Analysis of specific functions of Nkx5-1 and Nkx5-2 homeobox genes during neuronal differentiation and apoptosis

lnaugural-Dissertation
zur Erlangung des Grades
eines Doktor der Humanbiologie
des Fachbereichs Medizin

der Justus-Liebig-Universität Gießen

vorgelegt von

Robert Kramek

aus Poznań, Polen

Gießen (2014)

Gutachter: **Prof. Dr. Dr. Thomas Braun**

Gutachter: **PD Dr. Ulrich Gärtner**

Tag der Disputation: 27. Oktober 2015

This page is intentionally left blank.

1. INTRODUCTION

In the present work I investigated the role of two closely related proteins Nkx5-1 and Nkx5-2 in neuronal apoptosis and differentiation. These two proteins show high sequence conservation in several vertebrate species. It was postulated, that they play overlapping roles in the inner ear and nervous system development. Nkx5-1 and Nkx5-2 are expressed during inner ear development as well as during adult stages in the mouse. In addition to the inner ear structures, they are also expressed in post-mitotic neurons in several central and peripheral locations (Rinkwitz-Brandt et al., 1995). Nkx5-1 knockout leads to severe defects of the vestibular apparatus of the inner ear. However, singular Nkx5-1 gene knockout mice did not reveal any obvious neuronal phenotype (Hadrys et al., 1998; Wang et al., 2001). Based on the fact that double Nkx5-1/2 (also called Hmx2/3) knockout led to a severe postnatal lethal phenotype, redundant functions for both Nkx5 genes were postulated (Wang et al., 2004). In the double knockout mice defects in some hypothalamic functions were documented, however, no neuronal loss was observed in the functionally affected regions and the molecular basis of Nxk5 genes action remains unresolved (Wang et al., 2004).

To investigate molecular mechanisms of Nkx5-1 and Nkx5-2 genes functions in neuronal cells, PC12 rat pheochromocytoma cells (Green and Tischler, 1976) were used as an experimental model. This cell line is a commonly used system for the investigation of the neurogenesis and undergoes neuronal differentiation upon treatment with nerve growth factor NGF (Green and Kaplan, 1995). Recently, it was shown that BMP family members, BMP4 and BMP6, support NGF-mediated neuronal differentiation of PC12 cells (Allthini et al., 2003; Lönnet et al., 2005). In contrast to such coordinated action of BMP and NGF signalling, another BMP-family member, BMP2, is able to stimulate neurite outgrowth in PC12 without NGF (Iwasaki et al., 1996).

Interestingly, a multifunctional cellular regulator protein p53 was also recently demonstrated to be another player within the NGF-differentiation pathway: p53 knockdown inhibited NGF-induced differentiation (Zang et al., 2006). The high affinity NGF receptor TrkA was revealed as a direct target for p53-mediated transcriptional regulation (Yhang et al., 2006). Depending on the cellular context p53

may regulate TrkA either to induce cell cycle arrest and differentiation or apoptosis (Zhang et al., 2006; Lavoie et al., 2005).

To study the potential role of Nkx5-1 in the adult neuronal structures I used Nkx5-1 knockout mouse strain generated previously in our laboratory (Hadrys et al., 1998).

1.1. Nkx5-1 and Nkx5-2 genes

1.1.1. Nkx5-1 and Nkx5-2 functions in mouse development

The mouse Nkx5-1 and Nkx5-2 genes were first identified as homologs of the Drosophila S59/NK1 gene (Kim and Nirenberg, 1989; Dohrmann et al., 1990). Nkx5 homologous genes were identified in several species such as SpHmx in sea urchin (Martinez and Davidson, 1997), GH6 and Soho in chicken (Stadler and Solursh, 1994; Deitcher et al., 1994), H6 in human (Wang et al., 1990; Stadler et al., 1992), Nkx5-1 (Hmx1), Nkx5-2 (Hmx2), and Nkx5-3 (Hmx3) in mouse (Yoshiura et al., 1998; Bober et al., 1994; Rinkwitz-Brandt et al., 1995; Mennerich et al., 1999). Two different knockout mice were created to investigate the function of the Nkx5-1. The Nkx5-1 knockout mice generated in our laboratory exhibited behavioural abnormalities that resemble the typical hyperactivity and circling movements of the shaker/waltzer type mutants. That effect correlated with several malformations of the vestibular organ in Nkx5-1(-/-) mice. Nkx5-1(-/-) mice failed to develop the semicircular canals (Hadrys et al., 1998). Nkx5-1 gene transcription is first activated at embryonic day 8.5 (E8.5) in otic placode and exhibits dynamic changes of the expression pattern during otic vesicle formationNkx5-1 is first expressed in the rostral part of the otic placode and relocates during otic vesicle formation from the originally medial domain to the dorsolateral wall (Rinkwitz-Brandt et al., 1996). This later region gives rise to the vestibular apparatus of the inner ear (Li et al., 1978). Nkx5-2 shows similar expression in the inner ear and neuronal structures. Expression of this closely related gene was unchanged in Nkx5-1(-/-) mutants (Hadrys et al., 1998). Second knockout was generated by Thomas Lufkins' group. In this mouse model Nkx5-1 gene has been named Hmx3. This knockout also displayed abnormal circling behaviours. Comparison of the dissected labyrinths from Hmx3 wild-type, heterozygote and null animals did not reveal any discernible differences in either the

formation of the vestibular labyrinth or the cochlear duct (Wang et al., 1998). All of the semicircular ducts were present and appeared normal in the Hmx3 null inner ears, with the exception of the horizontal semicircular duct, which lacked both a horizontal crista and the associated horizontal ampullary chamber (Wang et al., 1998).

Knockout for Nkx5-2 (Hmx2) displayed behavioural similarity to Nkx5-1 knockout mouse such as hyperactivity, head tilting and circling activity. No defect was detected in central neuronal system. Lack of all three semicircular ducts as well as altered expression profiles of specific developmental regulators such as Bmp-4, Dlx5 and Pax2 were observed (Wang et. al., 2001). The highly similar expression patterns and close linkage on chromosome 7 suggested that Nkx5-1 and Nkx5-2 may share downstream regulatory targets (Wang et. al., 2001). Nkx5-1 and Nkx5-2 double mutant mice showed more severe defects in the inner ear than those displayed by either single knockout. In addition, abnormalities in the hypothalamic-neuroendocrine system, never observed in either of the single mutant mouse, confirmed the hypothesis that Nkx5-1/Hmx3 and Nkx5-2/Hmx2 also function redundantly to control embryonic development of the central nervous system (Wang et al., 2005).

1.1.2. Nkx5-1 and Nkx5-2 protein structure

Nkx5-1 and Nkx5-2 genes display nearly 85% identity within the homeobox. Thus, the Nkx5-1 and Nkx5-2 genes encode proteins with very similar homeodomains. The amino acid similarity within homeodomain is approximately 90% (Fig.1). This sequence is also closely related to homeodomains previously identified for other Nkx proteins and contains the conserved core motive responsible for binding to DNA target sequences.

It was showed that Nkx5 proteins can recognize the identical genomic DNA sequence CAATTAAGTG, but Nkx5-2 displayed weaker binding affinity to this sequence than Nkx5-1. An additional, novel and unrelated high affinity binding sequence could be identified for the Nkx5-2 protein (Mennerich et al., 1999).

Fig. 1. Similarity in homeodomain within Nkx5/Hmx protein family.
Amino acid residues identical in all three proteins are marked in red, the conservative amino acid exchanges in blue. Amino acid identical between Nkx5-1 and Nkx5-3 are marked in green.

1.2. PC12 cell line as a cell culture model to study gene interactions in neuronal development

PC12 cell line was established from a spontaneous rat pheochromocytoma derived from chromaffin cells of the suprarenal medulla (Greene LA, Tischler, 1978). PC12 cells have a potential to differentiate into sympathetic neurons in the presence of NGF and/or BMP2 and have been used extensively to study the mechanisms of neuronal differentiation. It was suggested that NGF and BMP signals are likely to interact with further downstream targets at the transcriptional level during neuronal differentiation of the PC12 cells (Althini et al., 2003). Below some of the best investigated factors that play critical roles in neuronal differentiation of PC12 cells, are shortly described.

1.3. NGF and BMP2 play essential roles in neuronal development and differentiation

During neuronal development differentiation and apoptosis are essential processes taking place in the neuronal cells. These processes are regulated by a complex array of molecular factors (Becker et al., 2003).

Members of the family of related growth factors, called neurotrophins, are required for differentiation, survival, development, and death of specific populations of neurons and also of non-neuronal cells: leukocytes, osteoblasts or fibroblasts. The best known member of this family is Nerve Growth Factor (NGF), which plays crucial roles in the differentiation and survival of neural cells. NGF has also been shown to be a strong inducer of neuronal phenotype in PC12 cells. PC12 cells, without exposure to NGF, are dependent on serum for survival, and withdrawal of serum initiates apoptosis. After about 7–10 days of NGF treatment, PC12 cells terminally differentiate into a neuronal phenotype, become dependent on NGF, and undergo apoptosis after NGF withdrawal even in the presence of serum. The hallmarks of neuronal differentiation of PC12 cells include inhibition of proliferation and outgrowth of neurites (Greene and Tishler, 1976).

As NGF is a secretory molecule, its effects can be exerted only after binding to specific receptors. Effects induced by NGF can be transmitted by high affinity TrkA, TrkB and TrkC thyrosine kinase receptors and the low affinity p75 neurotropin receptor (p75NTR) – all members of the TNF receptor superfamily. p75NTR acts as a Trk co-receptor that increases neurotrophin binding affinity (Esposito et al., 2001). TrkB signalling plays an important role in modulating the formation and maintenance of NMDA and GABAA receptor clusters at central synapses, and thus coordinately modulates these receptors as part of a mechanism that promotes the balance between excitation and inhibition in developing circuits (Elmariah et al., 2005). The expression of NGF and TrkA mRNA is regulated by interleukin (IL)-1beta. NGF uses a canonical signalling cassette, and the Raf mitogen-activated protein kinase (MEK) extracellular signal-regulated kinase (ERK) pathway to promote distinct outcomes, including neuritogenesis, gene induction, and proliferation. Pituitary adenylate cyclase-activating polypeptide (PACAP), a neurotransmitter that also causes differentiation including neuronal outgrowth, uses

the same canonical cassette as NGF but in a different way. The PACAP preferring receptor (PAC1) activates adenylate cyclase (AC), an enzyme catalysing the conversion of ATP to 3',5'-cyclic AMP (cAMP) and pyrophosphate. Growing level of cAMP activates protein kinase A (PKA), which in turn activates CBPCREB binding protein leading to activation of Trasin, TH and other genes involved in differentiation (Fig. 2). Neurotrophins and Trk receptors expressed in human periodontal tissue may contribute to regeneration as well as innervation of periodontal tissue through local autocrine and paracrine pathways. Recent data suggest that some functions of neurotrophins and Trk receptors relate to periodontal disease and periodontal tissue regeneration (Hidemi et al., 2003).

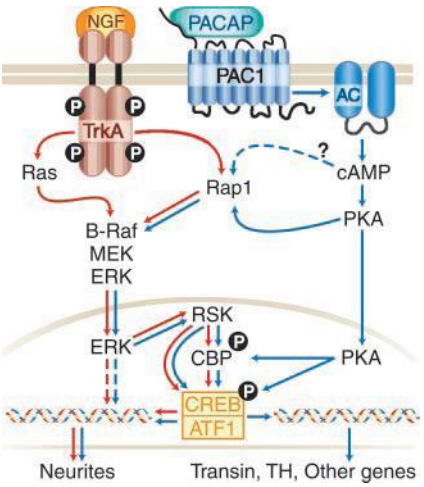

Fig. 2. Proposed model of NGF action in PC-12 cells (from Vaudry et al., 2002).
AC-adenylate cyclase;
ATF1-activating transcription factor 1; CBP- CREB binding protein; CREB; cAMP response element –binding protein;
ERK-extracellular signal regulated kinase; MEK-mitogen-activated protein kinase kinase
NGF-nerve growth factor; Pituitary adenylate cyclase-activating polypeptide (PACAP),
PAC1 -type 1 PACAP-preferring receptor; PKA- protein kinase A;
RSK-ribosomal S6 protein kinase; TH - tyrosine hydroxylase

BMP2 is another factor involved in neuronal differentiation. Even if NGF and BMP2 are different in structure and mechanism of action, they play overlapping functions during neuronal cells life. NGF and BMP2 were found to induce neuronal differentiation (Iwasaki et al., 1996). Upon treatment with BMP-2 or NGF changes in the morphology of PC12 cells indicating neuronal differentiation were observed (Fig. 3). The most prominent change was the formation of neurite-like processes. The process-inducing activity of BMP-2 was dose dependent and was maximal at a concentration of 30 ng/ml – 50ng/ml (1 nM). Although the majority (more than 85%)

of PC12 cells responded to BMP2 stimulation and started to extend processes within the initial 1~2 days, branching and intermingling of the processes in BMP2 treated PC12 cells were less conspicuous compared with those observed in NGF-treated cells. Bone morphogenetic proteins (BMPs) were shown to potentiate NGF-induced neuronal differentiation in PC12 pheo-chromocytoma cells grown on collagen under low-serum conditions. The mechanism by which BMP induces neuronal differention is relatively well studied (Iwasaki, et al., 1999). Employment of its inhibitor, Noggin, greatly contributed to further resolvement of BMP specific functions. Noggin has been described to be capable of binding bone morphogenetic proteins (BMPs) and inhibiting BMP signalling by preventing the interactions of BMPs with their receptors. BMP2 protein acts by its receptor (type I or II receptor for TGF-β), which recruits and phosphorylates several Smad transcription factors (Smad1, Smad5 or Smad8), which then translocate into the nucleus to regulate gene expression (Derynck et al., 1998). In contrast to NGF, BMP2 is able to induce neuronal differentiation of PC12 cells by a signalling pathway that is independent of MAP kinase or MEK cascade (Fig. 4). Activation of the TAK1-p38 kinase pathway is necessary for BMP-2-induced neuronal differentiation of PC12 cells (Iwasaki, et al., 1999) which is inhibited by Smad6 and Smad7 (Yanagisawa et al., 2001). However, the potential of BMP2 to induce differentiation of PC12 cells is relatively low. Much stronger neuronal induction could be achieved, when BMP2 treatment was combined with FGF even at subthreshold concentrations of FGF (Hayashi et al., 2001). Furthermore, bFGF and activin A were found to induce PC12 cell differentiation with moderate and low process formation, respectively. In contrast TGF-β1 and inhibin A possess no inductor potential (Fig. 3).

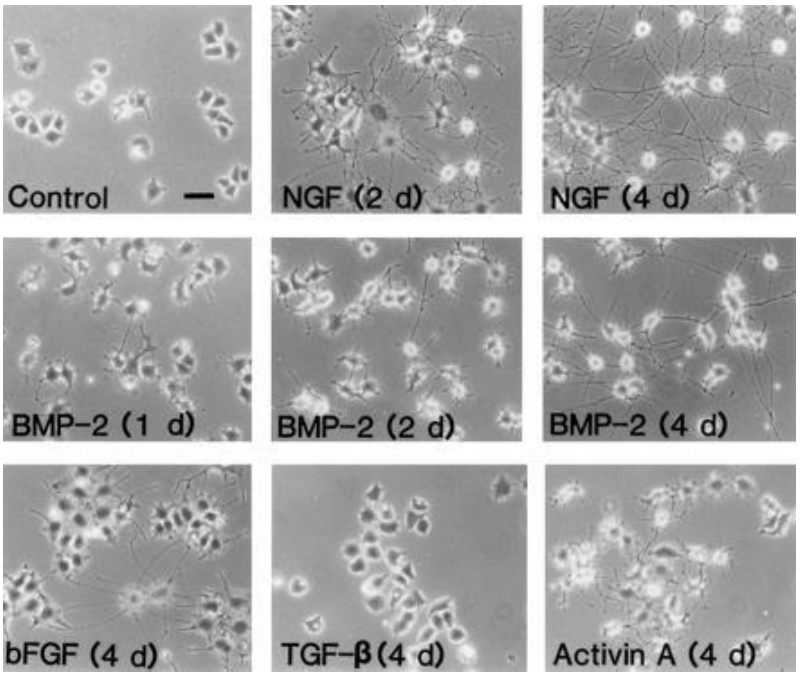

Fig. 3. Induction of neurite outgrowth in PC12 cells treated with various factors (from Iwasaki et al., 1996).

PC12 cells were treated with 20 ng/ml NGF, 30 ng/ml BMP-2, 10 ng/ml basic FGF, 10 ng/ml TGFβ1, or 30 ng/ml activin A for the indicated periods of time. Bar, 50 µm.

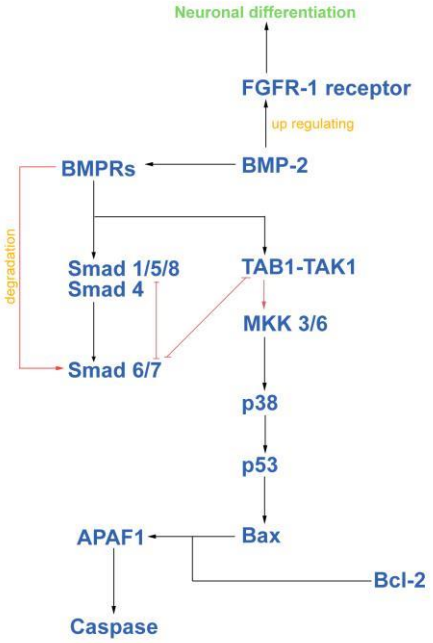

Fig. 4. Proposed model of BMP2 action in PC-12 cells (modified from Hayashi et al., 2003). APAF-1 apoptotic protease-activating factor-1, BMP2 – Bone Morphogenic Protein 2, BMPRs – BMP2 receptor, FGFR-1 receptor, MKK3/6-p38 mitogen-activated protein kinase, Smad 1/5/8/4/6/7 – proteins, TAB1transforming binding protein 1, TAK1 transforming activated kinase 1.

1.4. Apoptosis as a key process in neuronal differentiation and development

Neuronal apoptosis plays an indispensable role in neurogenesis. Apoptosis is a form of programmed cell death that occurs during development of the nervous system. The importance of apoptosis during neuronal embryonic development was demonstrated by genetic elimination of cell death. Knock outs of several apoptosis specific genes lead to embryonic mortality or gross anatomical malformations (Buss and Oppenheim, 2004). However, some of the apoptosis mutant animals develop normally but show excess of neurons and glia in the nervous system. Supernumerary neuronal progenitors may differentiate into functional neurons, however, such neurons show often size reduction, fail to differentiate properly, and/or lack normal connections with their targets. Changes in motor control and sensory processing are generally not observed, except for during the most complex of behaviours (Buss and Oppenheim, 2004). Examination of organisms where apoptotic genes have been genetically eliminated revealed that programmed cell death might play an important role in sculpting gross brain structure during early development of the neural tube. In contrast to the well investigated role of apoptosis during early embryonic development of the nervous system, the consequences of preventing neuronal cell death at later developmental stages (e.g. during vertebrate synapse formation) are just beginning to be understood (Buss and Oppenheim, 2004).

Apoptosis may also be responsible for neuronal death that occurs in neurological disorders such as stroke, Alzheimer's, and Parkinson's diseases (Culmsee et al., 2005). Here, cell loss via apoptosis is a key element causing neuronal degeneration.

1.5. P53 protein-mediated cell cycle arrest and apoptosis

The tumour suppressor protein p53 is a transcription factor that regulates the response to cellular insults such as DNA damage and growth factor withdrawal. Active p53 protein can induce cell cycle arrest to allow the cell to recover from damage. Alternatively, p53 is also able to induce apoptosis, especially in case of extensive or irreparable damage (Zhang et al., 2009). Transcriptional activity of p53 requires post-translational modification by phosphorylation and acetylation. P53

production is rapidly increased in neurons in response to a range of insults including DNA damage, oxidative stress, metabolic compromise and cellular calcium overload. Target genes induced by p53 in neurons include the pro-apoptotic proteins Bax and the BH3-only proteins PUMA and Noxa (Fig. 5). In addition, p53 may more directly trigger apoptosis by acting at the level of mitochondria, a process that can occur in synapses (synaptic apoptosis). Preclinical data suggest that agents that inhibit p53 may be effective therapeutics for several neurodegenerative conditions (Culmsee et al., 2005).

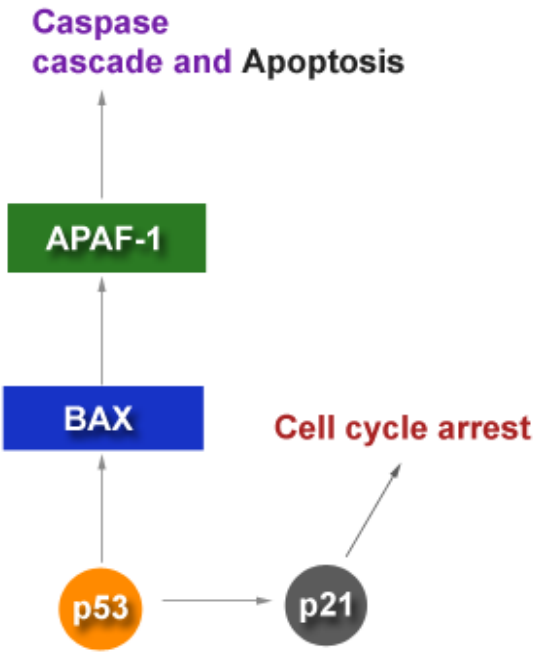

Fig. 5. P53 signalling – critical P53 – regulated factors leading to apoptosis or cell cycle arrest. (modified from Jian et al., 2003).

The role of p53 in neuronal apoptosis is still under debate and controversial data exist on its function and necessity for neuronal apoptosis.

The role of p53 in apoptosis in PC12 cells is also still discussed. It was suggested that p53 participates in the early phases of programmed cell death in PC12 cells through caspase3 activation. Consequently, absence of functional p53 resulted in a delay of apoptosis (Vaghefi et al., 2004).

Recent findings demonstrated that p53 plays a critical role in NGF-mediated neuronal differentiation in PC12 cells at least in part via regulation of TrkA levels (Zhang et al., 2006).

1.6. Aims of the project

The general purpose of this work was to investigate the role of Nkx5-1 and Nkx5-2 genes during the neuronal differentiation and their possible involvement in regulation of apoptosis using PC12 cells as an experimental model. At first I focused on examination of influence of Nkx5-1 and/or Nkx5-2 overexpression on neuronal differentiation and apoptosis in PC12 cells under different growing conditions. The next step was to identify pathway(s) involved in the induction of Nkx5-induced apoptosis in PC12 cells and the regions of Nkx5 protein(s) responsible for the apoptotic effect. For better understanding of the role of different factors in the regulation of Nkx5-1 gene transcription, cell culture experiments using LacZ reporter construct fused to the putative Nkx5-1 promoter sequences were performed. Finally, apoptosis and expression of neuronal differentiation markers were investigated in WT and Nkx5-1 knockout mouse embryos and adult animals to verify Nkx5-1 function in vivo.

2. RESULTS

It was shown previously that Nkx5 genes influence morphogenesis of the inner ear epithelium possibly by deregulation of cellular apoptosis (Merlo et al., 2002; Wang et al., 2004; Bober et al., 2003). In addition to the inner ear, Nkx5 genes are also expressed in specific neuronal structures (Rinkwitz et al., 1996). However, little is known about the function of Nkx5 genes during neuronal development or differentiation. In this work, PC12 cell culture system was used to investigate a potential role of Nkx5 genes during neuronal differentiation. To investigate whether the influence on apoptosis might be a general function of Nkx5 genes also in neuronal cells, Nkx5-1 and Nkx5-2 genes were overexpressed in PC12 cells and the cells were scored for apoptosis. Furthermore, the interrelationship between Nkx5-dependent apoptosis and known regulators of the apoptotic pathway were investigated using immunohistochemistry and RNA expression analysis. In the last part of this work the activation of Nkx5-1 promoter was investigated in PC12 cells using a plasmid containing Nkx5-1 promoter sequences and LacZ reporter gene.

2.1. Construction of the plasmids overexpressing Nkx5-1 and Nkx5-2 proteins

Nkx5 overexpressing plasmids were constructed using pCS2MTNLS (MalphaM) expression vector. To overexpress Nkx5-1 protein an Nkx5-1 SmaI-EcoRI 1.7 kb cDNA fragment, encompassing the entire coding sequence, was cloned into BamHI site of the pCS2MTNLS vector using blunt end ligation. For generation of the Nkx5-2 overexpresing construct an Nkx5-2 XbaI-SmaI 1.5 kb cDNA fragment was cloned into the blunt-end filled EcoRI site of the vector. This fragment also contained the entire protein coding information. Both constructions were fused in frame to vector sequences containing sequences of 6 Myc-epitops and the nuclear localization signal (NLS). The AUG start codon for translation was provided by the vector (see Fig. 6).

