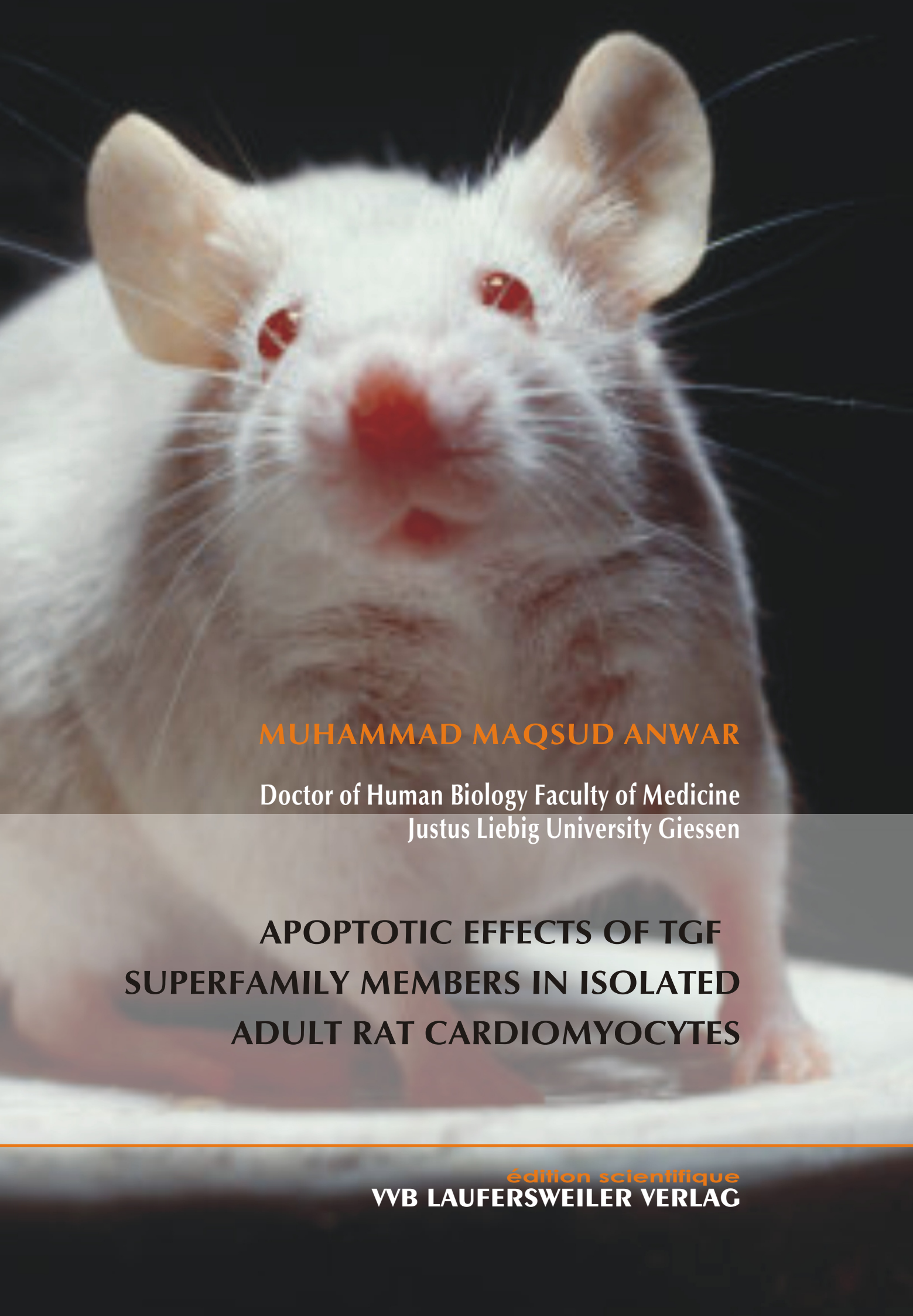

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Justus Liebig University

Giessen

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Vehari-Pakistan

Giessen 2005

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Day of the Examination:

March 13, 2006

dedicated to my parents

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Declaration

I hereby declare that the PhD thesis entitled **"Apoptotic effects of TGFβ superfamily members in isolated adult rat cardiomyocytes"** embodies my original work presented in this thesis report. This work was carried out under the supervision of PD. Dr. Gerhild Euler at the Institute of physiology, Justus Liebig University, Giessen, Germany.

Muhammad Maqsud Anwar

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At the end special thanks goes to my wife Attia Anwar. She has not only helped me through out the life but also she has kept me mentally and physically fit.

Abbreviations

% (vol/vol)	Volume percent
% (wt/vol)	Weight volume percent
AA/BAA	Acrylamide /Bisacrylamide
AP-1	Activator protein 1
APS	Ammonium persulphate
BMP-2	Bone morphogenetic protein-2
bp	Base pair
C	Control
$CaCl_2$	Calcium Chloride
cGMP	Cyclic Guanosin 3`,5´Monophosphate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylen diamine tetra acetic acid
FCS	Fetal calf serum
GDF15	Growth differentiation factor 15
HEPES	N-2-Hydroxyethyl piperazine-N-2-ethanesulphonic acid
Hoe	Hoechst dye H33285
KCl	Potassium Chloride
KH_2PO_4	Potassium dihydrogen phosphate
$MgCl_2$	Magnesium Chloride
n	Numbers
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium Chloride
NaF	Sodium Flouride
NO	Nitric oxide
PBS	Phosphate buffer saline
PI	Propidium iodid

PMSF	Phenyl methyl sulfonyl fluoride
SDS	Sodium dodecyl sulphate
SMAD	Small mother against decapentaplegic
SNAP	(±)-S-Nitroso-N-Acetylpenicillamin
TAE	Tris-acetate/EDTA-buffer
TE	Tris/HCl/EDTA-buffer
TEMED	N,N,N,N-tetra methyl ethylene diamine
TGFβ1	Transforming growth factor β1
Tris	Tris-hydroxymethly aminomethane

1. Introduction

1. Apoptosis

1.1. The development of the term apoptosis

In biology, apoptosis (from the Greek words apo = from and ptosis = falling, pronounced ap-a-tow'-sis) is one of the main types of cell death. Apoptosis is of Greek origin, having the meaning "falling off or dropping off", in analogy to leaves falling off trees or petals dropping off flowers. This analogy emphasizes that the death of living matter is an integral and necessary part in the life cycle of organisms.

