2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Reagents

Salts and buffer reagents were purchased from Sigma (Munich, D), Merck (Darmstadt, D), or Roth (Karlsruhe, D) unless otherwise stated. Solutions and solvents were purchased from Sigma (Munich, D), Merck (Darmstadt, D), Roth (Karlsruhe, D) and J. T. Baker (Deventer, NL).

Special agents were obtained as listed below:

17ß-estradiol	Sigma (Munich, D)
progesterone	Sigma (Munich, D)
testosterone	Sigma (Munich, D)
DHEA	Sigma (Munich, D)
GH	Sigma (Munich, D)
IGF-I	Sigma (Munich, D)

2.1.2 Cell culture media and solutions

Medium A

Sebomed®	Biochrom AG (Berlin, D)
10% fetal calf serum	Biochrom AG (Berlin, D)
50 µg/ml gentamicin	Sigma (Munich, D)
5 ng/ml epidermal growth factor (EGF)	Sigma (Munich, D)
1 mM $CaCl_2$	Sigma (Munich, D)

Medium B

Sebomed®	Biochrom AG (Berlin, D)
10% steroid-free fetal calf serum	cc pro GmbH (Neustadt, D)
50 µg/ml gentamicin	Sigma (Munich, D)
5 ng/ml epidermal growth factor (EGF)	Sigma (Munich, D)
1 mM $CaCl_2$	Sigma (Munich, D)
10^{-6} M retinol	Sigma (Munich, D)

10^{-7} M linoleic acid	Sigma (Munich, D)
0.1% human protease free albumin	Sigma (Munich, D)

Medium C

Dulbecco's modified Eagle's medium (DMEM)/ Ham's F12	Gibco BRL, Invitrogen, (Karlsruhe, D)
10% fetal calf serum	Biochrom AG (Berlin, D)
100 UI/ml penicillin/streptomicin	Sigma (Munich, D)
1 mM $CaCl_2$	Sigma (Munich, D)

Medium D

Defined keratinocyte-SFM medium	Gibco BRL, Invitrogen (Karlsruhe, D)
100 UI/ml penicillin/streptomicin	Sigma (Munich, D)
5 ng/ml epidermal growth factor (EGF)	Sigma (Munich, D)
50 µg/ml bovine pituitary extract	Gibco BRL, Invitrogen (Karlsruhe, D)

Freezing medium

40% Sebomed®	Biochrom AG (Berlin, D)
50% fetal calf serum	Biochrom AG (Berlin, D)
10% Dimethylsulfoxid (DMSO)	Sigma (Munich, D)

Other Substances:

Trypsin/EDTA 0.05/0.02% (w/v) in PBS	Biochrom AG (Berlin, D)
PBS-Dulbecco (1x) w/o Ca^{2+}, Mg^{2+}	Biochrom AG (Berlin, D)

2.1.3 Cell culture materials

blue & red cap tubes	Falcon, Becton Dickinson Labware, Franklin Lakes (NJ, USA)
cell culture flasks	Nunc (Wiesbaden, D)
centrifuge tubes	Nunc (Wiesbaden, D)
freezing vials	Falcon, Becton Dickinson Labware, Franklin Lakes (NJ, USA)

heparine injections	Becton Dickinson Labware, Franklin Lakes (NJ, USA)
96-well plates	Nunc (Wiesbaden, D)
serological pipettes	Becton Dickinson Labware, Franklin Lakes (NJ, USA)
sterile filters (0.2 µm)	B. Braun (Melsungen, D)
pipette tips	Biozym (Oldendorf, D)
cell scraper	Greiner (Frickenhausen, D)

2.1.4 Materials for cell biological techniques

Proliferation analysis

4-methylumbelliferyl heptanoate (MUH)	Sigma (Munich, D)

Detection of lipids

nile red	Sigma (Munich, D)

Detection of cytotoxicity

Cytotoxicity Detection Kit (LDH)	Roche Diagnostics (Mannheim, D)

2.1.5 Materials for RNA & DNA analysis

RNA isolation

RNeasy Midi Kit	Qiagen (Hildau, D)
RNase-Free DNase Set	Qiagen (Hildau, D)

cDNA synthesis

Ready To Go ™ T-Primed First-Strand Kit	Amersham Biosciences (Freiburg, D)

RT-PCR

The primers were purchased in lyophilized condition from TIB Molbiol (Berlin, D) and were diluted with bidistilled water to a concentration of 100 µM.

primers		Ta (°C)	length (bp)	cycles
IGF-IR				
insulin-like growth factor receptor *NM_000875*	up 5'-GTGTACGTTCCTGATGAGTGG GAG do 5'-GCC CCG TGT CAT CAG TTC CATGAT	65	300	24
IR				
insulin receptor *XM_048346*	up 5'-AAC CCG ACA ACT GTC CAG A do 5'-GGG CAC ATT CTC CAT GTC CT	64	195	35
ERα				
estrogen receptor α *NM_000125*	up 5'-CAG GGG TGA AGT GGG GTC TGC TG do 5'-ATG CGG AAC CGA GAT GAT GTAGC	62	483	35
ERβ				
estrogen receptor β *NM_001437*	up 5'-TGC TTT GGT TTG GGT GAT TGC do 5'-TTT GCT TTT ACT GTC CTC TGC	59	259	39
GHR				
growth hormone receptor *XM_003896*	up 5'-TCT CCT GAC CTC GTG ATC CA do 5'-AAG GTC TGC GAT AAA TGG GAA	64	194	35
AR				
androgen receptor *M34233*	up 5'-GAA GAC CTG CCT GAT CTG TG, do 5'-AAG CCT CTC CTT CCT CCT GT	63	269	35
c-Myc				
c-Myc proto-oncogene *X00364*	up 5'-CAG GCA AGC CCT CAG AAT AG do 5'-CAG AAA ACC CCC TAA TGC AA	56	681	35
FN1				
fibronectin 1 *X02761*	up 5'-GGATGGGGAGCAGAGTTTG do 5'-TCCATCTCACCAGGACAGTAGAA	65	627	28
Gusb				
β-glucuronidase *NM_000181*	up 5'-ACT TGA AGA TGG TGA TCG CTG do 5'-CCA GAC CCA GAT GGT ACT GCT	67	335	36

50xTITANIUM™ *Taq* DNA polymerase	Clontech, Invitrogen (Karlsruhe, D)
10xTITANIUM™ *Taq* PCR Buffer	Clontech, Invitrogen (Karlsruhe, D)