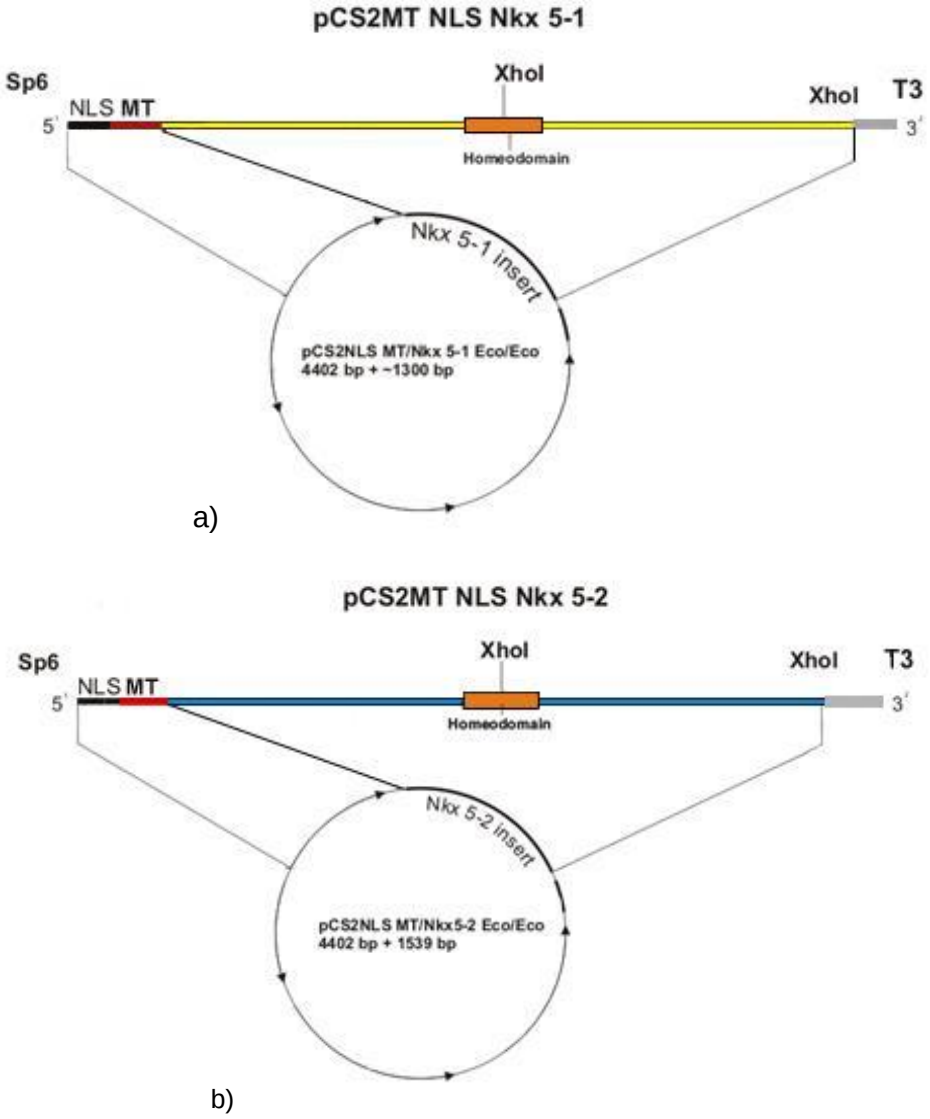

Fig. 6. Nkx5-1 and Nkx5-2 expression constructs.
Both constructs were based on pCS2MTNLS vector. This vector contains 6 copies of sequences encoding the Myc epitope (LEQKLISEEDLN SEQ ID NO:8) and the NLS (nuclear localisation signal).

a) Nkx5-1 SmaI-EcoRI fragment was subcloned into EcoRI –XhoI sites of pCS2NLS MT vector using blunt-end ligation.
b) Nkx5-2 XbaI-SmaI fragment was cloned into blunt-ended EcoRI site of the pCS2MT NLS vector.

The correct orientation was confirmed by sequencing and restriction analyses.

2.2. Investigation of influence of Nkx5 genes on apoptosis in PC12 cells

2.2.1. Nkx5-1 but not Nkx5-2 induces apoptosis in PC12 cells

It was previously demonstrated that Nkx5 genes influence apoptosis during inner ear development. To develop an in vitro model for investigation of apoptosis pathway and examine the role of Nkx5-1 and Nkx5-2 in apoptosis and neuronal differentiation, PC12 cells were used as a culture system. First, culture conditions were optimized by testing different cell density, concentration of differentiation inducing factors, and time of the cell culture. It is well known that NGF induces the neuronal fate of pheochromocytoma derived PC12 cells (Green and Tischler, 1976). In fact, addition of NGF induced neuronal differentiation (Fig. 7B, C). Similar effects could be achieved by supplementing of the cell culture medium with BMP2 (Fig. 7F). The most efficient neuronal differentiation was achieved by addition of BMP2 at a final concentration of 100ng/ml and NGF at a final concentration of 50ng/ml (Fig. 7B and F). The neuronal differentiation was confirmed by changes in cell morphology (Fig. 7) and by expression of neuronal markers (not shown). These estimated culture conditions were used as standard conditions in the following experiments.

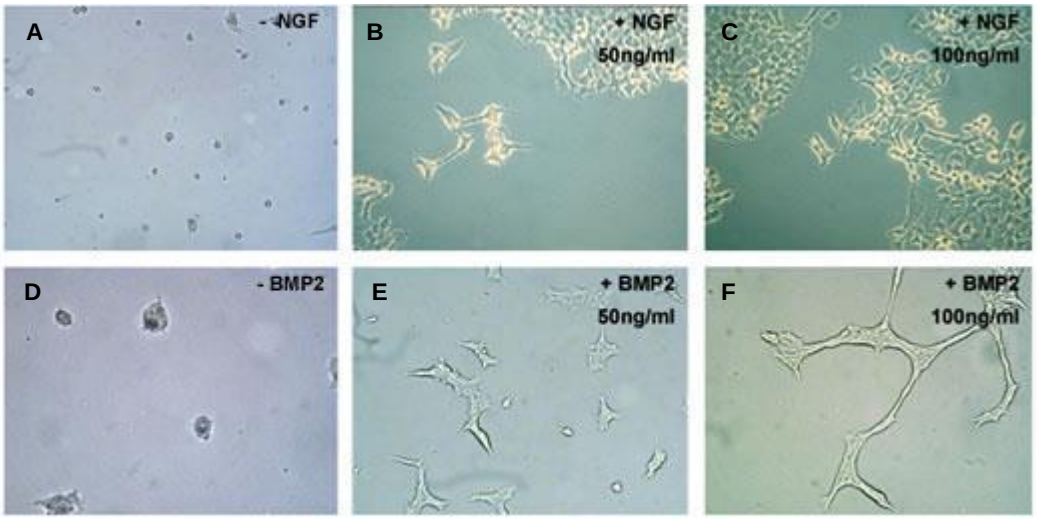

Fig. 7. Supplementation of the cell culture medium with NGF or BMP-2 induces neuronal differentiation.
PC12 cells were cultured under different growing conditions:
A,D – without any additional factor
B,C - with addition of NGF
E,F - with addition of BMP2

To estimate the basal level of apoptosis PC12 cells were cultivated under the standard conditions without DNA transfection and separately transfected with PCS2MT plasmid. As shown in Fig. 8 no apoptosis could be observed using the tunnel assay in untransfected cells (Fig. 8E-H). Similarly, transfection of PC12 cells with pCS2 plasmid did not induce any apoptosis (Fig. 8A-D).

To investigate the influence of Nkx5 proteins on apoptosis Nkx5-1 and Nkx5-2 plasmids were overexpressed in PC12 cells and the cells were scored for apoptosis two days after transfection. The presence of Nkx5 proteins encoded by the transfected plasmids was confirmed using anti-Myc tag antibody (Fig. 9B, F). The transfected cells were stained using tunnel assay to visualize apoptotic cells and the apoptosis induction was correlated to Nkx5-1 over-expression. In fact, almost all Nkx5-1 expressing cells underwent apoptosis (Fig. 9A-D). In contrast, Nkx5-2 transfection did not induce apoptosis at all (Fig. 9E-H). Since it was postulated that both Nkx5 genes play an overlapping role in neuronal development (Rinkwitz et. al., 1996), it is puzzling that we discovered a specific apoptosis inducing function only for Nkx5-1. Interestingly, Nkx5-1 has been reported as a potential target of BMP2 and BMP4 (Merlo et al., 2002). Since BMP proteins were already demonstrated to regulate apoptosis and neuronal differentiation, we were interested whether Nkx5-1-dependent induction of apoptosis might be modulated during neuronal differentiation. In following experiments I set to estimate the involvement of Nkx5-1 in signalling pathways known to regulate apoptosis and neuronal differentiation of PC12 cells.

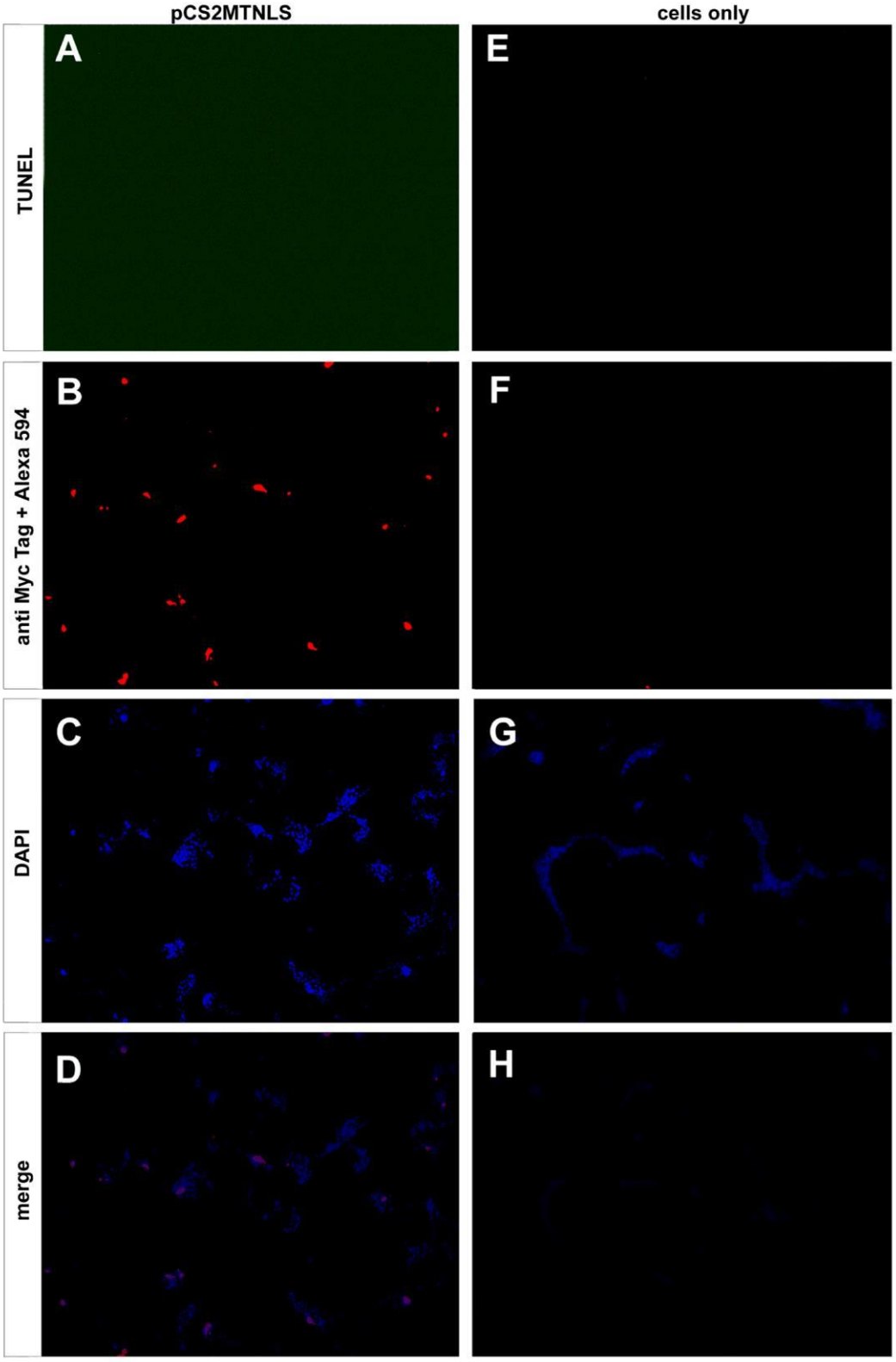

Fig. 8. Cells transfected with pCS2 vector and untransfected cells cultured under standard growing conditions do not undergo apoptosis.
A, E - TUNEL staining to indicate apoptotic cells.
B, F - The presence of vector transfected cells was confirmed using anti Myc-tag antibody.
C, G- DAPI staining was performed to visualize all nuclei.
B, D - Myc-tag was detected in pCS2MTNLS overexpressing cells.

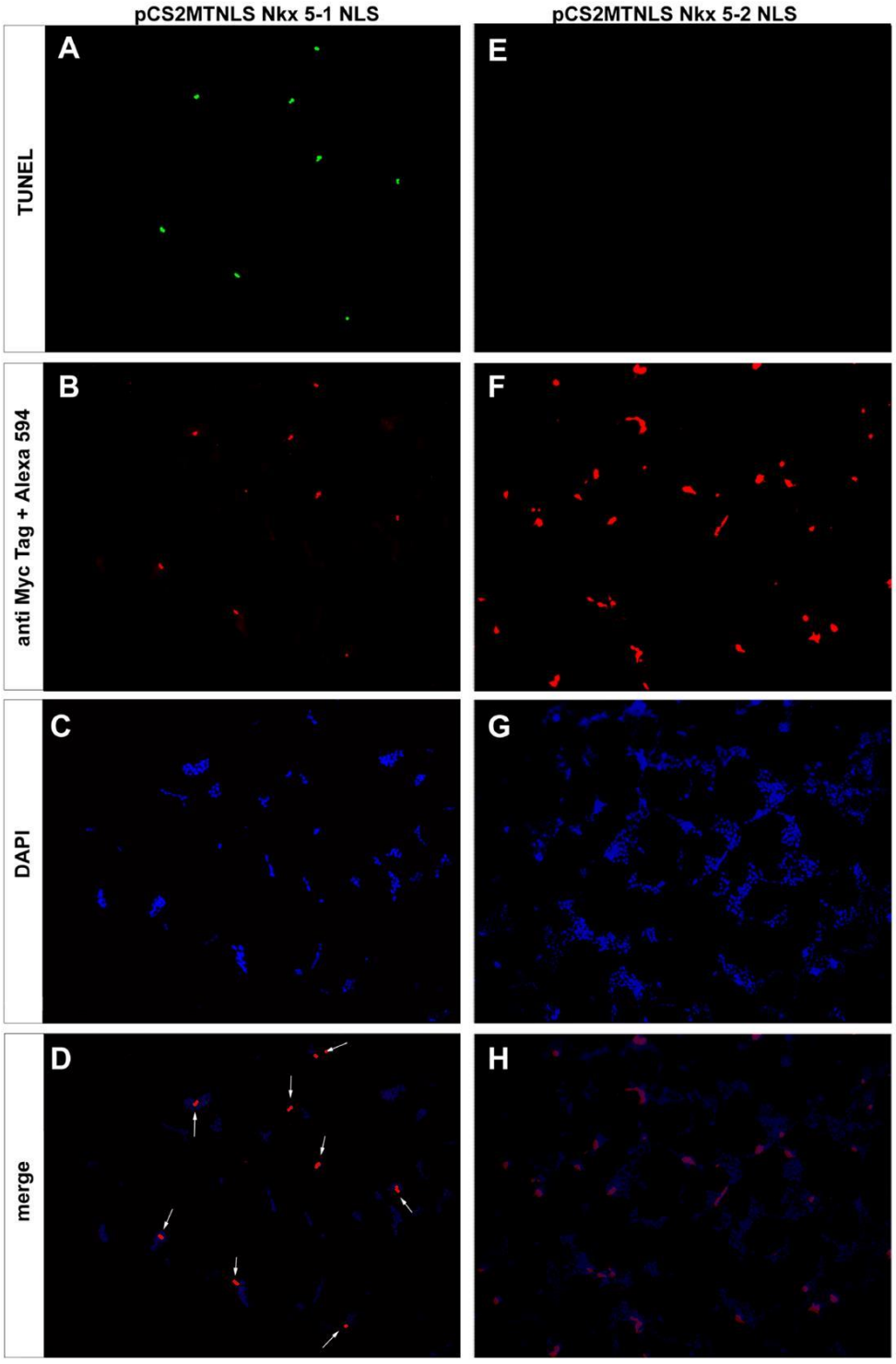

Fig. 9. Nkx5-1 but not Nkx5-2 induces apoptosis in PC12 cells.
A, E - TUNEL staining.
B, F - The presence of Nkx5 proteins encoded by the transfected plasmids was confirmed using anti Myc-tag antibody.
C, G - DAPI staining was performed to visualize all nuclei.
D - White arrows indicate TUNEL and Myc-tag positive cells.
H - No positive cells for TUNEL and Myc-tag were found in Nkx5-2 overexpressing cells.

2.2.2. NGF does not prevent Nkx5-1 induced apoptosis

NGF induces neuronal differentiation in PC12 cells and has been reported to act as a survival factor and to prevent apoptosis in these cells (Shimoke et al., 2001). To investigate whether NGF can prevent Nkx5-1 induced apoptosis or whether this apoptosis is an integral part of the differentiation program Nkx5 overexpression experiments in PC12 cells were repeated in the presence of NGF. PC12 cells were treated with 50 ng/ml NGF and transfected with Myc-tagged Nkx5-1 (Fig. 10A-D) and Nkx5-2 (Fig. 10E-H) expression constructs. The cells were harvested 48h after transfection and analysed for the presence of transfected proteins using anti Myc antibody (Fig. 10B, F) and for apoptosis using TUNEL assay (Fig. 10C, G). The nuclei were visualized by DAPI staining (Fig. 10A, E). D and H show cells positive for transfection and apoptosis in merged images. The obtained results strongly resemble those without NGF treatment: almost all Nkx5-1 transfected cells underwent apoptosis (arrows in Fig. 10D) while virtually no apoptosis was present in Nkx5-2 transfected cells (Fig. 10G, H). Does Nkx5-1 specifically induce apoptosis in cells undergoing NGF- dependent neuronal differentiation?

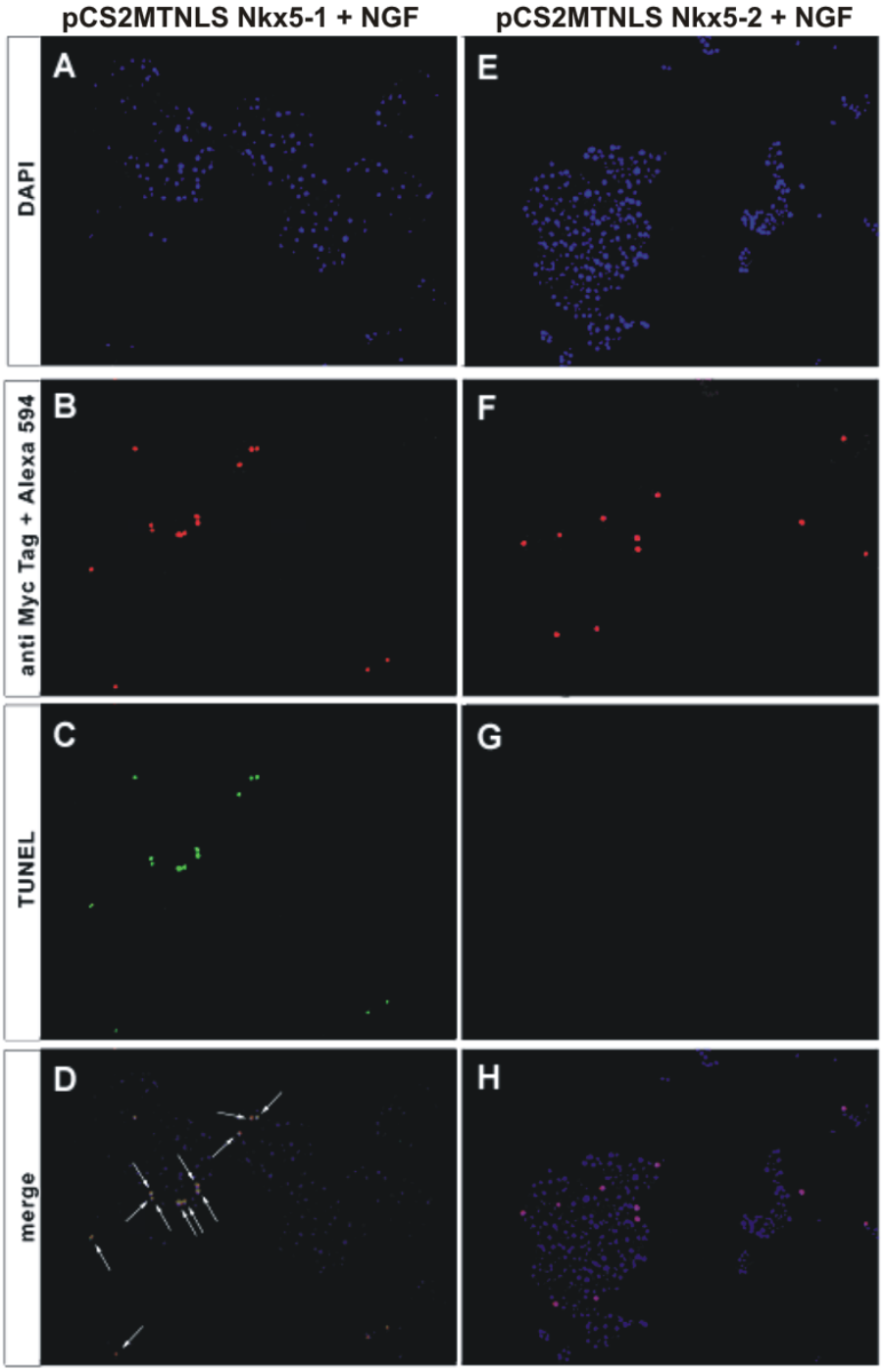

Fig. 10. Nkx5-1 induces apoptosis in PC12 cells. NGF does not prevent Nkx5-1-induced apoptosis.
Cells were transfected separately with each Nkx5 construct and treated with NGF in concentration of 50ng/ml. Transfected cells were identified using anti-Myc-tag antibody. Cells were stained using TUNEL assay to visualize apoptotic cells. DAPI staining was performed to visualize nuclei.
A,E – Nuclei were visualized by DAPI staining
B,F – Transfected cells were confirmed using anti-Myc-tag antibody.
C,G – TUNEL assay was performed to visualize apoptotic cells.
D,H – Almost all cells overexpressing Nkx5-1 and treated with NGF undergo apoptosis (arrows in D) in contrast to Nkx5-2 transfected cells.

2.2.3. BMP2 has no effect on Nkx5-1 induced apoptosis

In the further course of this work I wanted to investigate, whether Nkx5-1 induced apoptosis might be regulated by BMP2. Such regulation was already suggested in epithelial cells of the inner ear (Herbrand et al., man. in prep.).

PC12 cells were treated with BMP2 at the final concentration of 50 ng/ml and transfected again with Nkx5-1 and Nkx5-2 constructs (Fig. 11). The transfected cells were detected by an anti-Myc antibody staining (Fig. 11B, F). Apoptosis was visualized by TUNEL staining (Fig. 11C, G). In confirmation with previous results the majority of Nkx5-1 overexpresing cells underwent apoptosis (Fig. 11C, D). However, apoptotic cells were also found in PC12 cells transfected with Nkx5-2 construct. Under a more detailed scrutiny, it became apparent, that many cells undergoing apoptosis did not overexpress Nkx5-2 (arrows in Fig. 11H). Interestingly, some apoptotic cells were also found after Nkx5-1 transfection without Nkx5-1 overexpression (arrows in Fig. 11D). Thus BMP2, similarly to NGF does not prevent Nkx5-1-induced apoptosis. In addition, BMP2 is able to induce apoptosis in PC12 cells without Nkx5-1 or Nkx5-2 overexpression.

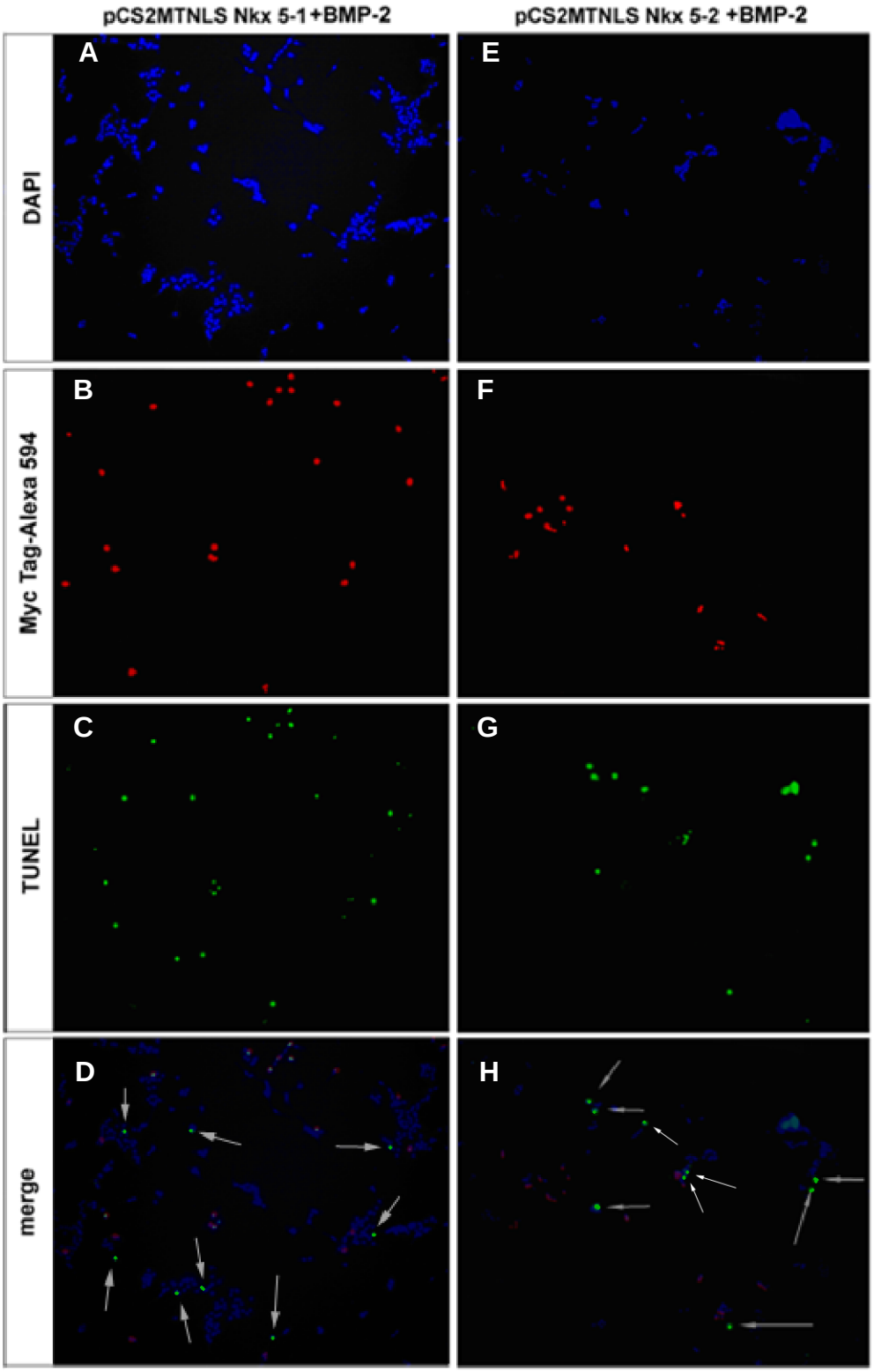

Fig. 11. Influence of BMP2 on apoptosis in Nkx5-1 and Nkx5-2 transfected PC12 cells
A,E – Nuclei were visualized by DAPI staining.
B,F – Transfected cells were confirmed using anti-Myc tag antibody.
C,G – TUNEL assay was performed to visualize apoptotic cells.
D,H – Merged pictures of anti-Myc and tunnel staing. Almost all cells overexpressing Nkx5-underwent apoptosis. Some apoptotic cells did not overexpress Nkx5-1 (arrows in D).
The majority of apoptotic cells at the Nkx5-2 transfection did not overexpress Nkx5-2 arrows in H.