Since the mid-nineteenth century, many observations have indicated that cell death plays a considerable role during physiological processes of multicellular organisms, particularly during embryogenesis and metamorphosis (Lockshin et al, 2001). The term programmed cell death was introduced in 1964, proposing that cell death during development is not of accidential nature but follows a sequence of controlled steps leading to locally and temporally defined self-destruction (Lockshin et al,2001).

Eventually, the term apoptosis had been coined in order to describe the morphological processes leading to controlled cellular self-destruction and was first introduced in a publication by Kerr, Wyllie and Currie (Kerr et al, 1972). The apoptotic mode of cell death is an active and defined process which plays an important role in the development of multicellular organisms and in the regulation and maintenance of the cell populations in tissues upon physiological and pathological conditions. It should be stressed that apoptosis is a well-defined and possibly the most frequent form of programmed cell death, but that other, non-apoptotic types of cell death, e.g. necrosis also are of biological significance (Leist et al, 2001).

1.2. The significance of apoptosis

The development and maintenance of multicellular biological systems depends on a sophisticated interplay between the cells forming the organism. It sometimes even seems to involve an altruistic behavior of individual cells in favor of the organism as a whole. During development many cells are produced in excess, which eventually undergo

programmed cell death and thereby contribute to sculpturing many organs and tissues (Meier et al, 2000).

A particularly instructive example for the implication of programmed cell death in animal development is the formation of free and independent digits by massive cell death in the interdigital mesenchymal tissue (Zuzarte-Luis et al, 2002). Other examples are the development of the brain, during which half of the neurons that are initially created will die in later stages when the adult brain is formed (Hutchins et al, 1998), as well as the development of the reproductive organs (Meier et al, 2000). Also cells of an adult organism constantly undergo physiological cell death, which must be balanced with proliferation in order to maintain homeostasis in terms of constant cell numbers. The majority of the developing lymphocytes die either during genetic rearrangement events during the formation of the antigen receptor, during negative selection or in the periphery, thereby tightly controlling the pool of highly efficient and functional but not self-reactive immune cells, and at the same time keeping lymphocyte numbers relatively constant (Rathmell et al, 2002). Taken together, apoptotic processes are of widespread biological significance; being involved in e.g. development, differentiation, proliferation/homoeostasis, regulation and function of the immune system and in the removal of defect and therefore harmful cells. Thus, dysfunction or dysregulation of the apoptotic program is implicated in a variety of pathological conditions. Defects in apoptosis can result in cancer, autoimmune diseases and spreading of viral infections, neurodegenerative disorders and AIDS (Fadeel et al, 1999).

Due to its importance in such various biological processes, programmed cell death is a widespread phenomenon, occurring in all kinds of metazoans (Tittel et al, 2000) such as in mammals, insects (Richardson et al, 2002), nematodes (Liu et al, 1999), and cnidaria (Cikala et al, 1999). Moreover, programmed cell death also might play a role in plant biology (Solomon et al, 1999), and apoptosis-like cell death mechanisms even have been observed and used as a model system in yeast (Fröhlich et al, 2000; Skulachev et al, 2002). Fascinating insights into the origin and evolution of programmed cell death might possibly be given by the fact, that programmed cell death is also an integral part of the life cycle of other unicellular eukaryotes (such as the kinetoplastid parasite Trypanosoma brucei, the ciliate Tetrahymena thermophila, and the slime mold Dictyostelium

discoideum), and that even prokaryotes (such as Bacillus subtilis, Streptomyces and Myxobacteria) sometimes undergo regulated cell death (Ameisen et al, 2002).

1.3. Morphological features of apoptosis

Apoptotic cells can be recognized by stereotypical morphological changes: the cell shrinks, shows deformation and loses contact to its neighbouring cells. Its chromatin condenses and marginates at the nuclear membrane, the plasma membrane is blebbing or budding, and finally the cell is fragmented into compact membrane-enclosed structures, called 'apoptotic bodies' which contain cytosol, the condensed chromatin, and organelles. The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response. Those morphological changes are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most notably the activation of proteolytic enzymes, which mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles (Saraste et al, 2000). Apoptosis can be distinguesh from necrotic mode of cell-death in which the cells suffer a major insult, resulting in a loss of membrane integrity, swelling and disrupture of the cells. During necrosis, the cellular contents are released uncontrolled into the cells environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue (Leist et al, 2001).

1.4. Apoptosis induction

In mammalian cells two main signal pathways of apoptosis are well characterised. One signal pathway depends on activation of receptors (Ashkenazi et al,1998), the other one is induced by different factors e.g. ROS, UV, NO. Mitochondria play a central role in this signaling pathway (Kroemer e al, 1997). However, both signalling pathways cannot be distinguished clearly from each other. Apoptosis induction by receptors particularly depends on Fas and TNF receptors. These death receptors contain a homologous cytoplasmic sequence termed the “death domain”. At this domain specific protein binding occurs after receptor activation, which activates the intracellular signal transduction cascades. In this cascade aspartat proteases “Caspases”play a major role (Ashkenazi et al,

1998). Caspases are able to cleave a set of cellular structural proteins and also contribute to the activation of endogenous DNAses (Enari et al, 1998).

The mitochondrial pathway of apoptosis is activated by different cytotoxic materials e.g. DNA-damaging agents, oncogenes (Fearnhead et al,1998) or the p53-protein (Soengas et al, 1999). Cytochrom C and other caspase activating substances are released from mitochondria (Kroeme et al, 1997). Downstream signals are also caspase dependent (Slee et al, 1999).

