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Optimized transient transfection: an approach to explore the function of signalling proteins regulating chondrogenesis in micromass cell cultures

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Introduction

The unique structure of hyaline cartilage covering bone surfaces in synovial joints manifests in the essential histological, anatomical and physical roles of articular cartilage. Throughout the lifespan of the individual, different mechanical loads affect the structure of hyaline cartilage without the capability of successful tissue regeneration. Because of this, several experiments were performed in the last decade to produce articular cartilage *in vitro*. In order to facilitate the development of these experimental procedures and to improve the therapeutic possibilities to treat degenerative diseases of articular cartilage, better understanding of the articular cartilage formation and the molecular biological background of chondrogenic differentiation is essential.

Cartilage belongs to the connective tissues of the human body. It can be divided into three subtypes: hyaline cartilage, elastic cartilage and fibrous cartilage. For all of them are avascular, the only possible way of their nutrition is diffusion from the periphery of the tissue. One of the essential characteristics of cartilage is the huge amount of extracellular matrix (ECM) between individual cells. Its complexity and the presence of several tissue specific macromolecules—such as aggrecan and type II collagen—provides the tissue with great resistance and tensile strength. Chondrocytes are located in the ECM within so-called lacunae, thus composing chondrons, the histological units of cartilage. Mature chondrocytes are non-proliferating, immortalized cells.

The primary mesenchymal cell culture system (HDC), established from chondrogenic mesenchymal cells that are isolated from limb buds of chicken embryos (Hamburger–Hamilton developmental stages 22–24), is a widely used experimental model of cartilage differentiation. Under normal circumstances, chondrogenesis occurs spontaneously in HD cultures, without adding differentiation and growth factors to the medium of the cells. Metachromasia can be observed from the beginning of the third day of culturing and abundant cartilage matrix is formed by day 6 of culturing. Detection of mRNA and protein

expression levels and the phosphorylation status of Sox9, as well as the monitoring of the expression of the core protein of aggrecan and collagen type II are reliable markers of *in vitro* chondrogenesis.

Steps of chondrogenesis

Differentiation of mesenchymal cells into chondrocytes is a multistep process. An important initial step of either *in vivo* or *in vitro* chondrogenesis is that chondroprogenitor mesenchymal cells undergo rapid proliferation and migrate to establish cell aggregates, which events occur during the first 2 days of culturing. Synchronized function of several cell-cell adhesion molecules (N-CAM, N-cadherin) is essential for formation of these aggregates. Final commitment of chondrogenic cells resulting in the activation of genes encoding cartilage matrix-specific macromolecules, *e.g.* type II collagen or aggrecan core protein occurs in these precartilage nodules; in our experimental model, this process takes place on day 3 of culturing.

Cartilage differentiation is regulated by specific transcription factors; the most relevant of which is Sox9 that activates *Col2a1, Col9a1, Col11a2* genes, thus induces the expression of collagen type II and the core protein of aggrecan. The more active phosphorylated form of Sox9 is not a prerequisite to differentiation but it augments the level of chondrogenesis. MAPK pathways consist of cascade systems and ERK1/2 is one of the main mediators of extracellular mitogen stimuli; furthermore, it plays an important role in the differentiation of several cell types, and chondrocytes are not exemptions. The Ca^{2+} sensitivity of the ERK1/2 pathway is due to its direct connection with PKCs, which also stimulates other Ca^{2+} dependent signal cascades.

Functions of PKC isoenzymes

Among a number of signalling molecules, members of the phospholipid-dependent Ser/Thr protein kinase C family are involved in signalling pathways governing crucial cellular events such as proliferation or apoptosis. The family

consists of 11 isoforms and can be divided into subfamilies; classic PKCs, novel PKCs, atypical PKCs and PKD isoenzymes. The expression of classic (PKCα and γ), novel (PKCε) and atypical (PKCζ, λ and ι) protein kinase C isoenzymes is described during chondrogenesis. Being a nPKC, the activation of PKCδ also requires lipid second messengers (*e.g.* DAG) or tumor-promoting phorbol esters (*e.g.* PMA), without the requirement of Ca^{2+}. One of its distinguishing characteristics is that unlike other PKC isoforms, PKCδ activity is also involved in negative regulation of various cellular processes, *e.g.* suppression of proliferation and survival. A polyphenolic compound, rottlerin is reported to inhibit PKCδ 30–40-fold stronger than other PKCs at 3–6 μM concentration.

Function of Ser/Thr phosphatases in chondrogenesis

PP2B (also known as calcineurin) is a Ser/Thr specific Ca^{2+}-calmodulin dependent protein phosphatase. It has a heterodimeric structure consisting of a catalytic subunit (CnA) and a regulatory subunit (CnB), which effectively binds four Ca^{2+} ions on its calcium binding site. In this way, calcineurin is one of the most important components of the Ca^{2+}-dependent signal transduction pathways. Regulation of the function of calcineurin is rather complex since it can also be activated by phosphorylation. The transcription factor NFAT (nuclear factor of activated T-cells) is reported to be the main target of calcineurin.