2.2.4. PFT alpha blocks apoptosis induced by Nkx5-1 protein

P53 plays an important role in cell differentiation, proliferation and apoptosis in PC12 cells (Zhang et al., 2006). We were interested whether Nkx5-1 induced apoptosis requires p53 pathway. Therefore PFT alpha has been used to block p53 transcription. The cells were transfected as previously but in the presence of PFT alpha at the concentration of 250 ng/ml. As a control untransfected cells were treated with PFT alpha (data not shown). TUNEL assay and MycTag staining were performed to visualize apoptotic and transfected cells (Fig. 12). No apoptosis was observed in the control cells after PFT alpha treatment (not shown). Interestingly, no apoptosis was detected by TUNEL assay after PFT alpha treatment of Nkx5-1 overexpressing cells (Fig. 12C). The successful inhibition of p53 was demonstrated by the lack of p53 protein in transfected cells after PFT alpha treatment (Fig. 13C, G). Without inhibitor, p53 could be easily detected in Nkx5-1 transfected cells using immunocytochemistry as detected by p53 antibody while endogenous p53 level and p53 expression in Nkx5-2 could not be detected (Fig. 15C, G; and results not shown). These findings suggested that Nkx5-1 induced p53 dependent apoptosis and that the induction of higher levels of p53 expression was essential for apoptosis induction in Nkx5-1 overexpressing cells.

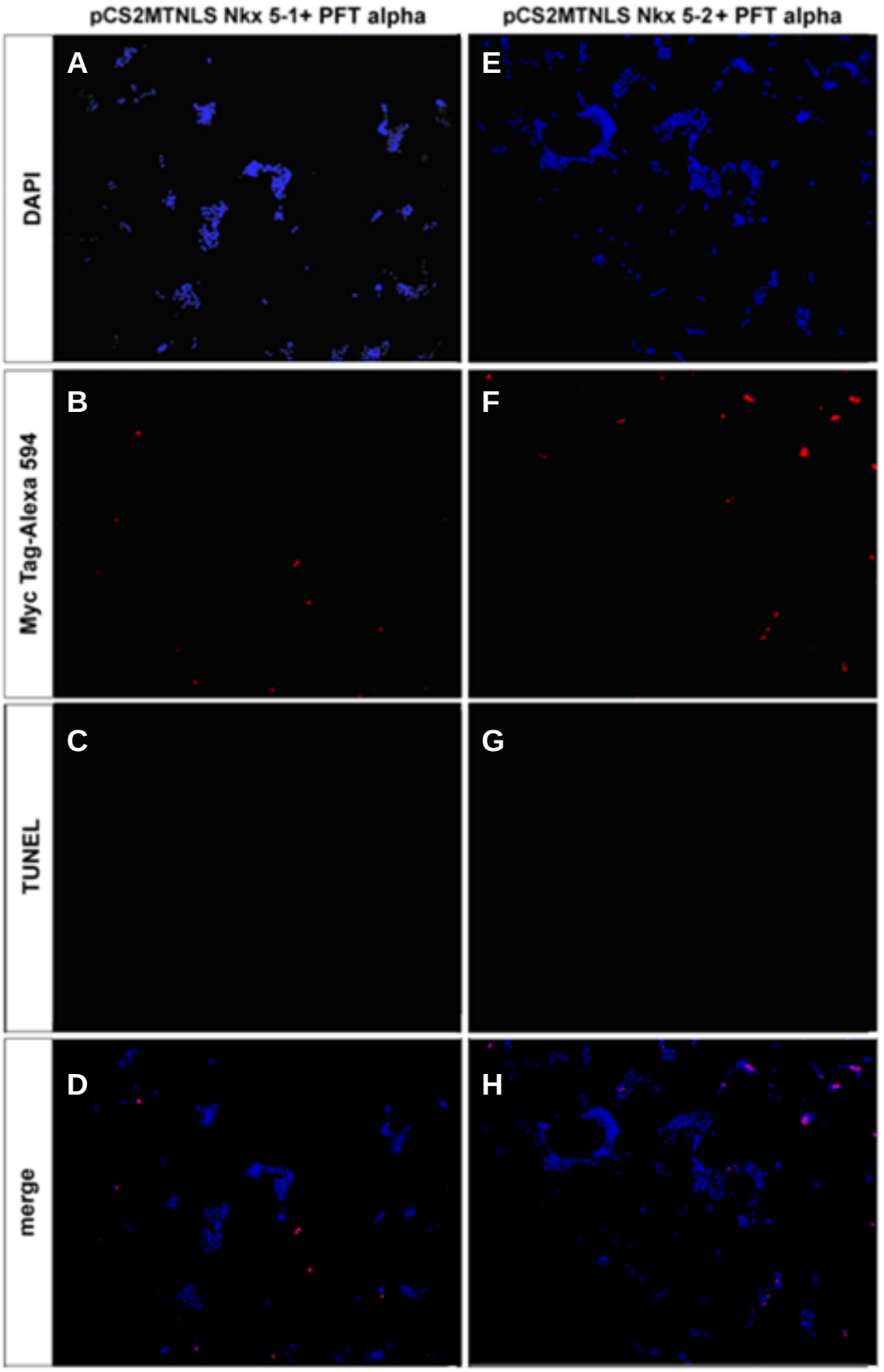

Fig. 12. Cells overexpressing Nkx5-1 and Nkx 5-2 were treated with PFT alpha as described.
A, E – Nuclei were visualized by DAPI staining.
B, F – Transfected cells were confirmed using anti-Myc tag antibody.
C, G – TUNEL assay was performed to visualize apoptotic cells.
D, H – Cells overexpressing Nkx5-1 do not undergo apoptosis.

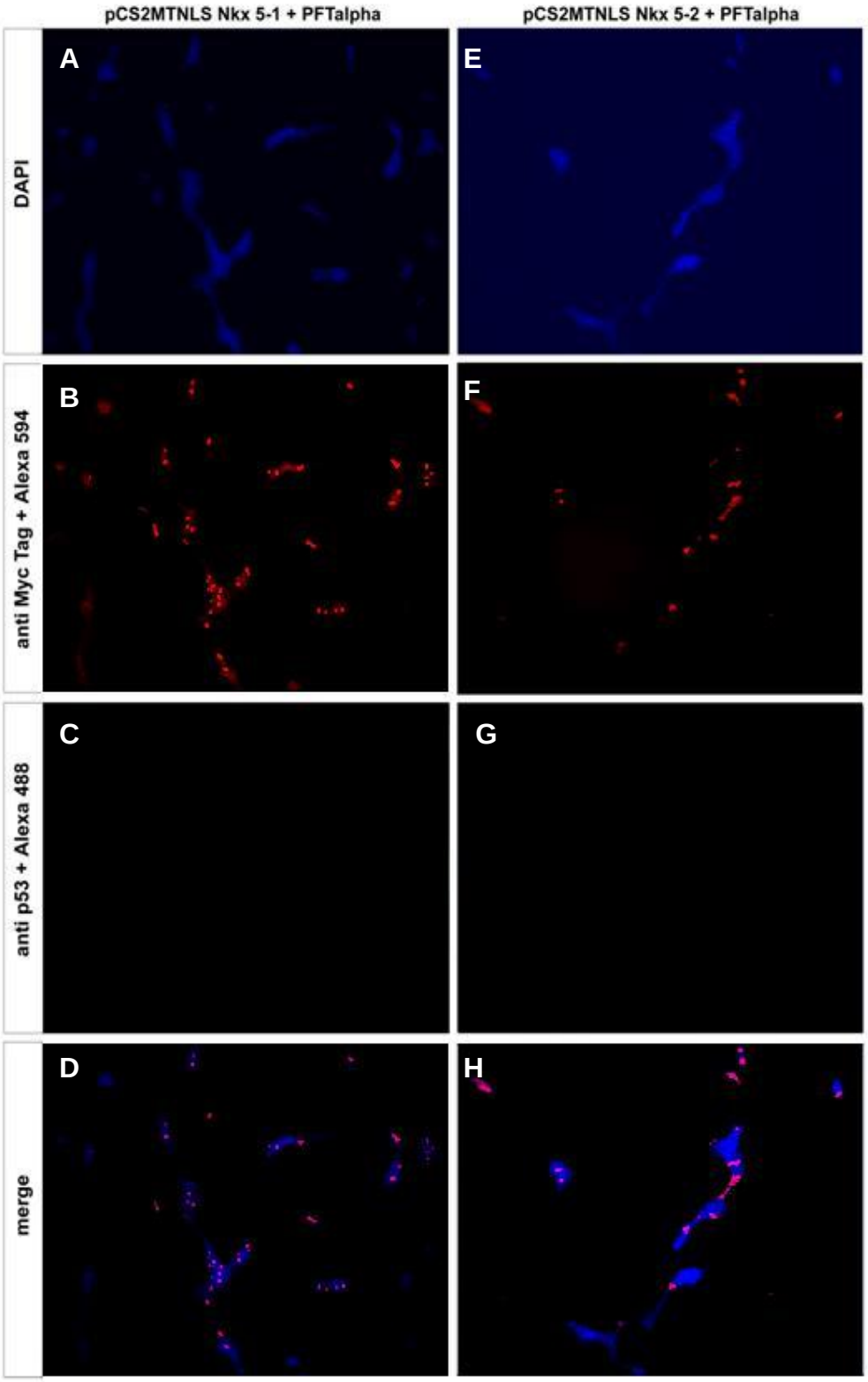

Fig. 13. PFT alpha blocks the activation of p53.
Cells were transfected with Nkx 5-1 and Nkx 5-2 expression constructs and cultivated in a medium with addition of 250ng/ml PFT alpha for 2 days. After that antibody staining was performed.
No p53 expression was detected in Nkx5-1 and Nkx5-2 overexpressing cells.
A, E – Nuclei were visualized by DAPI staining.
B, F – Transfected cells were confirmed using anti-Myc Tag antibody.
C, G – Anti-p53 antibody assay was performed to visualize p53 level in the cells.
D, H – Merged images.

2.2.5. BMP2 is able to induce apoptosis and p53 expression independently of Nkx5-1

As already demonstrated above (par. 2.2.3., page 25) BMP2 did not grossly affect apoptosis induction by Nkx5-1 overexpression. However, BMP2 was able to induce apoptosis in PC12 cells apparently without Nkx5-1 overexpression (see Fig. 11A-D, page 26). The question arises to what extent the down-stream apoptotic pathways activated independently by BMP2 and Nkx5-1 differ from each other. First, I wanted to investigate whether both apoptosis inducers activate p53. To address this issue Nkx5-1 and Nkx5-2 transfection experiments were performed in the presence of BMP2 as already described in par. 2.2.3. (page 25). The cells were harvested 48h after transfection and analysed for the presence of transfected proteins using the anti-Myc-tag antibody (Fig. 14B, F) and for p53 expression using anti-p53 antibody (Fig. 14C, G). Our data show again that Nkx5-1 overexpression strongly enhanced p53 level, while no increase in p53 could be observed in Nkx5-2 transfected cells (Fig. 14, compare C with G). In addition, BMP-2 lead to induction of p53 also in untransfected cells (white arrows in Fig. 15D pointing at cells positive only for p53 antibody). Such cells were also present in Nkx5-2 transfected cells (Fig. 14H, arrows). These cells were also undergoing apoptosis as shown already in Fig. 12. Therefore, BMP2 is able to induce p53 and apoptosis in PC12 cells without Nkx5-1 overexpression. However, interrelationship between endogenous BMP2, Nkx5-1, and p53 proteins in PC12 cells cannot, of course, be excluded and requires further investigation.

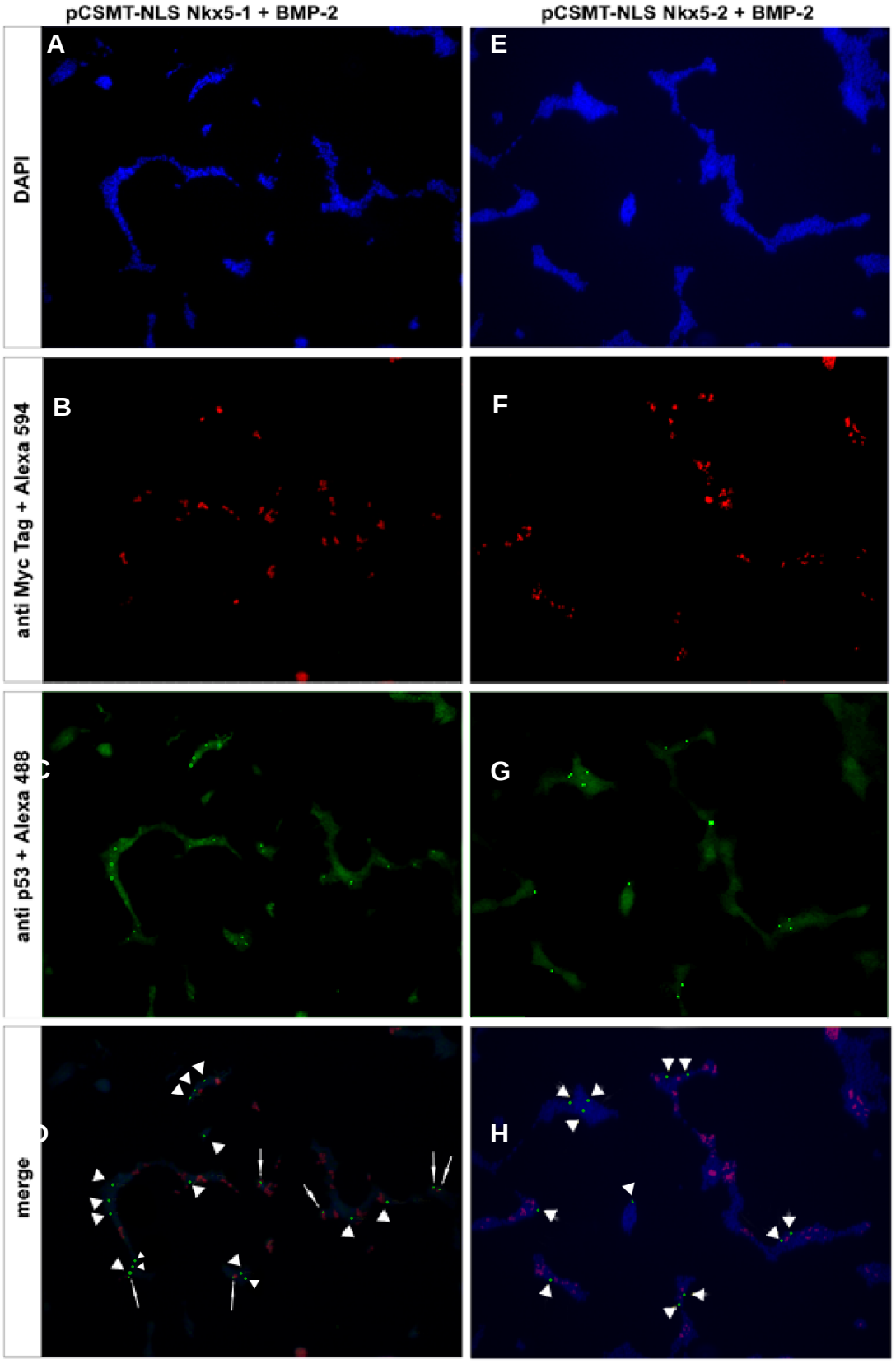

Fig. 14. BMP-2 induced apoptosis by p53 independent of Nkx5-1 overexpression. Cells positive both for p53 and Nkx5-1 overexpresion were observed in the culture after treatment with BMP-2.
A, E – Nuclei were visualised by DAPI staining.
B, F – Transfected cells were confirmed using anti-Myc Tag antibody.
C, G – Anti-p53 antibody assay was performed to visualize p53 level in the cells.
D, H – Merged images (arrows point at the untransected cells positive for p53).
Arrow heads point at cells positive for Nkx5-1 and apoptosis.

2.2.6. NGF does not interfere with p53 induction by Nkx5-1 overexpression

In the further course of this work the essential role of p53 activation for the Nkx5-1 induced apoptosis was confirmed in additional experiments. Since NGF did not prevent Nkx5-1 mediated apoptosis, I wanted to investigate whether p53 activation by Nkx5-1 overexpression does also take place in the presence of NGF. Fig. 15 demonstrates that despite the presence of high NGF concentration (100ng/ml) p53 is still activated in Nkx5-1 transfected cells (Fig. 15D, double-stained cells for anti-Myc and anti p53 antibodies are marked by arrows). However, a significant amount of Nkx5-1 overexpressing cells did not activate p53 expression high enough to allow immunohistochemical detection. Interestingly, treatment of Nkx5-1 transfected cells with NGF and PFT alpha almost completely abolished apoptosis (Fig. 16B, C). This experiment strongly suggests that p53 activation is required for Nkx5-1 induced apoptosis, even if the level of p53 expression escapes immunohistochemical detection.

To evaluate the importance of p53 activation in Nkx5-1 induced apoptosis more exactly, additional transfection experiments were performed and quantitatively analysed. The cells were transfected with Nkx5-1 overexpressing plasmid and treated with NGF, BMP2, PFT, or combinations as described. Further, in some experiments BMP2 signalling was inhibited by addition of noggin. The harvested cells were stained using p53 specific antibody to visualize p53 expressing cells (green) and anti-Myc tag antibody to detect positive cells for Nkx5-1 expression (red). Cells positive for either Nkx5-1 or p53 expression, as well as cells positive for both proteins has been counted on 5 different plates and on each plate 3 different areas were selected. Results are presented in histogram (Fig. 17) and summarized in Table 1.

The quantitative data generally confirmed the previous observations: under NGF treatment p53 expression is found essentially only in Nkx5-1 overexpressing cells, although approximately only a half up to 2/3 of Nkx5-1 positive cells switch on the p53 expression. After BMP2 treatment the majority of Nkx5-1 positive cells also activate p53 expression but, in addition, almost the same number of cells activates

p53 without Nkx5-1 overexpression. These particular cells are abolished after addition of noggin, thus confirming that BMP2 activates p53 independent of exogenous Nkx5-1. PFT alpha treatment generally leads to a substantial reduction of p53 positive cells.

Similar experiments were performed using Nkx5-2 overexpressing plasmid for transfections. As documented by quantitative data in Fig. 18 and Table 2, Nkx5-2 does not possess any potential for p53 activation.

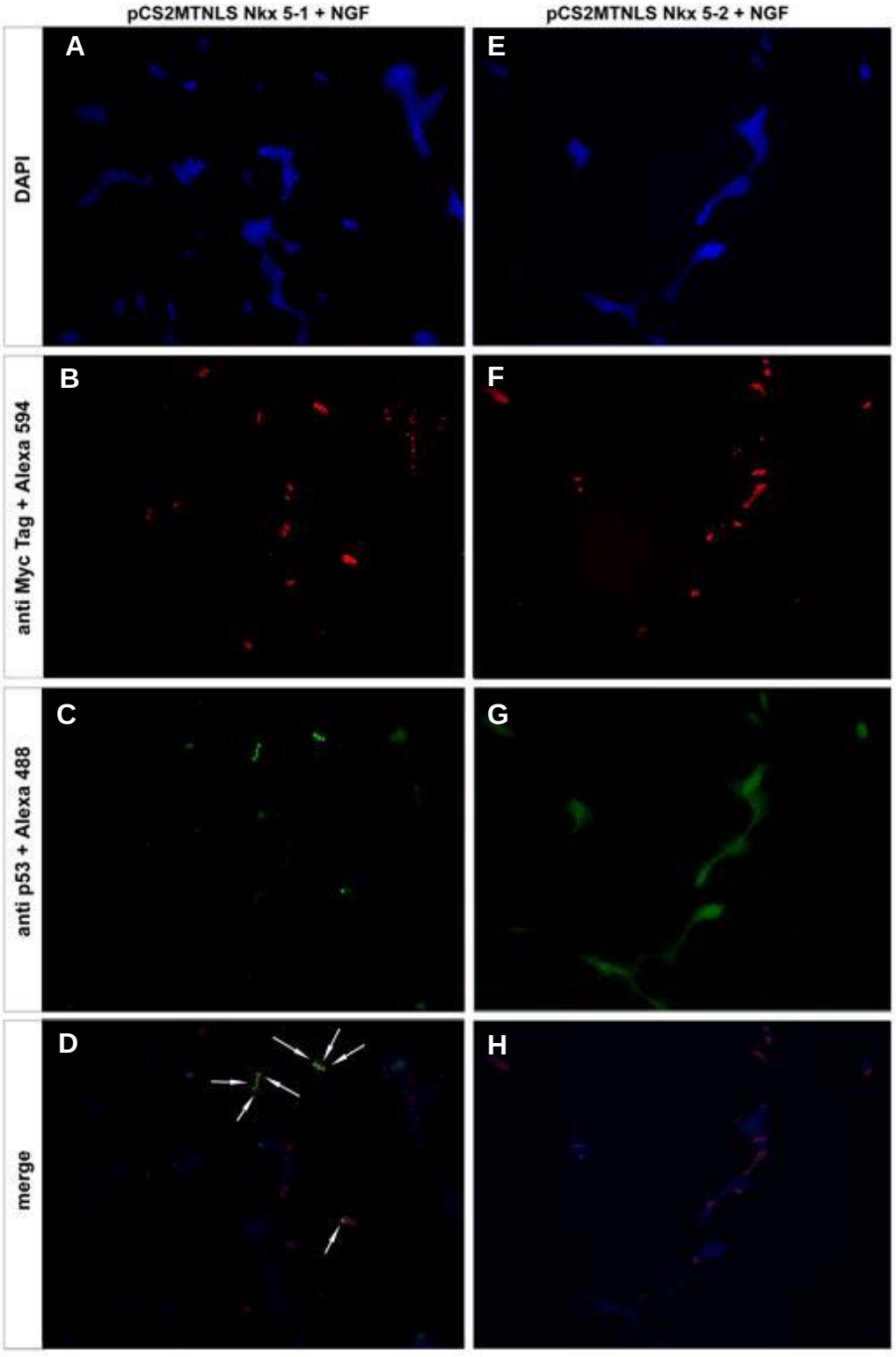

Fig. 15. NGF does not interfere with p53 inducion by Nkx5-1 overexpression.
A,E – Nuclei were visualized by DAPI staining.
B,F – Transfected cells were confirmed using anti-Myc Tag antibody.
C,G – p53 was visualized by anti-P53 antibody apoptotic cells.
D,H – Merged images (arrows points at cells positive for MycTag and p53).

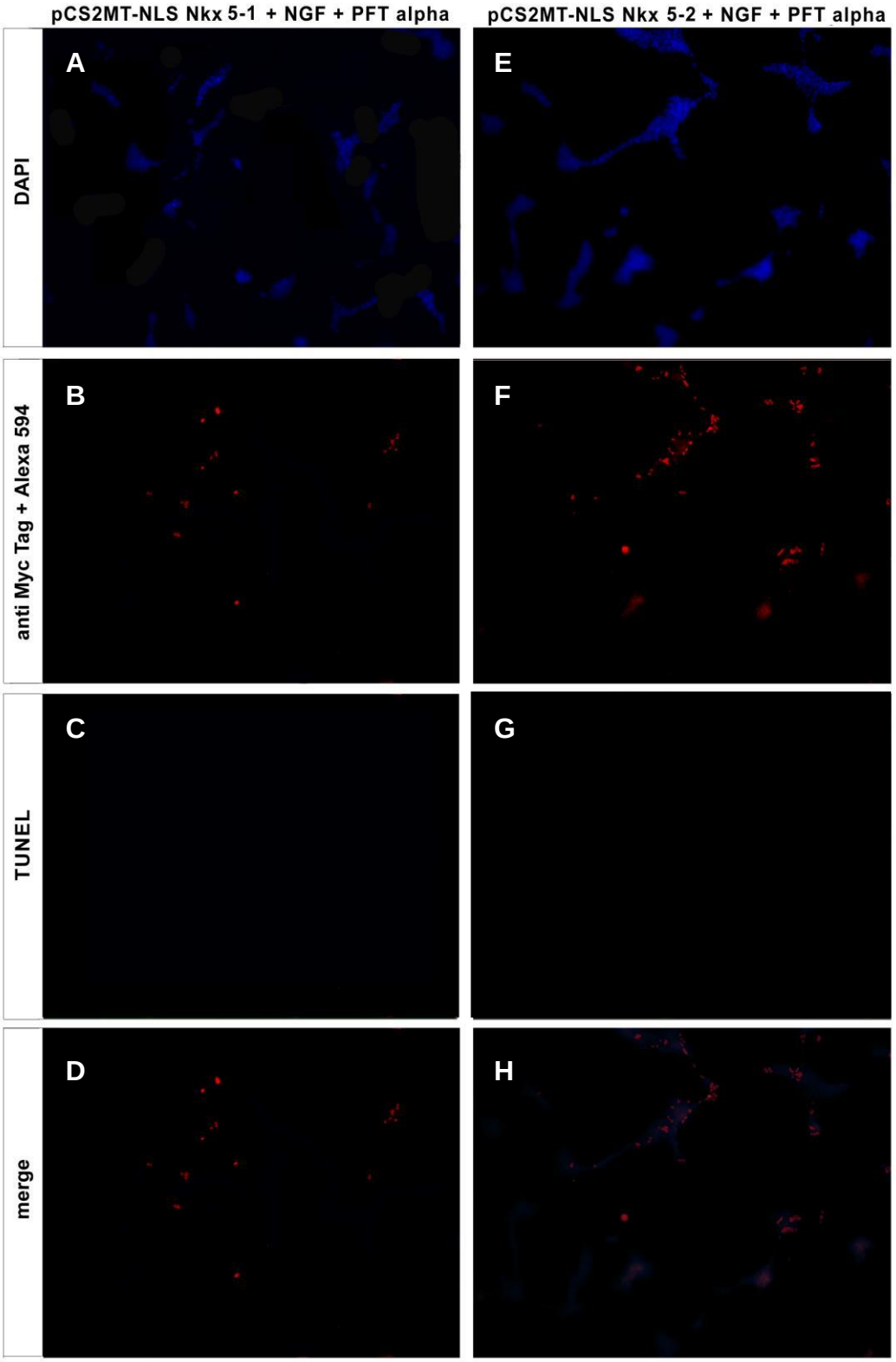

Fig. 16. Combination of NGF and PFT alpha abolishes apoptosis induction by Nkx5-1.
A,E – Nuclei were visualized by DAPI staining.
B,F – Transfected cells were confirmed using anti-Myc Tag antibody.
C,G – TUNEL assay was performed to visualize apoptotic cells.
D,H – Merged images.