1.5. Apoptosis in the heart

Numerous studies are related to the occurrence of apoptosis in the heart. Apoptosis was observed in autopsy material of heart muscle cells of patients in the final stage of cardiomyopathy (Narula et al, 1996) and also found after cardiac infarction (Saraste et al, 1997). Experimentally these findings could be confirmed in animal studies. After myocardial infarction (in vivo; rat) (Kajstura et al, 1997; Fliss et al, 1996) apoptosis was found. Anoxia alone does not induce apoptosis, other factors that are found increased after myocardial infarction may be involved i.e. NO, TGFβ.

1.6. Nitric oxide (NO) as an inducer of apoptosis in cardiomyocytes

Immunohistochemistry studies showed that after myocardial infarction a main source of iNOS expression are macrophages (Wildhirt et al, 1995). Parallel rising of apoptosis and iNOS after myocardial infarction could be observed. This correlation between increased NO formation and apoptosis induction indicates a functional connection. It could be proven in isolated cardiomyocytes that the NO donors (±)-S-Nitroso-N-Acetylpenicillamine (SNAP) or GSNO induce apoptosis (Taimor et al, 1999; Andreka et al, 2001).

1.7. Signaling pathway of NO induced apoptosis in cardiomyocytes

1.7.1. cGMP dependence of NO induced apoptosis

cGMP dependence of NO induced apoptosis was examined by Taimor et al, (2000) in isolated cardiomyocytes of adult rats. It is reported that the NO donor SNAP could

induce apoptosis under normoxic conditions. The induction of apoptosis could be blocked by ODQ, an inhibitor of the soluble guanylyl cyclase (Garthwaite et al, 1995) or by the protein kinase G-inhibitor KT5823 (Girder et al, 1993). Apoptosis was induced by incubation of cardiomyocytes with the cGMP- analogue 8-pCPT-GMP, an activator of protein kinase G (Sekhar et al, 1992), or by YC-1, an NO-independent activator of the soluble guanylyl cyclase (KO et al, 1994). These results show, that NO-induced apoptosis is cGMP-dependent in adult cardiomyocytes. The same results were reported by Shimojo et al; (1999) in their studies on the isolated neonatal myocytes of the rat.

1.7.2. Participation of "Transcription activator protein 1" (AP-1) in NO-induced apoptosis

It is well known from the literature, that NO functions as an activator of different mitogen activating protein kinases (MAPK). By use of pharmacological inhibitors or activators in cardiomyocytes it was shown that the "extracellular signal regulated kinase" (ERK) and c-jun-N-terminal kinase (JNK), both members of MAPK family, are necessary for NO-induced apoptosis (Taimor et al, 2001). The activation of both kinases is necessary for activation of the transcription factor AP-1 (activator protein 1). NO-induced apoptosis via AP-1 could be abolished by the use of AP-1decoy oligonucleotides (Taimor et al, 2001). The decoy oligos are double stranded DNA fragments forming the consensus binding sequences for AP-1. Transformation of cardiomyocytes with these oligos scavenge AP-1 intracellularly. A connection between AP-1 activation and SMAD (small mother against decapentaplegic) in apoptosis is already reported by Maulik et al, (2000) and Schneiders et al, (2005).

1.8. TGFβ superfamily.

1.8.1. TGFβs and pathophysiology

The Transforming Growth Factor-β (TGF-β) superfamily is composed of many multifunctional cytokines including TGF-βs, activins, inhibins, anti-müllerian hormones (AHM), bone morphogenetic proteins (BMPs), myostatin (Hao et al, 1999) and GDF15

(growth differentiation factor 15). Expression of TGFβ family members is elevated under pathophysiological conditions in the heart: Elevated levels of TGFβ**,** activin and myostatin are found after myocardial infarction (Liu et al, 1997; Chacko et al, 2004; Wu et al, 1997). TGFβs induce death associated proteins which induces apoptosis in certain cell types. (Ishisaki et al, 1999). Therefore, TGFβ family members may also contribute to apoptosis induction in cardiomyocytes and may contribute to the deleterious development of heart failure.

Increased expression of TGFβ also enhances left ventricular hypertrophy and fibrosis causing ventricular remodeling and heart failure (Wang et al, 2005). Therefore, elevated levels of TGFβ in the heart are related to bad prognosis for ventricular function. For the other members of TGFβ family members, myostatin and activin, similar correlations between their activation and development of ventricular remodeling have been found. Yet nothing is known about their involment in hypertrophy or apoptosis in the heart.

In contrast to the TGFβ/activin subgroup, BMP subfamily members have contrasting effects in different cells. The primary function of BMP is found in heart development. Addition of BMP to embryonic cells induces differentiation into cardiomyocytes. (Chen et al, 1998). BMP2 induces apoptosis in mouse hybridoma MH60 cells (Kimura et al, 2000) and in human myeloma cells (Kawamura et al, 2000). In contrast, BMP-2 shows anti-apoptotic effects in neonatal cardiomyocytes.

In conclusion, various effects of TGFβ superfamily members in the heart, including development, fibrosis, and growth can be found. Reasons for this may be the activation of different signaling molecules by different family members.

1.8.2. TGF-β cell signaling

Type I and type II serine/threonine kinase receptors are directly involved in signaling of TGF-β superfamily members. Five type II receptors and seven type I receptors, also termed receptor-like kinases (ALKs), have been identified (Arsura et al, 2003). The type II receptor is a constitutively active kinase, which upon ligand-mediated heteromeric complex formation phosphorylates particular serine and threonine residues in the type I receptor (Arsura et al, 2003). ALK-4 and ALK-5 are TGF-β type I receptors. ALK-2 is

most important for BMP signal transduction. The TGF-β receptors transduce their signals via SMAD proteins.

