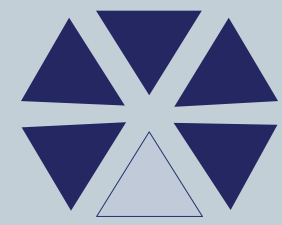


Influence of NMR Therapy on Metabolism of Osteosarcoma- and Chondrosarcoma Cell lines

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INTRODUCTION

Nuclear magnetic resonance (NMR) with weak magnetic fields (up to 2.3 mT, 100 kHz) has been shown to stimulate repair processes in cartilage and to influence pain signalling. Nowadays it has been proven that the therapeutical application of NMR has beneficial effects on different joint diseases and osteoporosis. On the contrary, strong magnetic fields (3 T) used for imaging purposes are suspected to have deleterious effects on chondrocytes and cartilage repair.

The aim of our study was to demonstrate positive or negative effects of the therapeutical used NMR on the metabolism in chondro- and osteosarcoma cells. The present investigations used NMRT with weak field strength adapted to the specific types of tissue. Testing its influence on cellular processes we used microarrays and quantitative PCR (qPCR) for expression profiling of NMRT-treated chondrosarcoma and osteosarcoma cells. In addition, functional effects on Ca²⁺ influx and Ca²⁺ release were studied by application of the Ca²⁺ imaging technique. Furthermore effects on protein functions involved in signal transduction pathways were studied using a luciferase reporter assay system.