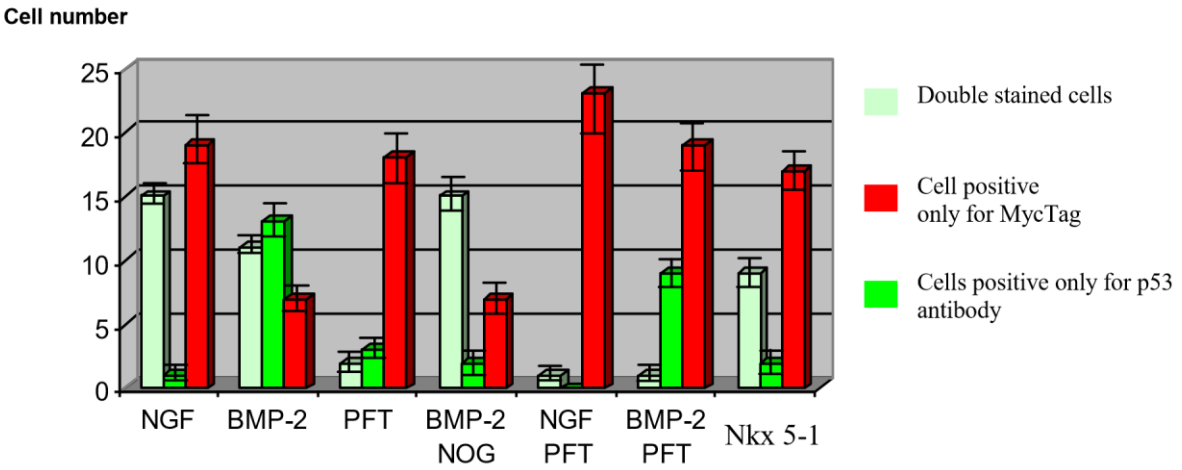

Fig. 17. Percent of cells positive for MycTag and p53 after pCS2MTNLS Nkx5-1 transfection.
Cells were counted under microscope. Three different fields were selected and counted on each plate of five individual transfections.

Treatment	cells labeled double g+r	cells labeled green	cells labeled red
+NGF	15	1	19
+BMP2	11	13	7
+PFT	2	3	18
+BMP2 NOG	15	2	7
+NGF PFT	1	0	23
+BMP2 PFT	1	9	19
+Nkx 5-2	9	2	17

Table 1. Average numbers of positive cells for Nkx5-1 (MycTag /red) and p53 (expression/green), and for double-stained cells.

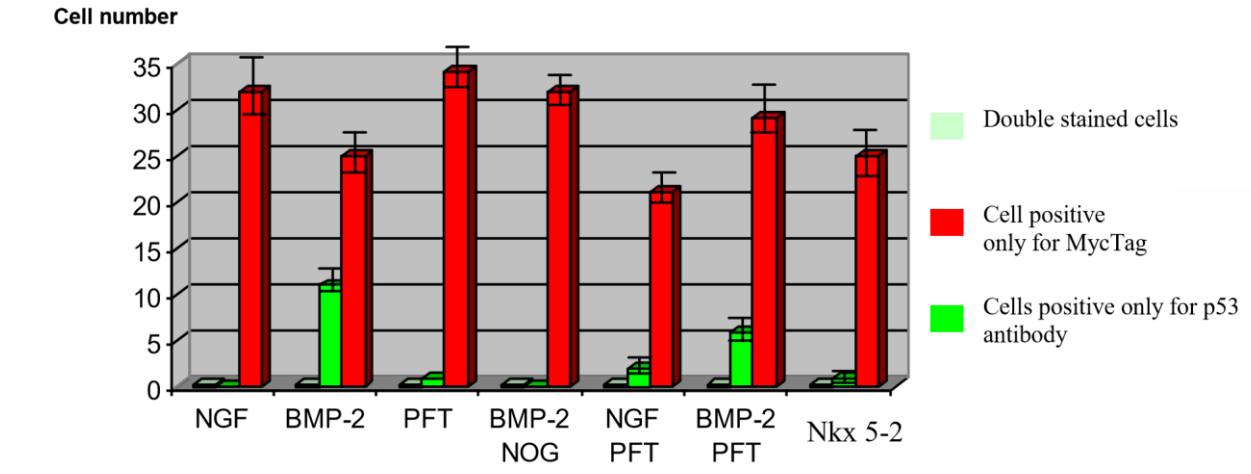

Fig. 18. Percent of cell positives for MycTag and p53 after pCS2MTNLS Nkx5-2 transfection.
Cells were counted under microscope on 5 different plates.

Treatment	cells labeled double g+r	cells labeled green	cells labeled red
+NGF	0	0	32
+BMP2	0	11	25
+PFT	0	1	34
+BMP2 NOG	0	0	32
+NGF PFT	0	2	21
+BMP2 PFT	0	6	29
+Nkx 5-1	0	1	25

Table 2. Average numbers of positive cells for Nkx5-2 (MycTag /red) and p53 (expression / green), and for double-stained cells.

2.2.7. Gene expression analysis in PC12 cells under different growing conditions

To get more insight into the molecular changes induced by overexpresion of Nkx5 genes and to correlate our findings with endogenous apoptosis pathways, gene expression was analysed by RT-PCR technique. RNA was isolated from PC12 cells cultivated under different conditions and RT-PCR analysis was performed to estimate expression of several genes involved in apoptosis (Fig. 19).

Interestingly, we observed an increased expression of the p53 gene as well as p53-regulated genes (p21, APAF-1) after overexpression of Nkx5-1. No increase in p53 expression was detectable in cells overexpressing Nkx5-2 (Fig. 18). The increased p53 expression was accompanied by activation of apoptosis specific genes such as caspase III and BAC-1. However, these genes were also expressed, albeit at the somewhat lower levels in cells overexpressing Nkx5-2.

In addition to overepression of Nkx5 proteins, the PC12 cells were treated with BMP-2 and NGF, BMP and p53 inhibitors, noggin and PFT, respectively. The influence of these factors on expression of Nkx5 and apoptosis related genes were examined by RT-PCR. Following conclusions could be drawn:

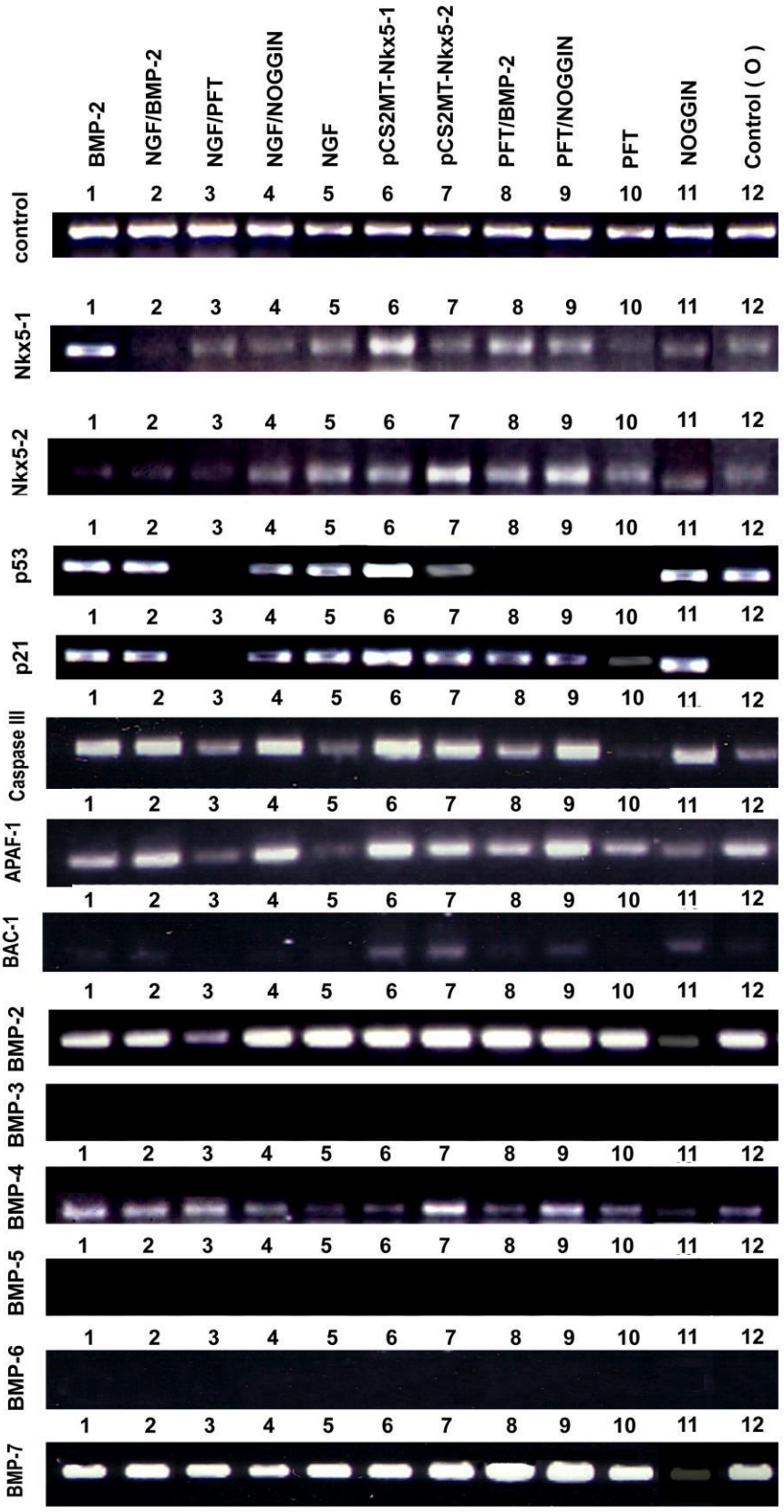

Fig. 19. RT-PCR analysis of different cDNAs isolated from PC12 cultivated under different conditions. Cells were treated for 2 days with NGF, BMP, NOGGIN, or transfected with Nkx5 overexpressing plasmids as indicated at the top of the figure. 25 cycles of RT-PCR were performed to detect expression of genes indicated on the left.

Nkx5-1 is activated by BMP-2 and NGF separately. However, combination of these two factors strongly inhibits Nkx5-1 expression.

Inhibition of p53 activity by PFT alpha leads to Nkx5-1 down-regulation. Combination of NGF, BMP-2 with PFT alpha also lowers Nkx5-1 expression as compared to higher Nkx5-1 expression induced by NGF or BMP-2 alone.

Nkx5-2 expression is up-regulated by NGF. In contrast, BMP-2 treatment does not activate Nkx5-2. Caspase III is strongly induced in cells transfected by Nkx5-1 confirming the ongoing apoptosis in Nkx5-1 overexpressing cells.

2.3. Estimation of Nkx5-1 protein domains conferring the induction of apoptosis

In the next step, we wanted to examine which part of the Nkx5-1 protein possesses the apoptosis-inducing activity. At the same time, we wanted to exclude that the lack of such activity in the Nkx5-2 protein was simply due to any faults in the experimental design or construction of Nkx5-2 overexpressing vector. Therefore, swapping expression constructs were cloned as illustrated schematically in Fig. 20 and the hybrid molecules were tested for their potential to induce apoptosis. A conserved XhoI restriction site, residing at the N-terminal part of the homeobox was used to generate two separate fragments of each Nkx5 cDNA. The correct orientation was confirmed by restriction analysis and sequencing (not shown). As shown in Fig. 21, construct overexpressing an Nkx5-1/2 hybrid molecule consisting of the Nkx5-1 N-terminus fused to the C-terminal part of Nx5-2, including the almost entire Nkx5-2 homeodomain (Fig. 20A), faithfully induced apoptosis, as it was the case for the full-length wild-type Nkx5-1 protein. An analogous Nkx5-2/1 construct expressing the N-terminus of Nkx5-2 joined to the Nkx5-1 C-terminus (Fig. 20B) did not show any apoptosis induction (Fig. 21E,H). These experiments clearly demonstrate that the non-conserved N-terminal domain of the Nkx5-1 protein harbours the apoptosis inducing activity. Such domain is obviously lacking within the Nkx5-2 molecule.

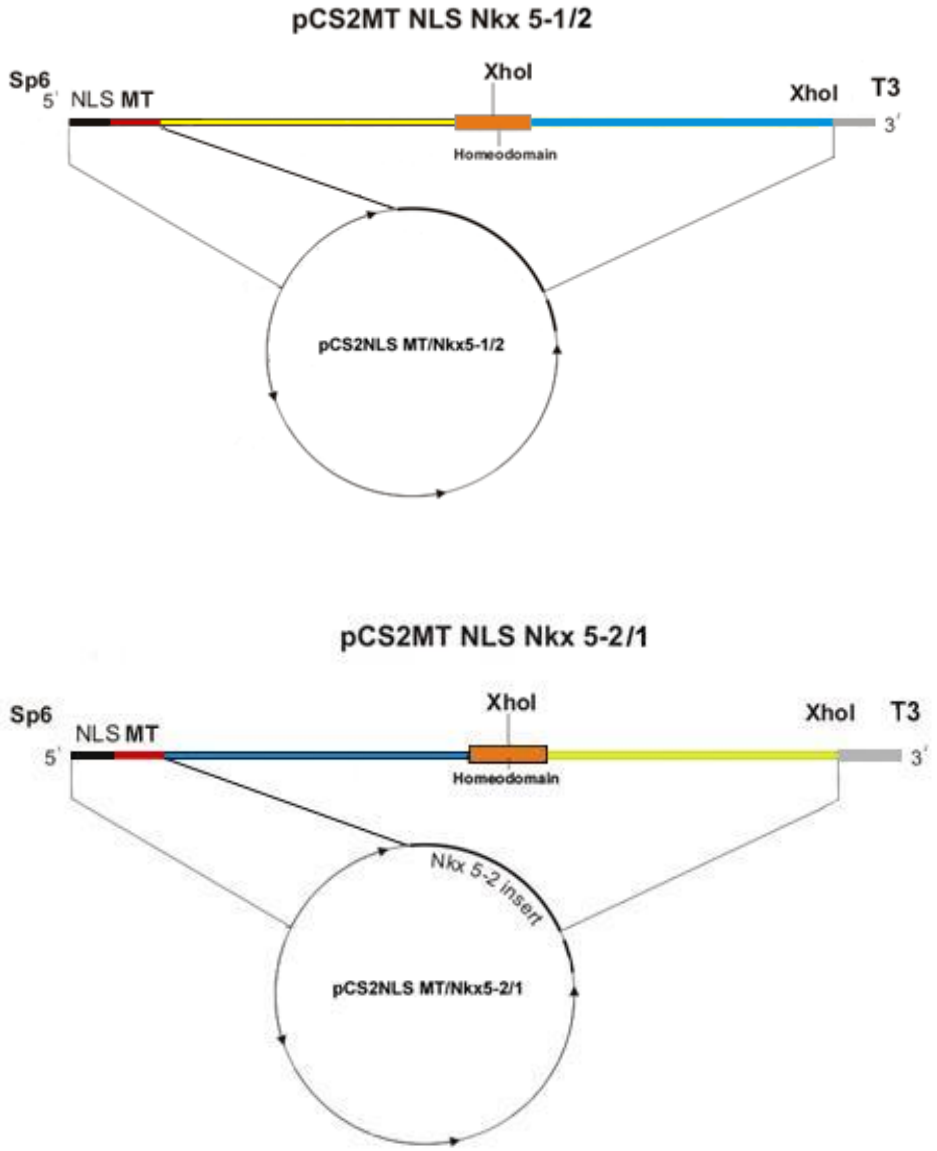

Fig. 20. Overexpression constructs for Nkx 5-1/ Nkx 5-2 – recombinant proteins.
Nkx5-1 specific fragments are marked by a yellow and Nkx5-2 specific sequences are indicated by a blue colour.

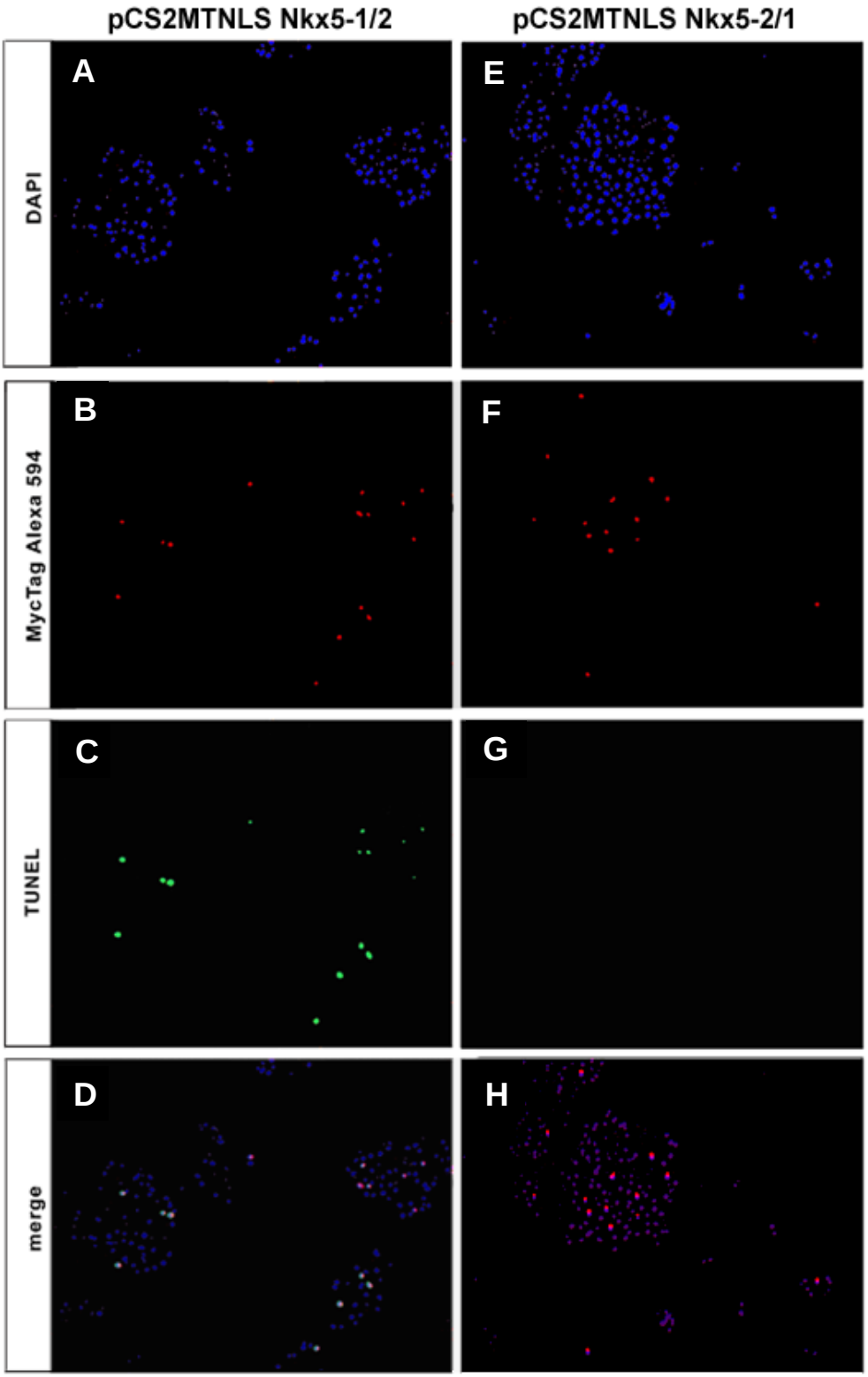

Fig. 21. Nkx 5-1/2 overexpression construct containing the N –terminus of Nkx5-1 protein induces apoptosis in contrast to Nkx5-2/1 construct containing the Nkx5-2 N-terminus.
A,E – Nuclei were visualized by DAPI staining.
B,F – Transfected cells were confirmed using anti-Myc Tag antibody.
C,G – Tunnel assay was performed to visualize apoptotic cells.
D,H – Merged images.

2.3.1. N-terminus of Nkx5-1 protein is sufficient to induce apoptosis but lacks p53-responsive elements

The results presented above (Fig. 21) clearly documented that sequences responsible for apoptosis induction are located in the Nkx5-1 region upstream of XhoI restriction site present within the homeobox (see Fig. 20A). These sequences were searched for potential similarity to motives known to be responsible for apoptotic effects in other genes using BLAST analysis. However, no similarities to known apoptotic sequences were found. Thus, further experiments are necessary to delineate the exact elements responsible for Nkx5-1-dependent apoptosis.

To investigate whether the Nkx5-1 N-terminus of the Nkx5-1/2 swapping construct contains entire sequence information responsible for apoptotic activity, further experiments were performed. First, apoptosis induction by overexpression of the native Nkx5-1 protein in PC12 cells was not influenced by NGF. Similarly, addition of NGF to the cell culture medium did not prevent apoptosis in the case when the cells were transfected with Nkx5-1/2 construct (results not shown).

Interestingly, different behaviour of cells transfected with Nkx5-1/2 construct as compared to wild-type Nkx5-1 transfection was observed after PFT alpha treatment. In contrast to previous observations, PFT alpha did not block apoptosis induced by Nkx5-1/2 protein. As shown in Fig.22A-D cells overexpressing Nkx5-1/2 construct underwent apoptosis even in the presence of PFT alpha. This observation suggested that apoptosis induced by Nkx5-1/2 hybrid protein was not p53-dependent. Alternatively, combination of PFT alpha and the Nkx5-1/2 hybrid protein might be toxic for the cells.

In next experiment BMP2 was added to the Nkx5-1/2 transfected cells. In this experiment Nkx5-1/2 induced apoptosis was not influenced by BMP2 (Fig. 22E-H). BMP2 was also able to induce apoptosis in cells, which were not transfected by the Nkx5-1/2 construct confirming the observation that BMP2-induced apoptosis did not require overexpression of Nkx5-1 or the hybrid Nkx5-1/2 protein (Fig. 22 H, arrows).

Transfection of PC12 cells with the swapping domain construct containing the Nkx5-2 N-terminus fused to the Nkx5-1 homeodomain, Nkx5-2/1 (Fig. 20B), did not induce apoptosis in the presence of PFT alpha or BMP2 (Fig. 23). The only apoptotic cells obviously induced by BMP2 treatment did not express Nkx5-2/1 construct (Fig. 23 E-H, see arrows in H).

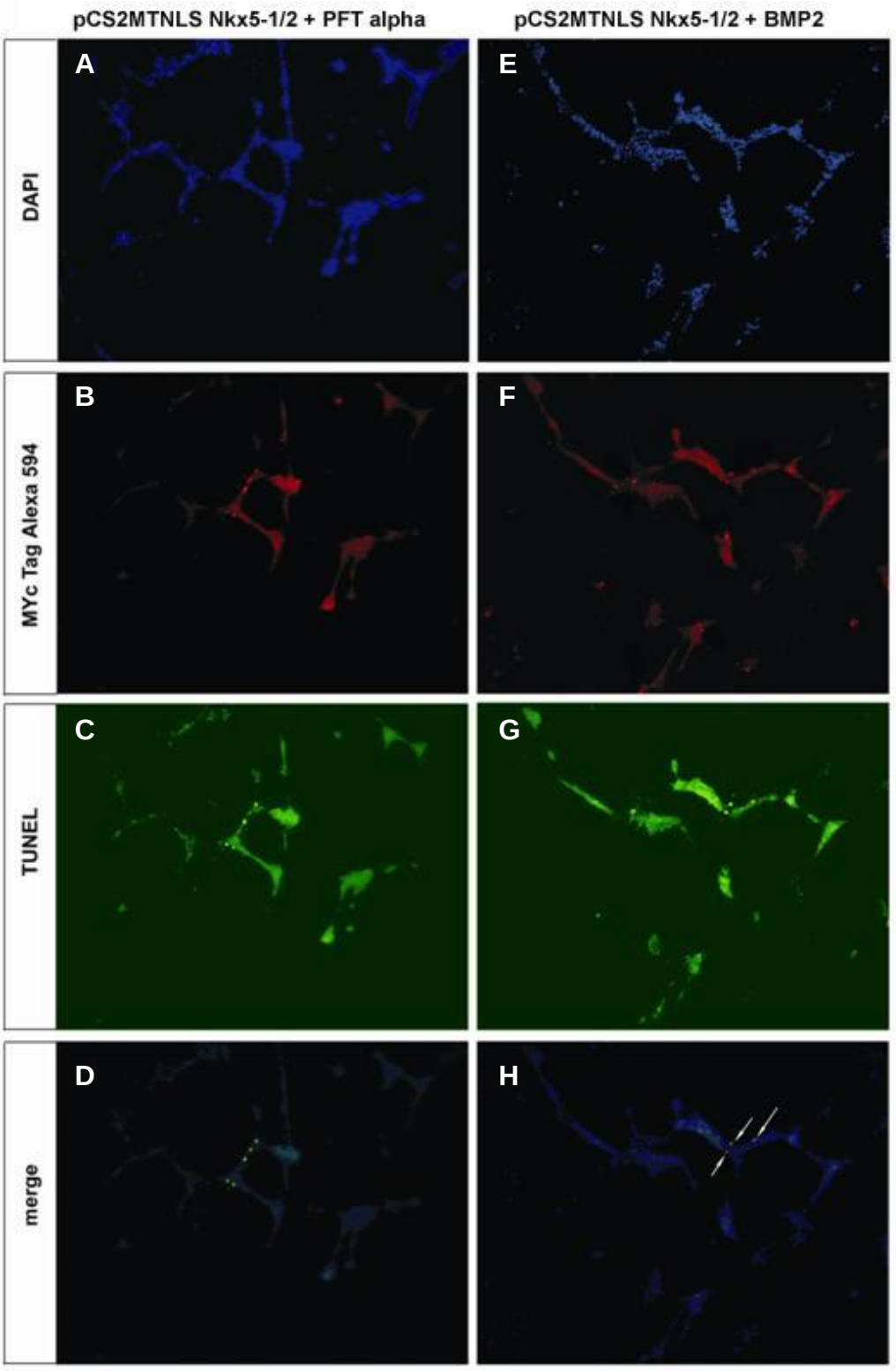

Fig. 22. PFT alpha and BMP-2 do not block apoptosis in PC12 cells transfected with Nkx5-1/2 vector.
A,E – Nuclei were visualized by DAPI staining.
B,F – Transfected cells were confirmed using anti-Myc Tag antibody.
C,G – Tunnel assay was performed to visualize apoptotic cells.
D,H – Merged images. Arrows in H indicate apoptotic cells without Nkx5-1/2 overexpression.