DNA agarose gel

DNA Typing Grade 50xTAE Buffer	GibcoBRL, Invitrogen (Karlsruhe, D)
Ultra PURE agarose	GibcoBRL, Invitrogen (Karlsruhe, D)
DNA molecular weight marker VIII (0.019-1.11kbp)	Roche (Mannheim, D)
glycerol gel loading buffer 5x	Amresco (Göttingen, D)
ethidium bromide	Sigma (Munich, D)

Northern blotting

Qiaquick gel extraction kit	Qiagen (Hildau, D)
Easyhyb hybridization solution	Clontech, Invitrogen (Karlsruhe, D)
radioactive agent [α-^{32}P] dCTP	NEN Dupont (Boston, USA)
RNA Ladder (0.24- 9.5 kb)	GibcoBRL, Invitrogen (Karlsruhe, D)
Ready-to-Go DNA labeling beads	Amersham Biosciences (Freiburg, D)

2.1.6 Materials for protein analysis

Protein isolation

Protease inhibitors:

pepstatin	Sigma (Munich, D)
leupeptin	Sigma (Munich, D)
aprotinin	Sigma (Munich, D)
PMSF	Sigma (Munich, D)

BCA protein determination kit	Pierce (Weiskirchen, D)

Western blotting

All antibodies were purchased by Santa Cruz Biotechnologies (Heidelberg, D).

Primary antibodies:

antigenes	species	dilution factor
IGF-IR	rabbit polyclonal	1/250
IR	rabbit polyclonal	1/250
ERα	mouse monoclonal	1/100
ERβ	goat polyclonal	1/100
GHR	goat polyclonal	1/250
AR	rabbit polyclonal	1/500
c-Myc	mouse monoclonal	1/100
Fibronectin	mouse monoclonal	1/500

Secondary antibodies:

Anti-antigenes	dilution factor
Anti mouse IgG_1-HRP	1/250
bovine anti-goat IgG-HRP	1/100
bovine anti-rabbit IgG-HRP	1/100

Full Range Rainbow marker	Amersham Biosciences (Freiburg, D)
NuPAGE 3-8% Tris-acetate gels	Novex, Invitrogen (Karlsruhe, D)
NuPAGE Tris acetate SDS buffer	Novex, Invitrogen (Karlsruhe, D)
NuPAGE LDS sample buffer (4x)	Novex, Invitrogen (Karlsruhe, D)
NuPAGE sample reducing agent	Novex, Invitrogen (Karlsruhe, D)
NuPAGE antioxidant	Novex, Invitrogen (Karlsruhe, D)
Hybond ECL	Amersham Biosciences (Freiburg, D)
non-fat milk powder	Biorad (Munich, D)
Tween-20	Sigma (Munich, D)
Immobilon-P (PVDF)	Millipore (Schwalbach, D)
video imaging system	TINA 2.0 (Fuji, Japan)

Elisa

estradiol detection Delfia Kit	PerkinElmer (Rodgau-Jügesheim, D)

human IGF-I Quantikine ELISA kit	R&D Systems (Wiesbaden, D)

2.1.7 Equipment

autoclave	Webeco (Bad Schwartau, D)
centrifuges	Biofuge pico, Heraeus (Osterode, D)
	Biofuge fresco, Heraeus (Osterode, D)
	Uni Vapo 100 H Uni Equip (Martinsried, D)
	Megafuge 1.0 Heraeus (Osterode, D)
CO_2- incubator	BB16, Heraeus (Osterode, D)
cytospin 2	Shandon (Frankfurt, D)
drying oven	T6 Heraeus (Osterode, D)
electrophoresis-power supplies	Power Pac 3000 Biorad (Munich, D)
	Power supply Model 200/2.0 Biorad (Munich, D)
elisa photometer	Dynatech MR5000 (Chantilly, VA, USA)
laminar flow bench	BSB 4A, Gelaire Flow Laboratories (Opera, I)
magnetic stirrer	MR 2000, Heidolph (Kehlheim, D)
	IKAMAG REG Jahnke & Kunkel (Staufen, D)
light microscope	IMT-2, Olympus (Berlin, D)
mixers	MS1 Minishaker IKA laboratories Jahnke & Kunkel (Staufen, D)
	Thermomixer comfort, Eppendorf (Hamburg, D)
	Vortex IKA VF2, Jahnke & Kunkel (Staufen, D)
PCR cycler	Mastercycler Gradient Eppendorf (Hamburg, D)
pH meter	pH 526, WTW (Weilheim i.OB., D)
camera	Polaroid (Cambridge, UK)
precision balances	Type 2662, Sartorius (Göttingen, D)

RNA calculator	Gene Quant II, AmershamPharmacia Biotech (Freiburg, D)
scientific imaging films	Hyperfilm Amersham Pharmacia Biotech (Freiburg, D) Bio Max MR, Kodak (New York, USA)
spectrophotometer	Ultrospec 2000, Amersham Pharmacia Biotech (Freiburg, D)

2.2 METHODS

2.2.1 Cell culture

2.2.1.1 Cell lines

Human immortalized SZ95 sebocytes

SZ95 sebocytes is an immortalized human sebaceous gland cell line (Zouboulis, Seltmann et al. 1999) derived from sebaceous gland cells of female facial skin, which shows the morphologic, phenotypic and functional characteristics of normal human sebocytes. The human facial sebaceous gland cells were transfected with a PBR-322-based plasmid containing the coding region for the Simian virus-40 large T antigen. The resulting proliferating cell cultures have been passaged over 50 times to date, have been cloned, and show no signs of senescence. The cells used for the experiments were between passages 26 and 30.

Human preputial skin fibroblasts

Fibroblasts were isolated from human foreskin coming from young children undergoing surgery. The skin was cut in 4x4 mm slices, was cleared from the fat and was digested with 0.25% trypsin solution overnight at 4ºC and then for 2 ½ h at 37ºC. This step allowed the dissociation of the epidermis from the dermis. After the dermis had been placed on a 100% FCS coated plate and had been dried for ¾ h at 37ºC, 8 ml of medium C (*see* 2.1.2) was added. Every 2 days the medium was changed. The fibroblasts were allowed to grow out and adhere on the plate ground for about 2 ½ weeks and then the cells were harvested and converted into 75 cm^2 culture flasks as describing in 2.2.1.3, where their cultivation was further proceeded. Fibroblasts used for the experiments were between passages 3 and 5.

Human preputial skin keratinocytes

Keratinocytes were also isolated from human foreskin coming from young children undergoing surgery. The dissociation of the epidermis from the dermis occurred as described above. The epidermis was collected from all skin slices and was tapped with tweezers in order to have keratinocytes released. Then, the cells were pelleted for 5 min at 1100xrpm. The supernatant was aspirated and the cells were resuspended in medium D (*see* 2.1.2). The

medium was replaced with fresh one every 2 days. Keratinocytes used for the experiments were between passages 3 and 5.