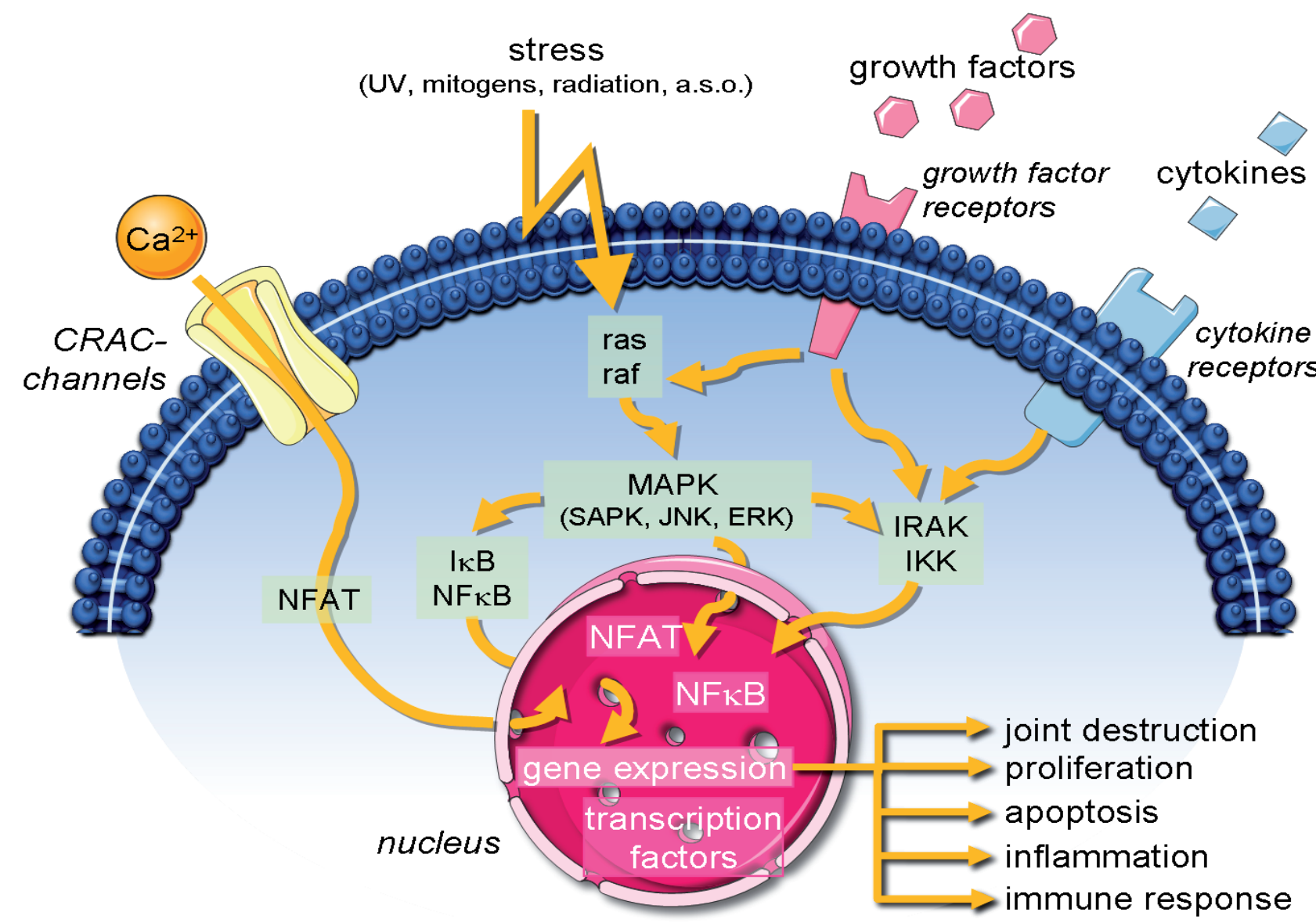


Fig. 1: In rheumatic diseases, certain signal cascades play a role in gene expression of factors with important functions in joint destruction, cell growth, apoptosis, inflammation, and immune response.

These pathways can be induced by stressors (e.g. UV radiation, mitogens, electromagnetic fields), by signals of growth factor receptors (e.g. IGF, TGF, EGF, HGH) and cytokines (e.g. IL-1, BAFF, IFN), as well as via Ca²⁺ signals of the NFAT pathway. Subsequently proto-oncogenes like ras, raf, etc. are upregulated in the cell, and various pathways are activated by kinases, for example the mitogene activated protein kinase (MAPK) signal pathway, the pathway of the nuclear factor of activated T-cells (NFAT). The IL-1 receptor associated kinase (IRAK) is generated via cytokine signals or MAPK, and the inflammation transcription factor NFkB is activated. Thus external signals are interlinked with internal signal pathways of the cell. In further consequence, activation or expression of various genes can be initiated by transcription factors like c-Jun, c-Fos, NFkB, NFAT, and gene promoters in the nucleus. In this way pathological processes in the joints can be regulated by chemokines, cytokines, RANKL, COX, metalloproteinases, etc.