Regulation of chondrogenesis by Ca^{2+}

The concentration of intracellular free Ca^{2+} is 100-fold lower than that of the extracellular space. This distribution provides potential for the influx of Ca^{2+} into cells through Ca^{2+}-sensitive channels where it can act as a second messenger. According to our previous results, changes of the free cytosolic Ca^{2+}-concentration show a specific pattern during chondrogenesis, which pattern follows the progress of differentiation. Moreover, cells of HDC exhibit rapid oscillatory changes of free cytosolic Ca^{2+}-concentration that is unique among non-excitable cells. In neurons, the entry of Ca^{2+} is at least in part regulated by

plasma membrane calcium channels. One of these channels is the NMDA receptor, which is a member of the glutamate receptor family and is predominantly permeable for Ca^{2+} ions. The functional NMDA receptor is heterotetrameric: it essentially consists of two NR1 subunits that associate with two of the NR2 or NR3 subunits. Recently, NMDA receptors have been demonstrated to be present in articular chondrocytes and they are suggested to playing role in mechanotransduction. However, no data are available concerning the role of these channels in the differentiation of chondrogenic cells.

Transfection methods of plasmid delivery

Introduction of DNA, plasmid, RNA, protein and antisense oligonucleotides by transfection into eukaryotic cells is a basic method to identify the function, structure and localization of different genes and enzymes both *in vitro* and *in vivo*. Primary cell cultures represent an important tool in mapping signaling pathways, facilitating therapeutic applications and drug development, however, they are sensitive to any subtle treatment or intervention and their differentiation can also be easily disrupted. In our experimental system, the chondrogenic mesenchymal cells have to be cultured in high density for spontaneous chondrogenesis and the transfection of these cell cultures is still a major challenge.

Aims of the study

We aimed to widen the molecular biological and biochemical knowledge about differentiating mesenchymal HD cultures. With application of transient transfection for overexpression or gene silencing, we tried to clarify the function of specific signaling molecules. For experimental purposes, it was necessary to optimize the specific gene delivery to this very sensitive, differentiating primary cell culture system.

Our aims were the following:

- Investigation of the efficiency versus preservation of sufficient chondrogenesis of different transfection methods during delivery of several plasmids, sh- or siRNAs;
- Transient transfection with PP2B vectors using the optimized method and investigation of the effects of overexpression on cartilage formation;
- Optimization of inducible PP2B shRNA transfection and investigation of the effects of gene silencing on cartilage differentiation;
- Clarification of the function of PKCδ in the regulation of chondrogenesis via inhibition with rottlerin and with application of an inducible shRNA;
- Investigation of the effects of NR1 gene silencing on cartilage formation and on different parameters of chondrogenic cells such as viability, proliferation and Ca^{2+} oscillations.

Materials and Methods

Cell culturing

The primary mesenchymal cell culture system established from chondrogenic mesenchymal cells that are isolated from chicken limb buds is a widely used experimental model of cartilage differentiation. Distal parts of the limb buds of chicken embryos (Hamburger–Hamilton developmental stages 22–24) were removed and primary micromass cultures of chondrifying mesenchymal cells were established from cell suspensions with a density of 1.5×10^7 cells/mL. Droplets (100 or 30 μL) of the suspension were inoculated onto the bottom of plastic Petri dishes and the adhesion period was 2 hours. Colonies were nourished with Ham's F12 medium, supplemented with 10% fetal calf serum, antibiotics and antimycotics. Afterwards, the colonies were cultured at 37°C in the presence of 5% CO_2 and 80% humidity in a CO_2 incubator.

Light microscopic morphology and fluorescence microscopy

High-density cultures were established from 30 µL droplets of mesenchymal cells of different experimental groups. The colonies were cultured on the surface of round coverglasses that were placed into wells of 24-well cell culture plates. Cell cultures were fixed in a 4:1 mixture of absolute ethanol and 40% formaldehyde on day 6 of culturing and were stained with 0.1% dimethyl-methylene blue dissolved in 3% acetic acid for 5 mins, washed in acetic acid and were mounted in gum Arabic.

Six-day-old cell cultures were washed in CMF-PBS, fixed in Khale‘s solution and stained with 0.1% toluidine blue that was dissolved in glycine-HCl buffer (pH 1.8) for 15 min, then the unbound toluidine blue was washed out in glycine-HCl buffer. Afterwards, the dye that was bound to highly sulphated proteoglycans and glycosaminoglycans was extracted in 8% HCl dissolved in absolute ethanol. Absorbance of samples containing the extracted toluidine blue was measured at 625 nm on a microplate reader.

PP2B vectors and their isolation

pEGFP-C1 vectors were used to determine the efficiency of transfection. The following cDNAs were cloned into pEGFP-C1. 1) CnAdelta, coding for a truncated form of calcineurin that mimics the proteolysed form of the PP known to have Ca^{2+}-independent, constitutively active phosphatase activity. 2) CnAgamma that codes for the complete phosphatase cDNA of the gamma isoform. The recombinant plasmids were amplified in competent *E. coli* using the One Shot chemical transformation kit and were isolated from kanamycin-resistant bacteria using MaxiPrep kit, according to the protocol of the manufacturer.

sh- and siRNA expression systems

PKCdelta and PP2B shRNA were cloned into GeneSwitch™, an inducible protein expression system by Invitrogen. Two vectors were transfected at the same time: the inactive vector containing the shRNA and the inducible vector.