At present eight distinct SMAD proteins are known. They can be divided into three different functional classes: (i) the receptor activated R-SMADs (SMAD 1, 2, 3, 5, and 8), (ii) the co-mediator Co-SMAD (SMAD 4) and (iii) the inhibitory I-SMADs (SMAD 6 and 7). In non-activated cells, R-SMADs are predominantly localized in the cytoplasm, Co-SMADs are equally distributed in the cytoplasm and the nucleus and I-SMADs are found mostly in the nucleus. Upon stimulation of receptors of the TGFβ superfamily R-SMADs become phosphorylated and activated. They undergo dimerization and form heterotrimers with Co-SMADs. This complex then translocates to the nucleus and influences the transcriptional regulation (Lebrin et al, 2005).

The TGFβ-family members comprise about 30 members in the mammalian system and can be devided into two groups, the TGFβ/activin family and the BMP group (bone morphogenetic protein). Each group is responsible for activation of different SMAD isoforms.

The recruitment of SMADs to DNA is regulated by cooperation with other transcription factors. These factors facilitate binding of SMADs to DNA. One transcription factor that can interact with SMAD and that is expressed in heart is AP-1. TGFβ is released under several pathophysiologic conditions like ischemia/reperfusion and cardiomyopathy (Poncelet et al., 2001), which simultaneously activate SMAD and AP-1. Both factors, AP-1 and SMAD mediate enhanced expression of TGFβ responsive genes, like collagen (Ross et al, 2004), c-Jun (Lopez-Rovira et al, 2000), endothelin-1 (Sanchez-Elsner et al, 2001). Another functional aspect of AP-1/SMAD signaling is the induction of apoptosis in TGFβ stimulated cells (Kon et al, 1999; Arthur et al, 2000; Euler et al., 2006) and involved in NO induced apoptosis in adult rat cardiomyocytes. Therefore, TGFβ family members may induce apoptosis in this pathway.

1.9. Aims of the study

Since various TGFβ family members are increased under pathophysiologic conditions in the heart it was the aim of this study to analyse:

1. If all members of the TGF-β superfamily act as pro-apoptotic molecules?

2. Which signaling pathways are involved in apoptosis?
3. If SMAD and AP-1 proteins are involved in apoptosis induction?
4. If any member of TGF-β superfamily does not induce apoptosis or acts anti-apoptotic?

2. MATERIALS

2.1. Chemicals

Milk powder	Applichem
Agarose	Invitrogen-UK
BMP2	R&D systems, Wiesbaden
Carbogene	Messer Griesheim, Krefeld
Carnitine	Sigma, Taufkirchen
Collagenase, Type CLS II	Biochrom, Berlin
Creatine	Sigma, Taufkirchen
Cytosin-β-Arabinofuranoside	Sigma, Taufkirchen
Cy3-dcTP-dye	Amersham Bioscience, Freiburg
Dithiothreitol	Sigma, Taufkirchen
DNAse-free RNAse	Qiagen, Hilden
Proteinase K	Merk Bioscience
Tris base	Roth, Karlsruhe
Glacial acetic acid	Merk Bioscience, Darmstadt
Ethanol	Merk Bioscience, Darmstadt
Ethidium bromide	Sigma, Taufkirchen
Fetal calf serum	PAA, Cölbe
Gentamycin	Invitrogen, Karlsruhe
HEPES	Invitrogen, Karlsruhe
Human activin A	R&D systems, Wiesbaden
Hoechst 33258 (Hoe 33258)	Sigma, Taufkirchen
Medium 199/ Earl's Salts	Biochrom, Berlin
Penicilline-Streptomycine	Invitrogen, Karlsruhe
Phenylmethylsulfonylfluorid	Sigma, Taufkirchen
poly-(dIdC)	Roche Diagnostics, Mannheim
Propidiumiodid	Sigma, Taufkirchen
Myostatin	R&D systems, Wiesbaden

TEMED	Roth, Karlsruhe
Terminale Transferase	New England Biolabs, Frankfurt am.Main.
$TGF\beta_1$	Merck Biosciences
Tris/HCl	Roth, Karlsruhe
Triton X-100	Serva, Heidelberg
Taurine	Sigma, Taufkirchen
Trichloroacetic acid	Merck Bioscience, Darmstadt
Sodium dodecyl sulphate	Merck Bioscience, Darmstadt
Sodium hydroxide	Roth, Karlsruhe

2.2. Decoy oligonucleotides

SMAD	Invitrogen, Karlsruhe
AP-1	Invitrogen, Karlsruhe

2.3. Antibodies

SMAD2/3	Santa Cruz Biotechnology, Heidelberg
SMAD4	Santa Cruz Biotechnology, Heidelberg
Actin	Santa Cruz Biotechnology, Heidelberg

All other chemicals used in this work were obtained with the best analytical quality by the following companies: Invitrogen (Karlsruhe), Amersham-Bioscience (Freiburg), Merck (Darmstadt) and Sigma (Taufkirchen).
All chemicals were dissolved and stored regarding the manufactures instructions. For the preparation of solutions, media and buffers millipore water was used, if not mentioned differently.

2.4. Equipments

2.4.1. General objects of utility

Gel electrophorosis	Biotec Fisher, Reiskirchen
Retardation gel chamber	Amersham Bioscience
Western blot apparatus	Biotec Fisher, Reiskirchen
Centrifuge (Type: 18)	Kendro, Hanau
Glass ware	Schott, Mainz
Magnetic stirrer with hot plate	Jahnke & Kunkel, Staufen
pH meter	WTW, Weilheim
Pipettes	Eppendorf-Netheler-Hinz, Hamburg
Thermo cycler	Techne, Wertheim Bestenheid
System for the production of distilled water.	Millipore, Eschborn
Waterbath (Julabo U3)	Julabo Labortechnik GmbH, Seelbach
Gel documentation system	INTAS, Göttingen
Thermoimager	Bio Rad

2.4.2. Special objects of utility

2.4.2.1. Cell culture

Dissection instruments	Aeskulap, Heidelberg
Incubator (Cytoperm)	Kendro, Hanau
Langendorff-Apparatus	University, Giessen
Nylon net (Pore size 200 µm)	Neolab, Heidelberg
Microscope (TMS-F)	Nikon, Japan
Sterile bench (Lamin Air® HBB2472)	Kendro, Hanau
Tissue chopper	Harvard Apparatus, March-Hugstetten