Quantification of Expression by Real Time PCR

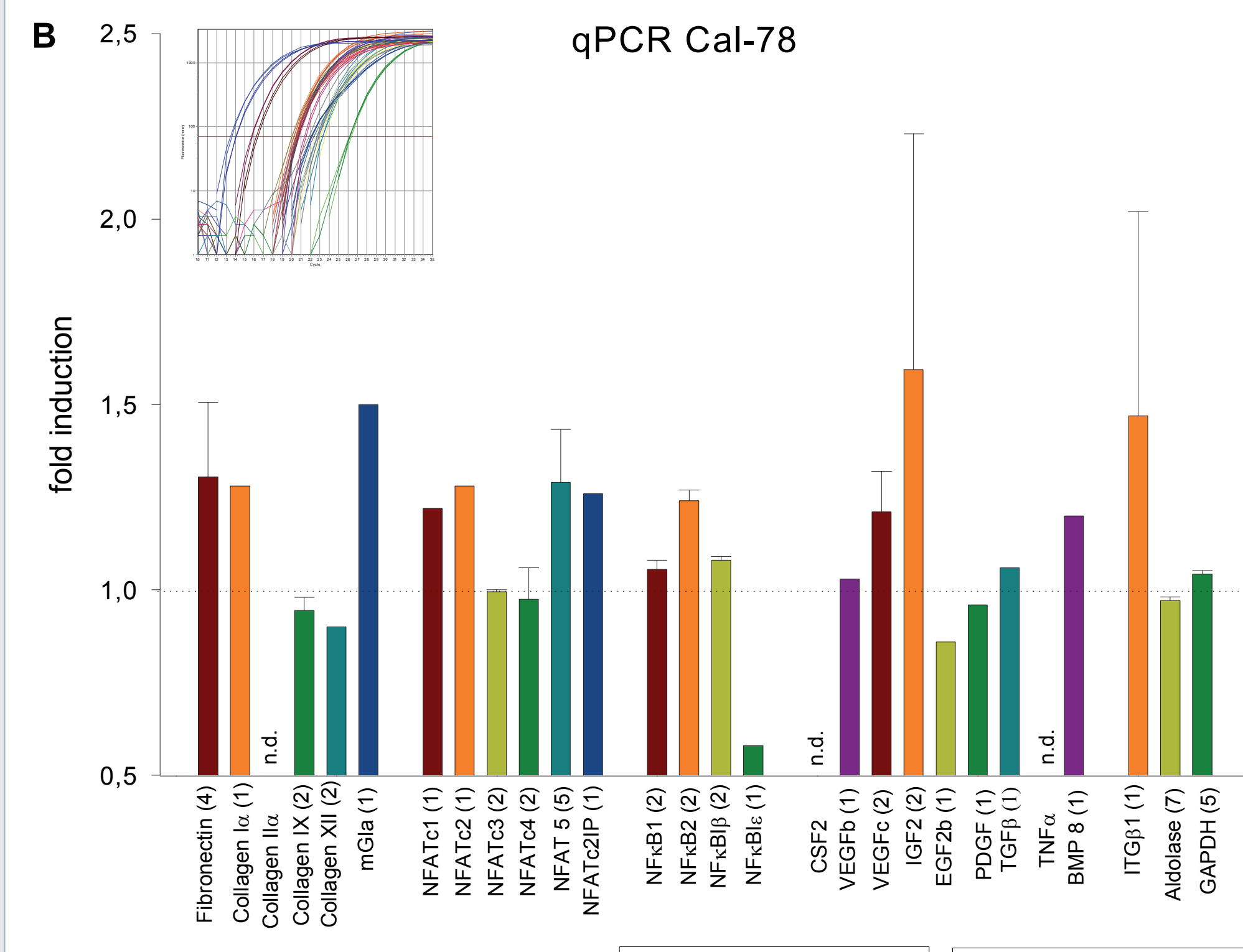