On day 2 of culturing, 1 μM mifepristone was added to the culture medium for 24 hours to activate the GeneSwitch™ System. mRNA sequence of chicken NMDA receptor subunit NR1 was downloaded from GenBank (accession number: AY510024) and a specific siRNA construct was designed for gene silencing.

Transfection of HD cultures

The transfection methods tested on chondrifying cell cultures were as follows: Lipofectamin 2000, SuperFect, DMRIE-C, Saint-Mix and the nucleofection protocol of Amaxa. Every protocol was performed in freshly isolated cell suspensions with a density of 1.5×10^7 cells/mL, however, the protocols were slightly modified for optimization. Transfection reagents and cell suspensions (100 or 30 μL) were inoculated onto the bottom of Petri dishes or into 24-well plates, respectively. After 2 hours, transfected colonies were nourished with Ham's F12 medium supplemented with 10% fetal calf serum. Nucleofection was performed using a modified nucleofection protocol by Amaxa as follows: 200 μL of 1.5×10^7 chondrogenic cell suspension was electroporated with the optimized C17 program. DharmaFect delivery system protocol was carried out according to the manufacturer.

Investigation of GFP Expressing Vectors with Fluorescent Microscopy

Effectiveness of different transfection methods was judged by approximating the number of GFP expressing cells in 1-day-old cultures with a Nicon Eclipse 800 fluorescent microscope at 488 nm wavelength.

Inhibition of PKCδ by the application of rottlerin

Rottlerin was dissolved in DMSO then kept at –20°C. Rottlerin was added into the culturing medium in 5 μM concentration on different days of culturing.

Measurement of cell viability with MTT assay

For investigation of cell viability on day 3 of culturing, 10 µL of MTT reagent (3-/4,5-dimethylthiazolyl-2/-2,5-diphenyltetrazolium bromide) was added into each well of the 96-well microtiter plates in which the cells were cultured. Cells were incubated for 2.5 hours at 120 rpm and 37°C in an orbital shaker and following the addition of 100 µL of MTT solubilizing solution, the absorption was measured at 570 nm using a Chameleon microplate reader.

Measurement of cell proliferation with ^{3}H-thymidine labeling

15 µL droplets of cell suspension were inoculated into wells of 96-well Wallac LSC microtiter plates. Medium containing 1 µCi/mL ^{3}H-thymidine (185 GBq/mM ^{3}H-thymidine) was added to the wells for 16 hours on day 3. After washing with PBS, proteins were precipitated with ice-cold 5% trichloroacetic acid for 20 mins, then washed with PBS again. Colonies were dried for a week, and radioactivity was measured using Chameleon liquid scintillation counter.

Measurement of apoptosis and transfection efficiency by Flow Cytometry

The rate of apoptosis was measured on day 2 or day 3 of culturing using Annexin V DY647 kit; necrosis was determined using PI; and transfection efficiency was judged by the detection of GFP. Mock-transfected cells (cultures treated only with the transfection reagent) or untreated cultures were used as control. After washing twice in CMF–PBS, cells were incubated with 10 µL Annexin V DY647 at room temperature for 10 mins. Before harvesting with 0.25% trypsin, cells were washed with Annexin binding buffer. Afterwards, cells were washed in CMF–PBS, 2 µL PI was added to the wells, then the plates were placed in dark chamber for 10 mins at room temperature. Cell pellets were resuspended in 500 µL FACS buffer (PBS supplemented with 1% BSA and 0.05% NaN_3) and measured in a CyFlow® space Flow Cytometer. PI was monitored at 430 nm, Annexin DY647 at 670 nm and GFP at 508 nm. Analysis

was performed using WinMDI 2.8 freeware software. Results were illustrated on density plots.

Measurement and analysis of spontaneous calcium transients

Spontaneous calcium transients were monitored using LSM 510 META Laser Scanning Confocal Microscope. Prior to measurements, cells of high density micromass cell cultures were incubated for 1 hour at 37 °C in 10 μM Fluo-4-AM dissolved in Ham's F12 medium. Calcium imaging was performed in normal Tyrode's solution. Images were taken using a 63× water immersion objective. Fluo-4-loaded cells were excited with a 488 nm argon ion laser and the emitted fluorescence was collected at 500–570 nm. Spontaneous Ca^{2+} events were investigated with x–y analysis by taking 10 images from the 50-second-long measurement period.

Preparation of cell extracts

Two- or three-day-old cell cultures were harvested after being washed in physiological NaCl solution. After centrifugation at 2000 rpm, cell pellets were suspended in 100 μL homogenization buffer. Samples were stored at –70°C until utilization. For RT-PCR analysis of cartilage colonies, cells were washed three times with RNase-free physiological sodium chloride solution then the cultures were stored at –70°C.

