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Protein kinase CK2 is necessary for the adipogenic differentiation of human mesenchymal stem cells

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ABSTRACT

CK2 is a serine/threonine protein kinase, which is so important for many aspects of cellular regulation that life without CK2 is impossible. Here, we analysed CK2 during adipogenic differentiation of human mesenchymal stem cells (hMSCs). With progress of the differentiation CK2 protein level and the kinase activity decreased. Whereas CK2α remained in the nucleus during differentiation, the localization of CK2β showed a dynamic shuttling in the course of differentiation. Over the last years a large number of inhibitors of CK2 kinase activity were generated with the idea to use them in cancer therapy. Our results show that two highly specific inhibitors of CK2, CX-4945 and quinalizarin, reduced its kinase activity in proliferating hMSC with a similar efficiency. CK2 inhibition by quinalizarin resulted in nearly complete inhibition of differentiation whereas, in the presence of CX-4945, differentiation proceeded similar to the controls. In this case, differentiation was accompanied by the loss of CX-4945 inhibitory function. By analysing the subcellular localization of PPARγ2, we found a shift from a nuclear localization at the beginning of differentiation to a more cytoplasmic localization in the presence of quinalizarin. Our data further show for the first time that a certain level of CK2 kinase activity is required for adipogenic stem cell differentiation and that inhibition of CK2 resulted in an altered localization of PPARγ2, an early regulator of differentiation.

1. Introduction

Adipogenesis denotes the development of differentiated adipocytes from multipotent mesenchymal precursor cells. The multipotent mesenchymal stem cells (MSCs) are adult stem cells with a fibroblastoid shape and a high ability for self-renewal [11]. They can be isolated – among others – from adipose tissue and bone marrow. They are characterized by different surface markers, *i.e.* they lack the haematopoietic markers CD23 and CD45, but express high levels of CD29, CD44, CD73, CD90, CD105 and CD106 [49]. By diverse extracellular signals MSCs are prompted to differentiate into several lineages like osteoblasts, chondrocytes, myoblasts or adipocytes *in vivo* and *in vitro* [60]. Many findings concerning the events during differentiation of stem cells were obtained from model cell lines like the pluripotent stem cell line C3H10T/1/2 [51] or the preadipocyte cell line 3T3-L1 [20,21] which have been proven reliable models for the processes occurring during differentiation. During differentiation hMSCs undergo defined phases which begin with mitotic clonal expansion, commitment to a certain lineage, lineage progression, terminal differentiation, and finally maturation [6,7]. Lineage determination seems to be regulated by a large network of extracellular signalling factors which exert an impact on the promoters of lineage specific transcription factors. Balancing these different signalling molecules and pathways determines the fate of the MSC ([66] and literature therein). In the late phase of adipogenic differentiation adipocyte specific proteins like perilipin, lipoprotein lipase, glycerol phosphate dehydrogenase, leptin and adiponectin are expressed which support the characteristics of an adipocyte phenotype [44].

The activity of several signalling molecules and transcription factors during differentiation is controlled by phosphorylation [27,33,68]. Also the commitment of human mesenchymal stem cells to an osteogenic or adipogenic lineage is regulated by protein kinases [26,28,34]. One of the kinases which might also be implied in the regulation of differentiation is protein kinase CK2.

CK2 is one of the most pleiotropic protein kinases with hundreds of substrates already known [37,52]. It is a ubiquitous heterotetrameric serine/threonine kinase, consisting of two catalytic α- or α′-subunits and two non-catalytic β-subunits, which is deeply implicated in a multitude of cellular processes including embryonal development and differentiation. A lack of the catalytic α- or the regulatory β-subunit is not compatible with life [5,35].

During early embryogenesis of the mouse, catalytic as well as the non-catalytic subunits of CK2 are particularly expressed in neuronal tissues, in later stages CK2 is found everywhere in the body [40]. Similar

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observations have been made in chicken [36] and in the nematode *Caenorhabditis elegans* [23]. The enhanced expression goes along with an enhancement of the CK2 activity which reaches a maximum at day 11 during mouse embryogenesis and is then subsequently down-regulated [55]. Changes in activity depending on the embryonic stage were confirmed for rat and for *C. elegans* [22,46].

CK2β is discussed as a positive regulator for the proliferation of neuronal progenitor cells and their multipotency [75]. Knocking out the gene for the regulatory β-subunit in mice leads to a diminished cell proliferation and the development of the murine embryos stops at the blastocyst stage with the resorption of the embryo. CK2β$^{-/-}$ blastocysts are not able to generate an inner cell mass [5]. Mouse embryos with a lack of CK2β in neuronal stem cells show a disturbed cell proliferation and severe defects in brain development [24].

When knocking out the α-subunit of CK2, mouse embryos die during embryogenesis and exhibit structural injuries in the heart and neural tube [12,35]. The lack of the catalytic α′-subunit is compatible with life; however, it is detrimental for spermatogenesis and male α′-knock-out mice are infertile [14,73].

In a number of different genetic experiments it has been demonstrated that mice with combined loss of CK2α and CK2α′ are viable as long as one of the two CK2α alleles is present [31]. Furthermore, these experiments have demonstrated that a combined loss of one CK2α allele or both CK2α′ alleles leads to abnormalities in growth and development of the offspring. In particular, CK2α$^{+/-}$ CK2α′$^{-/-}$ mice differ in weight but not in size compared to their wild-type litter mates. In fact, knock-out mice were found to have about 50% of the body fat compared to mice of other genotypes. There are some observations about a distinct role of CK2 upon signalling during differentiation in several stem cell models for haematopoietic [8,13,65], osteogenic [3,4] or neuronal differentiation [19,70]. In these publications there are examples for a positive as well as a negative role for CK2 in differentiation. There are only few indications that CK2 might be employed in the differentiation of established stem cell or preadipocyte lines to adipocytes [41,72]. In the preadipocyte line 3T3-L1 CK2 is necessary for the early stages of differentiation. Upon inhibition of CK2 preadipocytes fail to differentiate into mature adipocytes [72]. In later phases the expression as well as the activity of CK2 has to be down-regulated to enable a successful differentiation.