2.4.2.2. Microscopy

Phase Contrast microscopy (IX 70)	Olympus, Japan

2.4.2.3. Consumables

Culture dishes (Falcon 3001 -3004)	Becton Dickinson, Heidelberg
Gloves	NOBA pvt. Wetter
Pipette-Tips	Sarstedt, Nümbrecht
Reaction tubes (0.5/ 1.5/ 2.0 ml)	Eppendorf-Netheler-Hinz, Hamburg

3. METHODS

3.1. Isolation of ventricular cardiomyocytes

3.1.1. Laboratory Animals

Ventricular cardiomyocytes were isolated from two to three months old male Wistar rats. These 300 to 400 g rats were breeded in the animal house of the Institute of Physiology at the Justus-Liebig-University, Giessen.

3.1.2. Preparation of isolated ventricular cardiomyocytes from rat hearts (Piper et al., 1982)

The following solutions were used for the preparation of cardiomyocytes.

Calcium-stock solution:

$CaCl_2$	100 mM

Powell-Medium: (Carbogen gased):

NaCl	110 mM
$NaHCO_3$	25 mM
Glucose	11 mM
KCl	2.6 mM
KH_2PO_4	1.2 mM
Mg_2SO_4 x H_2O	1.2 mM

Collagenase-buffer:

Powell-Medium	40 ml
Collagenase	25 mg
Calcium-Stock solution	12.5 µl

3.1.3. Procedure for the preparation of cardiomyocytes

At the beginning of the preparation, the Langendorff perfusion system was flushed with powell medium and then filled bubble free with 80 ml powell medium and warmed up to 37°C. To obtain a constant pH value, powell medium was gased with Carbogen throughout the whole preparation. Wistar rats (appr. 300 g) were anaesthetised for 1-2 min with diethyl ether. Chest was opened and diaphragm dissected. The hearts together with the lungs were transfered into a large petridish containing ice-cold salt solution (0.9 % NaCl). Lung, esophagus, trachea and thymus were removed. The flow of perfusion were started with 1 drop per sec. The hearts were mounted on cannula of the Langendorff apparatus, by slipping the aorta over the cannula, and fixed it with a clamp. The appended heart was flushed blood free with 40 ml powell medium and then retrograde perfused with 50ml recirculating collagenase buffer.

After perfusion aorta and atria were removed and ventricles were chopped in pieces with a tissue chopper (slitting with 0.7 mm). Chopped tissue was digested with 30 ml carbogen gased collagenase buffer for 5 min at 37 °C. To separate single cells, suspension was pipetted up and down several times with a sterile 5 ml pipette.The material was filtered through a nylon mesh. The suspension was spined down at 25 x g for 3 min, and the resulting pellet was resuspended in powell medium containing 200 µM calcium chloride to adapt cells to calcium. Thereafter cells were centrifugated at 25 x g for 2 min, the cell pellet was resuspended in powell medium containing 400 µM calcium chloride solution. Test tubes were filled with 1 mM calcium chloride solution and resuspended cardiomyocytes were added. Intact myocytes were collected after a centrifugation at 15 x g for 1 min. The supernatant was removed and the pellet resuspended in culture medium resulting in 40-60 % intact cells.

3.2. Culturing of cardiomyocytes

The following solutions were used for cultivating isolated cardiomyocytes.

CCT-Medium (sterile filtered, pH 7.4):

M199 / HEPES	x ml
Creatine	5 mM
Taurine	5 mM
Carnitine	2 mM
Cytosin-β-Arabinofuranoside	10 µM

Preincubation Medium

M199/HEPES (sterile filtered, pH 7.4)	x ml
FCS	4 % (vol/vol)
Penicilline	100 IE/ml
Streptomycine	100 µg/ml

CCT-Culture medium

CCT-Medium (sterile filtrated, pH 7.4)	x ml
Penicilline	100 IU/ml
Streptomycine	100 µg/ml

3.2.1. Preincubation of culture plates

In order to allow cardiomyocytes to attach culture dishes were incubated at least 2 hours at 37°C with preincubation medium. The medium was removed before plating cells.

3.2.2. Plating of cardiomyocytes

Isolated cardiomyocytes from one heart were suspended in 24 ml of CCT-culture medium, mixed homogeneously and plated at a density of approximately 5 x 10^4 cells per 35 mm culture dish. In each culture dish the portion of rod shaped living cells ranged from 40 to 60 %.

3.2.3. Culturing of cardiomyocytes

Plated cells were cultured for 2-4 hours at 37°C under CO_2 free conditions and then washed three times with CCT-culture medium to remove round and non-attached cells. This results of 90 % living, intact, rod-shaped cells. When cells should be incubated for 24 hours, gentamicin (10 µg/ml) was added to the CCT culture medium.

3.2.4. Treatment of cardiomyocytes

After washing of cardiomyocytes with CCT-culture medium, the cells were treated according to the different protocols e.g., TGFβ1 (1ng/ml), myostatin (10 ng/ml), BMP-2 (80 ng/ml), Activin (100 ng/ml), and GDF15 (3 ng/ml), were added and cells were incubated according to respective protocols at 37°C, 95 % humidity. For inhibition of JNK pathway cells were incubated with JNK specific inhibitor (10 µM) for 30 min before addition of myostatin. For controls non treated cells were used. Afterwards all treated and non treated cells were incubated at 37°C, 95 % humidity. Added substances were left on the culture dishes.

3.3. Transformation of cardiomyocytes

3.3.1. Hybridization of decoy-oligonuleotides

For transformation of cardiomyocytes the following oligonucleotides were used.

Decoy-oligonucleotides

SMAD-Decoy	5’-GTACATTGTCA**GTCTAGACA**TACT-3’
AP-1-Decoy	5’-**TGACGT**CA**TGACGT**CA**TGACGT**CA-3’

The decoy oligos are double stranded DNA fragments and contained consensus binding sequences. The underlined sequences are consensus binding sequences. Decoy oligos are capable of scavenging intracellular specific transcription factors.