Human breast area skin fibroblasts

Breast area skin fibroblasts were isolated from a 60-y-old lady undergoing surgery as described above. The cells used for the experiments were between passages 3 and 5.

2.2.1.2 Cell cultivation

All work with the cells was done under the laminar flow. SZ95 sebocytes, fibroblasts and keratinocytes were grown in medium A, C and D, respectively in 75 cm^2 tissue culture flasks, in a humified atmosphere with 5% CO_2 at 37ºC, because the medium used was buffered with sodium bicarbonate/carbonic acid and the pH should be strictly maintained. Culture flasks had loosened caps to allow for sufficient gas exchange and the cells were left out of the incubator for as little time as possible. Every 2 days the medium was removed and replaced with warm, fresh medium.

SZ95 sebocytes and human fibroblasts, which were maintained under hormone-substituted conditions were grown in medium B (*see* 2.1.2). The cells were incubated with a mixture of hormones, namely 17β-estradiol, progesterone, testosterone, DHEA, GH, IGF-I, in concentrations similar to the median serum values in healthy 20-y-old men and women, and to the median serum values in healthy 60-y-old men and women, respectively (Table 2.2.1). On day 0 the cells were treated with the mixture of hormones in levels of 20-y-old men and women, accordingly. On day 2, and day 4 half of the cells were incubated at levels of 20-y-old men and women, accordingly, and the other half at levels of 60-y-old men and women. On day 6 the cells were collected for protein and RNA isolation.

2.2.1.3 Subculturing of cells

Cells are harvested when they have reached a population density which suppresses growth. Ideally, cells are harvested when they are in a semi-confluent state and are still in log phase. Cells that are not passaged and are allowed to grow to a confluent state can sometime lag for a long period of time and some may never recover.

Processing:

For subculturing, 70-90% confluent cells containing mitotic features were rinsed with 10 ml PBS twice, and subsequently incubated with 2 ml Trypsin/EDTA (T/E) for 5 min. Trypsin is an enzyme which disrupts peptide bonds, and helps the detachment of the cells from the ground of the flask, whereas EDTA is a novel molecule for complexing metal ions and allows the separation of the cells. As cells detached from the flask, they were incubated for 5-15 min with T/E at 37ºC until they became rounded and loosen when flask was gently tapped with the side of the hand. Then, 8 ml medium containing 10% FCS was added to the flask. FCS was used in order to stop the proteolytic activity of trypsin. Subsequently, the content of the flask was transferred to a 50 ml red cap and was spinned down at 1100xrpm for 5 min. The supernatant was aspirated and the cells were resuspended in Sebomed®/10% FCS and counted. 10,000 cells/cm^2 were then seeded in new 75 cm^2 flasks.

Table 2.2.1 Median hormone concentrations in serum of healthy 20- and 60-y-old men and women added to the culture medium of SZ95 sebocytes

Hormones	20-y-old men	60-y-old men	20-y-old women	60-y-old women
17β-estradiol (pmol/l)	100	100	1000	74
progesterone (nmol/l)	0.5	0.5	50	1
testosterone (nmol/l)	20	10	2	0.7
DHEA (nmol/l)	20	5	20	5
GH (ng/ml)	8	2	8	2
IGF-I (ng/ml)	400	92	400	84

2.2.1.4 Counting of cells

The viability of cells can be observed visually using an inverted phase contrast microscope. Live cells are phase bright, and adherent cells like SZ95 sebocytes, fibroblasts and keratinocytes form projections, when they attach to the growth surface. Viability can also be assessed using the vital dye, trypan blue, which is excluded by live cells but accumulates in dead cells. Cell numbers are determined using a hemacytometer. A hemacytometer consists of

a thickened glass slide into which a small chamber has been cut to allow the introduction of cells to be counted. The floor of the chamber is divided into nine sections, but in cell counting usually four corner sections are used. With a coverslip in place, each square of the chamber represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is approximately equivalent to 1 ml, the cell concentration per ml and the total number of cells can be determined.

Processing:

A cell suspension was prepared from the cell culture as described above (*see* 2.2.1.3). Then, 20 µl of cells was combined with 20 µl of trypan blue suspension (0.4%), mixed thoroughly and allowed to stand for 5-15 min. With the cover slip in place, a small amount of trypan blue/cell suspension was transferred into one of the two counting chambers of the hemacytometer by carefully touching the edge of the cover slip with the pipette tip and allowing each chamber to fill by capillary action. Then, all the cells were counted in each of the four corner square. Viable and non-viable cells were counted separately. Cells on top and left touching middle line were not included. The cell count was calculated using the equation:

$$\text{cells/ ml} = (n) \times 10^4,$$

n = the average cell count per square of the four corner squares counted.

Finally, the volume of the cell suspension was multiplied by the cells/ml value calculated above in order to determine the total number of cells in the total suspension volume.

2.2.1.5 Freezing of cells

Freezing can be lethal to cells due to the effects of damage by ice crystals, alterations in the concentration of electrolytes, dehydration, and changes in pH. To minimize the effects of freezing, several precautions are taken. First, a cryoprotective agent which lowers the freezing point, such as glycerol or DMSO, is added. In addition, it is best to use healthy cells that are growing in log phase and to replace the medium 24 h before freezing. The cells are slowly cooled from room temperature to -80ºC to allow the water to move out of the cells before it freezes. The optimal rate of cooling is 1-3ºC per min.

Processing:

Subconfluent cells were washed twice with 10 ml PBS and trypsinized as described above. After having aspirated the supernatant, the cells were resuspended in 1 ml freezing solution and the cell suspension was transferred in 1 ml freezing vial. The vials were then placed in a low tech device, which was filled with 200 ml of isopropanol at room temperature and then it was placed in the -80ºC freezer. The effect of the isopropanol is to allow the tubes to come to the temperature of the freezer slowly, at about 1ºC per minute. Once the container had reached -80ºC (overnight) the vials were removed from the device and immediately placed in the liquid nitrogen storage tank. Cells were stored in nitrogen temperatures because the growth of ice crystals could be retarded below -130ºC.

2.2.1.6 Thawing of cells

As cryopreserved cells are very delicate, one should thaw them and return them to culture as quickly as possible with minimal handling. Every freezing vial was thawed at a time and other vials were kept in liquid nitrogen until ready for use.