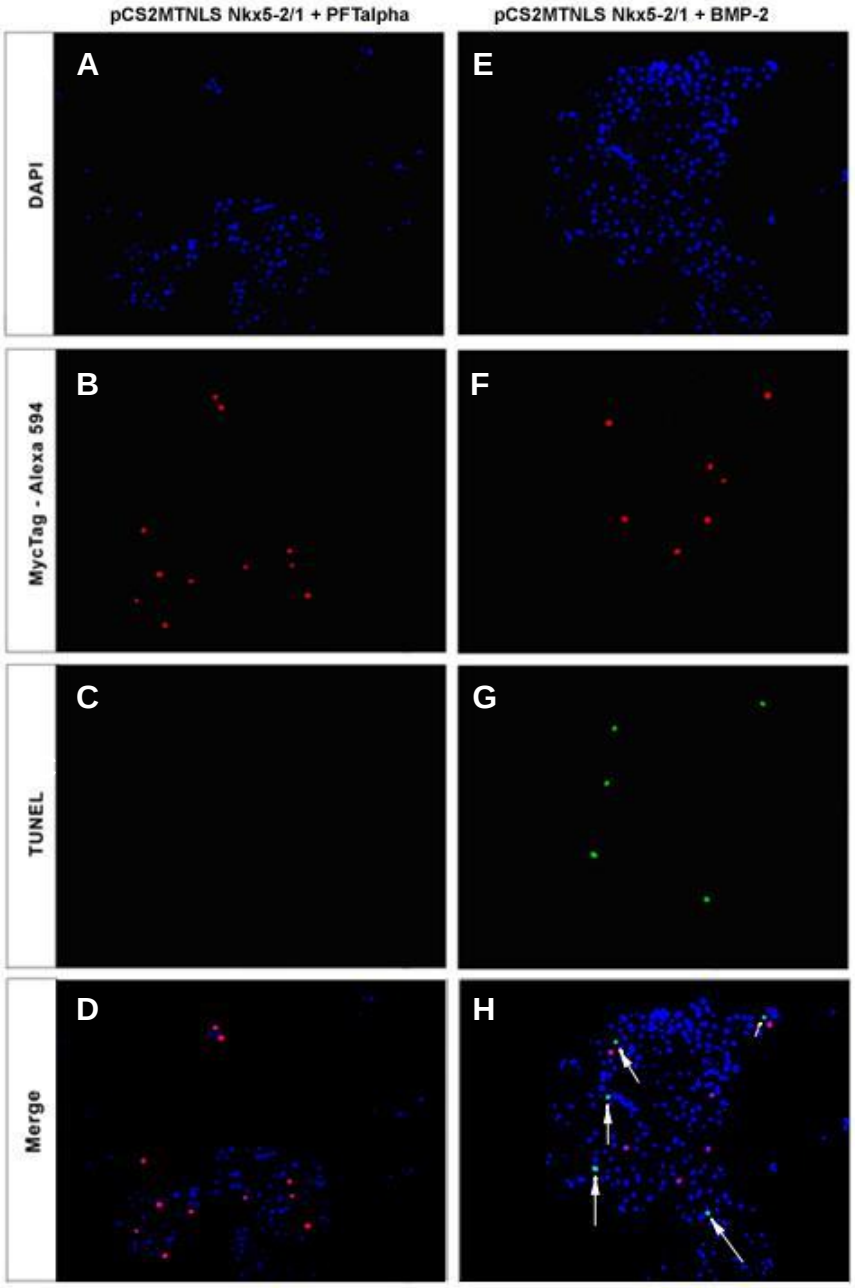

Fig. 23. Nkx5-2/1 hybrid protein does not induce apoptosis after PFT alpha or BMP-2 treatment.
A,E – Nuclei were visualized by DAPI staining.
B,F – Transfected cells were confirmed using anti-Myc Tag antibody.
C,G – Tunnel assay was performed to visualize apoptotic cells.
D,H – Merged images. Arrows indicate apoptotic cells due to BMP-2 treatment and negative for Nkx5-2/1 expression.

2.4. Identification of Nkx5-1 promoter region and analysis of its activity in neuronal cells

2.4.1 Generation of Nkx5-1 promoter construct

To investigate Nkx5-1 promoter activity a construct encompassing Nkx5-1 gene sequences upstream of the transcription start site was generated. 10 kb BamHI-KpnI Nkx5-1 genomic fragment containing the first 10,5 kb of Nkx5-1 upstream sequences of the protein coding region was fused in frame to the fragment encoding LacZ reporter gene (Fig. 24).

To analyse whether the cloned Nkx5-1 genomic fragment contains regulatory sequences responsible for Nkx5-1 gene activity, the construct was transfected into PC12 cells and the cells stained for β-Gal activity under different conditions (see next chapter).

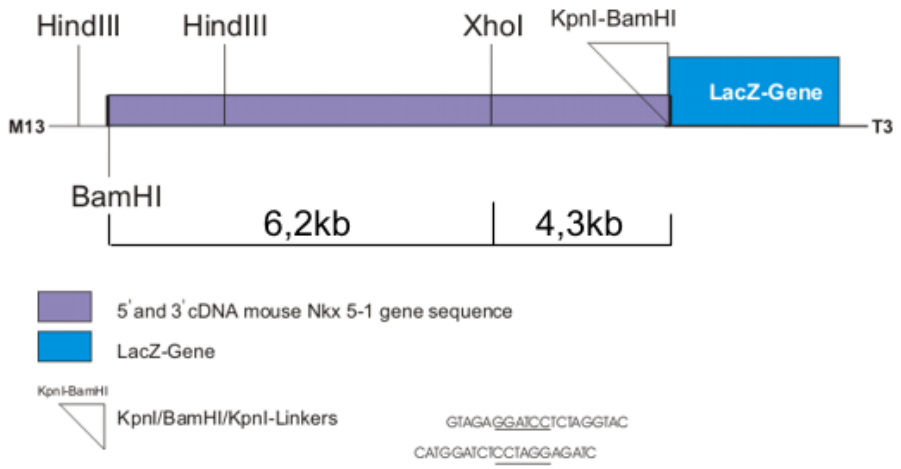

Fig. 24. Promoter construct with reporter gene LacZ for investigation of Nkx5-1 gene activity.

2.4.2. Nkx5-1 promoter construct is active and regulated by NGF and BMP2 in PC12 cells

To investigate whether the Nkx5-1 genomic sequences cloned into the LacZ-reporter plasmid can activate transcription of the reporter sequences, the Nkx5-1 promoter construct was transfected into PC12 cells under standard conditions. The cells were harvested 48 hours after transfection and stained for ß-gal activity to visualize transcriptional activity. However, only very weak ß-gal staining was observed in cells transfected with the Nkx5-1 promoter construct as compared with

cells transfected with vector without additional insertions (see Fig. 25A, D, respectively).

As was shown before, NGF and BMP2 activate expression of the endogenous Nkx5-1 gene, when added to PC12 cells separately. In contrast, addition of both factors simultaneously, led to inhibition of Nkx5-1 transcription (see chapter 2.2.7 and Fig. 19). In fact, cells transfected with the Nkx5-1 promoter construct showed high intensity ß-gal staining, when NGF or BMP2 were added to the culture medium (Fig. 25B, C). Consistent with previous observation on endogenous Nkx5-1 activity, simultaneous addition of NGF and BMP2 strongly inhibited the activity of Nkx5-1 promoter leading to a decrease of ß-gal staining to basal levels (Fig. 25E). The specificity of BMP2-dependent activation of Nkx5-1 promoter was confirmed by treatment of transfected cells with BMP2 and its inhibitor noggin. Addition of noggin strongly reduced ß-gal activity as demonstrated in Fig. 25F.

Since NGF strongly promotes neuronal differentiation I wanted further to investigate whether the higher Nkx5-1 promoter activity observed after supplementation of culture medium with NGF correlates also with neuronal differentiation. Thus, PC12 cells transfected with Nkx5-1 promoter construct and cultivated in the presence of NGF were stained for ß-gal and for ß-tubulin III expression using anti-tubulin antibody (Fig. 26). In fact, the highest ß-gal staining intensity was observed in cells also positive for the neuronal differentiation marker ß-tubulin III (Fig. 26B), while cells transfected with Nkx5-1 promoter construct without any additional treatment displayed only weak, basal-level ß-gal activity and no ß-tubulin III immunoreactivity (Fig. 26A). Similar results were obtained when the cells were treated with BMP2. Here, strong ß-gal activity correlated also with positive ß-tubulin III signals (Fig. 26C). Simultaneously addition of NGF and BMP2 strongly induced neuronal differentiation as evidences by positive ß-tubulin staining (Fig. 26D). The activity of Nkx5-1 promoter in these cells was, however, strongly suppressed (Fig. 26D).

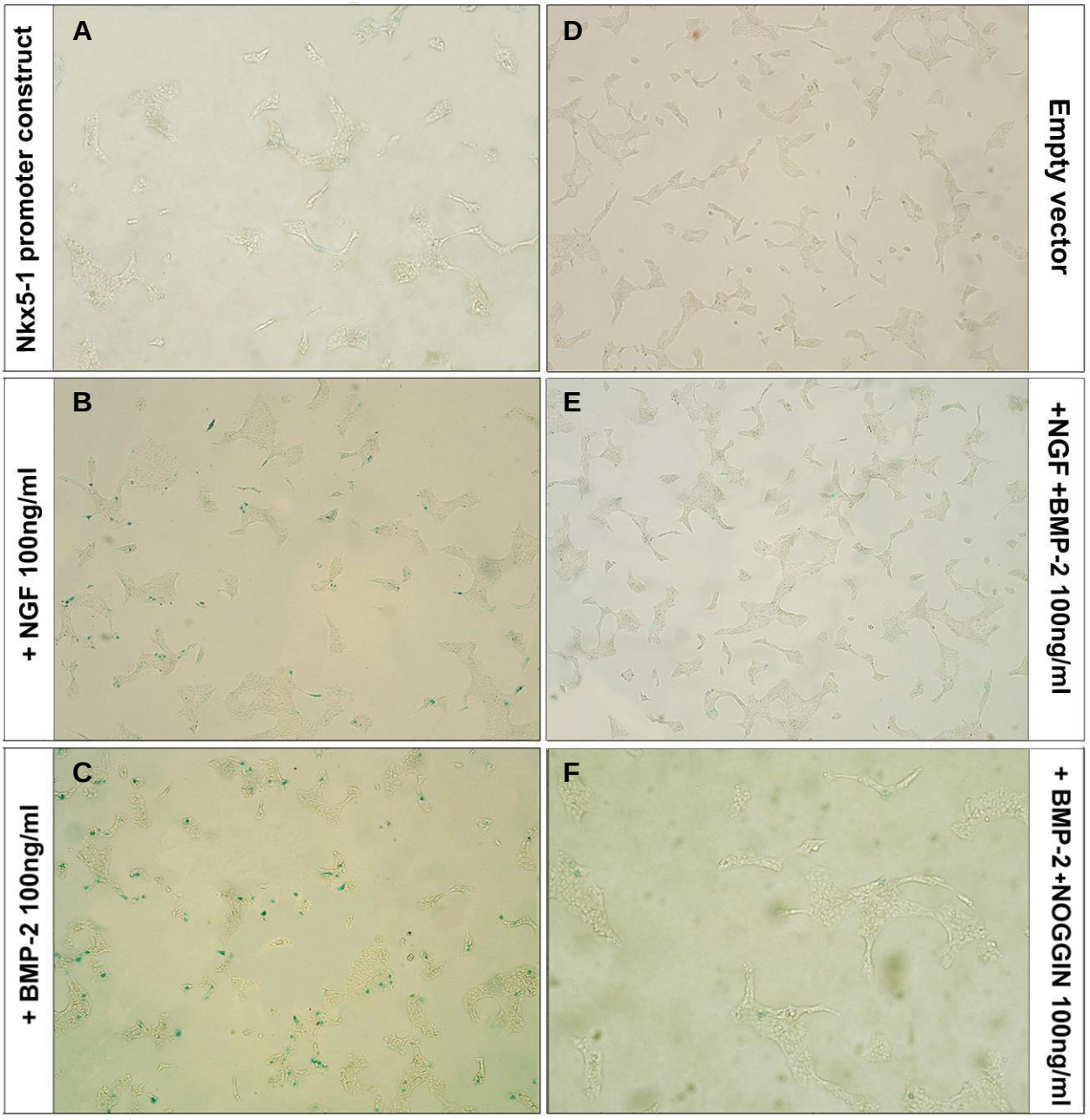

Fig. 25. Activity of LacZ reporter gene under the control of Nkx5-1 promoter.
PC12 cells were transfected with the Nkx5-1 promoter construct (A – C, E, F) or LacZ reporter construct without any promoter sequences (D). Cells were cultivated under standard conditions (A, D) or in presence of NGF (B), BMP2 (C), NGF and BMP2 (E) or BMP2 and noggin (F). All substances were added at a final concentration of 100ng/ml. 48 hours after transfection cells were stained for ß-gal activity and representative areas were photographed.

Taking together, these experiments clearly document the presence of sequences governing the basal Nkx5-1 promoter activity on the genomic fragment used for the construction of Nkx5-1-LacZ reporter plasmid. In addition, regulatory elements responsible for the activation of Nkx5-1 gene transcription by NGF and BMP2 are also present within these sequences. Significantly, a higher Nkx5-1 activity correlates with neuronal differentiation in NGF or BMP2 treated cells but not in neuronal cells treated by combination of both factors.

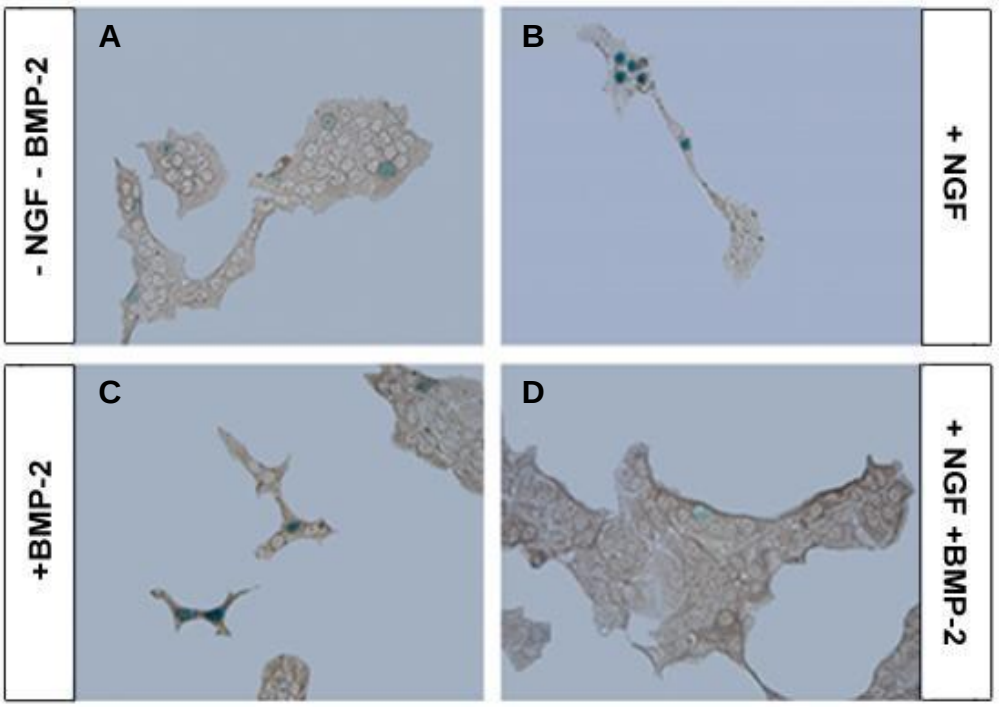

Fig. 26. Nkx5-1 promoter activity correlates with neuronal differentiation.
PC12 cells were transfected with Nkx5-1 promoter construct and cultivated under standard conditions (A) or with addition of NGF (B), BMP2 (C), or combination of both (D). After transfection cells were stained for ß-gal activity (blue) and ß-tubulin III immunoreactivity (brown).

2.5. Apoptosis and neuronal differentiation in Nkx5-1 knockout mouse in comparison to wild type

The results achieved in in vitro PC12 cells system suggested that Nkx5-1 may be involved in neuronal development. Therefore, expression of essential neuronal markers was investigated by PCR analysis in vivo in Nkx5-1 knockout and wt control brains. Mice brains from three different age stages (3 months, 6 months and 1 year) have been isolated and whole brain RNA isolated and analysed by RT-PCR. However, no significant differences in the neuronal marker expression have been detected. In each age stage the intensity of RT-PCR signal for Ng1A, Ng1B and THA and THB was comparable in Nkx5-1 and wt samples as exemplified in Fig. 27 for samples at the stage of 6 months.

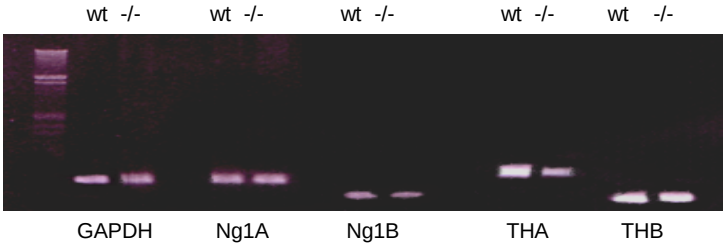

Fig. 27. RT-PCR analysis of different neuronal markers in brain from wt and Nkx5-1 knockout mouse at the age of 6 months.

Ng1A – neurogenin 1 alpha, Ngn1B – neurogenin 1 beta, THA – tubulin alpha, THB – tubulin beta. GAPDH was used as a loading control.

Expression of Nkx5-1 has been reported in brain during embryonic development. It was also reported that Nkx5-1 is expressed in rat salivary glands in postnatal ages (Shaw et. al., 2003). Since no reports on Nkx5 genes expression in an adult brain are available, in situ hybridization using Nkx5-1 antisense sequence was performed on sections of adult mouse brain, however no significant hybridization signal could be detected (results not shown). Since in situ hybridization might not be suitable for detection of low level Nkx5-1 expression, RT-PCR analysis was performed using adult brains of 4, 8 and 18 months old mice. Three Nkx5-1 -/- knockout mice and three white type ICR mice at the age of 4 months of postnatal development and three Nkx5-1 -/- and three white type mice at the age of 8 months and the same numbers for 18 months old mice. Presence of Nkx5-1 expression was detected on all

investigated adult stages by RT-PCR, suggesting a potential function for Nkx5-1 (Fig. 28). In contrast, no Nkx5-2 was detected (Fig. 28).

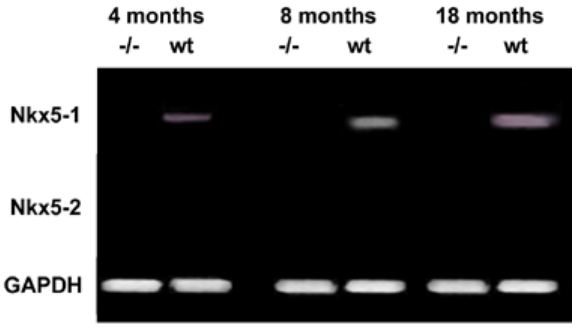

Fig. 28. RT-PCR analysis preformed on adult mouse brain – derived RNA samples (4, 8 and 18 months).
Nkx5-1 positive signals were detected at all analysed stages in wt samples. No Nkx5-2 expression was detected.

Apoptosis was analysed on brain frozen-sections using TUNEL assay and in brain-derived RNA samples using RT-PCF (Fig. 29). Using TUNEL assay several positive areas (indicated by numbers on overview brain in Fig. 29) could be identified on sections from wt control brains (Fig. 29D, F, H). Apoptotic signals in corresponding areas of knockout brains were always weaker or beyond detection (Fig. 29C, E, G). Interestingly, RT-PCR analysis of apoptosis-related genes expression revealed lower activation of a p53 target P21 in knockout brain tissue. Although no Cas6 and APAF expression could be detected, expression of another apoptosis promoting gene Bax was higher in the wt brain (Fig. 29I). Summing up, the in vivo data generally confirmed pro-apoptotic Nkx5-1 activity.

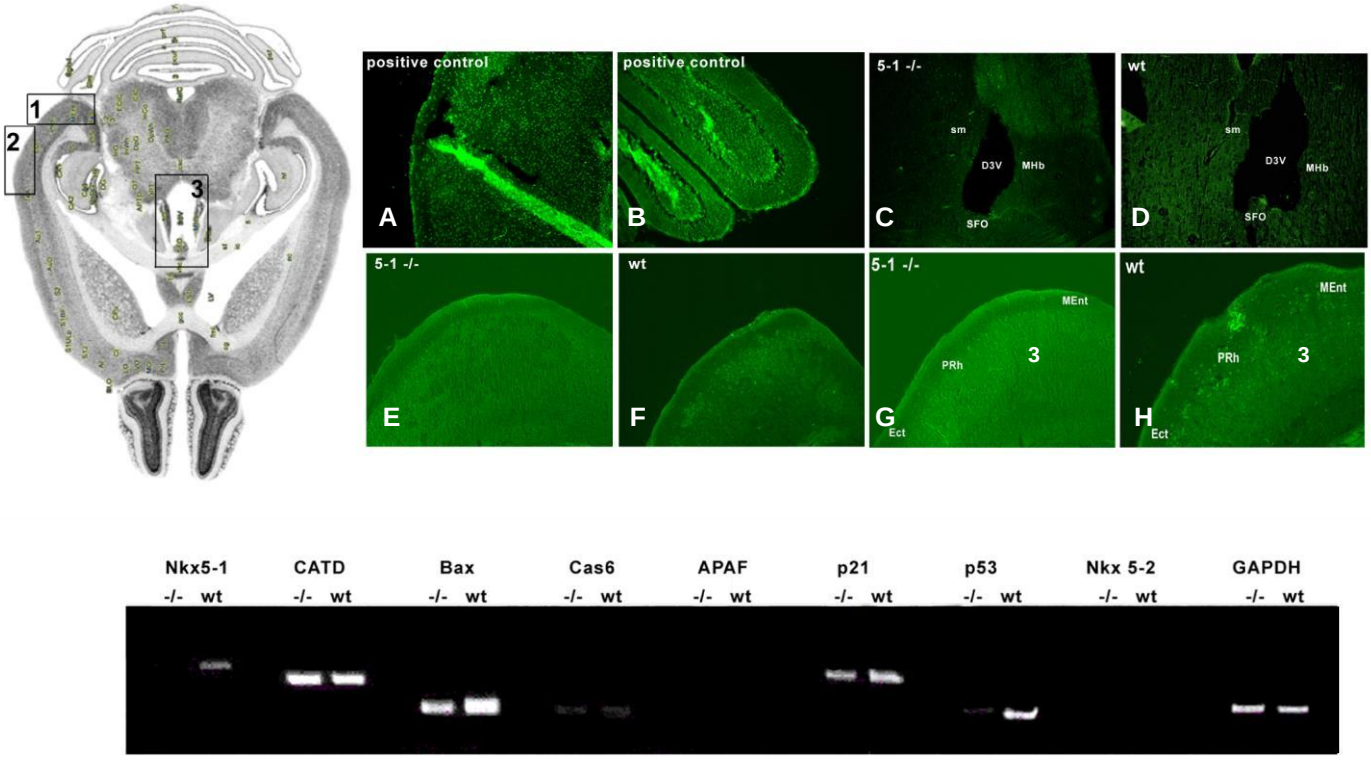

Fig. 29. Analysis of apoptosis in Nkx5-1 knockout and wt control brains.
Tunel stained sections from 3 brain areas indicated on the shin are shown (A-B corresponding to region 2, E-H correspond to region 2 and C-D corresponding to region 3. As a positive control DNAase I was used (A-B). Sections from Nkx5-1 knockout (C,E,G) and wt control brains are shown. RT-PCR analysis of genes expression(genes name as indicated) in Nkx5-1 knockout and wt. D3V - dorsal 3rd ventricle, Ect - ectorhinal cortex, MEnt - entorhinal cortex, medial part, MHb - medial habenular nucleus, PRh - perirhinal cortex, SFO - subfornical organ, sm -stria medullaris, I –RT-PCR.

3. REAGENTS AND CHEMICALS

Chemicals were purchased from the following companies: Amersham, AppliChem, Biomol, Eurogentech, Invitrogene, Merck, Jena Biosciences, New England Biolabs, Pierce, Promega, Roche, Roth, Santa Cruz, Seromed, Serva, Sigma and Stratagene. Consumables came from Amersham, Beckman, Biozym, Costar, Eppendorf, Falcon, Gilson, Greiner, Kodak, Pharmacia, Qiagen, Sarstedt, Machery Nagell and Whatman. Restriction enzymes were purchased from Jena Biosciences and New England Biolabs. Oligonucleotides were purchased from Roth.