Each decoy oligonucleotide was dissolved in Tris-HCl/EDTA buffer. To hybridize both complementary strands equimolar amounts (100 µM) were mixed and heated in the cycler at 95°C. Reaction was then slowly cooled down for several minutes to room

temperature. In this time complementary strands passed through the melting point and hybridized as double strand DNA fragments.

3.3.2. Transformation of cardiomyocytes with decoy oligonucleotides

To increase stability of oligonucleotides against intracellular exonucleases the last 5 bases on each end were provided with phosphothioester. The hybriadized double stranded DNA is capable to intracellular scavenge specific transcription factors. For transformation of cells decoy oligonucleotides (500 nM) were added to the medium after washing of the cells with CCTculture medium, the cells were incubated 5 hours at 37°C, 95 % humidity.

3.4. Retardation assay

Retardation assay is a technique to determine protein-DNA interaction. The interaction of proteins with DNA is central for the control of many cellular processes including transcription. Retardation assay technique is based on the observation that protein-DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide electrophoresis. Because the rate of DNA migration is shifted or retarded upon protein binding. The DNA is lablled with fluorescent dye and can be detected by scanning in a fluorimager.

3.4.1. Fluorescence labelling of oligonucleotides

The following oligonucleotides were labelled with fluorescent dye.

SMAD 5’-GTACAT TGTCA**GTCTAGACA**TACT-3’

AP-1 5’-ATCCGCTTGA**TGAGTCA**GCCGGAA-3’

The underlined sequences are consensus binding sequences and bind with specific transcription factors in the retardation assay.

3.4.2. Fluorescence labelling buffer

NEB buffer No. 4 (10x)	10 µl
$CoCl_2$ (2.5 mM)	10 µl
oligonucleotides (100µM)	X µl

Cy3-dCTP (10mM)	5 µl
Terminal Transferase (20 U/µl)	2 µl
Deionised H_2O	Y µl
Total	100 µl

The oligonucleotides were commercially purchased.

3.4.3. Procedure of fluorescence labelling of oligonucleotides

All labelling reactions were performed in brown eppendorfs tubes to protect the dye against light. The reaction tube was incubated for 15 minutes at 37°C. The reaction was stopped by addition of 20µl 0.2 mM EDTA pH 8.0 and stored at -20°C. These labeled oligonucleotides bind to specific proteins and can be visualized with fluorescence imager.

3.4.4. Nuclear extraction

3.4.5. Nuclear extraction buffers

For the nuclear extraction following buffers were used:

1x swelling buffer

Tris-hydroxymethylaminomethan (Tris)/HCl (pH 7.9)	10 mM
KCl	10 mM
$MgCl_2$	1 mM
Dithiothreitol	1 mM

1x homogenising buffer

Sucrose	300 mM
Tris/HCl (pH 7.9)	10 mM
$MgCl_2$	1,5 mM
DTT	1 mM
Triton X-100	0,3 % (wt/vol)

10x storage buffer

NaCl	300 mM
KCl	50 mM
HEPES (pH 7.5)	10 mM
Dithiothreitol (DTT)	1 mM
Phenyl methyl sulfonyl fluorid (PMSF)	1 mM
Glycerol	20 % (wt/vol)

3.4.6. Nuclear extraction procedure

Cardiomyocytes with different treatments were harvested with 1x PBS as described above. For generation of nuclear extracts 2 dishes of cells were harvested and cells dissolved in 400 µl of 1x swelling buffer. After incubation for 1h on ice, nuclei were pelleted by centrifugation at 900-x g for 10 min at 4°C. The upper phase was removed and pellets were suspended in 400 µl 1 x homogenizing buffer for 10 min on ice, homogenized with 8 strokes in a glass homogenizer, and again centrifuged as above. To the pellet 40 µl of 1x-storage buffer was added and incubated on ice, every 5 min vortexed. After 30 minutes extracts were centrifuged at 13000 x g for 10 min at 4°C. The resulting supernatants contained the nuclear extract and were stored at -80°C.

3.4.7. Binding reaction of oligonucleotides with specific proteins

Binding Buffer

Glycerine	10 % (vol/vol)
Tris/HCl	20 mM
KCl	5 mM
$MgCl_2$	5 mM
DTT	3 mM
PMSF	0.2 mM

10 µl of nuclear extract was mixed with 4.5 µl binding buffer and 0.5 µl of nonspecific DNA (poly (dIdC); 1mg/ml), and incubated at 30°C for 15 minutes. Thus nonspecific DNA binding proteins could be intercepted. 1 µl of fluorescence labelled oligonucleotides together with 1.5 µl of binding buffer were added to the above reaction

tube and incubated at 30°C for 30 minutes. During this time specific proteins will bind to their consensus binding sites of the oligos

3.4.8. Retardation assay gel electrophoresis

This method is based on the changed electrophoretic mobility of a protein DNA complex compared with a marked DNA fragment or oligonucleotide.
For gel electrophoresis the following solutions were used.

3.4.9. Solutions for retardation gel electrophrosis

Distilled water	35 ml
Acrylamid/Bisacrylamid (80%, 79:1)	4 ml
Glycerine	1 ml
100x RA-buffer	400 µl
Ammoniumpersulphate 10 % (wt/vol)	300 µl
TEMED	34 µl

100x RA-buffer, pH 7.9

Tris/HCl	670 mmol/l
Sodium accetate	330 mmol/l
EDTA	100 mmol/l

10x marker dye Tris-acetat/EDTA (TAE)-buffer

Tris-accetat	40 mM
EDTA	1 mM
Bromophenol blue/xylene cyanol	1 % (vol/vol)

3.4.10. Retardation assay gel

The reaction mix was resolved on 4% denaturing polyacrylamide gels. For preparation of these gels first two glass plates were cleaned with detergent and degreased with alcohol (100 %). Subsequently, one of the plates was treated with Acrylase. Between the plates now two spacers were placed. The plates were clamped in a gel casting device and sealed at the bttom.

