           |     |     |
| Adiponectin|       |     |     |
| β-actin|             |     |     |
fig. 5

A

signalling pathways

β-Catenin
CamKII
JNK

B

anti-wnt-5a

+  -

P-CamKII
CamKII

loading control
fig. 6

A

anti-wnt-5a

|       | +  | -  |
|-------|----|----|
| cytosolic β-Catenin |     |     |
| β-actin          |     |     |

B

LICl

+AB    -AB

C

RLU

+AB    -AB
fig. 7

A

anti-wnt-5a

+  -

P-JNK

JNK

B

C 200x

anti-wnt-5a

+  -  +  -

C

RLU

*  

+AB  -AB
fig. 8

A

\[
\begin{array}{c|c|c}
\text{anti-wnt-5a} & + & - \\
\hline
\text{P-JNK} & \text{image} & \text{image} \\
\text{JNK} & \text{image} & \text{image} \\
\end{array}
\]

B

\[
\begin{array}{c|c|c}
\text{anti-wnt-5a} & + & - \\
\hline
\text{cytosolic} & \text{image} & \text{image} \\
\beta\text{-Catenin} & \text{image} & \text{image} \\
\end{array}
\]

C

\[
\begin{array}{c|c|c}
\text{anti-wnt-5a} & + & - \\
\hline
\text{P-CamKII} & \text{image} & \text{image} \\
\text{CamKII} & \text{image} & \text{image} \\
\end{array}
\]

D

\[
\begin{array}{c|c|c|c}
& \text{untreated} & \text{unspec. AB} & \text{anti-wnt-5a AB} \\
\hline
\text{P-JNK} & \text{image} & \text{image} & \text{image} \\
\text{JNK} & \text{image} & \text{image} & \text{image} \\
\end{array}
\]
fig. 9

A

| pXP-OPN | + | + | + | + |
| pcDNA3.1-wnt-5a | + | + | + | + |
| SP600125 | + | + | + | + |

B

- Control
- SP600125
- +AB

**p-JNK**

**JNK**

**p-ERK**

**ERK**

***ns***
Role of wnt-5a in the determination of human mesenchymal stem cells into preadipocytes

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