3.1. Reagents

Alkalize Phosphates	SIGMA
NGF	SIGMA
Proteinase K	
Restriction nucleases	(Jena Bioscience, New England Biolabs)
Reverse Transcriptase Superscript	(Invitrogen)
RNase A	
RNasin (Ribonuklease Inhibitor)	(Promega)
SuperScript II Reverse Transcriptase	(Invitrogen)
Taq DNA Polymerase	(Eppendorf)
TRIzol	(Invitrogen)
Tripsin 2,5% (10x)	(Invitrogen)
Tripsin (EDTA) (0,5% Tripsin with EDTA 4Na) 10x	
T4 DNA Ligase	(Promega)
Vectabond	(Vector Laboratories)
X-Gal (5-bromo-4chloro-3-idolyl ß-D-galactopiranosyde	(Roth)
Polyfreeze Tissue freezing medium	(Polysciences)
RQ1 RNAase-Free-DNase	(Promega)
Vectastatin ABC Kit (mouse IgG or rabit IgG)	(Vector Laboratories)

3.2. Kits

TUNEL Assay	(Roche)
Qiagex II Gel Extraction Kit	(Qiagen)
Vectasatin ABC Kit	(Vector Laboratories)

3.3. Antibodies

α-c-myc tag antibody 9E10

ß-tubulin alpha

3.4. Growing Factors and Inhibitors

BMP2 (SIGMA B 3555)	(SIGMA)
NOGGIN	(SIGMA)
PFT-alpha (pifithrin-alpha)	(SIGMA)

3.5. Vectors and Primers

If not otherwise indicated, all vectors listed code for resistance to ampicillin.

pGEM-T easy (Promega)

System for cloning of PCR products with single 3´ thymidine overhangs at the insertion site (Promega). Contains T7 and SP6 RNA polymerase initiation sites flanking a multiple cloning region within the coding region of β–galactosides.

pCS2 + MT+NLS (Strategene)

Contains 6 copies of myc tag epitope recognized by 9e10 monoclonal antibody; constructed for production of epitope –tagged fusion proteins contains nuclear localization signal.

pBluscript II KS+ (Stratagene)

pBluescript II phagemids (plasmids with a phage origin) are cloning vectors designed to simplify commonly used cloning and sequencing procedures, including the construction of nested deletions for DNA sequencing, generation of RNA transcripts in vitro and site-specific mutagenesis and gene mapping. The pBluescript II phagemids have an extensive polylinker with 21 unique restriction

enzyme recognition sites. Flanking the polylinker are T7 and T3 RNA polymerase promoters that can be used to synthesize RNA in vitro. The choice of promoter used to initiate transcription determines which strand of the insert cloned into the polylinker will be transcribed

pDSRED (Clontech)

pDsRed-Express is a prokaryotic expression vector that encodes DsRed-Express, a variant of *Discosoma sp.* red fluorescent protein (DsRed; 1)

Primers sequence

Name	Sequence	Annealing Temperature
GAPDH forward	ACTGCACCCTCCCCCGATGCACCCATGTTTGT	control PCR
GAPDH reverse	TGGAGGCAACCAGGGCAACCACCACAGCTACA	control PCR
Nkx5-1 rat forward	GCACTACCTGGAGCGCTCCCC	62ºC
Nkx5-1 rat reverse	CCGAGCTGCTCAGGTAGCGTTTC	62ºC
Nkx5-2 rat forward	CTGCGGCTCGGAGCGCACGCCTTTCC	58ºC
Nkx5-2 rat reverse	GGGGTAATAGAGCGGAGCCGG AAAGGCG	58ºC
BMP2 rat forward	CCAGACTATTGGACACCAGGTTAGTGAC	60ºC
BMP2 rat reverse	GGGTGCCTTTTGCAGCTGGACTTAAGACG	60ºC
BMP3 forward	CGAAAGCAGTGGGTCGAACCTCGGAAC	60ºC
BMP3 reverse	GGTTATCTACAAGCACAGGAGTCGACTG	60ºC
BMP4 forward	GGGACCAGTGAGAGCTCTGCTTTTC	60ºC
BMP4 reverse	GGGTTGCTTTTCCCGGGTCCATCGAAGG	60ºC
BMP5 forward	GGGAGAGATCCAACGTGAGTGGAAAACG	57ºC
BMP5 reverse	CCGGAATTCAGCTGCCGTCACTGCTTC	57ºC
BMP6 forward	GAATTCAGCTGCCGTCACTGCTTC	56ºC
BMP6 reverse	CAATGACATCCACAAGCTCTCACAACC	56ºC
BMP7 forward	GGTGGCTTTCTTCAAGGCCACGGAGGTTC	58ºC
BMP7 reverse	CAGGATGACGTTGGAGCTGTCGTCGAAG	58ºC
p53 forward	ATGCTGAGTATCTGGACGAC	59,4ºC
p53 reverse	TTCAGCTCTCGGAACATCTC	59,4ºC

p21 rat forward	ACCTTCCAGCTCCTGTAACATACT	62,4ºC
p21 rat reverse	GTCTAGGTGGAGAAACGGGAA	62,4ºC
Bax-1 rat forward	GGGTGGCAGCTGACATGTTT	60ºC
Bax-1 rat reverse	TGTCCAGCCCATGTATGGTTC	60ºC
Apaf-1 rat forward	TCCTGGTCATTCGATGGAAC	58ºC
Apaf-1 rat reverse	TCCAGATCTTGGCGGTCTTAT	58ºC
Caspase 6 forward	GACTGGCTTGTTCAAAGGAG	62ºC
Caspase 6 reverse	CCAGCTTGTCTGTCTGATGAT	62ºC
NGN 1 A forward	CCCGGTGCCCAGGACGAAGAG	60ºC
NGN 1 A reverse	GGGCAGGCCAGGAAAGGAGAAAAG	60ºC
NGN 1 B forward	GCGACCTGTCCAGCTTCCTCAA	58ºC
NGN 1 B reverse	AAGCCTTGCCATTGTATTGTCAGC	58ºC
THA forward	CTAAGGAGCGCCGGATGGTGTG	60ºC
THA reverse	AGTTCTGTGCGTCGGGTGTCTGA	60ºC
THB forward	AGCGCCGGATGGTGTGAGGACT	60ºC
THB reverse	TACTGTCTGCCCGTGATTTTCTGG	60ºC

3.6. Solutions and media

- Ethidiumbromid Solution (0,01%); 10 mg Ethidiumbromid in 100 bidest. H_2O
- LB-Agar: LB-Medium with 15g/l Agar
- LB-Amp-Selective Medium (agar medium after 60ºC cooling, ampiciline where added in concentration 50mg/ml)
- LB Medium: 5g/L Yeast Ekstrakt; 5g/l NaCl and 10g/l Bactotryptone – ph to 7.5 where calibrated with NaOH
- PBS 10x: 1,5 M NaCl; 0,03 M KCl; 0,08 M Na_2HPO_4 x $2H_2O$; 0,01 M KH_2 PO_4
- TAE- Bufor 50x: 2 M Tris-Base; 1M CH_3COOH; 0,1 M EDTH with HCl ph 8,3
- TE Bufor (10/1): 10mM Tris-HCl pH 8,0; 0,1 mM EDTH, pH 8,0
- PFA: PFA-40g in 1 litre of 1xPBS, pH=7.0; heated to 60ºC, 2M NaOH added to solubilize PFA; filter sterilized
- Ampiciline Stocks 50 mg/ml H_2O

- Chloropan – Solution: 50 ml Tris-HCl, pH 8.0; 250 ml Phenol; 240 ml Chlorophorm; 10 ml isoamyloalkohol; 0,5 g 8-Hydroxychinolin,
- DNA –Laderbuffer (10ml): 1 ml Bromophenolblue, 2,5%ig in H_2O; 1ml Xylencyanol, 2,5 % in H_2O; 2,5g gFicoll Type 4000

4. METHODS

All standard molecular methods were performed according to protocols described in “Molecular Cloning” (Sambrook et all. 1989). All cloning steps as well as RT-PCR products were confirmed by DNA restriction analysis and sequencing reactions.

4.1. Eukaryotic cell culture methods

4.1.1. Cell lines

PC12 were a kind gift from Prof. T. Braun. The neural crest-derived, rat pheochromocytoma cell line PC12 is a widely used model of the sympathetic and sensory nervous system (Greene, L.A., and Tischler, A.S. 1976) that responds to nerve growth factor NGF and bone morphogenic protein BMP2.

4.1.2 General components for cell culture

DMEM (Gibco) +0.11 g/l Sodiumpyruvate, with Pyridoxine
RPMI 1640 (Gibco) + L-Glutamine
Penicillin, Streptomycin (Cytogen) 10.000 U/ml, 10 mg/ml
Trypsin/EDTA (Cytogen) 0,05/0,02% in PBS
FCS (Gibco) heat-inactivated for 30 min at 56°C
Horse Serum
G418 (Sigma) 67 mg/ml stock
PBS (1x) 137 mM NaCl
2.7 mM KCl
6.6 mM $Na_2HPO_4x\ 2H_2O$
1.5 mM KH_2PO_4

4.1.3. Passages and Cryoprotection of the cells

For conservation, cells were resuspended in 1 ml of the appropriate cell culture medium with 20% FCS and 10 % DMSO, transferred into a cryotube and frozen in a polystyrene box at -20°C. After 4 h, the cells were transferred to -80°C for 24 h and

subsequently stored in liquid nitrogen. PC12 cell lines were frozen in FCS containing 10% DMSO. For recovery, cells were thaw for 10 min at 37°C, washed with 10 ml medium and transferred to a tissue culture flask.

4.1.4. Growing of the PC12 cells line

PC12 adheres poorly to plastic, and tends to grow in small clusters. They were grown on standard tissue culture plastic dishes without addition of collagen or poly – L – lysine, in 10% FCS, 5% horse serum, DMEM, 100µg/ml penicillin and streptomycin. The doubling time of PC12 is quite long 2,5-4 days. Cells were kept in humified air with 10% CO_2 at 37°C. Insect cells were cultured at 37°C and were split at 60% confluence.

4.1.5. Treatment with the factors

PC12 was treated whit different factors.

Parameters:

Differentiation and treatment was achieved by placing PC12 cells in Petri dishes at 20% density (about 2.5 x 10^4 cells/cm^2) in the presence of 50 ng/ml NGF (SIGMA), 100 ng/ml BMP2 and 250ng/ml PFT alpha in normal growing medium containing 1% serum.

4.1.6. Transient transfection of plasmid DNA

Cells were transfected with FUGENE 6 reagent (ROCHE). Different conditions for the transfection were tested. The best results were achieved by following procedure. After splitting cells were spin off and plated at 20 % confluence and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 5% fetal bovine serum, L-glutamine (final concentration of 4mM), 10 IU penicillin, 10µg/ml streptomycin, PC12 cells were grown at 37ºC with the atmosphere of 10% CO_2 and 90 % air for one day. On the next day transfection mixes were prepared. For effective transfection different proportion between FUGEN 6 reagent, DNA and OPTIMEM Medium were tested. Finally the best

results were achieved by combination of 3µl FUGEN 6 reagent and 1 µg DNA to a total volume of 100 µl. To OPTIMEM medium first DNA was added then FUGENE and mixed delicate with pipette. Mix was incubated in the cell culture chamber up to one hour. For co-transfection amount of transfection reagent was increased in proportion to the amount of total µg DNA. Then normal medium was replaced with DMEM medium containing 0.1% Horse Serum and 10 IU penicillin, 10µg/ml streptomycin. The transfection mix was added to the cells by drop to drop as well as other factors (BMP2, PTF alpha, NGF). Cells were treated with the factors for 2 days.

4.2. Prokaryotic cell culture

4.2.1. Bacterial strains

Two different bacterial strains were used:

SURE E.coli (Stratagene)

XL-1 Blue E.coli (Stratagene)

4.2.2. Cryoconservation of bacteria

500 µl of overnight culture were added to 500 µl of glycerol (87%) in a cryo tube and stored at -80°C.

4.2.3. Preparation of competent cells and transformation

Competent bacteria were prepared using the rubidium chloride method. An overnight culture was diluted 1:100 in LB medium and grown at 37°C and 200 rpm to an OD600 between 0.6 and 0.8. From now on, all steps were performed at 4°C and buffers were ice cold. Cells were kept on ice for 15 min, centrifuged at 1.000 x g for 10 min and cell pellets were resuspended gently and thoroughly in 20 ml RF_1 buffer per 50 ml of starting culture. After a 15 min incubation on ice, cells were centrifuged as above, resuspended in 2 ml RF_2 per 50 ml of starting culture and aliquoted at 200 µl. After 15 min incubation on ice, cells were competent and were used for transformation or stored at -80°C.

For each transformation, 200 µl of competent bacteria were mixed with DNA (e.g. 100 ng plasmid DNA) in a 1.5 ml tube, incubated on ice for 1 h and subsequently heat shocked for 45 sec at 42°C and put on ice for 5 min. After addition of 800 µl LB medium prewarmed to 42°C and an incubation period of 45 min at 37°C and 200 rpm, 100 µl of cell suspension was plated onto LB agar plates containing the appropriate antibiotic for selection. Plates were incubated overnight at 37°C.

RF1: 100 mM RbCl2 RF2: 10 mM MOPS

30 mM K acetate 75 mM CaCl2

10 mM CaCl2 10 mM RbCl2

50 mM MnCl2

15 % (v/v) glycerol 15% (v/v) glycerol

pH 5.8 with acetic acid pH 6.5 with KOH

4.2.4. Culture media and growth conditions

LB-medium:

1% (w/v) Trypton (Becton Dickinson)

0.5% (w/v) Yeast extracts (Difco)

1% (w/v) NaCl

pH 7.0 with NaOH

Agar-plates:

LB-Medium with 1.5% (w/v) Bacto-Agar (Becton Dickinson)

Overnight cultures were usually grown in LB medium at 37°C and 220 rpm. The medium was inoculated with bacteria kept on agar plates at 4°C or from cryoconserved cultures (2.4.3).The medium/agar plates, depending on the properties of the plasmid being introduced, were supplemented with a final concentration of one or several of the following:

Ampicillin 100 µg/ml

Kanamycin 25 µg/ml

Chloramphenicol 20 µg/ml

IPTG 0.5 mM

X-Gal 100 mM

4.2.5. Phenol- chloroform extraction of circular DNA

Equal volumes of phenol: chloroform: isoamylalcohol (P:C:IAA ration of 25:24:1; pH8.0) was added to the cell lysates and mixed. Mixtures were spun for 2 min at top speed in a table top centrifuge. Top aqueous layers containing plasmid DNA were transferred to clean tubes and mixed with equal amounts of P:C:IAA and proceeded as mentioned above. This was repeated until no protein precipitate was visible at the interphase (but at least three times). NaAcetate solution (pH 5.2) was added to the supernatants to a final concentration of 0.3M. 2.5 volumes of ethanol (100%) were added and samples were incubated at –20°C for at least 30 min to precipitate DNA. Samples were centrifuged at 4°C at top speed in a table top centrifuge (Eppendorf) for 20 min. Supernatants were removed, and the remaining precipitates were washed once with 500 µl of Ethanol (70%) pre-chilled to -20°C. Samples were centrifuged for 5 min, supernatants removed and the pellets air dried in a heating block at 50 °C. Pellets could then be resuspended in H_2O containing RNAaseA (100 µg/ml).

4.2.6. Preparation of the RNA from cell culture

For a RNA preparation TRIZOL reagent were used. All steps were performed according to manufacturer's protocol. Concentration of RNA was determined with Eppendorf BioPhotometer by measuring absorbance at 260 nm.

Cells were denaturing by TRIZOL reagent then all substances were removed to a new tube. The first step was removing DNA from RNA.

3 µg RNA

1 µl RQ RNAase – Free DNase 10x buffer

1 µl RQ RNAase – Free DNase (1U/µl)

and water to 10µl

Incubation was carried out at 37°C for 30 minutes followed by incubation at 65°C for 10 minutes. After incubation 1µl of RNA threated with DNAase was reversed transcribed in mixture containing the following elements:

3,5µl DNase- treated RNA

1µl oligo d(T)

5µl dNTP's (2mM)

3µl H_2O

---------- Total

12,5 µl

The mixture was incubated at 65°C for 10 min. and then immediately replaced to ice. Following reagents were added to the above mix.

4µl DNase- treated RNA
2µl DTT(0.1M)
0.5µl RNAasin (40U/µl)
Super Script II Reverse Transcriptase (200U/µl)
1µl Transcriptase
---------- Total
20,0 µl

RNA was reverse transcribed for 1 h. at 42°C followed by incubation at 70°C for 15 minutes. Obtained cDNA was immediately used for RT-PCR or stored at -20°C.

4.2.7. Electrophoresis of the RNA

To check a quality of obtained RNA electrophoresis was performed at 140V.

4.2.8. Sequencing of the positives clones

For the sequencing analysis the following cocktail was prepared:

Setting up the reaction mix (total volume 10 µl):

- Ready Reaction Premix 4µl (A, C, G, T and AmpliTaq® DNA Polymerase)
- 5x Big Dye Sequencing Buffer 2µl
- Primer /Sp6 or T7/ 3.2pmol
- Plasmid temple /200ng/
- H_2O up to 10 µl

Synthesis of the probe – following reaction conditions were used

96ºC – 2 min

96ºC – 30 sec

52ºC – 15 sec for T7 sequence primer for the T3 and 46ºC for Sp6 annealing temperature was used

60ºC – 4 min

Program was repeated 25 times

Hold temperature at 4ºC

Cleaning up the reaction by ethanol precipitation:

- add 5 µl 125 mM EDTA to reaction
- add 75 µl 96% Ethanol to the tube
- mix by inverting sever time
- incubate at room temperature for 15 min
- Spin down: 13 000rpm, 4ºC, 15 min
- Carefully remove the supernatant
- Add 70 µl 70% Ethanol
- Incubate at room temperature 5 min
- Spin down 13000 rpm, 4ºC, 5 min
- Carefully remove the ethanol
- Air dry at room temperature for 5 min

4.2.9. Enzymatic modification of DNA

Digestion of DNA with specific restriction endonucleases, dephosphorylating with alkaline phosphates and ligations were performed with enzymes and the suitable provided buffers from PROMEGA or New England Biolabs according to the manufacturer's instructions.

4.2.10. Amplification of DNA

Standard PCR reactions were carried out in a final volume of 50 µl with the following components: 200 ng of template DNA, two specific primers (5 pmoles each), 250 µM of each dNTPs, 3 mM $MgCl_2$, 1x polymerase buffer, 5 U Taq/Pfu polymerase and H_2O in a 0.2 ml PCR tube. DNA fragments were amplified with the appropriate oligonucleotide primers listed in table 2 in an Eppendorf thermocycler. The length of denaturation, annealing and elongation were dependent on the characteristics of the DNA template (e.g. genomic DNA or plasmid DNA, GC-content of the fragment), the primers (the optimal annealing temperature was usually estimated experimentally starting with a value calculated according to the formula 4x (G/C) + 2x (A/T) - 5) and the length of the amplified PCR product.

4.2.11 RT PCR

For each reaction RNA was isolated with TRIZOL reagent from the cells and probe was suspended in DEPC water. To ensure purity and to measure RNA concentration, it was analysed spectrometrically and an aliquot was run on a 1% agarose gel.
1µl RNAasin was added to each probe. After that 3µg of the each were treated with 1 µl DNAaseI RNAase free (PROMEGA) in total 10µl mix for 30 min.
DNAaseI were deactivated at 65ºC for 15 min. Reaction mix was prepared -3.5 µl RNA /DNA free/ + 1µl oligo dT + 5µl 2mMdNTP + 1.5µl H_20. Reaction were incubated at 65ºC for 5 min and immediately put on ice for annealing of the oligo dT primers. To each tube 4µl 5x reaction buffer, 4µl DTT, 1µl RNAasin and 1µl reverse Transcriptase Superscript II (Invitrogen) were added. Total 20µl reaction mixes were incubated for 60min at 42ºC for cDNA synthesis.

4.3. Tissue sections

4.3.1. Paraffin embedded tissue sections

1. After isolation tissues were embedded in paraffin solution
2. Left overnight in paraffin and 4% PFA
3. After incubation washed in PBS and then 1h 25% EtOH, 1h 50% EtOH, 1h 75% EtOH, 1h 96% EtOH, 2x10 min in 100% EtOH and left over night.
4. On the next day tissues were washed in Xylol 2x1h RT and then in mixture Xylol/Parafin 2h 60ºC and again 1h at 60ºC.
5. Tissues were cut from paraffin blocks on Leica microtome and mounted from warm water (42ºC) onto Vectabond slides. Sections were allowed to dry on heating block (40ºC) and left for 6h and then stored at room temperature.

4.3.2. Immunohistochemistry

PC12 cells were plated for transient transfection onto 2,5cm plate dish in six well plates. 48 h after transfection cells were washed once with PBS and fixed for 20 min at RT with 3% paraformaldehyde (PFA) in PBS. Cells were washed three times with PBS, permeabilised with 0.2% Triton X100 in PBS for 10 min, residual PFA was inactivated with 100mM Glycin in H_2O for 10 min. Subsequently, cells were incubated with 10% FCS in PBS for 10 min to block unspecific protein binding sites. The primary antibodies were diluted in 2% FCS in PBS and incubated with the cells for 1 h at 37°C in a humid chamber (100 µl per cover slip). Afterwards, cells were washed three times with PBS and incubated with the secondary antibodies diluted in 2% FCS in PBS for 45 min at 37°C, subsequently washed three times with PBS. Finally, the DNA was stained with DAPI in PBS (1 µg/ml, 10 min, RT) and cells were washed three times with PBS.

4.3.3. Antibodies

Anti Myc Tag antibody was purchased from Hybridoma Bank and used in dilution 1:1000.

Anti p53 antibody.

Rabbit Polyclonal Antibody anti p53 was used in 1:1000 dilution and purchased from Novacastra Laboratories Ltd.

Anti Tubulin alpha antibody - was used in 1:1000 dilution and purchased from Developmental Studies Hybridoma Bank University of Iowa, Department of Biology.

4.3.4. Immunofluorescence and fluorescence microscopy

Efficiency of transfection was analysed under fluorescent microscope. Cells were plated for transient transfection onto six well plates. After adhering overnight, cells were changed to differentiation medium and treated with factors for 48 hours. Immunofluorescent staining occurred in four steps. 48 h after transfection cells were washed once with PBS and fixed for 20 min at RT with 3% paraformaldehyde (PFA) in PBS. Cells were washed three times with PBS, permeabilised with 0.2% Triton X100 in PBS for 10 min, residual PFA was inactivated with 100mM Glycin in H_2O for 10 min to block unspecific protein binding sites. Primary antibody was applied overnight at 4ºC; 1:2 anti-MycTag served as experimental conditions, whereas parallel experiments with 1:500 mouse IgG served as negative controls. Afterwards, cells were washed three times with PBS and incubated with the secondary antibodies diluted in 2% FCS in PBS for 45 min at 37°C in a humid chamber, subsequently washed three times with PBS. Finally, the DNA was stained with DAPI in PBS (1 µg/ml, 10 min, RT) and cells were washed three times with PBS. PBS was removed by a short washing step in distilled water and the cover slips were mounted in 30 µl Mowiol containing 25 mg/ml DABCO.

Blocking solution:

10% (v/v) FCS in PBS

3% (w/v) Paraformaldehyde in PBS with 0.1 mM $CaCl_2$ and 0.1 mM MgCl2, pH 7.4

0.1-0.2% (v/v) Triton X-100 in PBS

Mowiol 4-88 (Calbiochem), DABCO (Sigma) 25 mg/ml, DAPI (1 mg/ml stock)

Secondary antibodies: Alex 594 anti-rabit mouse (1:1000)

4.3.5. TUNEL analysis

TUNEL analysis was performed on second day after transfection. For TUNEL analysis I have used ROCHE diagnostic kit and following procedure.

1. Cells were fixed with a freshly prepared Fixation solution (4% Paraformaldehyde in PBS, pH 7.4, freshly prepared) for 30 min at 15-25ºC.
2. Plates were rinsed with PBS.
3. Incubated with blocking solutions for 10 min at 15-25ºC.
4. Rinsed twice with PBS.

For a positive control:

Incubated, fixed and premeabilized cells with DNAaseI I (100µg/ml in PBS) for 10 min at 15-25ºC to induce DNA strand breaks, prior to labelling procedures. During this time TUNEL reaction mix were prepared.

Preparation of TUNEL reaction mixture

Sol A: Dilute 50µl of vial I (enzyme solution) with 100µl dilution buffer

Sol B: Add 550µl of PBS to vial 2 (label solution), then transfer 200µl to an extra tube for the negative control and finally add 50µl of Sol A to the rest of vial 2. This will yield 950µl.

The TUNEL reaction mixture should not be stored. Keep TUNEL reaction mixture on ice until use.

5. Rinsed twice with PBS.
6. Added TUNEL reaction mixture (as much as needed; about 100µl) on positive control samples and real test samples.

For a negative control:

Incubated, fixed and permeabilized cells in Label Solution (without terminal transferase) instead of TUNEL reaction mixture.

7. Add lid and incubate for 60 min at 37ºC in a huminidfied atmosphere in the dark.
8. Plates were rinsed 3 times with PBS.
9. Samples were analyzed in PBS under a fluorescence microscope.

4.3.6. LacZ staining

Followed solutions were used:

Solution A – PBS (100 mM, pH=7,4)

Solution B – (0,2% gluteraldehyde (GDA) in PBS)

Solution C – 0,01% Na desoxycholate and 0,02% Nonidet P-40 in PBS containing 5mM EGTA and 2mM $MgCl_2$.

Solution D stain: 0,5 mg/ml X-gal, 10mM K_4 $(Fe(CN)_6)$ and 10 mM K_4 $(Fe(CN)_6)$ in solution C

Procedure:

1. Cells were washed 3 times in PBS.
2. Cells were fixed in solution B for 5 min at RT.
3. Then 5 min washed with solutions C at RT.
4. Solution C were replaced with solution D and incubated at 37ºC.
5. After staining for 24 hours samples were washed in solution C.
6. Samples were analyzed under light microscope.

5. DISCUSSION

5.1. Nkx5-1–specific potential to induce apoptosis in PC12 cells

In this work I demonstrated a new function for the homeodomain-containing transcription factor, Nkx5-1, in the neuronal PC12 cells.