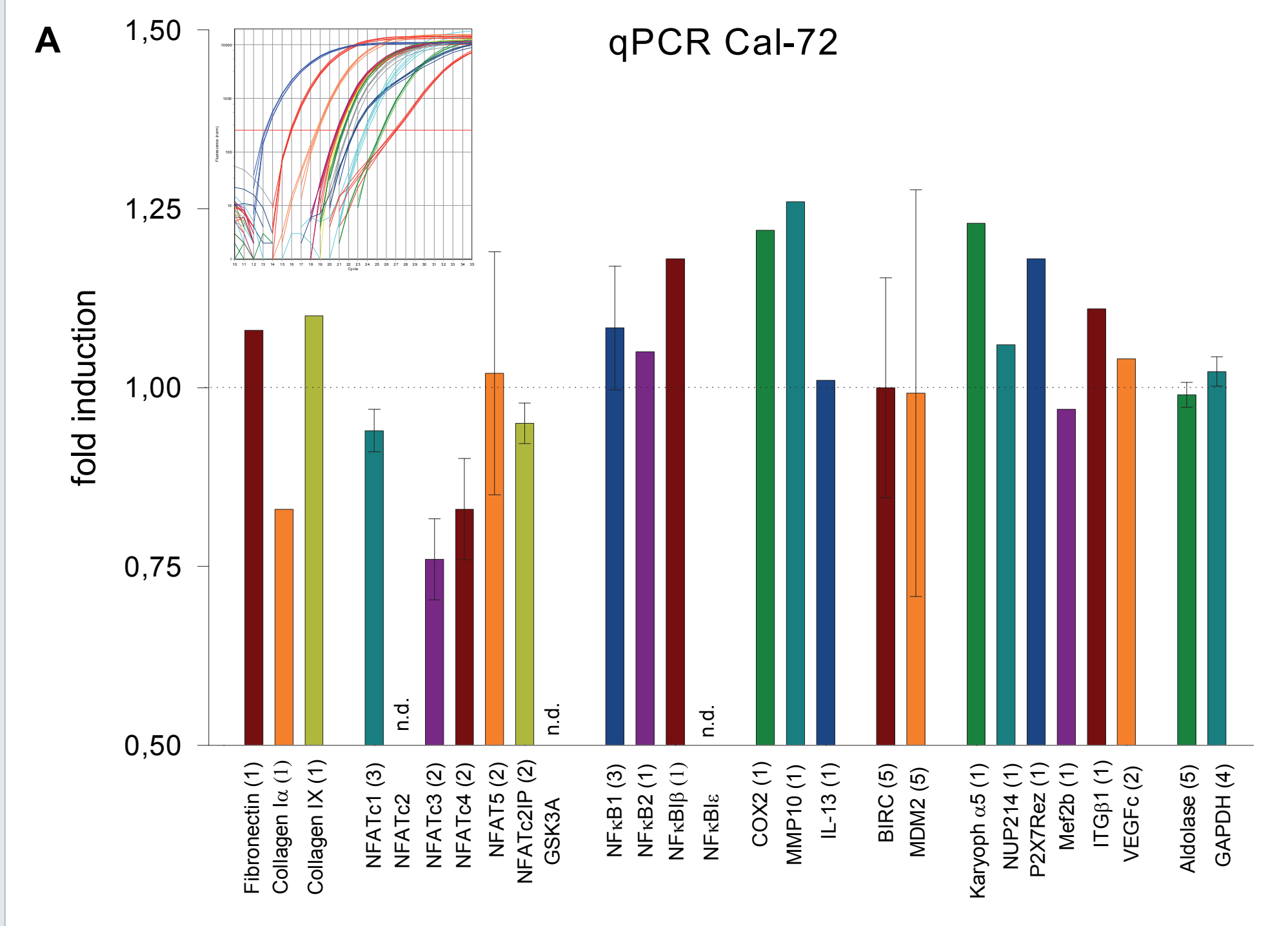
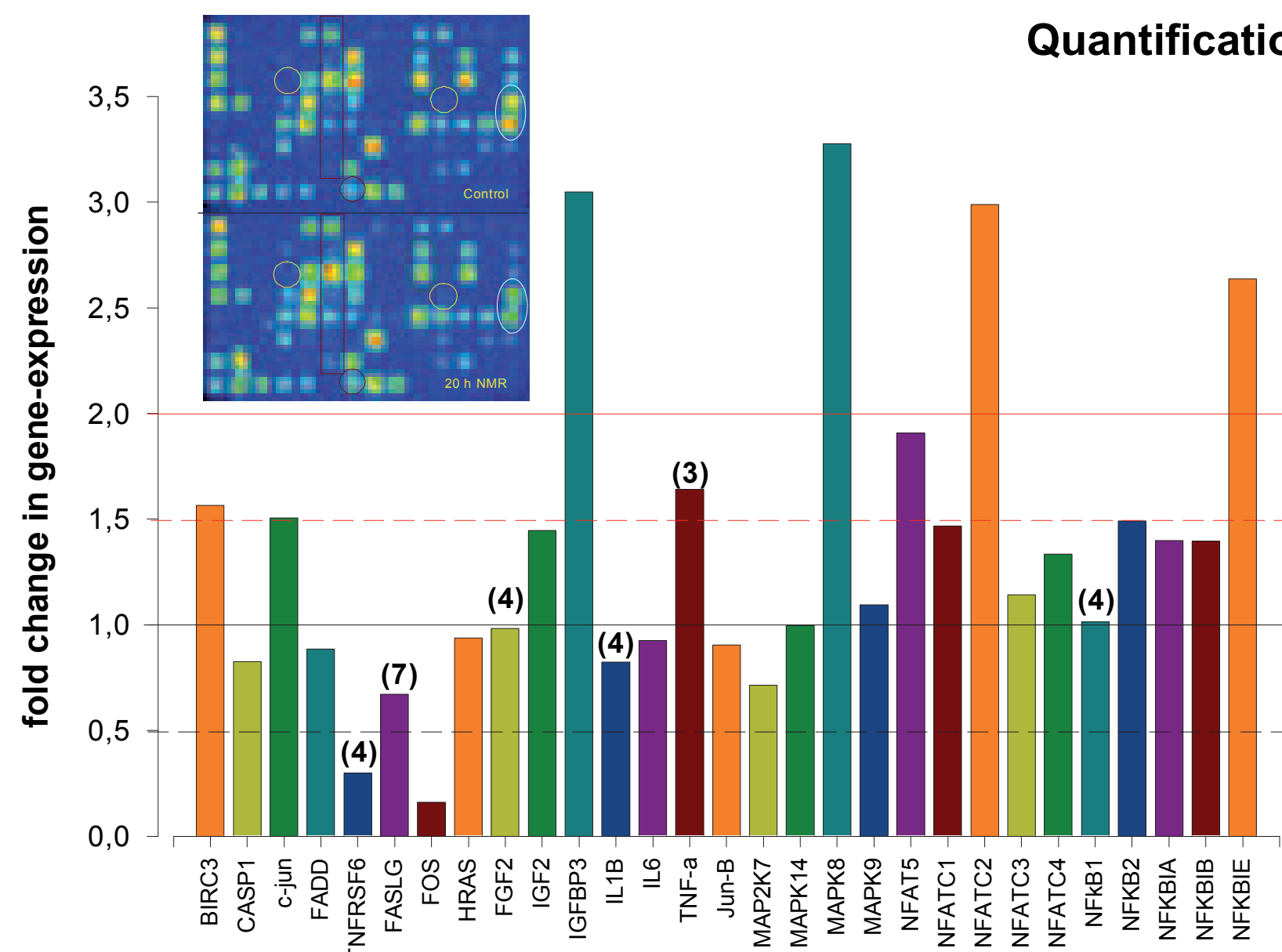