RT-PCR Analysis

Cell cultures were dissolved in Trizol, 20% RNase-free chloroform was added and the samples were centrifuged at 4°C and 10,000 ×*g* for 15 mins. Each sample was incubated in 500 μL of RNase-free 2-propanol at –20°C for 1 h, total RNA was suspended in RNase-free water and stored at –20°C. The assay mixture for reverse transcription contained 2 μg RNA, 0.112 μM oligo(dT), 0.5 mM dNTP and 200 units of High Capacity RT in 1× RT buffer. The reactions took place in a programmable thermocycler as follows: 95°C, 2 min, (94°C, 1 min, 54°C depending on primers, 30 sec, 72°C, 1.5 min) and then 72°C, 10 min.

PCR products were analyzed by electrophoresis at 90 V in 1.5% agarose gel containing ethidium bromide. Images were recorded with a gel documentation system.

Western blot analysis

After the determination of the protein concentrations, 80 μg protein was separated in 7.5% SDS-PAGE gel at 120 V. Blotting was preformed for 90 mins at 300 mA. After blocking in 5% non-fat dry milk in PBST, the membranes were washed and exposed to the primary antibodies overnight at 4°C. After washing two times for 15 mins with PBST, membranes were incubated with the secondary antibodies in PBST containing 1% non-fat dry milk for 1 hour at room temperature and 200 rpm. Signals were detected using enhanced chemiluminescence according to the manufacturer's instructions. 8-bit gray images were taken with a CCD camera integrated in the gel-doc system. Optical density of signals was measured by using ImageJ 1.40g freeware and results were normalized to the optical density of control cultures.

Calcineurin enzyme activity assay

For calcineurin enzyme activity assays, cells were harvested and after centrifugation at 10,000× rpm for 15 min at 4°C, the supernatants were used for measurements. The assay mixture (30 μL) containing 50 mM Tris HCl buffer (pH 7.0), 0.16 mM dithiothreitol, 3.4 μg/mL Gordox, 3.4 μg/mL leupeptin, 1 mM phenylmethylsulphonyl (PMSF), 1.6 mM benzamidine, 3.4 μg/mL trypsin inhibitor, 40 μg/mL calmodulin, 0.2 mM $CaCl_2$, 100 nM okadaic acid, 2 nM protein phosphatase inhibitor-2 and ^{32}P-labelled protein phosphatase inhibitor-1 was added to the lysates which was followed by an incubation period at 30°C for 20 mins. The ^{32}P-content of the supernatant fraction was determined by Cerenkov counting in a liquid scintillation counter.

Protein kinase C enzyme activity measurements

For PKC activity assays, cells were harvested and after centrifugation at 10,000×*g* for 10 mins at 4°C, supernatants were used for enzyme activity measurements. PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]-ATP into histone IIIS. The reaction mixture (40 µL) contained 50 mM Tris HCl buffer (pH 7.5), 1 mg/mL histone IIIS, 0.8 mM $CaCl_2$, 0.5 mM DTT, 1 µg/mL Gordox, 1 µg/mL leupeptin, 0.1 mM phenylmethylsulphonyl-fluoride, 0.5 mM benzamidine, 1 µg/mL trypsin inhibitor as a protease inhibitor, 100 µg/mL phosphatidylserine, 4 µg/mL phorbol-myristate-13-acetate, 0.12 mM ATP, 6 mM Mg-acetate and [γ-^{32}P]-ATP the quantity of which was adjusted to approximately 1 million cpm/reaction mixture. Activity measurements were performed at 30°C. Determination of ^{32}P incorporation into histone IIIS was carried out by pipetting 30 µL of the reaction mixture on filter paper squares (2×2 cm) followed by 20 mins of reaction time. All papers were washed three times in 0.5% phosphoric acid, dried and their radioactivity was measured in a liquid scintillation counter. For measurements of PKCdelta activity, rottlerin (10 µM) was administered into the reaction mixtures. The difference caused by the PKCdelta inhibitor rottlerin in the measured radioactivity was considered to be the contribution of PKCdelta to the overall PKC activity.

Statistical analysis

Statistical comparisons between control and test samples were analyzed using Student's paired *t* test, changes were considered significant if $p < 0.05$.

Results

Transfection efficiency optimization of High Density Cultures

Owing to the primary nature and short culturing period of HDC, only transient overexpression or transient gene silencing can be performed in this chondrogenesis model. For the optimization of transfection procedures, "empty"

vectors (encoding only GFP) were used. Since transfection of cells after attachment to the surface of the culturing dishes yielded very low efficacy, experiments were carried out in the cell suspension of freshly isolated cells with a few modifications of the original protocols. 16 hours after transfection, numerous GFP-expressing cells were detected with fluorescent microcopy. The highest GFP expression was detected after electroporation. Transfection methods based on lipofection showed various results. Transfection with DMRIE-C or SuperFect reagents, which are recommended for transfection of adhering cells, yielded very low efficiency. Moreover, after the application of SuperFect, cells detached into the medium by the end of day 2. The transfection efficiency of Lipofectamin 2000 and Saint-Mix reagent were significantly higher as revealed by levels of GFP expression. Data of flow cytometry also supported the effectiveness of nucleofection with Amaxa (95.4% transfection), but the apoptotic rate following electroporation (47.3% apoptosis) was extremely high. Very low apoptotic rate was detected in the presence of DMRIE-C or SuperFect reagents (0.5% and 0.7%). Higher apoptotic rate was recorded with DMRIE (12.7%) and Superfect (6.9%). The apoptotic effect of Lipofectamin 2000 was more pronounced (25.2%) than that of the Saint-Mix reagent (7.2%). Either Saint-Mix (61.7%) or Lipofectamin 2000 (62%) resulted in an acceptably high number of GFP-expressing cells.