In PC12 cells Nkx5-1 displays a potential to very efficiently (almost 100% of Nkx5-1 overexpressing cells) induce apoptosis. It is striking that a neuronal differentiation and survival factor, NGF, is not able to prevent apoptosis induction caused by the forced Nkx5-1 expression. A role in apoptosis induction was already postulated for Nkx5-1 during early development of the inner ear. There, Nkx5-1 acts in concert with BMP4, and, possibly with another homedomain-containing transcription factor, Dlx5, to induce apoptosis within clearly defined regions of the epithelial cells forming the inner ear vesicle (Merlo et al., 2002; Bober et al., 2003; Herbrand et al., 2007). In addition, during inner ear development Nkx5-1 positively influences expression of netrin-1, a molecule involved in neuronal survival, axon guidance and chemotaxis (Salminen et al., 2000; Bober et al., 2003). This interaction appears especially interesting in connection with our demonstration, that another molecule, a cellular regulator p53, is necessary for the apoptotic function of Nkx5-1. p53 has been shown to directly activate transcription of human netrin-1 dependence receptor, Unc5B (Arakawa, 2005). It was postulated that p53 might create a state of cellular dependence on netrin-1 for survival: in the absence of netrin-1, p53 induced Unc5B behaves as a death receptor and leads to apoptosis, while in the presence of ligand (netrin-1) survival prevails (Arakawa, 2005). The exact molecular interrelationships between Nkx5-1, p53, and netrin-1, as well as their significance in neuronal cells remain to be established.

In previous investigations the possibility of redundant functions of the two closely related Nkx5-1 and Nkx5-2 genes was discussed. Especially, the lack of any apparent neuronal phenotype in Nkx5-1/Hmx3 knockout mice seemed to indicate that both genes might functionally substitute for each other (Hadrys et al. 1998; Wang et al., 2004). Unexpectedly, our results revealed that the apoptosis induction in PC12 cells could be executed specifically by the Nkx5-1. The region responsible for this

function was localized to the non-conserved N-terminal part of the Nkx5-1 protein and logically, the Nkx5-2 protein cannot substitute for this function.

The question remains why no neuronal defects could be detected in Nkx5-1 deficient mice despite the fact, that this protein fulfils specific function in neuronal cells? One possibility is that Nkx5-1 plays a minor role in a subpopulation of neurons and the defect possibly caused by Nkx5-1 deficiency is too discrete for an easy detection. In fact, a minor decrease in apoptosis in adult brain tissues of Nkx5-1 knockout mice was observed using RT-PCR gene expression analysis and TUNEL staining on brain sections (see Fig. 29). Another, more plausible explanation is the possibility that the apoptotic Nkx5-1 function gets into action first after neuronal cells are challenged with neurotoxic or other kinds of stresses. Such a possibility would agree with known p53 function, a molecule, which is obviously necessary for Nkx5-1 to exert its apoptotic function. P53 can prompt PC12 cells to undergo neuronal differentiation under favourable conditions but under neurotoxin treatment p53 is responsible for removal of defected cells by apoptosis (Zhang et al., 2006). Nkx5-1 might be also involved in both functions. Nkx5-1 endogenous expression is detectable at low levels already in growing PC12 cells but its expression increases significantly during neuronal differentiation indicating a functional relevance of Nkx5-1 in this process. Such a function of Nkx5-1 protein has still to be elucidated by future experiments. The here demonstrated role of Nkx5-1 in apoptosis induction might have physiological relevance under stress conditions, since the neuronal PC12 cells expressing normal endogenous Nkx5-1 levels do not undergo apoptosis. Apoptosis is first induced by supraphysiological amounts of Nkx5-1 protein in the cell. Whether and under what circumstances such an induction of Nkx5-1 takes place in vivo remains to be demonstrated.

5.2. Nkx5-1 apoptotic activity resides within the non-conserved N-domain

Functional analyzes using constructs expressing a combination of different parts of Nkx5-1 and Nkx5-2 proteins (swapping constructs) revealed that apoptosis inducing activity is confined to the N-terminus of the Nkx5-1 molecule. For the generation of the swapping constructs a conserved XhoI restriction site, residing at the N-terminal part of the homeobox was chosen to produce two separate fragments of each Nkx5 of cDNAs. The Nkx5-1/2 construct expressing a hybrid molecule consisting of the Nkx5-1 N-terminus fused to the C-terminal part of Nx5-2 was found to induce apoptosis at the efficiency similar to the full-length wild-type Nkx5-1 protein. An analogous Nkx5-2/1 construct expressing the N-terminus of Nkx5-2 joined to the Nkx5-1 C-terminus did not show any apoptosis induction. These results clearly document that sequences responsible for apoptosis induction are located in the Nkx5-1 region upstream of XhoI restriction site present within the homeobox. Since the small part of the Nkx5-1 homeobox protein, which was retained in the Nkx5-1/2 construct was identical to Nkx5-2 it is clear that the apoptotic potential is encoded by the non-conserved N-terminal part of the Nkx5-1 protein. This entire region does not show any sequence similarity between both Nkx5 proteins. Moreover, the Nkx5-1 N-terminal region (132 amino acids) is much longer than that of the Nkx5-2 (78 amino acids: see fig. 1, introduction). To find potential domain or sequences responsible for an apoptotic effect computer analysis of the existed databases was performed. However, no sequence motives which could be potentially responsible for an apoptotic effect were identified.

To investigate whether the Nkx5-1 N-terminus of the Nkx5-1/2 swapping construct contains entire sequence information responsible for apoptotic activity further experiments were performed. As mentioned above apoptosis induction by overexpression of the native Nkx5-1 protein in PC12 cells was not influenced by NGF. Similarly, addition of NGF to the cell culture medium did not prevent apoptosis in the case when the cells were transfected with Nkx5-1/2 construct. Interestingly, different behaviour of cells transfected with Nkx5-1/2 construct as compared to wild-type Nkx5-1 transfection was observed after PFT alpha treatment. In contrast to PFT effects observed after wild-type Nkx5-1 transfection PFT alpha

did not block apoptosis induced by Nkx5-1/2 protein. Cells overexpressing Nkx5-1/2 construct underwent apoptosis even in the presence of PFT alpha. This observation suggested that apoptosis induced by Nkx5-1/2 hybrid protein was not p53-dependent. Summing up, our results indicate that sequences responsible for apoptosis of Nkx5-1 protein reside within the non-conserved N-domain, however the mechanism of action and additional factors involved in these processes need to be elucidated in the future.

5.3. Apoptosis induced by Nkx5-1 and Nkx5-1/2 swapping constructs is not influenced by NGF and BMP-2

It is well known that NGF exerts a strong survival promoting and anti-apoptotic function in neuronal cells (Truong LX Nguyen et al., 2010). However, our data indicated that almost all Nkx5-1 transfected cells underwent apoptosis even in the presence of NGF. This is a surprising and unexpected finding, because NGF is known to possess strong anti-apoptotic and pro-surviving activities. NGF binds to at least two classes of receptors: p75 LNGRFR (low affinity nerve growth factor receptor) and TrkA (high-affinity tyrosine kinase receptor). Binding of the receptor leads to its phosphorylation, which in turn activates down-stream effectors such as PI3 kinase, ras and PLC signalling pathway (Iannone et. al., 2002). Lack of apoptosis inhibition by NGF in Nkx5-1 and Nkx5-1/2 overexpressing cells could be explained by the fact that there is no direct connection between pathways regulated by these two proteins. Endogenous Nkx5-1 expression in PC12 cells is increased under treatment with NGF, however this higher NGF-induced Nkx5-1 expression does not promote apoptosis (see above). This might indicate that Nkx5-1 has also functions in neuronal differentiation in addition to apoptosis induction. Such function has still to be elucidated in future experiments.

Considering the previously discussed interrelationships between Nkx5-1 and BMPs in apoptosis I also investigated the influence of BMP2 on Nkx5-1 induced apoptosis in PC12 cells. Similarly to NGF, BMP2 does not grossly affect apoptosis induction by Nkx5-1 overexpression. However, BMP2 was able to induce apoptosis in PC12 cells apparently without Nkx5-1 overexpression. The potential mutual influence between endogenous BMP2 and Nkx5-1 proteins in PC12 cells cannot, of course, be excluded and requires further investigation. Based on the current data, the possible

involvement of BMP-signalling in the Nkx5-1 dependent apoptosis in PC12 cells is still unclear. Nevertheless, BMP-signalling seems to be very important for the Nkx5-1 gene activity, since treatment of PC12 cells with the BMP-inhibitor, noggin, reduces Nkx5-1 transcription.

Moreover, it is known that activation of the p38 MAP kinase pathway is necessary for BMP-2-induced neuronal differentiation of PC12 cells. The activation of the p38 MAP kinase pathway alone can induce the neuronal differentiation of PC12 cells (Iwasaki et al., 1999). Even if the new data provided by this work does not allow placing of Nkx5-1 within the signalling pathway controlled by NGF and BMP2 in PC12 cells, they provide a basis to plan future experiments investigating the exact molecular role of Nkx5-1 in these pathways.

5.4. p53 as a potential target for Nkx5-1?

P53 plays an important role in cell differentiation, proliferation and apoptosis in PC12 and other cells. In un-stressed cells p53 is inactivated by its regulator, mdm2. Upon DNA damage or other stress, various pathways will lead to the dissociation of the p53 and mdm2 complex (Brady et al., 2005). Once activated, p53 will either induce a cell cycle arrest to allow repair and survival of the cell or apoptosis to discard the damaged cell (Edward Estli et al., 2011). P53 is necessary for the elimination of neural cells inappropriately differentiated or in response to various stimuli (Edward Estli et al., 2011). Recent data showed that nerve growth factor (NGF)-mediated differentiation in PC12 cells is enhanced by overexpression of wild-type p53 but inhibited by knockdown of endogenous wild-type p53 (Zhang et al., 2006). Interestingly, p53 knockdown or overexpression of a dominant negative p53 mutant attenuates NGF-mediated activation of TrkA, the high-affinity receptor for NGF and consequently inhibits of the mitogen-activated protein kinase pathway (Zhang et al., 2006). In addition, p53 knockdown reduces the constitutive levels of TrkA, which renders PC12 cells inert to NGF. Taken together this data demonstrate that p53 plays a critical role in NGF-mediated neuronal differentiation in PC12 cells at least in part via regulation of TrkA levels (Zhang et al., 2006). How does p53 make a choice between activation of differentiation pathways or apoptosis induction is currently unknown. Without inhibitor, p53 could be easily detected in Nkx5-1

transfected cells using immunohistochemistry, while no p53 expression in Nkx5-2 overexpressing cells could be detected. These findings suggested that Nkx5-1 induced p53-dependent apoptosis and that the induction of higher levels of p53 expression was essential for apoptosis induction in Nkx5-1 overexpressing cells. Moreover, NGF does not interfere with p53 induced by Nkx5-1 overexpression. Under NGF treatment p53 expression is found essentially only in Nkx5-1 overexpressing cells, also approximately only a half up to 2/3 of Nkx5-1 positive cells switch on the p53 expression. After BMP2 treatment the majority of Nkx5-1 positive cells also activate p53 expression but, in addition, almost the same number of cells activates p53 without Nkx5-1 overexpression. These particular cells are eliminated after addition of noggin, thus confirming that BMP2 activates p53 independently of exogenous Nkx5-1. PFT alpha treatment generally leads to a substantial reduction of p53 positive cells.

Similar experiments were performed using Nkx5-2 overexpressing plasmid for transfections. Nkx5-2 does not possess any potential for p53 activation. These results undermine that Nkx5-1 specific function uncovered in this work. Interestingly, different target sequences were already reported for Nkx5-1 and Nkx5-2, what suggested that specific non-overlapping functions exist for these two closely related homebox genes (Mennerich et al., 1999).

5.5. Activation of Nkx5-1 promoter in PC12 cells by NGF and BMP2 correlates with neuronal differentiation

Proper tissue development and homeostasis require a balance between apoptosis and cell proliferation. All somatic cells proliferate via a mitotic process determined by progression through the cell cycle. Apoptosis (programmed cell death) occurs in a wide variety of physiological settings, where its role is to remove harmful, damaged or unwanted cells (Alenzi, 2004). Apoptosis and cell proliferation are linked by cell-cycle regulators and apoptotic stimuli that affect both processes. Cell proliferation, differentiation and death are fundamental processes in multicellular organisms, and several lines of evidence link apoptosis to proliferation (Alenzi, 2004). A number of dominant oncogenes (e.g. c-Myc) appear to induce apoptosis, which suggests that the cell proliferation and apoptosis pathways are closely linked (Alenzi and Faris, 2004).

Development of the semicircular canals in the vestibular part of the inner ear requires the independent control of several homeobox genes, which appear to exert their function via tight regulation of BPM4 expression and the regional organization of cell differentiation, proliferation, and apoptosis (Merlo et al., 2002). The linkage between proliferation and apoptosis can also be seen in PC12 cells on example of Nkx5-1 promoter activity. The activity of Nkx5-1 promoter construct in PC12 is positively regulated by NGF, a critical regulator of neuronal survival and differentiation. Another differentiation promoting factor, BMP2, is also able to induce Nkx5-1 promoter activity in PC12 cells. In line with these findings, NGF and BMP2 also activate expression of the endogenous Nkx5-1 gene, when added to PC12 cells separately. In contrast, addition of both factors simultaneously led to inhibition of Nkx5-1 transcription suggesting negative cross-regulatory circuits between these two proteins and Nkx5-1 promoter activation. The specificity of BMP2-dependent activation of Nkx5-1 promoter was confirmed by treatment of transfected cells with BMP2 inhibitor noggin. Addition of noggin strongly reduced the activity of the ß-gal reporter indicating inhibition of the Nkx5-1 promoter. These results clearly document the presence of sequences convening the basal Nkx5-1 promoter activity on the genomic fragment used for the construction of Nkx5-1-ß-gal reporter plasmid. In addition, regulatory elements responsible for the activation of Nkx5-1 gene transcription by NGF and BMP2 are also present within these sequences.

In addition to its survival promoting function NGF strongly induces neuronal differentiation. In fact, the highest ß-gal staining intensity was observed in cells positive for the neuronal differentiation marker ß-tubulin III. In contrast, cells transfected with Nkx5-1 promoter construct and cultivated without NGF displayed only weak, basal-level of the ß-gal activity and no ß-tubulin III immunoreactivity.

Summing up, our results indicate that NGF and Nkx5-1 play essential role during apoptosis and differentiation of neuronal cells. Recent data postulate a role for Nkx5-1 and Nkx5-2 as cell autonomous, redundant factors required for cell fate specification and differentiation during inner ear and lateral line development in zebrafish (Feng and Xu, 2010). Knockdown of both Nkx5-1 and Nkx5-2 in zebrafish, disrupts formation of the mechanosensory neuromasts and also leads to impaired vestibular function in which utricular maculae fail to develop and the

utricular otolith gradually fuses with the saccular otolith. They have also been shown to function redundantly to control embryonic development of the central nervous system (Feng and Xu, 2010).

5.6. Proposed Nkx5-1 function in neural development in connection with p53

This work describes a novel role of Nkx5-1 in neuronal differentiation and apoptosis in addition to the already well-studied Nkx5-1 function during inner ear morphogenesis. Using the established PC12 cell line, which is the convenient in vitro model to study neuronal differentiation in dependence on various factors such as NGF and BMP-2, we could place the Nkx5-1 in the neurogenesis-specific signalling pathways as schematically summarized in Fig. 24. The main novel finding concerns a double role of Nkx5-1 in activation of neuronal differentiation on one side and activation of apoptosis on the other. I found that both Nkx5 genes transcription can be increased by NGF-signalling, however neurogenesis-promoting activity is specific only for Nkx5-1. Another Nkx5-1 specific function is induction of apoptosis, partly in cooperation with BMP-2 and p53. Further elucidation of the exact mechanisms of Nkx5-1 apoptotic and neurogenesis-promoting effects will lead to a better understanding of the role of other factors (BMPs, p53, p21) in neuronal development.

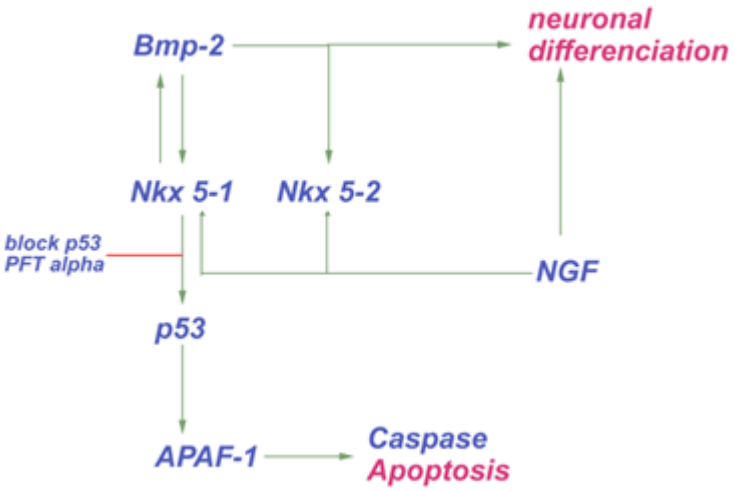

Fig.24. Schematic summary of Nkx5-1 function in neuronal differentiation and apoptosis and cooperation with NGF, BMP-2 and p53.
See text for further explanations.

6. SUMMARY

Nkx5-1 and Nkx5-2 are two strongly related proteins, which play important roles in the inner ear and neuronal development. Expression pattern and in vivo consequences of elimination of Nkx5 genes in the mouse are well described. In contrast, little is known about their regulation and function in neuronal cells. The main aim of this work was investigation of Nkx5-1 and Nkx5-2 functions during differentiation of neuronal cells.

PC12 pheochromocytoma cell line derived from rat chromaffin cells of the suprarenal medulla, which can be easily induced to neuronal differentiation, was used as an experimental system. In addition to the endogenous Nkx5 genes expression, overexpression of both Nkx5 tagged proteins was employed. Furthermore, an Nkx5-1 promoter activity was monitored using promoter-LacZ-reporter construct.

The main finding of this work was discovery of an Nkx5-1 specific function in induction of apoptosis. Overexpression of Nkx5-1 protein in PC12 cells led to apoptosis induction while overexpression of Nkx5-2 had no effect on apoptosis. This was the first description of a molecular function specific for only one of both Nkx5 proteins. Thus far, functional redundancy was postulated for both proteins.

In the further course of this work I analyzed the modalities of the Nkx5-1 apoptosis promoting function. Since apoptosis is regarded as an integral part of the neuronal differentiation program I investigated the possible cross-regulation with neuronal differentiation factors NGF and BMP. Surprisingly, NGF and BMP did not prevent apoptosis induction by Nkx5-1. My results indicate that Nkx5-1 induces apoptosis by utilization of the p53-dependent pathway.

I further performed mutation analysis using different Nkx5-1 and Nkx5-2 domain swapping constructs to delineate the protein region responsible for the Nkx5-1 specific function. An N-terminus of the Nkx5-1 protein was identified as responsible for the apoptosis induction. This part encompasses sequences, which are not conserved between the two Nkx5 proteins. In agreement with an importance of apoptosis during

neuronal differentiation, I observed higher Nkx5-1 expression in cells induced to differentiate. This observation was confirmed by a higher activity of the Nkx5-1 LacZ-promoter construct during neuronal differentiation. My results document that DNA sequences within 10.5 kb upstream of the transcription site of the Nkx5-1 gene bear regulatory elements responsible for the Nkx5-1 activity in neuronal cells. This construct contains also sequences responsive to the NGF and BMP signals.

In the final part of this work I performed experiments on the Nkx5-1 knockout mice to find out, whether my in vitro results correlate with the Nkx5-1 function in vivo. In fact, I observed diminished apoptosis signals on the brain sections of Nkx5-1 knockout mice as compared with wild type controls. Moreover, a lower level of expression of neuronal marker genes was detected in Nkx5-1 knockout mice. These results confirm, that Nkx5-1 exerts regulatory role in neuronal tissues in vivo.

7. ZUSAMMENFASSUNG

Nkx5-1 und Nkx5-2 sind zwei eng verwandte Proteine, die wichtige Rollen in der Entwicklung des Innenohrs und der neuronalen Strukturen spielen. Die Expression der Nkx5 Gene sowie die Folgen deren Ausschaltung in der Maus sind bereits beschrieben. Es ist jedoch wenig über die Regulation und Funktion der Nkx5 Gene in neuronalen Zellen bekannt. Das Hauptanliegen dieser Arbeit ist daher eine funktionelle Untersuchung der Nkx5-1 und Nkx5-2 Gene während der Differenzierung von neuronalen Zellen.

PC12 Pheochromocytoma Zelllinie wurde als Untersuchungssystem gewählt. Diese Zelllinie wurde ursprünglich von Chromaffin-Zellen der Suprarenal Medulla der Ratte etabliert. Neben der Untersuchung der Expression der endogenen Nkx5 Gene wurden Nkx5 überexprimierende Konstrukte verwendet. Des weiteren wurde ein LacZ-reporter Konstrukt zur Untersuchung von Nkx5-1 Genpromotors-Aktivität generiert.

Den wichtigsten Befund dieser Arbeit stellt die Aufdeckung einer Nkx5-1 spezifischen Funktion bei der Induktion der Apoptose dar. Während eine Überexpression des Nkx5-1 Proteins in den PC12 Zellen die Apoptose induzierte, löste dagegen eine Überexpression des Nkx5-2 Proteins keinen derartigen Effekt aus. Mit diesem Befund wurde zum ersten Mal eine molekulare Funktion, die spezifisch für nur eins der beiden Nkx5 Proteine ist, definiert. Bislang wurde eine funktionelle Redundanz der beiden Proteine postuliert.

Im weiteren Verlauf dieser Arbeit habe ich die pro-apoptotische Wirkung des Nkx5-1 Proteins genauer untersucht. Da Apoptose als ein wesentlicher Bestandteil des neuronalen Differenzierungsprogramms betrachtet wird, habe ich die potentiellen Wechselwirkungen mit den neuronalen Differenzierungsfaktoren NGF und BMP untersucht. Überaschenderweise haben weder NGF noch BMP mit der Nkx5-1 induzierten Apoptose interferiert. Meine Daten weisen darauf hin, dass die Nkx5-1 induzierte Apoptose im p53-abhängigen Signalweg erfolgt.

Um die Proteinbereiche, die für die Nkx5-1 spezifische Funktion verantwortlich sind zu definieren, habe ich s. g. „domain swapping“ (Domänenaustausch) Konstrukte verwendet. In diesen Experimenten konnte der N-Terminus des Nkx5-1 Proteins als verantwortlich für die pro-apoptotische Wirkung identifiziert werden. Dieser Proteinbereich beinhaltet Sequenzen, die unter den beiden Nkx5 Proteinen nicht konserviert sind. Die endogene Nkx5-1 Expression steigt während der neuronalen Induktion und spiegelt möglicherweise die wichtige Rolle des Apoptosevorgangs in der neuronalen Differenzierung wieder. Die erhöhte transkriptionelle Aktivität des Nkx5-1 Gens in differenzierten Zellen wurde zusätzlich durch die Bestimmung der Nkx5-1 Promoter-Aktivität mittels LacZ-Reporter Konstrukts bestätigt. Meine Ergebnisse zeigen, dass sich die regulatorischen Promotorelemente, die für die Nkx5-1 Genaktivität in neuronalen Zellen verantwortlich sind, in einem 10,5 kb Bereich 5´von der Transkriptionsstartstelle befinden. Dieses Genfragment beinhaltet ebenso Sequenzen, die durch NGF und BMP Signale reguliert werden.

Im letzten Teil dieser Arbeit habe ich Apoptosevorgänge sowie Expression verschiedener neuronaler Marker im CNS der Nkx5-1 knockout Maus durchgeführt. Ich habe eine verminderte Apoptose in verschiedenen Gehirnregionen der Nkx5-1 -/- Maus dokumentiert. Weiterhin, wurden niedrige Expressionslevel von neuronalen Markergenen gefunden. Diese Daten deuten auf eine regulatorische Rolle von Nkx5-1 im neuronalen Gewebe in vivo.

8. ABBREVIATIONS

bp	base pair
BMP2	Bone Morphogenetic Protein 2
BSA	Bovine Serum Albumine
cDNA	DNA complementary to RNA
CNS	Central Neuron System
EtOH	Ethanol
FCS	Fetal Calf Serum
kb	kilo base pair
min.	minute
mRNA	messenger RNA
NGF	Neuron Growing Factor
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
TUNEL	Terminal deoxynucleotidyl transferase mediated UTP Nick End
Wt	Wild type
PC12	cell line

9. REFERENCES

Agar A, Yip SS, Hill MA, Coroneo MT.
Pressure related apoptosis in neuronal cell lines.
J Neurosci Res. 2000 May 15;60(4):495-503.

Akassoglou K.
Nerve growth factor-independent neuronal survival: a role for NO donors.
Mol Pharmacol. 2005 Oct;68(4):952-5. Epub 2005 Jul 26.

Akin ZN, Nazarali AJ.
Hox genes and their candidate downstream targets in the developing central nervous system.
Cell Mol Neurobiol. 2005 Jun;25(3-4):697-741.

Alenzi FQ.
Links between apoptosis, proliferation and the cell cycle.
Br J Biomed Sci. 2004;61(2):99-102.

Althini S.; Usiskin D., Kylberg A.
Bone morphogenetic protein signalling in NGF-stimulated PC12 cells
Biochem Biophys Res Commun. 2003 Aug 1;307(3):632-9.

Alves da Costa C., Mattson M.P., Ancolio K., Checler F.
The C-terminal fragment of preselin 2 triggers p53-mediated staurosporine-induced apoptosis, a function independent of the presenilinase-derived N-terminal caunterpart,
J.Biol.Chem. 278 (2003) 12064-12069

Arthur DB, Akassoglou K, Insel PA.
P2Y2 receptor activates nerve growth factor/TrkA signaling to enhance neuronal differentiation.
Proc Natl Acad Sci U S A. 2005 Dec 27;102(52):19138-43. Epub 2005 Dec 19.

Asha L. Bhakar, Jenny L. Howell, Christine E. Paul, Amir H. Salehi,
Esther B. E. Becker, Farid Said, Azad Bonni, and Philip A. Barker
Apoptosis Induced by p75NTR Overexpression Requires Jun Kinase-Dependent Phosphorylation of Bad
The Journal of Neuroscience, 2003, 23(36):11373-11381

Bhakar AL, Howell JL, Paul CE, Salehi AH, Becker EB, Said F, Bonni A, Barker PA.
Apoptosis induced by p75NTR overexpression requires Jun kinase-dependent phosphorylation of Bad.
J Neurosci. 2003 Dec 10;23(36):11373-81.