Fig. 3: Possible NMR induced changes in gene expression identified by gene array technology were further studied by quantitative PCR performed with cDNA from isolated RNA of control and NMR treated cells. Figures plot changes analyzed by PCR in A) Cal-72 osteosarcoma cells and B) Cal-78 chondrosarcoma cells. High amount of a specific cDNA in the sample is characterized by an early onset of fluorescence development. A two-fold difference in cDNA concentration results in a shift of one PCR cycle at a certain threshold value (CT value). For calculation of expression values, CT values of genes of interest were normalized to housekeeping genes (aldolase and GAPDH). Inserts represent the increase of fluorescence due to increasing concentrations of the amplicons.

fold-change of gene-expression in Cal-72 osteosarcoma cells tested by gene-array technique	fold-change of gene-expression in Cal-78 chondrosarcoma cells tested by gene-array technique
NAIP/BIRC1	0.230
BIRC2	2.408
CSF2	1.221
CSF3	0.521
FN1	1.243
GSK3A	2.102
ITGB1	0.988
KPNA5	6.217
MDM2	3.709
MEF2B	2.122
MEF2D	1.350
MMP10	1.467
P2RX7	2.036
PPP3CB	0.825
PPP3CC	2.035
PPP3R1	1.225
PTGS2/Cox2	1.987
TGFb1	1.463
VEGFC	0.458
COL12A1	0.329
COL1A1	n.d.
COL1A2	0.610
COL2A1	0.841
COL9A2	4.649
CSF2	1.330
CSF3	3.545
EGF	6.768
FN1	1.146
IGF2	0.579
ITGB1	1.986
TGFb2	4.551
TNF	42.523
VEGFB	0.130
VEGFC	0.899