The pores formed during electroporation probably caused such serious alterations that both the mitochondrial activity and GFP expression further inhibited the metabolic activity of chondrifying mesenchymal cells. The application of SuperFect and DMRIE decreased the viability of cells to 71% and 62% of the control, respectively. GFP expression did not have further effects on viability of HDC using the above mentioned transfection methods: Lipofectamin 2000 and Saint-Mix also decreased the metabolic activity of cells to 71% and 75%, respectively, but these methods had the least harmful side effects. To the contrary, GFP expression caused further reduction of mitochondrial activity in

Lipofectamin 2000-transfected cells. Nucleofection and the SuperFect reagent completely blocked the proliferation of chondrifying cells, while DMRIE-C had a moderate proliferation inhibitory effect even when it was applied together with GFP expression. Inhibition of proliferation was low in case of the application of Lipofectamin 2000, but GFP decreased it to 30%. Cells transfected with Saint-Mix reagent showed the least affected mitochondrial activity and proliferation rate, irrespectively of the presence of GFP.

We were almost unable to detect any metachromatic staining in cell cultures treated with electroporation, and GFP expression caused a total loss of metachromasia. Since chondrogenic cultures treated with SuperFect detached from the surface of Petri dishes on day 2 of culturing, no cartilage formation occurred. Cultures treated with DMRIE showed low metachromasia (35% of the control) and expression of GFP further suppressed cartilage formation to 11% of the control. The reduction of cartilage formation after transfection by Lipofectamin 2000 was moderate (76% of control), but GFP expression of Lipofectamin 2000-transfected cells resulted in a decrease of the area of metachromatic cartilage nodules to 23%. Saint-Mix was unique with respect to metachromasia and GFP transfection efficiency compared to the other methods used in our experimental protocol, and resulted in the best-preserved chondrogenic capacity.

Since cells of HDC cultures treated with Amaxa detached on the second or third day of culturing, we did not use this method in the following set of experiments. The other transfection reagents did not cause significant changes in the expression level of sox9 mRNA either in the absence or presence of GFP. In contrast, the mRNA expression of aggrecan core protein did increase when Lipofectamin 2000 or SuperFect with pEGFP were applied.

Overexpression of calcineurin

As the Saint-Mix reagent seemed to be an efficient method of gene transfer, transfection of pEGFP-C1 vectors encoding a constitutively active form

(CnAdelta) or the gamma isoform (CnAgamma) of calcineurin have been tested. According to the results of FACS analyses, the transfection efficiency reached 25% in case of both vectors (encoding CnAdelta and CnAgamma) parallel to very low apoptotic rate. mRNA expression of calcineurin showed 50–60% elevation in case of CnAdelta and 30–40% in case of CnAgamma. Interestingly, besides the introduced forms of calcineurin, the basal expression level of the enzyme also increased. Both constructs slightly elevated the viability of chondrifying cells, suppressed the proliferation ratio and caused a pronounced decrease of the cartilage matrix formation. Activity of calcineurin was significantly higher in the CnAdelta-expressing experimental group, while it was at the same level in CnAgamma expressing cultures than that of control cultures. Inhibition of cartilage formation was observed in both vector-expressing experimental groups: mRNA expression of cartilage-specific molecules such as aggrecan and sox9, and also the protein expression of Sox9 as well as the level of the more active P-Sox9 were all diminished.

Gene Silencing of calcineurin

For transient gene silencing, a vector coding for PP2B shRNA cloned into inducible GeneSwitch system was used. Cells treated with Lipofectamin and/or pGene/V5HisA “empty” vector were regarded as control. Transfection efficiency was determined by the detection of mRNA expression of calcineurin by RT-PCR. The mRNA expression of PP2B decreased to 50% of control cultures, while at the protein level, calcineurin was not detectable. The activity of PP2B was found to be elevated at the presence of Lipofectamin 2000, and decreased to the control level with the application of “empty” vector. The administration of shRNA caused a significant reduction of the activity of PP2B compared to pGene/V5HisA. PP2B gene silencing completely diminished cartilage formation. Mitochondrial activity of chondrifying cells was found to be increased under the effect of shRNA, but the proliferation of HDC was reduced

to 50% in cultures transfected with the "empty" vectors. The mRNA expression of aggrecan showed more pronounced decrease (70%) than that of Sox9 (30%). The protein expression of Sox9 and its phosphorylated form became occasionally detectable.

Investigation of PKCδ during chondrogenesis

mRNA expression of chicken PKCδ was uniform on all culturing days, while the protein expression profile followed a peak-like pattern and showed an almost four-fold elevation of PKCδ by days 2 and 3 compared to day 0. Under control conditions, the enzyme activity of PKCδ in cells of HCD exhibited a pattern which was well correlated with the protein expression: the enzyme activity reached its peak on day 3 with a two-fold elevation compared to day 0.