Processing:

After removing the freezing vial of cells from storage, the cap was twisted a quarter turn to relieve the internal pressure and was then retightened. Subsequently, the vial was dipped in a 37ºC water bath and swirled gently for 1-2 min until its content was thawed. In a sterile field the content was diluted 1 to 10 in warm culture medium and centrifuged at 1000xrpm for 5 min. The supernatant was aspirated, the cell pellet resuspended in 20 ml culture medium and then distributed in two 75 cm^2 flasks. The cells were ready to be used for the experiments after one week.

2.2.2 Cell biological methods

2.2.2.1 Proliferation analysis

Cells initially go through a quiescent or lag phase that depends on the cell type, the seeding density, the media components and previous handling. Then, they go into exponential growth (log phase), where they have the highest metabolic activity, and finally, they enter into stationary phase, where the number of cells is constant, and is characteristic of a confluent

population. MUH (Fig. 2.2.2.1) is used as a biocatalyst and for documenting cell proliferation. It is a fluorescent substrate for esterase and lipase and can only be incorporated in viable cells. In the cytoplasm it gets split by these enzymes and emits fluorescence at $\lambda_{cm} = 460$ nm.

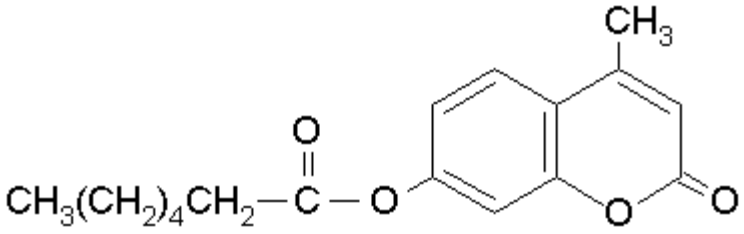

Fig.2.2.2.1 The structure of 4- methylumbelliferyl heptanoate ($C_{17}H_{20}O_4$)

Solutions used:

10 mg/ml MUH diluted in DMSO (per 96-well plate)	0.1 ml
PBS	9.9 ml

Processing:

Cells were cultured in 96-well tissue culture plates for 48 h. The wells were then washed with PBS, and the cells were treated with the compounds. Cell proliferation was measured after 48 h and 96 h treatment. On the day of the evaluation the medium was removed, the cells were washed twice with PBS, and 100 µl of a 100 µg/ml MUH solution in PBS was added to each well. The plates were then incubated at 37ºC for 30 min, and the released fluorescence which is representative for cell numbers was read on a Molecular Devices SPECTRAmax Gemini spectrofluorometer using 355 nm excitation and 460 nm emission filters. Experiments were performed in triplicate with 10 wells evaluated for each data point in each experiment.

2.2.2.2 Detection of lipids

The production of lipids was assessed by the nile red fluorescence assay. This is an assay which is based on the measurement of fluorescence by a spectrofluorometer after staining the cells with nile red (Fig. 2.2.2.2), a dual emission lipid dye (Zouboulis, Seltmann et al. 2002). Fluorescence is a member of the ubiquitous luminescence processes, in which susceptible molecules emit light from electronically excited status by either a physical (for example,

absorption of light), mechanical (friction) or chemical mechanism. After staining with nile red, when the cells are viewed for gold-yellow fluorescence (excitation: 450-500 nm; emission > 528 nm), neutral lipids such as triglycerides are detected and when the cells are viewed for red fluorescence (excitation: 515-560 nm; emission > 590 nm) polar lipids indicative of phospholipid containing organelles are detected (75).

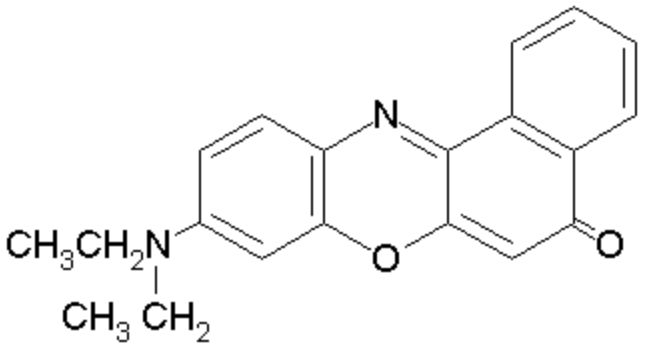

Fig.2.2.2.2 The structure of nile red ($C_{20}H_{18}N_2O_2$)

Solutions used:

10 mg/ml nile red diluted in DMSO (per 96-well plate)	0.1 ml
PBS	9.9 ml

Processing:

The cells were cultured in 96-well tissue culture plates for 48 h. The wells were then washed with PBS, and the cells were treated. After 48 h treatment the medium was harvested, the wells were washed twice with PBS, and 100 µl of a 100 µg/ml nile red solution in PBS was added to each well. The plates were then incubated at 37ºC for 30 min, and the released fluorescence was read on a Molecular Devices SPECTRAmax Gemini spectrofluorometer. The results are presented in the absolute fluorescence units in comparison with the controls, using 485 nm excitation and 565 nm emission filters for neutral lipids and 540 nm excitation and 620 nm emission filters for polar lipids. Experiments were performed in triplicate with 10 wells evaluated for each data point in each experiment.

2.2.2.3 Fluorescence microscopy

Fluorescence microscopy is also used for the detection of intracellular lipid droplets after they have been stained with substances such as nile red (*see* 2.2.2.2), however, in this case, lipid production is documented by a specifically developed fluorescence microscope.

Processing:

SZ95 sebocytes were seeded on 18 mm coverslips in culture dishes at an approximately 40% confluence. Then, SZ95 sebocytes were treated, whereas control cells received no treatment. After 24 h incubation the medium was harvested, the cells were washed twice with PBS, and 100 µl of a 100 µg/ml nile red solution in PBS was added. The plates were then incubated at 37ºC for 10 min, and the released fluorescence was documented byAxiophot SIP 31182 fluorescence microscope (Carl Zeiss, Göttingen, Germany).

2.2.2.4 Detection of cytotoxicity

Cell necrosis, in contrast to apoptosis, which is a normal physiological process for replacement of old or dysfunctional cells, is caused by physical disruption of the cell through injury, chemicals, radiation, invasion of microorganisms or nutritional deprivation. The cell structure is disrupted and the cell loses its functional control. Osmotic pressure causes swelling in organelles, the chromatin clumps, and the cell eventually bursts. As a consequence of the membrane disruption, intracellular enzymes like the lactate dehydrogenase (LDH) can be measured in the culture supernatant and can be used for quantification of the cell lysis. LDH is an oxidoreductase which catalyzes the conversion of lactate to pyruvate and is present in all cells. In these experiments the cytotoxicity of the used reagents was evaluated with the 'Cytotoxicity Detection Kit (LDH)' from Roche Diagnostics, which measures the LDH activity from the cytosol of damaged cells into the culture media. It is based on a two-step enzymatic reaction. First, NAD^+ is reduced to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate. Then, the catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt INT (2-[4-iodophenyl]-3-[4-nytrophenyl]-5-phenyltetrazolium chloride), which is reduced to red colored formazan. The formazan dye shows an absorption maximum at 492 nm and its amount correlates to the increased LDH activity after cell lysis.