Quantification of Expression by Gene array



Gene	Function
BIRC3	Baculoviral IAP repeat-containing 3
CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
c-jun	Jun oncogene
FADD	Fas (TNFRSF6)-associated via death domain
FAS/TNFRSF6	Fas (TNF receptor superfamily, member 6)
FASLG	Fas ligand (TNF superfamily, member 6)
FGF2	Fibroblast growth factor 2 (basic)
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog
HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog
IGF2	Insulin-like growth factor 2 (somatomedin A)
IGFBP3	Insulin-like growth factor binding protein 3
IL1B	Interleukin 1, beta
IL6	Interleukin 6 (interferon, beta 2)
Jun-B	Jun B proto-oncogene
MAP2K7	Mitogen-activated protein kinase 7
MAPK14	Mitogen-activated protein kinase 14
MAPK8	Mitogen-activated protein kinase 8
MAPK9	Mitogen-activated protein kinase 9
NFAT5	Nuclear factor of activated T-cells 5, tonicity-responsive
NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
NFATC2	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2
NFATC3	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3
NFATC4	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)
NFKB3	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NFKB4	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta
NFKB5	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon
TNF-a	Tumor necrosis factor (TNF superfamily, member 2)

Fig. 2: Testing for long-term effects of NMR stimulation, different gene arrays (GEArray technology, SABiosciences) were probed with RNA isolated from Cal-72 and Cal-78 cells treated by NMR for 20 h. Hybridization probes were made up from pools of RNAs in detail isolated from eight Cal-72 and six Cal-78 stimulation experiments, respectively. Concerning Cal-72, duplicates of studied arrays were tested in two different experiments. The insert illustrates pseudo-colour pictures of gene arrays with high (red) and low (blue) levels of expression. Pictured by the bar chart NMR stimulation on Cal-72 cells influences some but not all expression levels of genes worthwhile to be discussed in this content. The bars show the average gene values of two experiments – respectively a higher number in brackets – resulting from values of various gene arrays. Abbreviations and/or functions of analyzed genes are given in the table.

METHODS

Cell culture: CAL-72 human osteosarcoma (ACC 439) established from a chemotherapy-resistant tumor sample and CAL-78 human chondrosarcoma (ACC 449) established from the recurrence of a dedifferentiated chondrosarcoma (grade III) were purchased from DMSZ (German Collection of Microorganisms and Cell Cultures). Cells used for RNA-isolation were treated with an NMR therapy device (MBST®, MedTec, Wetzlar, Germany) for 20 h within 4 days (3 days 6 h each and 2 h on the last day). The extracted RNA was labelled with biotin and hybridized to membranes carrying different gene markers. The expression of specific genes was quantified with bioluminescence. In addition, quantitative PCR was performed to support the results from gene arrays. RNA of cells incubated for the same time at room temperature functioned as control. Free Ca²⁺ concentration in living cells was determined fluorimetrically with cells loaded with fura 2 AM for 60 min. Ca²⁺ release was triggered in osteosarcoma and chondrosarcoma cells by application of different concentrations of histamine. Osteosarcoma cells were transfected with DNA constructs corresponding to luciferase reporter genes to test for up and/or down regulation of the activation of regulatory proteins involved in signal transduction pathways.

IMPORTANT OSTEOLOGIC/RHEUMATOLOGIC FACTS

- Stress activated protein kinases (SAPK/JUN amino terminal kinases /JNK) are members of the MAPK family and are activated by a variety of environmental stress factors, inflammatory cytokines and growth factors.
- c-Jun signalling in together with NFAT is crucial for RANKL-regulated osteoclast differentiation! (IKEDA et al., 2004)
- NFAT has an important role within the transcription program of osteoblasts.
- NF-KB is activated by RANKL and plays an essential role in osteoclastogenesis.
- The Fas Ligand (FasL) serves as a key death factor in the immune system by inducing apoptosis.
- The ras/raf/MAPK/ERK pathway negatively regulates IGF stimulated PG synthesis in chondrocytes.
- Apoptosis inhibitor (IAP) family of proteins prevent cell death. The expression of BIRC-3 is increased following action of NF-KB by TNF receptor.
- Glycogen synthase kinase 3 (GSK-3) activity can dictate how cells will respond to glucocorticoids (GALLIHER-BECKLEY et al., 2008)
- Several growth factors (e.g. IGF, TGF, VEGF) regulate the behavior of cells in bone and cartilage and have a role in the inflammatory immune response.