Subsequently, the freshly prepared retardation gel solution was poured bubble free into area between the two plates. Then the comb was set. The gel polymerized in approximately 30 min at room temperature.

The comb was removed, the glass plates were transferred from the gel casting device into the electrophoresis chamber. The chamber was filled in such a way with 1x RA-buffer that the gel had both above and down a bubble free contact to the buffer. Now the labeled reaction samples and distance indicator Bromophenol blue were loaded, and the electrophoresis chamber was attached to the power supply unit. Electrophoresis was performed at 4°C and 150 V for approx. 2 h. Gels were scanned and images were evaluated.

3.5. Apoptosis assay

3.5.1. Quantitative apoptosis assay with Hoechst 33258/Propidium iodide double staining

About 20 h after apoptosis induction, cardiomyocytes were double stained with Hoechst 33258 (Hoe 33258) (5 mg/ml) and propidium iodide (pi) (1 mg/ml) successively given to the culture medium. The staining materials in the medium were distributed by gently shaking. Afterwards the cultures were incubated for 15 min at 37°C. The cells were analysed in the fluorescence microscope.

Hoe 33258 is a cell membrane permeable DNA staining dye, which stains DNA in all cells. Thus, the late phase of apoptosis in which a typical condensation of chromatin appears, can be particularly well recognized by the intense staining of the nuclei. Hoe 33258 was analysed at wavelengths of 340-360 nm and showed blue fluorescence. Propidium iodide is not a cell membrane permeable DNA staining dye. Therefore, it only stains nuclei of necrotic cells, whose cell membranes are already damaged. Propidium iodide stain becomes visible at a spectrum of 510-550 nm and shows an intensively red fluorescence. Per value about 200 cells from different places in the culture dish were analysed.

3.5.2. DNA laddering analysis

Lysis buffer

NaCl	100 mM
Tris pH 8.0	50 mM
EDTA	10 mM
SDS	1%

TE solution

Tris-HCl pH 8.0	10mM
EDTA	1mM

RNAse

DNAse-free RNAse	5 mg/ml

Proteinase K

Proteinase K pH 8	100 mg/ml

3.5.3. Isolation of genomic DNA

After 24 hours treatment cardiomyocytes were harvested by addition of 1 ml of lysis buffer to the dishes. The cells were transferred into new tubes and Proteinase K (100 mg/ml) was added and incubated overnight at 55°C. Then 1 ml of phenol/chloroform (1:1, V/V) was added, tubes were vortexed for 15 seconds, and centrifuged at 13000 X g for 15 min. The upper phase was transferred to new 2 ml eppendorf tubes. 1 ml of chloroform was added and again centrifuged for 15 min at 13000 X g. The upper DNA containing phase was transferred to a new 2 ml eppendorf tube. For precipitation of DNA an equal volume of isopropanol was added and centrifuged at 4°C for 30 min at 13000 X g. The supernatant was removed and 500 µl of 70% ethanol were added, again centrifuged for 15 min at 13000 X g. The supernatant was removed and pellet was dried at room temperature for 30 min. 50 µl of TE buffer was added. The pellet was dissolved at 30°C overnight. 5 µl of DNAse-free RNAse was added and incubated at 37°C for 1 hour. Again the DNA was precipitated by the addition of ethanol. After centrifugation the pellet was resuspended in TE buffer. DNA concentration was determined spectrophotometrically at a wavelength of 280 nm.

3.5.4. Agarose gel analysis

50xTAE buffer (1L)

2 M Tris base	242 g
1 M Glacial acetic acid	57.1 ml
0.5M EDTA pH8	100 ml
The final pH 7.2 was adjusted	

DNA loading buffer

Sucrose / 1x TAE buffer	1:1-V/V

2 % agarose gel was prepared by boiling 2 grams of agarose in 100 ml of 1x TAE buffer. After cooling the gel solution to 60-70°C, ethidium bromide was added (0.5 µg/ml). DNA loading buffer (20 µl) was added to each sample. 10 µg of DNA probes were loaded on the gel and the gel was run in 1x TAE buffer at 3-5 V/cm for 1 hour. The gel was analyzed under UV light gel-documentation system and image was saved.

3.6. Western blot

RIPA buffer

Tris pH7.5	50 mM
NaCl	150 mM
Nonidet P-40	1 %
Deoxycholat	0.5 %
SDS	0.5 %
PMSF	1 mM
EDTA	1 mM
NaF	50 mM
β-glycerophosphat	40 mM
Pepstatin	1 µg/ml

1 xPBS

KCl	2.7 mM
KH_2PO_4	1.5 mM
NaCl	150 mM
Na_2HPO_4 x$2H_2O$	6.5 mM

The pH was adjusted to 7.5

3.6.1. Harvesting of cells

Plates (35 mm) were placed on ice. Cells were washed twice with 1 ml ice-cold 1x PBS. 200 µl of RIPA buffer was added. After an incubation time of 10 minutes on ice, cells were scraped and transferred into a fresh tube. 4 µl of magnesium chloride (100 mM) and 5 µl benzonase (1: 10) were added and lysates were incubated on ice for 30 minutes. Cell lysates were centrifuged at 13000 rpm for 30 minutes at 4°C. Supernatant was transferred into a fresh tube and frozen at -80°C.

3.6.2. 10% Polyacrylamide gel

Stacking gel

Water	6.75 ml
30 % acrylamide (29:1 AA/BA)	1.675 ml
1 M Tris pH 6.8	1.25 ml
10 % SDS	100 µl
10 % APS	100 µl
TEMED	20 µl

Resolving gel

Water	8.4 ml
30 % acrylamide (29:1 AA/BA)	6.6 ml
1.5 M Tris pH 8.8	5 ml
10 % SDS	200 µl
10 % APS	50 µl
TEMED	40 µl

10x Lämmli buffer

Glycin	144 g
Tris	30.275 g
Water was added up to 1000 ml	

Running buffer

Lämmli buffer	1x
SDS	0.1%

4x Lämmli protein loading buffer

1 M Tris pH 6.8	2.5 ml
20% SDS	4 ml
Glycerol	2 ml

DTT	154 mM
Bromophenol blue	1 mg
Water	1.5 ml

Preparation of samples

RIPA sample	30 µl
Lämmli-protein- loading buffer	10 µl

Samples were incubated for 10 minutes at 95°C and then loaded on gel.