Solutions used:

Reaction buffer (per 96-well plate):

Catalyst solution (ready to use)	0.25 ml
Dye solution (ready to use)	11.25 ml

Processing:

Cells were cultured in 96-well tissue culture plates at a density of 20,000 cells/well for 48 h. The wells were then washed with PBS, and the cells were treated with the indicated compounds. After 24 h, the plates were centrifuged at 1000xg for 5 min, in order to remove cell detritus. 100 µl of the resulting supernatant of each sample was transferred into another 96-well plate and 100 µl of the reaction buffer were added. After 15 min incubation at room temperature, the absorption at 492 nm was measured by the ELISA photometer. Experiments were performed in triplicate, with 10 wells evaluated for each data point in each experiment and absorption values of controls were set at 100%. The LDH release from the cells was calculated as percentage of control.

2.2.3 Molecular biological methods

2.2.3.1 RNA isolation and DNase treatment

As RNA is a chemical unstable product and the presence of ribonucleases (RNases) in very small amounts is already sufficient to degrade it, strict precautions must be taken with all RNA work. Solutions should be treated with strong reduction means e.g. β-mercaptoethanol, and diethylpyrocarbonate (DEPC), reagents and plasticware should come from fresh packages set aside for RNA work.

Solutions used:

RLT-buffer (ready to use)

RW1-buffer (ready to use)

RPE-buffer (ready to use)

Lysis buffer: 10 µl β-mercaptoethanol/ml RLT

H_20 $_{DEPC}$

DNase I, RNase-free (ready to use) in 550 µl H_20_{DEPC}
Buffer RDD (ready to use)

Processing:

Total RNA was isolated using the RNeasy Midi Kit. With the RNeasy procedure all RNA molecules longer than 200 nucleotides could be isolated. The cells, when they were about 80-85% confluent, were first washed twice with warm PBS and lysed in the presence of 2 ml lysis buffer, which was a highly denaturating guanidine isothiocyanate (GITC) containing buffer, and could immediately inactivate RNAses and ensure isolation of intact RNA. After transferring them to centrifuge tubes, lysates were vortexed for 10 sec and homogenized through an 18-20 gauge needle attached to a plastic syringe. Then, 1 volume of 70% ethanol (2 ml) was added to each lysate and mixed carefully, in order to provide appropriate binding conditions. Each lysate was applied to an RNeasy midi column with a silica-gel membrane, where the total RNA could selectively bind, and was centrifuged 5 min at 3000xg so that contaminants could efficiently wash away. The flow-through was removed. Then, the column was washed with 2 ml of RW1 buffer, centrifuged for 5 min at 3000xg to wash and the flow-through was discarded. In order to provide efficient on-column digestion of DNA during the RNA purification, the QIAGEN® RNase-Free DNase Set was used. The complete DNA removal was necessary for future use of the collected RNA in Real-Time PCR and cDNA microarrays. 20 µl DNase I stock solution was added to 140 µl RDD buffer, mixed gently by flicking the tube and was briefly centrifuged. Then, the DNase I mix was pipetted directly into the RNeasy silica-gel membrane and was placed on the benchtop (20-30ºC) for 15 min. Afterwards, 2 ml of RW1 was pipetted into the column and placed on the benchtop for 2 min. Then, the column was centrifuged 5 min at 3000xg and the flow-through was discarded. 2.5 ml RPE buffer was added two times to the column, centrifuged for 2 min at 3000xg and the flow-through was discarded. After being washed, RNA was eluted in RNase-free water, H_2O_{DEPC}. The RNeasy column was then transferred to a new 15 ml collection tube, 150 µl RNase-free water was pipetted directly to the RNeasy silica-gel membrane, which was then placed for 1 min on the benchtop and centrifuged for 3 min at 3000xg. The last step was repeated, so that 300 µl of RNA was eluted.

2.2.3.2 Measurement of RNA

The amount of RNA is measured photometrically and is based on the extinction of the azotic bases at 260 nm. At 230 and 280 nm the extinction rates provide the contamination of the samples with polysaccharides and proteins. The extinction ratios at 260/230 nm e.g. 260/280 nm should be between 1.8-2.0, which speaks for the satisfactory purification of RNA. At 260 nm an absorbance of 1.0 corresponds to 40 µg/ml RNA. The amounts of RNA are calculated with the following relation of Lambert-Beer:

$$A^{260} \times \varepsilon^{RNA} \times DF \times d$$

(A= absorbance at 260 nm, ε^{RNA}= 40, DF= dilution factor, d= cuvette gauge)

Processing:

The samples were diluted 1 to 50 -responding to 2 µl of the RNA probe and 98 µl H_2O_{DEPC}- were put into a 100 µl cuvette and the RNA amount was measured via the Gene Quant II spectrophotometer. For the calibration 100 µl H_2O_{DEPC} was used.

2.2.3.3 cDNA synthesis

RNA was transcribed into full-length first-strand cDNA using a Ready-To-Go™ T-Primed First-Strand Kit. The T-Primed First-Strand Kit utilizes Moloney Murine Leukemia Virus reverse transcriptase and an oligo(dT) primer to generate first-strand cDNA (Frohman, Dush et al. 1988).

Processing:

5 µg of total RNA was brought to a volume of 33 µl in a RNase-free microcentrifuge tube using DEPC-treated water. Then, the sample was heated to 65ºC for 5 min and transferred to a 37ºC bath for another 5 min. The First-Strand Reaction Mix was placed at 37ºC for 5 min, and the RNA solution was transferred to the Mix and incubated at 37ºC for 5 min. After vortexing and centrifuging, the mixture was incubated at 37ºC for 60 min.

2.2.3.4 Polymerase chain reaction (PCR)

PCR allows the production of more than 10 million copies of a target DNA sequence from only few molecules (Mullis and Faloona 1987). One has to choose two synthetic oligonucleotides (primers), which are complementary to the desired DNA sequence, and with the help of the *Taq* polymerase the target sequence can be duplicated. This happens in three major steps repeated for 30 or 40 cycles on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time. During the first step, the DNA double strand opens to single stranded DNA at 94°C and all enzymatic reactions stop. Then, at the primer annealing temperature (Ta), which must be optimized for each primer, ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. Primers that fit exactly build more stable bonds, which last longer, and on that little piece of double stranded DNA (template and primer) the polymerase can attach and starts copying the template (Vosberg 1989). Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore. Finally at 72°C the primers already have a strong ionic attraction, the bases which are complementary to the template are coupled to the primer on the 3' side and the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side.