To analyze the function of PKCδ during chondrogenesis, we administered its pharmacological inhibitor rottlerin to the culture medium of HDC. Rottlerin strongly inhibited *in vitro* chondrogenesis when it was added to the culturing medium at 5 μM concentration on days 2 and 3 for 4 hours (metachromasia of these cultures decreased to 11% of the untreated control cultures). Moreover, it caused a significant decrease both in metabolic activity and proliferation of cells in HDC. Rottlerin treatments did not result in the elevation of apoptotic or necrotic rate in cells of HDC according to FACS analyses.

Interestingly, exposure to rottlerin resulted in a marked decrease in the mRNA expression of PKCδ, and a less pronounced reduction was also observed in its protein expression level. A significant decrease in the mRNA levels of both aggrecan core protein and Sox9 was detected under the effect of rottlerin treatments. Western blot analyses showed that the inhibitor only caused a slight reduction of the protein level of Sox9, whereas a significant decrease was observed in its phosphorylation level. Administration of 5 μM rottlerin caused inconsistent alterations in the activity of PKCδ assayed in day 3 cell cultures.

Since MAP-kinases, particularly ERK1/2 is one of the key regulators that influence *in vitro* chondrogenesis, we wanted to examine whether the observed decrease in cartilage matrix production was related to the ERK1/2 dependent pathway. Application of rottlerin did not alter the mRNA expression level of ERK1/2 and its protein expression was also only slightly modified, but exposure to 5 μM rottlerin significantly reduced the level of phosphorylated ERK1/2.

To selectively inhibit the expression of PKCδ, we applied a specifically designed shRNA with the same conditions as in the previous experiments. Cultures treated with either Lipofectamin 2000 or with the "empty" pGene/V5His A vectors were used as controls. PKCδ mRNA expression only decreased to a lesser extent as a result of shRNA expression, however, it exhibited a marked inhibition at the protein level (to 30% of cultures transfected with the empty vector) reflecting on the efficiency of PKCδ gene silencing. Administration of either the empty or the PKCδ shRNA-containing vector resulted in a significant decrease compared to mock-transfected cultures (75% reduction with shRNA) in the PKCδ enzyme activity of cells in HDC measured on day 3 of culturing. Introduction of the shRNA-coding GeneSwitch vector resulted in a very strong inhibition of cartilage formation (5% of mock-transfected control cultures), and the metachromatic cartilage matrix virtually disappeared. In spite of the pronounced inhibition of cartilage matrix production, mitochondrial activity and proliferation of cells transfected with either empty or PKCδ shRNA-coding GeneSwitch constructs remained unchanged compared to mock-transfected control cells. Cellular viability and apoptosis assays performed by measuring the ratio of propidium iodide and Annexin V DY647 stained cells using FACS analysis revealed that neither transfection with the empty vector nor introduction of the PKCδ shRNA vector caused any significant change in these parameters.

Whereas PKCδ gene silencing did not change the mRNA expression of sox9, and the mRNA level of aggrecan core protein only exhibited slight

alterations, the protein level of Sox9 showed a marked decrease as a result of the introduction of PKCδ shRNA. Although PKCdelta gene silencing did not affect either the mRNA or the protein expression of ERK1/2, a significant (two-fold) increase in its phosphorylated form was observed.

Effects of NMDA Receptor Subunit Silencing on Cartilage Formation

For the gene silencing of NR1, a specific siRNA construct was designed. As a result of gene silencing, both the mRNA and the protein expression of NR1 decreased to 20% compared to the control. Metachromatic extracellular matrix production decreased to approximately 15% of control cultures, although the transfection reagent also inhibited the cartilage formation (30% reduction). 48 hours after the introduction of NR1 siRNA into cells of HDC, cultures showed decreased level of proliferation compared with untransfected control cultures as revealed by radioactively labelled thymidine incorporation assays, but the viability of chondrifying cells remained unchanged. mRNA and protein expression of both sox9 and aggrecan were reduced after the application of the siRNA. Although the protein expression of Sox9 was lowered, the phosphorylated form of Sox9 only showed a slight decrease. Introduction of the siRNA and consequent transient gene silencing of the NR1 subunit almost completely abolished spontaneous Ca^{2+} oscillations in cells of HDC and only 10% of cells exhibited periodic Ca^{2+} oscillations.

Discussion

Transfection optimization of HD cultures

Overexpression or transient gene silencing by the transfection of different expression vectors is among the most valuable experimental tools to investigate the function of various signalling molecules. Exploring the insights of the regulation of chondrogenesis may help to understand better this multistep process and can lead to the development of more successful restorative treatment methods of degenerative cartilage diseases. HD cell culture system,