Over the last 8 years the development of a great number of inhibitors of the kinase activity of CK2 with an increasing specificity has provided new tools for studying the role of CK2 for many biological processes [10,50,53]. Here we used two highly specific CK2 inhibitors – CX-4945 [47] and quinalizarin [9] – for the investigation of the fate of human mesenchymal stem cells (hMSCs) with respect to adipogenic differentiation.

2. Material and methods

2.1. Isolation of human mesenchymal stem cells

Human mesenchymal stem cells (hMSCs) were isolated from bone marrow of the *caput femoris* from patients undergoing hip replacement surgery. Briefly, bone fragments were minced and isolated cells were pooled in α-MEM. Mononuclear cells (MNC) were extracted by ficoll density gradient centrifugation at 350 ×g for 30 min. Collected cells were seeded in complete medium (α-MEM, 15% foetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin) and cultured in a humidified atmosphere and 5% CO_2 at 37 °C. The medium was changed twice a week. Before reaching confluence, cells were harvested with trypsin/EDTA and seeded at 1×10^3 cells/cm^2 for further expansion. Prior to their usage the stem cell potential of hMSC was verified by the expression of cell surface markers CD29, CD44, CD73, CD90, CD105, CD106 and HLA-ABC as well as absence of CD34, CD45, CD133 and HLA-DR and their ability to differentiate adipogenically and osteogenically.

2.2. Cell cultivation, differentiation and treatment

Characterized hMSCs were cultivated in complete medium and kept at a maximum confluence of 70–80%. For adipogenic differentiation cells were seeded into six-well plates or in 60 mm culture dishes at a density of 1.5×10^4 cells/cm^2. When cells reached confluence the culture medium was replaced by the differentiation mix (α-MEM, 10% heat-inactivated FCS, 500 μM isobutylmethylxanthine (IBMX), 100 nM dexamethasone, 200 μM indomethacin and 100 ng/ml insulin) (corresponds to day 0 of the differentiation). Differentiation medium was replaced twice a week.

The CK2 inhibitors CX-4945 (Selleckchem, Munich, Germany) or quinalizarin (1,2,5,8-tetrahydroxyanthraquinone) (Labotest OHG, Niederschöna, Germany) were dissolved in dimethyl sulfoxide (DMSO) to a 20 mM stock solution, which was used to treat the cells with a final concentration of 20 or 30 μM, unless otherwise stated. The inhibitors were added with every change of the differentiation medium. The same volume of the solvent DMSO was used in control experiments without CK2 inhibitors.

2.3. Staining of lipid droplets in hMSC

After 3 weeks of differentiation cells were washed with PBS, fixed in 3.7% formaldehyde in PBS for 15 min at room temperature and then washed again. Cells were permeabilized with 0.2% Triton X-100, 5% bovine serum albumin in PBS for 15 min on ice. Then, cells were washed twice and incubated with the lipid staining dye Bodipy® (493/503) (Invitrogen, Karlsruhe, Germany) (50 μl/ coverslip, 30 μg Bodipy®/ml PBS) for 1 h at room temperature in a humidified chamber in the dark. To stain the nuclei, cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI) (50 μl, 5 ng/ml) at 37 °C for 15 min. Finally, cells were washed again and analysed under a fluorescence microscope (excitation wave length: Bodipy®: 488 nm, DAPI: 340 nm).

Alternatively, lipids were visualized with OilRedO (Sigma Aldrich, Munich, Germany). After removing the medium and washing with PBS, cells were fixed in 10% formaldehyde in PBS for 30 min at room temperature and then washed twice with deionized H_2O. Cells were incubated for 3 min with 60% isopropanol. OilRedO was dissolved in isopropanol (0.3 g/100 ml). A fresh 60% staining solution of OilRedO was prepared with deionized H_2O, incubated for 10 min at room temperature and subsequently filtered through a folded filter (3MM, Whatman, United Kingdom). Cells were incubated with the staining solution for 5 min at room temperature. After incubation, the OilRedO solution was removed and cells were washed three times with distilled water. The staining of the lipid droplets was visualized by phase contrast microscopy.

2.4. Immunofluorescence analysis

For immunofluorescence analysis hMSCs were seeded in 8 well Permanox slides (Nalge Nunc International Corp., Naperville, USA) at a density of 12,000 cells per well and differentiation was started in the presence or absence of 30 μM quinalizarin or 20 μM CX-4945 when cells had reached confluence. Every third day immunofluorescence analysis was performed as described [15]. For the identification of CK2α we used the mouse monoclonal antibody 1A5 [56], for the identification of CK2β the polyclonal rabbit serum #32 [16]. The PPARγ2 isoform was detected with the anti-PPARγ2 antibody ab45036 (Abcam, Cambridge, UK), the marker perilipin was identified with the specific antibody D418 (Cell Signaling Technology, Schwalbach, Germany). All primary antibodies were incubated overnight.

2.5. Extraction of cells

Cells were washed twice with cold PBS, scraped off the plate, centrifuged (250 ×g, 4 °C) and the pellet was extracted with RIPA-

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