Reagents and solutions used:

Reagent	Final concentration	[µl] / sample
sterile deionized water	-	36.75
10 x TITANIUM™ Taq PCR buffer with 15 mM $MgCl_2$	1 x TITANIUM™ Taq PCR buffer with 1.5 mM $MgCl_2$	5.0
40 mM dNTP mix	0.8 mM dNTP mix	1.0
10 µM forward primer	0.5 µM forward primer	2.5
10 µM reverse primer	0.5 µM reverse primer	2.5
50 x TITANIUM™ *Taq* DNA polymerase	0.25 x TITANIUM™ *Taq* DNA polymerase	0.25
Template DNA	-	2

Processing:

To perform several parallel reactions it was necessary to prepare a master mix containing water, buffer, nucleotides (dNTP's), primers and Taq DNA polymerase in a single tube, which was then aliquotted into individual tubes 48 µl each. 2 µl of DNA solution was then added into each tube. The conditions used were optimized for each primer (*see* 2.1.5 Materials for RNA & DNA analysis). As most of the PCR reagents were temperature sensitive, all reagents were kept on ice.

2.2.3.5 DNA agarose gel electrophoresis

DNA fragments can be unsewed according to their size in an electrical field applied across an agarose gel. Agarose is a polymer extracted from seaweed that can be cast in whatever shape is desired. When cool, the agarose gel contains pores through which DNA can be drawn. The size of the pores is a function of the concentration of agarose used in forming the gel. The electrical field is applied so that the negative pole (cathode) is at the same end of the gel where the DNA has been loaded, and the positive pole (anode) is at the other end of the gel. Because DNA is negatively charged, the loaded fragments will migrate towards the positive pole-though the pores in the agarose. The larger the DNA molecule, the more friction there is on a fragment as it attempts to move through the pores of the agarose gel slab, hence the more slowly it will migrate.

Processing:

In 10 µl of each amplified PCR product 2 µl loading buffer was added. Each sample was then loaded into a well of a 2% agarose gel (range of separation: 0.1-2 kbp long), which was already stained with ethidium bromide (10 mg/ml) and the gel run for 45 min at 80 V in 1xTAE Buffer. For the determination of the DNA fragments size a DNA Molecular weight marker was also applied into a well. After the electrophoresis, the DNA bands were visualized under UV light and photos were taken.

2.2.3.6 Northern blotting

Via Northern blotting the size and the amount of specific mRNA molecules of total or poly $(A)^+$ RNA are determined (Alwine, Kemp et al. 1977). The RNA is separated according to

size by electrophoresis through a denaturing agarose gel and is then transferred to activated cellulose (Seed 1982), nitrocellulose (Thomas 1980), glass or nylon membranes (Bresser, Hubbell et al. 1983). The RNA of interest is then located by hybridization with radiolabelled DNA or RNA with the help of a DNA polymerase, called Klenow enzyme, followed by autoradiography (Feinberg and Vogelstein 1983).

Solutions used:

5xformaldehyde gel-running buffer:	0.1 M 3-(-N- morpholino) propanesulfonic acid (MOPS) (pH 7.0) 40 mM sodium acetate 5 mM EDTA (pH 8.0)
Formaldehyde gel-loading buffer:	50% glycerol 1 mM EDTA (pH 8.0) 0.25% bromophenol blue 0.25% xylene cyanol FF
20xSSPE Puffer:	3 M NaCl 200 mM $NaH_2PO4.H_2O$ 20 mM EDTA pH 7.4
3M NaCl 10% SDS hybridization solution:	Express Hyb Hybridization Solution, Clontech
T/E buffer:	10 mM Tris HCl 1 mM EDTA pH 8
20xSSC:	3 M NaCl

0.34 M Na-Citrat x $2H_20$

pH 7.0

Processing:

Agarose/ Formaldehyde Gel Electrophoresis of RNA

Each sample was prepared by mixing 4.5 µl RNA (20 µg), 2 µl 5xformaldehyde gel-running buffer, 3.5 µl formaldehyde and 10 µl formamide in a sterile microfuge tube. It incubated for 15 min at 65ºC and then chilled on ice. After centrifuging the sample in order to deposit all of the fluid in the bottom of the microcentrifuge tubes, 2 µl of sterile, DEPC-treated formaldehyde gel-loading buffer was added. The samples were loaded on a 1% agarose gel containing 2.2 M formaldehyde and ran for 2 h in 1xformaldehyde gel-running buffer at 3-4 V/cm.

Transfer of RNA from gel to membrane

After the electrophoresis, the gel was rinsed in several changes of DEPC-treated water to remove formaldehyde and then it was soaked for 20 min in 0.05 N NaOH in order to hydrolyze the RNA and improve the efficiency of transfer. Afterwards, it was rinsed in RNase-free water and soaked for 45 min in 20xSSC. Then, it was placed in contact with the nitrocellulose membrane over night, where RNA could be transferred by capillary elution as described in Fig. 2.2.3.6. After the transfer, the membrane was rinsed in 5xSSPE, placed on a 3 MM Whatman paper and was left to dry.

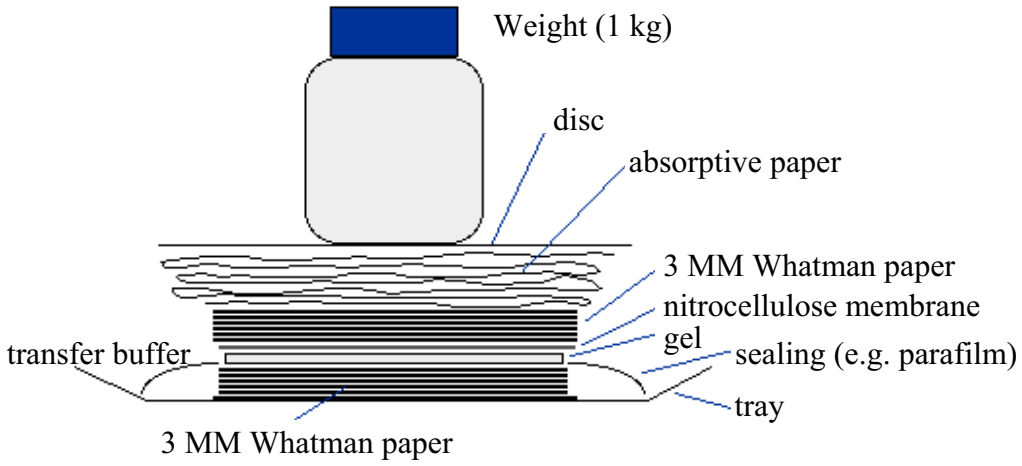

Fig. 2.2.3.6 Capillary transfer of RNA from agarose gel to nitrocellulose membrane