which is established from freshly isolated chondrogenic cells of chick limb buds, is the best-known chondrogenesis model,. Due to the short culturing period, the multi-layered nature of the cultures and the rapid progression of the chondrogenesis, to achieve a successful level of the introduction of any expression vector into a reasonably high number of cells in HDC without disturbing their precisely timed differentiation program is indeed a big challenge. We applied several transfection methods in order to select those that best satisfy these demands. Electroporation with Amaxa using a protocol given for primary cells showed high efficiency, however, the expression of GFP resulted in a very serious apoptosis and loss of cartilage formation. Owing to electroporation, small injuries could have occurred on the cell membrane inducing apoptosis in most of the cells, and due to the subsequently low cell density, mesenchymal cells could have lost their chondrifying characteristics. Three types of liposome-based reagents: Lipofectamin 2000, DMRIE-C and SuperFect—each characterized by similar chemical structure and transfection mechanism—have been tested in our experimental system. SuperFect resulted in detachment of cells, while application of DMRIE showed low efficiency with a moderate apoptotic effect. Although DMRIE did not influence the viability of cells or the expression of sox9 and aggrecan, it decreased cell proliferation and only a few metachromatic cartilage nodules developed. Lipofectamin 2000 had low apoptotic effect and the efficiency of transfection was also acceptable. However, overexpression of GFP increased apoptosis and reduced both mithochondrial activity and proliferation rate of differentiating mesenchymal cells with the expression of chondrogenic markers remained unaffected. These findings suggest that Lipofectamin 2000 itself did not cause serious damage to mesenchymal cells, but introduction of a GFP-expression vector is moderately cytotoxic, thus lowers cartilage formation. We managed to successfully transfect approximately 50% of the chondrifying cells without inducing apoptosis with the application of the Saint-Mix reagent, even in the presence of GFP. The

mRNA level of aggrecan and sox9, as well as Sox9 and p-Sox9 protein levels remained unaffected. According to these results, Saint-Mix reagent and Lipofectamin 2000 were chosen for our further experiments. Moreover, in these sets of experiments we have also demonstrated that the presence of GFP is cytotoxic for cells of HD cultures.

Effects of calcineurin overexpression and gene silencing on chondrogenesis

As calcineurin is a positive regulator of chondrogenesis, we aimed to demonstrate the effects of the elevated expression of this signal molecule during chondrogenesis. Saint-Mix was chosen as the transfection reagent to introduce CnAdelta and CnAgamma calcineurin variants into chondrifying mesenchymal cells. Surprisingly, introduction of these vectors increased the basal calcineurin expression and inhibited cartilage formation. Inhibition of cartilage formation at least in part could be the result of the inhibition of cell proliferation, but Sox9 expression and phosphorylation were also reduced. Transfection with the vector encoding the entire CnAgamma did not have any significant effect on calcineurin enzyme activity. Elevation of the calcineurin activity by the expression of the constitutively active CnAdelta was less pronounced than it was predicted on the basis of the protein expression level data. These findings suggest that activity of calcineurin should be kept in a narrow range for proper cartilage formation and endogenous calcineurin inhibitors might become activated. Moreover, as PP2B is believed to be responsible for the anabolic and catabolic balance in different cells, the overexpression of calcineurin may interfere with these cellular processes and lead to the inhibition of chondrogenesis.

As the above-mentioned results were inconsistent, we decided to apply transient gene silencing of calcineurin. Since the majority of chondrogenic cells differentiate on day 3 of culturing, we needed to apply a method that enabled maximum silencing efficiency on this day. Therefore GeneSwitch, an inducible

transfection system was chosen to deliver the calcineurin shRNA-encoding vector into cells of HDC. The fact that both of transfection and plasmid induction were successful was demonstrated by the 50% decrease of calcineurin mRNA and total loss of protein expression. Interestingly, while the lack of calcineurin protein expression caused a complete loss of metachromatic cartilage areas, it also resulted in an increased proliferation rate of cells. Previous results of our laboratory suggest that calcineurin is one of the (indirect) regulators of the phosphorylation level of ERK1/2. This MAPK is reported to regulate early steps of the commitment of chondrogenic cells and proliferation in HDC. Transient silencing of calcineurin expression at the critical time of chondrogenesis may lead to an improper function of ERK1/2 and a consequent imbalance in the regulation of proliferation versus differentiation. Our results also highlight the negative regulatory role of calcineurin in the cellular proliferation of HDC.

Effect of PKCδ inhibition on HDC

It has long been known that various PKC isoenzymes are involved in the chondrogenic differentiation of chicken limb micromass cultures. Recently, it has been demonstrated that protein expression of PKCδ is essential in the regulation of the formation of prechondrogenic nodules. Consistent with these data, our results also showed a marked increase in PKCδ protein expression at the beginning of the 6-day-long culturing period, followed by a decrease in its expression level. We also confirmed these findings by PKCδ enzyme activity assays, with the maximum levels of activity corresponding to the highest levels of protein expression on culturing days 2 and 3. Correspondence of these patterns to the onset of chondrogenic differentiation of cells in HDC supports our idea that PKCδ is a good candidate among PKC isoforms to be involved in the regulation of signalling mechanisms leading to *in vitro* cartilage formation.

In the subsequent set of experiments rottlerin, described as a pharmacological inhibitor of PKCδ activity in many publications was administered to cells of HDC. Rottlerin treatments resulted in a decrease of cartilage matrix production in a concentration and time dependent manner. These results are in agreement with the findings described in the literature. While the cellular viability only decreased to a lesser, but still significant, extent, a marked decrease in the rate of proliferation was also observed. The latter result is consistent with the antiproliferative effect of rottlerin. Administration of rottlerin did not induce either apoptosis or necrosis in cells of HDC. As rottlerin is described some recent publications as a mitochondrial uncoupler of different mammalian cell types rather than a direct PKCδ inhibitor, these findings may reflect on a difference in mitochondrial sensitivity to rottlerin of chicken compared to mammalian cells. In our experiments neither the mRNA nor the protein expression of ERK1/2 was altered after treatments with rottlerin, but its phosphorylated form was almost completely diminished. Our result is in agreement with that of another study carried out on pancreatic acinar cells, where rottlerin also proved to inhibit MAPK-activation.