Barde YA.
Neurotrophins: a family of proteins supporting the survival neurons.
Prog. Clin. Biol. Res. 1194, 390:45-56

Barker PA, Shooter EM
Disruption of NGF binding to the low affinity neurotrophin receptor $p75^{LNTR}$ reduces NGF binding to trkA on PC12 cells.
Neuron 13: 203-215

Bamji SX, Majdan M, Pozniak CD, Belliveau DJ, Aloyz R, Kohn J, Causing CG, Miller FD
The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death.
J Cell Biol 140 (1998): 911-923.

Beckera Esther B.E, Azad Bonnia
Cell cycle regulation of neuronalapoptosis in development and disease .
Progress in Neurobiology Volume 72, Issue 1, January 2004, Pages 1–25

Mark Brady, Nikolina Vlatković, and Mark T. Boyd
Regulation of p53 and MDM2 Activity by MTBP
Mol Cell Biol. 2005 January; 25(2): 545–553.

Brann AB, Tcherpakov M, Williams IM, Futerman AH, Fainzilber M
Nerve growth factor-induced p75-mediated death of cultured hippocampal neurons is age-dependent and transduced through ceramide generated by neutral sphingomyelinase.
J Biol Chem (2002) 277: 9812-9818

Brann DW, Unda R, Mahesh VB.
Role of the progesterone receptor in restrained glutamic acid decarboxylase gene expression in the hypothalamus during the preovulatory luteinizing hormone surge.
Neuroendocrinology. 2002 Nov;76(5):283-9.

Brown KS, Hill CC, Calero GA, Myers CR, Lee KH, Sethna JP, Cerione RA.
The statistical mechanics of complex signaling networks: nerve growth factor signaling.
Phys Biol. 2004 Dec;1(3-4):184-95.

Bunone G, Mariotti A, Compagni A, Morandi E, Della VG
Induction of apoptosis by p75 neurotrophin receptor in human neuroblastoma cells.
Oncogene 14 (1997): 1463-1470.

Buss RR, Oppenheim RW.
Role of programmed cell death in normal neuronal development and function.
Anat Sci Int. 2004 Dec;79(4):191-7.

Culmsee C., Zhu X., Yu Q.S., Chan S.L., Camandola S., Guo Z., Greig N.H., Mattson M.P.
A synthetic inhibitor of p53 protects neurons against death induced by ischemic and excitotoxic insults and amyloid beta-peptide, J.Neurochem. 77 (2001) 220-228.

Culmsee C, Mattson MP.
p53 in neuronal apoptosis
Biochem Biophys Res Commun. 2005 Jun 10;331(3):761-77.

Chien CL, Liu TC, Ho CL, Lu KS.
Overexpression of neuronal intermediate filament protein alpha-internexin in PC12 cells.
J Neurosci Res. 2005 Jun 1;80(5):693-706.

Davies AM, Lee KF, Jaenisch R
p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins.
Neuron 11 (1993): 565-574

Derynck, R., Zhang, Y. and Feng, X. H. (1998).
Smads: transcriptional activators of TGF-beta responses.
Cell 95, 737-740.

Drummond HA, Furtado MM, Myers S, Grifoni S, Parker KA, Hoover A, Stec DE.
ENaC proteins are required for NGF-induced neurite growth.
Am J Physiol Cell Physiol. 2006 Feb;290(2):C404-10. Epub 2005 Sep 28.

Elmariah SB, Hughes EG, Oh EJ, Balice-Gordon RJ.
Neurotrophin signaling among neurons and glia during formation of tripartite synapses.
Neuron Glia Biol. 2005;1:1-11.

Epa WR, Markovska K, Barrett GL.
The p75 neurotrophin receptor enhances TrkA signalling by binding to Shc and augmenting its phosphorylation.
J Neurochem. 2004 Apr;89(2):344-53.

Edward Estlin, Richard Gilbertson, Rob Wynn
Pediatric Hematology and Oncology: Scientific Principles and Clinical Practice
20Jun2011

Erhardt JA, Legos JJ, Johanson RA, Slemmon JR, Wang X.
Expression of PEP-19 inhibits apoptosis in PC12 cells.
Neuroreport. 2000 Nov 27;11(17):3719-23.

Frade JM, Barde YA.
Microglia-derived nerve growth factor causes cell death in the developing retina. Neuron 20: (1998) 35-41.
Frade JM, Barde YA.
Nerve growth factor: two receptors, multiple functions.
Bioessays. 1998 Feb;20(2):137-45.

Forget C, Stewart J, Trudeau LE.
Impact of basic FGF expression in astrocytes on dopamine neuron synaptic function and development.
Eur J Neurosci. 2006 Feb;23(3):608-616.

Francois F, Godinho MJ, Dragunow M, Grimes ML.
A population of PC12 cells that is initiating apoptosis can be rescued by nerve growth factor.
Mol Cell Neurosci. 2001 Oct;18(4):347-62.

Gilman C.P., Chan S.L., Guo Z., Zhu X., Greig N., Mattson M.P.
p 53 is present in synapses where it mediates mitochondrial dysfunction and synaptic degeneration in response to DNA damage, and oxidative and excitotoxic insults, Neuromol. Med. 3 (2003) 159-172

Glebova NO, Ginty DD.
Growth and survival signals controlling sympathetic nervous system development.
Annu Rev Neurosci. 2005;28:191-222.

Goldberg JL, Klassen MP, Hua Y, Barres BA
Amacrinesignaled loss of intrinsic axon growth ability by retinal ganglion cells.
Science 296 (2002): 1860–1864

Goldshmit Y, Walters CE, Scott HJ, Greenhalgh CJ, Turnley AM.
SOCS2 induces neurite outgrowth by regulation of epidermal growth factor receptor activation.
J Biol Chem. 2004 Apr 16;279(16):16349-55. Epub 2004 Feb 4.

Gotz R, Karch C, Digby MR, Troppmair J, Rapp UR, Sendtner M.
The neuronal apoptosis inhibitory protein suppresses neuronal differentiation and apoptosis in PC12 cells.
Hum Mol Genet. 2000 Oct 12;9(17):2479-89

Greene LA, Tischler AS
Establishment of a noradrenergic clonal line of rat adrennal pheochromocytoma cells which respond to nerve growth factor. Proc Nat1 Acad Sci USA 73:2424-2428 (1976).

Tischler AS, Greene LA.
Morphologic and cytochemical properties of a clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor.
Lab Invest. 1978 Aug;39(2):77-89.

Hadrys, T., Braun, T., Rinkwitz-Brandt, S., Arnold, H. H., and Bober, E.
Nkx5-1 controls semicircular canal formation.
Development 125 (1998): 33-39

Harrington AW, Kim JY, Yoon SO
Activation of Rac GTPase by p75 is necessary for c-jun N-terminal kinase-mediated apoptosis.
J Neurosci 22 (2002): 156-166.

Hayashi H., Ishisaki A., Suzuki M. and Imamura T.
BMP2 augments FGF-induced differentiation of PC12 cells through upregulation of FGF receptor-1 expression
Journal of Cell Science 2001 114, 1387-1395

Harish C., Joshi and Don W. Cleveland
Differential Utilization of ß-tubulin Isotypes in Differentiating Neurities
The Journal of Cell Biology, Volume 109, 663-673

Hayashi H, Ishisaki A, Imamura T.
Smad mediates BMP2 -induced upregulation of FGF-evoked PC12 cell differentiation.
FEBS Lett. 2003

Vaghefi H., Allison L. Hughes, and Kenneth E. Neet
Nerve Growth Factor Withdrawal-mediated Apoptosis in Native and Differentiated PC12 Cells through p53/Caspase-3-dependent and -independent Pathways
THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 15, Issue of April 9, pp. 15604–15614, 2004

Iannone F.De Bari C., Dell'Accio F., Covelli M., Patella1 V., Lo Bianco1 L. and Lapadula G.
Increased expression of nerve growth factor (NGF) and high affinity NGF receptor (p140 TrkA) in human osteoarthritic chondrocytes
Rheumatology (2002) 41 (12): 1413-1418.

Iwasaki, S., Hattori, A., Sato, M., Tsujimoto, M. and Kohno, M. (1996).
Characterization of the bone morphogenetic protein-2 as a neurotrophic factor.
Induction of neuronal differentiation of PC12 cells in the absence of mitogenactivated protein kinase activation.
J. Biol. Chem 1996. 271, 17360-17365.

Iwasaki S. , Iguchi M., Watanabe K., Hoshino M, Tsujimoto M., Kohno M.
Specific Activation of the p38 Mitogen-activated Protein Kinase Signaling Pathway and Induction of Neurite Outgrowth in PC12 Cells by Bone Morphogenetic Protein-2
J Biol Chem. 1999 Sep 10;274(37):26503-10.

Jian Yu, , Lin Zhang
No PUMA, no death: Implications for p53-dependent apoptosis
Cancer Cell, Volume 4, Issue 4, October 2003, Pages 248–249

Leist M, Nicotera P.
Apoptosis, excitotoxicity, and neuropathology.
Exp Cell Res. 1998 Mar 15;239(2):183-201. Review.

Liu DX, Biswas SC, Greene LA.
B-myb and C-myb play required roles in neuronal apoptosis evoked by nerve growth factor deprivation and DNA damage.
J Neurosci. 2004 Oct 6;24(40):8720-5.

Liang Y, Yan C, Nylander KD, Schor NF.
Early events in Bcl-2-enhanced apoptosis.
Apoptosis. 2003 Dec;8(6):609-16.

Liang Y, Nylander KD, Yan C, Schor NF.
Role of caspase 3-dependent Bcl-2 cleavage in potentiation of apoptosis by Bcl-2.
Mol Pharmacol. 2002 Jan;61(1):142-9.

Kaufmann JA, Perez M, Zhang W, Bickford PC, Holmes DB, Taglialatela G.
Free radical-dependent nuclear localization of Bcl-2 in the central nervous system of aged rats is not associated with Bcl-2-mediated protection from apoptosis.
J Neurochem. 2003 Nov;87(4):981-94.

Komarov P.G., Komarova E.A., Kondratov R.V., Christov-Tselkov K., Coon J.S., Chernov M.V., Gutkow A.V.
A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy, Science 285 (1999) 1733-1737

Kurihara H, Shinohara H, Yoshino H, Takeda K, Shiba H.
Neurotrophins in cultured cells from periodontal tissues.
J Periodontol. 2003 Jan;74(1):76-84.

Lönn P., Zaia K., Israelsson C., Althini S., D. Usoskin, A. Kylberg, and Ebendal T.
BMP Enhances Transcriptional Responses to NGF During PC12 Cell Differentiation
Neurochemical Research Vol. 30, Nos. 6/7 (2005).

Li, C. W., Van De Water, T. R. and Ruben, R. J.
The fate mapping of the eleventh and twelfth day mouse otocyst.
J. Morph. 157, 249-268 (1978).

Lee J, Kim MS, Park C, Lim YS, Lee I, Moon BS, Lee HS, Park R.
Protective effects of Debo on serum-deprived apoptosis of PC12 cells via inhibition of H2O2 generation and caspase 3-like protease activity.
Immunopharmacol Immunotoxicol. 2002 May;24(2):227-43.

Mattson M.P.
Cellular actions of beta-amyloid precursor protein and its soluble and fibrillogenic derivatives, Physiol. Rev. 77 (1997) 1081-1132

Massaad CA, Taglialatela G.
Bcl-2 phosphorylation is not required for its effects on NFkappaB activity.
Neuroreport. 2003 Jun 11;14(8):1167-70.

Mennerich D, Hoffmann S, Hadrys T, Arnold HH, Bober E.
Two highly related homeodomain proteins, Nkx5-1 and Nkx5-2, display different DNA binding specificities.
Biol Chem. 1999 Sep;380(9):1041-8.

Melissa T. Jack, Richard A. Woo, Atsushi Hirao, Alison Cheung, Tak W. Mak, and Patrick W. K. Lee
Chk2 is dispensable for p53-mediated G1 arrest but is required for a latent p53-mediated
apoptotic response.
Proc Natl Acad Sci U S A. 2002 Jul 23;99(15):9825-9. Epub 2002 Jul 3.

Merlo GR, Paleari L, Mantero S, Zerega B, Adamska M, Rinkwitz S, Bober E, Levi G.
The Dlx5 homeobox gene is essential for vestibular morphogenesis in the mouse embryo through a BMP4-mediated pathway.
Dev Biol. 2002 Aug 1;248(1):157-69.

Mihara M., Erster S., Zaika A., Petrenko O., Chittenden T., Pancoska P., Moll U.M., p 53 has a direct apoptogenetic role at the mitochondria, Mol. Cell 11 (2003) 577-590

Molina R., Sequi M.A., Climent M.A., Bellmut J., Albanelll J., Fernandez M., Fillela X., Jo J., Gimenez N., Iglesias E., Mirales M., Alonso C., Peiro G., Perez-Picanol E., Balesta A.M.
p53 oncoprotein as a prognostic indicator in patients with breas cancer, Anticancer Res. 18 (1998) 507-511

Niederhauser O, Mangold M, Schubenel R, Kusznir EA, Schmidt D, Hertel C.
NGF ligand alters NGF signaling via p75(NTR) and trkA.
J Neurosci Res. 2000 Aug 1;61(3):263-72.

Ohyashiki T, Satoh E, Okada M, Takadera T, Sahara M.
Nerve growth factor protects against aluminum-mediated cell death.
Toxicology. 2002 Jul 15;176(3):195-207.

Park JG, Yuk Y, Rhim H, Yi SY, Yoo YS.
Role of p38 MAPK in the regulation of apoptosis signaling induced by TNF-alpha in differentiated PC12 cells.
J Biochem Mol Biol. 2002 May 31;35(3):267-72.

Perfetini J.L., Roumier T., Castedo M., Larochette N., Boya P., Rayal B., Lazar V., Ciccosanti F., Nardacci R., , Penninger J., Piacentini M., Kroemer G.
NF-kappaB and p53 are the dominant apoptosis-inducing transcription factors elicited by the HIV-1 envelope,
J.Exp. Med. 199 (2004) 629-640

Petratos S, Butzkueven H, Shipham K, Cooper H, Bucci T, Reid K, Lopes E, Emery B, Cheema SS, Kilpatrick TJ
Schwann cell apoptosis in the postnatal axotomized sciatic nerve is mediated via NGF through the low-affinity neurotrophin receptor.
J Neuropathol Exp Neurol (2003) 62: 398-411.

Porat S, Simantov R.
Bcl-2 and p53: role in dopamine-induced apoptosis and differentiation.
Ann N Y Acad Sci. 1999;893:372-5.

Rinkwitz-Brandt, S., Arnold, H.-H. and Bober, E**.** Regionalized expression of Nkx5-1, Nkx5-2, Pax2 and sek genes during mouse inner ear development. Hearing Research 99**,** (1996)129-138.

Rohn TT, Cusack SM, Kessinger SR, Oxford JT.
Caspase activation independent of cell death is required for proper cell dispersal and correct morphology in PC12 cells.
Exp Cell Res. 2004 Apr 15;295(1):215-25.

Romagnoli A, Oliverio S, Evangelisti C, Iannicola C, Ippolito G, Piacentini M.
Neuroleukin inhibition sensitises neuronal cells to caspase-dependent apoptosis.
Biochem Biophys Res Commun. 2003 Mar 14;302(3):448-53.

Rong P, Bennie AM, Epa WR, Barrett GL.
Nerve growth factor determines survival and death of PC12 cells by regulation of the bcl-x, bax, and caspase-3 genes.
J Neurochem. 1999 Jun;72(6):2294-300.

Rukenstein A., Rydel RE, and Greene LA
Multiple agents rescue PC12 cells from serum-free cell death by transition- and transcription-independent mechanisms.
J. Neurosci 11:2552-2563.

Ryden M, Hempstead B, Ibanez CF
Differential modulation of neuron survival during development by nerve growth factor binding to the p75 neurotrophin receptor.
J Biol Chem 1997 272: 16322-16328.

Scott CW, Sobotka-Briner C, Wilkins DE, Jacobs RT, Folmer JJ, Frazee WJ, Bhat RV, Ghanekar SV, Aharony D.
Novel small molecule inhibitors of caspase-3 block cellular and biochemical features of apoptosis.
J Pharmacol Exp Ther. 2003 Jan;304(1):433-40.

Schimmelpfeng J, Weibezahn KF, Dertinger H.
Quantification of NGF-dependent neuronal differentiation of PC12 cells by means of neurofilament-L mRNA expression and neuronal outgrowth.
J Neurosci Methods. 2004 Oct 30;139(2):299-306.

Shacka JJ, Sahawneh MA, Gonzalez JD, Ye YZ, D'alessandro TL, Estevez AG.
Two distinct signaling pathways regulate peroxynitrite-induced apoptosis in PC12 cells.
Cell Death Differ. 2006 Jan 20;

Shacka JJ, Roth KA.
Regulation of neuronal cell death and neurodegeneration by members of the Bcl-2 family: therapeutic implications.
Curr Drug Targets CNS Neurol Disord. 2005 Feb;4(1):25-39.

Shaw PA, Zhang X., Russo AF, Ament BA, Henderson S., Wiliams V.
Homeobox protein, HMX3, in postnatally developing rat submandibular glands
J.Histochem Cytochem 2003, Mar;51(3): 385-96

Song Q, Mehler MF, Kessler JA.
Bone morphogenetic proteins induce apoptosis and growth factor dependence of cultured sympathoadrenal progenitor cells.
Dev Biol. 1998 Apr 1;196(1):119-27.

Song BJ, Soh Y, Bae M, Pie J, Wan J, Jeong K.
Apoptosis of PC12 cells by 4-hydroxy-2-nonenal is mediated through selective activation of the c-Jun N-terminal protein kinase pathway.
Chem Biol Interact. 2001 Jan 30;130-132(1-3):943-54.

Stolzing A, Grune T.
Neuronal apoptotic bodies: phagocytosis and degradation by primary microglial cells.
FASEB J. 2004 Apr;18(6):743-5. Epub 2004 Feb 6.

Takeda K, Hatai T, Hamazaki TS, Nishitoh H, Saitoh M, Ichijo H.
Apoptosis signal-regulating kinase 1 (ASK1) induces neuronal differentiation and survival of PC12 cells.
J Biol Chem. 2000 Mar 31;275(13):9805-13.

Tabakman R, Jiang H, Levine RA, Kohen R, Lazarovici P.
Apoptotic characteristics of cell death and the neuroprotective effect of homocarnosine on pheochromocytoma PC12 cells exposed to ischemia.
J Neurosci Res. 2004 Feb 15;75(4):499-507.

Tamagno E., Parola M., Guilielmotto M., Santoro G., Bardini P., Marra L., Tabaton M., Danni O.
Multiple signalling events in amyloid beta-induced, oxidative stress-depended neuronal apoptosis
Free Radic Biol Med. 2003 Jul 1,35(1):45-58

Truong LX Nguyen, Chung Kwon Kim, Jun-Hee Cho, Kyung-Hoon Lee, and Jee-Yin Ahn
Neuroprotection signaling pathway of nerve growth factor and brain-derived neurotrophic factor against staurosporine induced apoptosis in hippocampal H19-7 cells.
Exp Mol Med. 2010 August 31; 42(8): 583–595.

Troy CM, Rabacchi SA, Friedman WJ, Frappier TF, Brown K, Shelanski ML.
Caspase-2 mediates neuronal cell death induced by beta-amyloid.
J Neurosci. 2000 Feb 15;20(4):1386-92.

Torocsik B. and Szeberenyi J.
Anisomicin affects both pro- and antiapoptotic mechanisms in PC12 cells
Biochem Biophys Res Commun 278: 550-556

Vaudry D, Chen Y, Hsu CM, Eiden LE.
PC12 cells as a model to study the neurotrophic activities of PACAP.
Ann N Y Acad Sci. 2002 Oct;971:491-6.

Valavanis C, Hu Y, Yang Y, Osborne BA, Chouaib S, Greene L, Ashwell JD, Schwartz LM.
Model cell lines for the study of apoptosis in vitro.
Methods Cell Biol. 2001;66:417-36.

W. Wang, Edwin K. Chan, S.Baron, T.Van De Water, T.Lufkin
Hmx2homeobox gene control of murine vestibular morphogenesis
Development 128, 5017-5029 (2001)

Wang, W., Van De Water, T., and Lufkin, T. (1998)
Inner ear and maternal reproductive defects in mice lacking the Hmx3 homeobox gene. Development 125: 621-34

Wang W., Edwin K. Chan, Shira Baron, Thomas Van De Water and Thomas Lufkin
Hmx2 homeobox gene control of murine vestibular morphogenesis
Development 128, 5017-5029 (2001)

Wang W., Lufkin T.
Hmx homeobox gene function in inner ear and nervous system cell-type specification and development

Wang W, Dow KE, Riopelle RJ, Ross GM.
The common neurotrophin receptor p75NTR enhances the ability of PC12 cells to resist oxidative stress by a trkA-dependent mechanism.
Neurotox Res. 2001 Oct;3(5):485-99.

Weber YG, Geiger J, Kampchen K, Landwehrmeyer B, Sommer C, Lerche H.
Immunohistochemical analysis of KCNQ2 potassium channels in adult and developing mouse brain.
Brain Res. 2006 Feb 23;

Wright KM, Linhoff MW, Potts PR, Deshmukh M.
Decreased apoptosome activity with neuronal differentiation sets the threshold for strict IAP regulation of apoptosis.
J Cell Biol. 2004 Oct 25;167(2):303-13.

West T, Atzeva M, Holtzman DM.
Caspase-3 deficiency during development increases vulnerability to hypoxic-ischemic injury through caspase-3-independent pathways.
Neurobiol Dis. 2006 Feb 8;

Vaghefi H, Hughes AL, Neet KE.
Nerve growth factor withdrawal-mediated apoptosis in naive and differentiated PC12 cells through p53/caspase-3-dependent and -independent pathways.
J Biol Chem. 2004 Apr 9;279(15):15604-14.

Vaghefi H, Neet KE.
Deacetylation of p53 after nerve growth factor treatment in PC12 cells as a post-translational modification mechanism of neurotrophin-induced tumor suppressor activation.
Oncogene. 2004 Oct 21;23(49):8078-87.

Vaisid T, Kosower NS, Barnoy S.
Caspase-1 activity is required for neuronal differentiation of PC12 cells: cross-talk between the caspase and calpain systems.
Biochim Biophys Acta. 2005 Apr 15;1743(3):223-30.

Yanagisawa M., Nakashima K., Takeda K., Ochiai W., Takizawa T., Ueno M., Takizawa M., Shibuya H., Taga T.
Inhibition of BMP2-induced, TAK1 kinase-mediated neurite outgrowth by Smad6 and Smad7.
Genes Cells. 2001 Dec;6(12):1091-9.

D. Vaudry, P. J. S. Stork, P. Lazarovici, L. E. Eiden
Signaling Pathways for PC12 Cell
Differentiation: Making the Right Connections
Science 2002, Vol. 296

Vilar M, Murillo-Carretero M, Mira H, Magnusson K, Besset V, Ibanez CF
Bex1, a novel interactor of the p75 neurotrophin receptor, links neurotrophin signaling
to the cell cycle.
EMBO J. 2006 Feb 23

Vyas S, Juin P, Hancock D, Suzuki Y, Takahashi R, Triller A, Evan G.
Differentiation-dependent sensitivity to apoptogenic factors in PC12 cells.
J Biol Chem. 2004 Jul 23;279(30):30983-93.

Yan C, Liang Y, Nylander KD, Schor NF.
TrkA as a life and death receptor: receptor dose as a mediator of function.
Cancer Res. 2002 Sep 1;62(17):4867-75.

Yang E, Kim H, Lee J, Shin JS, Yoon H, Kim SJ, Choi IH.
Overexpression of LIM kinase 1 renders resistance to apoptosis in PC12 cells by inhibition of caspase activation.
Cell Mol Neurobiol. 2004 Apr;24(2):181-92.

Yoon SO, Casaccia-Bonnefil P, Carter B, Chao MV
Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival.
J Neurosci 18 (1998): 3273-3281.

Zhang J, Krishnamurthy PK, Johnson GV.
Cdk5 phosphorylates p53 and regulates its activity.
J Neurochem. 2002 Apr;81(2):307-13.

Zhang HN, Zhou JG, Qiu QY, Ren JL, Guan YY.
ClC-3 chloride channel prevents apoptosis induced by thapsigargin in PC12 cells.
Apoptosis. 2006 Mar 2

Zhang J, W Yan, X Chen
p53 is required for nerve growth factor-mediated differentiation of PC12 cells via regulation of TrkA levels
Cell Death Differ. 2006 May 26

Zhang XP, Liu F, Cheng Z, Wang W.
Cell fate decision mediated by p53 pulses.
Proc Natl Acad Sci U S A. 2009 Jul 28;106(30):12245-50. Epub 2009 Jul 15.

Zhu X., Yu Q.S., Cutler R.G., Cilmsee C.W., Holloway H.W., Lahrini D.K., Mattson M.P., Greig N.H.
Novel p53 inactivators with neuroprotective action: syntheses and pharmacological evaluation of 2-imino-2,3,4,5,6,7-haxahydrobenzoxazole and 2-imino-2,3,4,5,6,7 – hyxahydrobenzoxazole derivatives

10. ERKLÄRUNG

"Ich erkläre: Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der "Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" niedergelegt sind, eingehalten."

Bad Nauheim, 07th April 2014 Robert Kramek

11. ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Eva Bober for initiating this project, her unrestricted support, her patience, academic guidance and confidence throughout every phase of my PhD study.

Further, I would like to thank Prof. Thomas Braun at Max Planck Institute for Heart and Lung Research in Bad Nauheim for helpful and constructive discussions and providing the organisational framework.

I thank Katja Kolditz for outstanding support and technical introduction.

Furthermore, I thank Michal Mielcarek and Izabella Piotrowska for the support.

I am grateful to Tomasz Loch for spending weekend's time for thousands of minipreps with a great jazz music.

Special and warm thanks to Gabriele Hoang for her work and being optimistic every day.

I am deeply grateful to all people at the Institute of Physiological Chemistry the University of Halle-Wittenberg and Max Plank Institute in Bad Nauheim, for solving many, many problems and introducing me to techniques and equipment.

….and my wife Anna for the sun even on a cloudy days.