Hybridization analysis

After UV-crosslink of the blot at 254 nm, it was prehybridized for 2 h with the hybridization solution Easyhyb at 60ºC. The DNA probes were prepared by PCR and then purified by a Qiaquick Gel Extraction kit according to the manufacturer's instructions. Each probe was labelled with [α-^{32}P] dCTP by using Ready-to-Go DNA labelling beads and the blot was hybridized with the respective DNA probe and the hybridization solution for 16 h at 60ºC. 100 µg/ml denatured salom sperm DNA was also added in order to bind all non-specific sites. After the hybridization the blot was washed for 4x5 min with 2xSSC buffer, then for 30 min with 2xSSC/1% SDS buffer and finally with 0.1xSSC/1% SDS buffer for another 30 min. Every blot was also hybridized with β-glucuronidase as control, in order to adjust for differences in the amount of RNA which was loaded in each lane. The blots were quantitated by the storage phosphor imaging technique and quantitated using image analyzing software (TINA 2.04).

2.2.3.7 cDNA microarray

A cDNA microarray consisting of 15,529 annotated human cDNAs was performed by the group of Dr. James Adjaye at the Max Planck Institute for Molecular Genetics, Department of Vertebrate Genomics as previously described (Adjaye 2005).

2.2.4 Protein analysis methods

2.2.4.1 Protein isolation

Protein isolation requires very few steps compared to isolation of DNA or RNA and special precautions are not needed. However, while isolating proteins one should always work on ice or at 4°C in order to avoid their degradation. Moreover, the lysis buffer used for the protein extraction contains protease inhibitors, which do not last very long and as a consequence they should be added immediately before starting the experiment.

<u>*Solutions used:*</u>

Lysis buffer:

Tris-HCl	0.01 M
NaCl	0.144 M

Nonidet P-40	0.5%
SDS	0.5%
Pepstatin	5 µg/ml in distilled H_2O
Leupeptin	10 µg/ml in distilled H_2O
Aprotinin	0.1% in distilled H_2O
PMSF (phenylmethansulfonyl fluoride)	2 mM in DMSO

Processing:

1-2x10^7 cultured cells were washed twice in PBS at room temperature and incubated with cold lysis buffer for 5 min. Then, they were collected on ice by scraping and passed through a 21 gauge needle to shear the DNA. After being transferred to microcentrifuge tubes, they were centrifuged at maximum speed for 15 min at 4°C in order to sedimentate cell debris and nuclei. The supernatant fluid was the total cell lysate.

2.2.4.2 Protein quantitation

For the determination of the protein concentration, the BCA™ Protein Assay (Pierce, Weiskirchen, D) can be used, which is based on the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium, also called biuret reaction (Smith *et al* , 1985). Cu^{1+} can then react with two molecules of bicinchoninic acid (BCA) and build a purple-colored chelate complex, which exhibits an absorbance at 562 nm analogue to the protein concentration over 20-2000 µg/ml. The protein concentrations are determined with reference to standards of bovine serum albumin (BSA) diluted in H_2O (0-1500 µg/ml).

Solutions used:

Working solution (per well):

Reagent A (ready to use)	196 µl
Reagent B (ready to use)	4 µl

Processing:

First, the working solution was prepared by mixing Reagent A and B (50:1). 10 µl from each cell lysate and each standard rank probe was transferred to a 96-well-plate in triplicate and 200 µl/well of the working solution was added. After incubating the plate at 37ºC for 30 min the absorption was measured at 562 nm with an ELISA photometer. The protein was then calculated using the standard rank curve.

2.2.4.3 Western blotting

In the first stage of Western blotting, polyacrylamide gel electrophoresis (PAGE) is used to separate proteins. Polyacrylamide is a polymer that forms a gel with pores that are small enough to separate proteins. Two types of PAGE can be done. One is Native PAGE which is performed under conditions of high pH, a condition that causes almost all proteins to have a negative charge. Native PAGE allows separation of proteins by their surface charge and their size. The second type of PAGE gel is called an SDS PAGE. In this system negatively charged SDS coats individual proteins negating the charge on the proteins. In this type of gel the proteins separate on the gel, based on size alone. A molecular weight can be used to tell the sizes of various proteins. In the second stage, proteins from the gel are transferred onto a membrane using electrophoresis and finally, specific antibodies are used in order to detect the desired proteins (Laemmli 1970). For the experiments the NuPAGE® Pre-Cast Gel system was used, consisting of the NuPAGE® Novex® Tris-Acetate Gels and specially optimized buffers.

Solutions used:

Running buffer A:	50 ml NuPAGE® Tris Acetate SDS Buffer 950 ml distilled H_2O
Running buffer B:	250 ml Running buffer A 500 µl NuPAGE® Antioxidant
5xTransfer buffer:	58.15 g Tris (base) 21.75 g glycin

	3.7 g SDS 20% methanol ad 1600 ml distilled H_2O
Washing buffer:	19.1 g PBS 2000 ml distilled H_2O 2000 µl Tween-20
Blocking buffer:	3 g non-fat dry milk 100 ml washing buffer
Stripping buffer:	21 g citric acid ad 100 ml distilled H_2O pH 1.5

Processing:

Sample preparation

40 µg of total protein from each sample was brought to 18 µl with distilled H_2O. Then, 5 µl of LDS sample buffer and 2 µl of reducing agent, which provides complete reduction of disulfides, were added and the protein samples were incubated for 10 min at 70°C in order to denature.

Polyacrylamide gel electrophoresis and protein blotting

The protein samples were loaded onto a Nupage Tris acetate gel 3-8% and the gel run for 1 h at 150 V. Then, the gel was equilibrated for 10 min at room temperature in transfer buffer. A transfer sandwich consisting of the protein gel and a nitrocellulose membrane was assembled. The proteins were electrophoretically transferred from the gel to the nitrocellulose membrane for 2.5 h at 100 V in cooled transfer buffer.

Blocking and incubation with antibodies

The membrane was blocked by 3% non fat dry milk in 0.1% PBS-Tween-20 over night at 4°C. After 3 washes in PBS-Tween-20 the membranes were incubated for 1 h at room temperature with the antibody optimized diluted in PBS-Tween-20 (*see* 2.1.6 Materials for

protein analysis). After 3 washes, they were incubated for 60 min at room temperature with horseradish peroxidase-conjugated anti-antibody diluted 1:5000 in PBS-Tween-20. After a final wash, protein bands were detected by an enhanced chemiluminescence system (ECL). The protein band intensities were quantified densitometrically using a video imaging system TINA 2.0.