Since rottlerin has been described as a factor having both PKCδ dependent and independent effects often leading to contradictory results on various tissue and cell types, we applied targeted PKCδ mRNA silencing. Transfection with PKCδ shRNA almost completely blocked *in vitro* chondrogenesis. Neither mitochondrial activity, nor cellular proliferation rate were significantly affected by the introduction of either the empty or the PKCδ shRNA-containing vectors into cells of HDC, and PKCδ gene silencing did not induce significant apoptotic and/or necrotic cell death rate of chondrogenic cells. Although neither the mRNA nor the protein expression of ERK1/2 showed any alterations as a result of PKCδ gene silencing, the level of its phosphorylated form exhibited a two-fold increase. The elevated ERK1/2 activity could be another factor involved in the complete block of *in vitro* cartilage matrix

production observed following PKCδ gene silencing. However, application of gene silencing of PKCδ had variable effect on MEK-ERK1/2 signalling pathway in different systems, but the majority of the investigations describes PKCδ as a negative regulator of the MEK-ERK1/2 pathway. As we failed to detect any elevation in the phosphorylation of ERK1/2, instead, we found a decreasing pattern following the application of rottlerin, we suppose that this compound is probably not a PKCdelta inhibitor in HDC. This idea is further supported by the fact that we were unable to detect any consistent change of PKCdelta enzyme activity in our experiments when rottlerin was applied to the reaction mixtures of enzyme activity assays. Although worked properly in *ex vivo* enzyme activity assays, because of its diverse and probably non-specific effects, rottlerin should not be chosen as a means for investigating the role of PKCdelta in chondrifying high density micromass cultures.

Absence of NMDA receptors strongly inhibits Ca^{2+} oscillations of HDC

Calcium ion is a ubiquitous cellular signal for normal life cycle of eukaryotic cells as it can act as a second messenger and regulate many signalling pathways. Various stimuli promote the movement of Ca^{2+} through different channels from the extracellular space into the cytosol. NMDA receptor is one of the three major ionotropic glutamate receptors of excitable cells and is primarily permeable to Ca^{2+}. NMDA receptors are assembled as heterotetramers, and presence of the NR1 subunit is indispensable to the membrane delivery and functionality of the channel. In the last few years, NMDA receptors were identified in chondrocytes, but their function is not clarified yet. Previous results of our laboratory demonstrated a characteristic pattern of the changes of the basal cytosolic Ca^{2+}-concentration in cells of HDC, which showed a very strong correlation to the onset of the differentiation of chondrogenic cells. Moreover, we also described high frequency cytoplasmic Ca^{2+} oscillations in these cells. To explore the contribution of NMDA receptors to these Ca^{2+} concentration

changes, we applied gene silencing of the NR1 subunit. The efficiency of gene silencing was 80–90% as revealed by RT-PCR and Western blot analyses, and a considerable inhibition of cartilage formation was observed. NR1 gene silencing also resulted in a significant decrease of the proliferation in HDC, but viability of cells remained unaffected. Moreover, the spontaneous Ca^{2+} oscillations almost totally disappeared from HDC in the absence of NMDA receptors. As the result of NR1 gene silencing, the mRNA expression of aggrecan and Sox9 diminished, moreover,the protein expression of Sox9 was also decreased, but the phosphorylated form remained at a constant level.

In our experiments we provided evidence that the NMDA receptor is one of the essential Ca^{2+} channels regulating chondrogenesis and at least in part they are responsible for Ca^{2+} oscillations during differentiation.

Summary

In this work, we studied the transient transfection possibilities of chondrifying mesenchymal cultures and after optimization we aimed to transfect three different types of constructs and investigated their effects on chondrogenesis.

- We demonstrated that the efficiency of transient transfection of HDC highly depends on the transfection method and on the size of the delivered vector. Saint-Mix was found to be suitable for transfection of larger vectors, while Lipofectamin 2000 was better applicable for the delivery of smaller shRNAs.

- For siRNA transfection, the specific DharmaFect delivery system is required.

- Manipulation of the PP2B activity either by overexpression. or gene silencing by inducible shRNA vectors diminishes cartilage formation,

which suggests a very precisely set calcineurin activity during proper chondrogenesis.

- We proved that rottlerin is not a PKCδ-specific inhibitor in chondrogenic cells.
- Application of a PKCδ-specific shRNA inhibited chondrogenesis probably via increasing the activity of ERK1/2.
- The gene silencing of NR1 subunits of NMDA receptor by siRNA decreased the number of chondrogenic cells exhibiting repetitive Ca^{2+} transients and completely blocked cartilage formation, which suggest a crucial role of this receptor in the Ca^{2+} homeostasis and chondrogenesis of HDC.

Publications

This thesis is based on these *in extenso* publications:

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