CONCLUSION

- As shown with the microarray technique, various genes are regulated to different extent compared to control.
- Obviously, the genes of the NFAT-pathway are regulated in a uniform manner.
- qPCR of the most noticeable genes didn't show significant changes compared to control.
- Luciferase reporter gene assays definitely didn't show any distinct increase but rather a slight decrease of NF-KB or MAPK activity compared to untreated cells. This might be of importance because high levels of NF-KB and MAPK support the persistence of inflammatory processes in rheumatic diseases.
- Intracellular Ca²⁺ signalling was not significantly changed due to the influence of NMR.
- From qPCR it can be concluded that NMR in therapeutic dosage does not induce apoptotic mechanisms in Cal-72 and Cal-78 cells.
- Arrays concerning apoptosis, cell cycle and osteogenesis are effective tools to evaluate responses to potential stresses – No oxidative-, metabolic-, repair-, or heat shock stress during NMR exposure appeared.
- Our results imply that NMR of a magnetic field strength up to 2,3 mT and therapeutic exposure time has no deleterious effects on osteo- and chondrosarcoma cells.

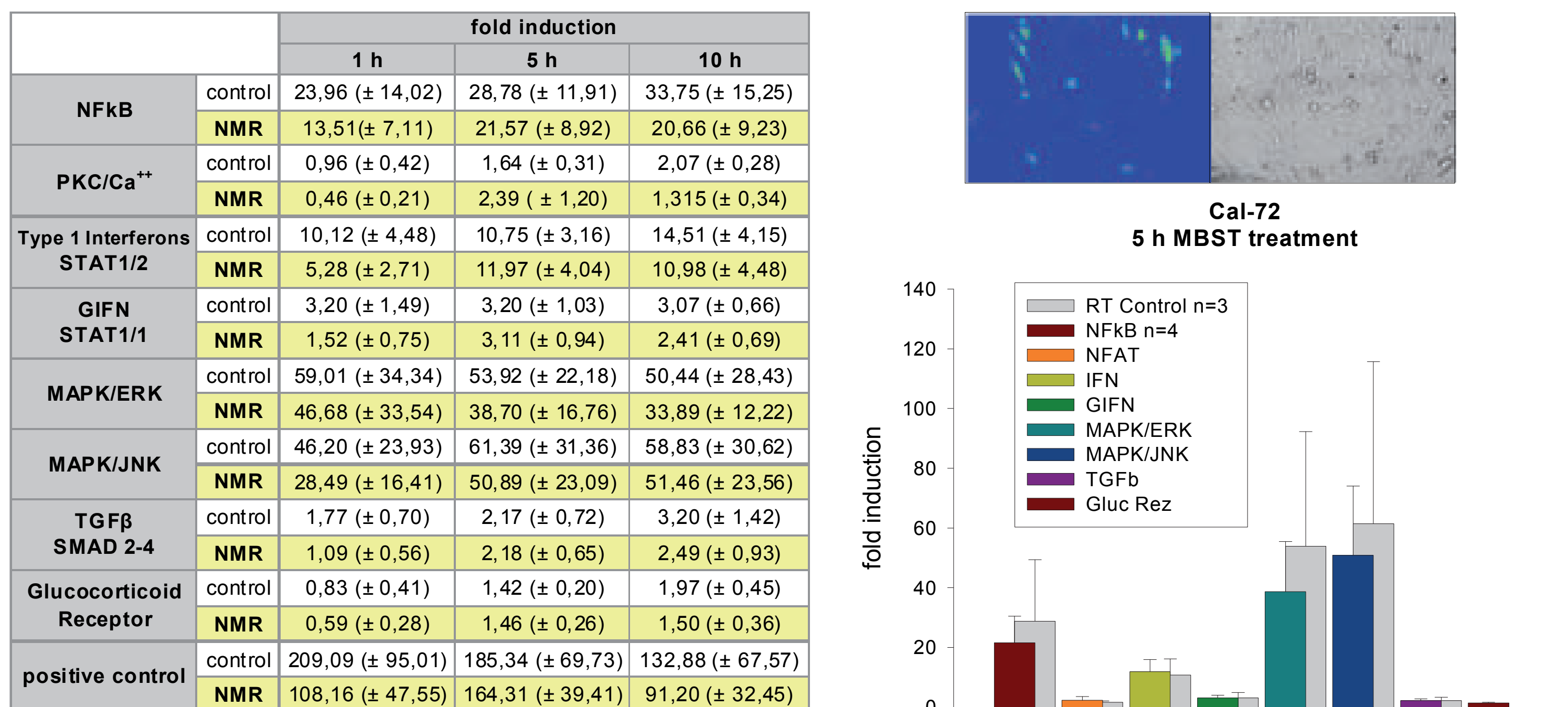


Fig. 4: The time course of the influence of NMR on pathway signaling activity was implemented by use of the Signal Finder™ 10-Pathway Reporter Array (Immune Response Reporter Array, SABiosciences), the ten reporter assays tested are listed above (Left: table, column 1). Cal-72 cells transfected with different pathway-focused transcription factor-responsive firefly luciferase constructs were stimulated by NMR for three different time intervals (1h, 5h and 10h). The table further represents the averaged values out of four experiments for NMR treatment and three for control, the standard error is given in parenthesis. Right: the insert depicts Cal-72 cells effectively transfected by positive control DNA based on a GFP construct. The summarized data for the second time point (5 h) are presented by the bar chart.

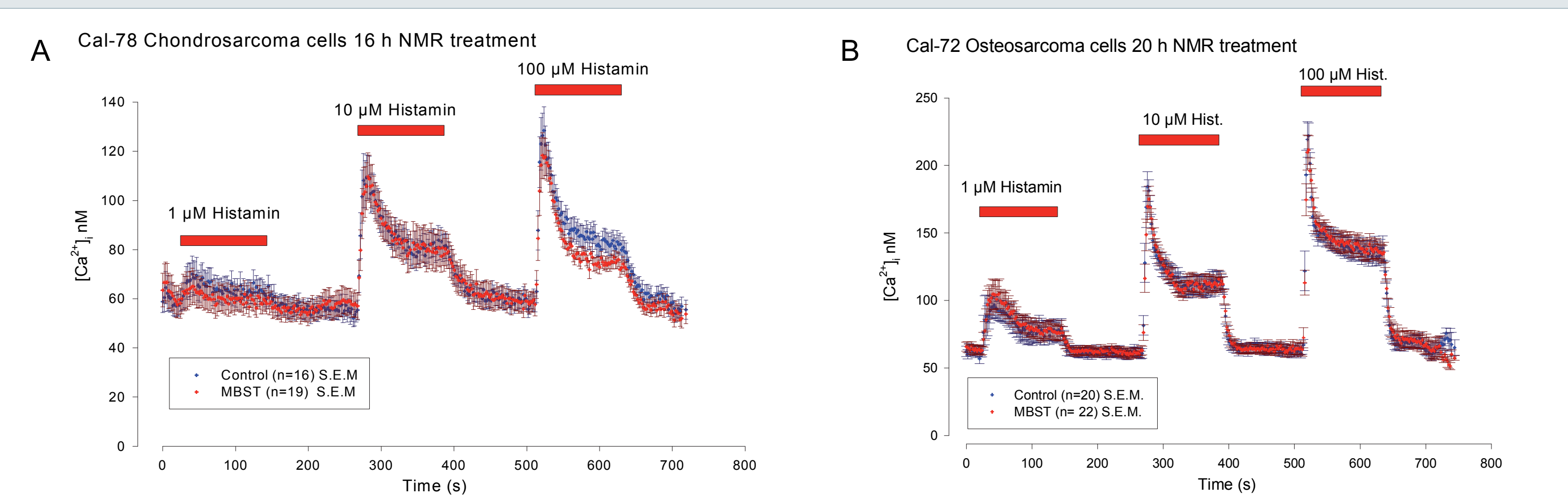


Fig. 5: Time course of intracellular Ca²⁺ concentration measured in: A) chondrosarcoma cells, B) osteosarcoma cells. NMR treatment was before the measurement. Ca²⁺ release was triggered in Cal-72 and Cal-78 cells by application of histamine.