2.2.4.4 APAAP staining

The APAAP staining is a technique, which allows the detection of researched antigens in the cells by utilizing a preformed soluble enzyme-anti-enzyme immune complex (Cordell et al., 1984). The staining sequence consists of an unconjugated primary antibody, a secondary antibody, the soluble alkaline phosphatase-antialkaline phosphatase complex and substrate solution. The primary antibody and the antibody of the enzyme immune complex must come from the same species.

Solutions used:

Wash buffer:	4.5 g Tris-Base
	34.25 g Tris/HCl
	43.90 g NaCl
	ad 5000 ml distilled H_20
	5 ml Tween-20
Diluted RPMI medium:	50 ml RPMI
	50 ml FCS
	0.5 g sodium acid
	ad 450 ml distilled H_20
	pH 7.4-7.6
Conjugated buffer:	2 ml diluted RPMI medium
	250 µl human serum
Development buffer:	4. 9 g Tris-Base

	1.5 g Tris/HCl
	8.7 g NaCl
	ad 1000 ml distilled H_20
Propandiol solution:	21 g propandiol
	ad 1 l distilled H_20
New fuchsin solution:	1 g new fuchsin
	20 ml 2 M HCl
Nitrite/new fuchsin:	10 mg sodium nitrite
	ad 250 µl distilled H_2O
	100 µl new fuchsin
NAB phosphate:	250 mg Naphtol AS-BI-phosphate
	3 ml N, N-dimethylformamid
Fuchsin solution:	35 ml development buffer
	12.5 ml propandiol solution
	pH 9.75
	20 mg Levamisol
	350 µl nitrite/new fuchsin
	300 µl NAB-phosphate
	pH 8

Processing:

A cell suspension was prepared and microscopic slides were mounted with paper pads and cuvettes in a metal holder. Then, 200 µl of the cell suspension was loaded in each cuvette and was spinned for 3 min at 800 rpm in a Shandon Cytospin 2 on the slides. The preparations were fixed in methanol at -20ºC for 5 min and were incubated with the antibodies (*see* 2.1.6 Materials for protein analysis), which were diluted in PBS/1% fetal calf serum albumin, for 5 min. They were washed 3 times for 5 min with wash buffer and incubated with the second

antibody, which was diluted in conjugated buffer, for 30 min. After washing 3 times with the wash buffer for approx. 5 min, the slides were incubated with the APAAP complex diluted 1:50 in RPMI 30 min at room temperature. They were washed 3 times for 5 min and developed by incubation with the fuchsin solution for 20 min and by counterstaining with hematoxylin for 1 min. Finally, the slides were washed with distilled H_2O for 5 min, mounted with Aquamount and covered with a cover slip. Triplicate slides were stained for each data point.

2.2.4.5 Human IGF-I immunoassay

The Quantikine IGF-I Immunoassay is used to determine relative mass values for naturally expressed IGF-I in supernates. It is a solid phase ELISA, which is based on the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IGF-I is pre-coated onto a microplate. The samples are pipetted onto the wells and any IGF-I present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IGF-I is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IGF-I bound in the initial step. The color development is stopped and the intensity of the color is measured.

Reagents and solutions used:

IGF-I microplate	
IGF-I conjugate	
IGF-I standard:	ad 1 ml H_2O
Assay Diluent RD1-53	
Calibrator Diluent	
Pretreatment A	
Pretreatment B:	ad 10 ml distilled H_2O
Wash Buffer solution:	20 ml Wash Buffer Concentrate
	ad 480 ml distilled H_2O
Substrate solution:	50% Color Reagent A
	50% Color Reagent B

Stop solution

Processing:

After 48 h and 72 h treatment the cell culture supernates were collected and centrifuged in order to remove debris. For the standard row the IGF-I standard was diluted first 1:10 and then 1:2 with the Calibrator Diluent RD5-22 in polypropylene tubes. Then, 150 µl of Assay Diluent RD1-53 50 µ1 of each standard and each sample was loaded onto the wells of the precoated microplate and was incubated for 2 h at 2-8ºC. After having aspirated the solution, each well was washed four times with 400 µl Wash Buffer and then the plate was inverted and blotted against clean towels. 200 µl of cold IGF-I Conjugate was added to each well. After 1 h incubation at 2-8ºC the plate was washed again as mentioned above. 200 µl of Substrate Solution was added to each well and the plate was left for 1 h at room temperature. Finally, 50 µl of Stop Solution was added to each well and the samples were read at 450 nm on a Dynatec MR 500 plate reader.

2.2.4.6 Estradiol fluoroimmunoassay

The Delfia Estradiol assay is a solid phase fluoroimmunoassay, which is used to determine the estradiol values in supernates and is based on competition between euphorium-labelled estradiol and sample estradiol for polyclonal anti-estradiol antibodies. Samples which contain estradiol inhibit the binding of the euphorium-labelled estradiol to the antibody molecules. There is also a second antibody which is coated to the solid phase and can bind the IgG-estradiol complex and separate the antibody-bound and free antigen. Then, the Enhancement Solution has the ability to dissociate euphorium ions from the labelled estradiol into solution, where they form highly fluorescent chelates. The fluorescence for each sample is inversely proportional to the concentration of estradiol in the sample.

Reagents and solutions used:

Anti-rabbit IgG microtitration strips

Estradiol standards

Estradiol-Eu tracer

Estradiol Antiserum

Wash solution:	40 ml Wash concentrate
	960 ml distilled H_2O
Estradiol Assay Buffer	
Enhancement Solution	

Processing:

After 48 h and 72 h treatment the cell culture supernates were collected and centrifuged in order to remove debris. Then, 25 µl of the Estradiol Standards and of the samples were pipetted into the strip wells. 100 µl of the Estradiol Antiserum solution was added to each well and incubated for 30 min at room temperature with slow shaking. After having added 100 µl of the Estradiol-Eu solution to each well, the plate was incubated for 2 h at room temperature with slow shaking. The plate was washed and 200 µl Enhancement Solution was added. After 5 min shaking fluorescence was measured via a Delfia 1234 fluorometer and the MultiCalc protocol was used.

Statistical analysis

Values represent the mean values ± Standard Deviation (SD) of at least three experiments. Statistical significance was calculated by the two-sample independent-groups *t-test*. Mean differences were considered to be significant when $p<0.05$. Data and graphs were produced with MS Excel 2000 (Microsoft Corporation, USA).