3.6.3. Preparation of gel

The above mentioned resolving and stacking gel reagents were for one 7x10 cm gel, 0.75-1.00 mm thick. Glass plates were assembled by placing a spacer at both sides and sealed at bottom. The ammonium persulphate (APS) and TEMED were added just prior to pouring the gel, as these reagents promote and catalyze the polymerization of the acrylamid. The resolving gel mix was poured into the assembled gel plates leaving sufficient space for the stacking gel to be added later. The gel was gently overlayed with 0.1% SDS, and the gel was allowed to polymerize for 15-30 minutes. After polymerization, SDS overlay was removed and the surface of the resolving gel was rinsed with water to remove any unpolymerized acrylamide. The remaining space was filled with stacking gel and comb was inserted immediately. After the stacking gel was polymerized, the comb was removed and the wells were rinsed with running buffer to remove the unpolymerized acrylamide. At least 1 cm of stacking gel was present between the bottom of the loading wells and the resolving gel.

The samples were prepared and loaded, together with protein markers and run at 200 volts for 4 hours.

3.6.4. Blotting of proteins

Blotting buffers

Western blot buffer A (cathode buffer)

Tris (3.03g)	25 mM
6-amino-hexanoic acid (5.25g)	40 mM
Methanol	20%

Water was added to a total volume of 1000 ml and pH was adjusted to 9.4.

Western blot buffer B (anode buffer)

Tris (3.63g)	30 mM
Mthanol	20%

And pH 10.4 was adjusted.

Western blot buffer C (10x anode buffer)

Tris (36.3g)	300 mM
Methanol	20%

And pH was adjusted to 10.4.

3.6.5. Preparation of blotting chamber and transfer of protein to membrane

9 pieces of whatman papers were cut as the size of gel. 3 papers were soaked with buffer C and placed at the bottom of chamber, above 3 papers socked with buffer B were placed. Nirocellose membrane of equal size as the gel was socked in buffer B, placed above. Gel was placed above membrane and 3 papers socked with buffer A were placed at the top. Blot was run at 200 mA (~1.5mA/cm^2) current and maximum 50 V. After 3 hours gel was removed and membrane was stained with ponceau, destained with water and a photograph was taken of stained bands. Membrane was washed 3 times with 1x TBS.

Blocking buffer

TBS buffer	1x
Tween	0.1%

Milk powder	5%

Washing buffer

TBS	1X
Tween	0.1%

Membrane was incubated in blocking buffer at room temperature for 1 hour and washed 3 times with washing buffer.

3.6.6. Antibody dilutions

Smad4 and P-Smad 3 antibody dilution

Smad 4 or P-Smad 3 antibody	1:1000
TBS	1X
Tween	0.1%
BSA	5%

P-Smad 2 dilution

P-Smad 2 antibody	1:1000
TBS	1X
Tween	0.1%
Milk powder	5%

Dilution of Actin antibody

Actin antibody	1:2000
TBS	1X
Tween	0.1%
BSA	5%

Dilution of second antibody

Second antibody	1:2000
TBS	1X
Tween	0.1%
Milk powder	5%

Membrane was incubated with first antibody over night at 4°C by shaking. Membrane was washed 3 times with 1xTBS/0.1% Tween for 15 minutes for each washing Step. Membrane was incubated for SMADs with anti rabbit HRP conjugated antibody and for actin with anti mouse HRP conjugated antibody for 1-2 hours at room temprature. Membrane was washed 3 times with 1xTBS/0.1% Tween for 15 minutes for each washing step.

The blot was incubated in ECL solution for 1 minute and several photographs were taken over 15 min.

3.7. Statistics

Results are presented as mean ± standard error from different culture preparations.

Statistical comparisons were performed by ANOVA (One-Way analysis of variance).

Differences with $p<0.05$ were taken as statistically significant.

The data were analysed with Microsoft Excel 2000® as well as SPSS® version 11.5.1. (SAS of institutes Inc., Cary, N.C., and the USA).

4. Results

4.1. Involvement of TGFβ1 in apoptosis induction in adult rat cardiomyocytes

4.1.1. SMAD-binding activity under TGFβ1

Cardiomyocytes were stimulated with TGFβ1 (transforming growth factor β1) (1 ng/ml) for 2 h. Nuclear extract were isolated and retardation assay was performed to determine the SMAD (small mother against decapentaplegic)-binding activity. Therefore the nuclear extracts were incubated with fluorescence labeled SMAD-decoy oligonucleotides, which have SMAD binding sequences. When binding active SMAD protein is present in the nuclear extract it will bind to these decoy oligos when run on retardation assay gel and results in band shifts. TGFβ1 induced cells showed a strong band shift as compared to the control. This showed that TGFβ1 induces SMAD binding activity in cardiomyocytes (fig.4.1.1.A).

C **TGFβ1**

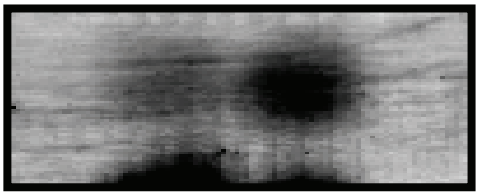

Fig: 4.1.1.A. Identification of SMAD binding activity in TGFβ1 stimulated cardiomyocytes. Cardiomyocytes were stimulated in the presence of TGFβ1 (1 ng/ml) for 2 h. This is a representative retardation assay gel for SMAD activation.

Density metric analysis of band shifts revealed that SMAD binding increased 114.1 $\pm$ 14.8 % as compared to control values.

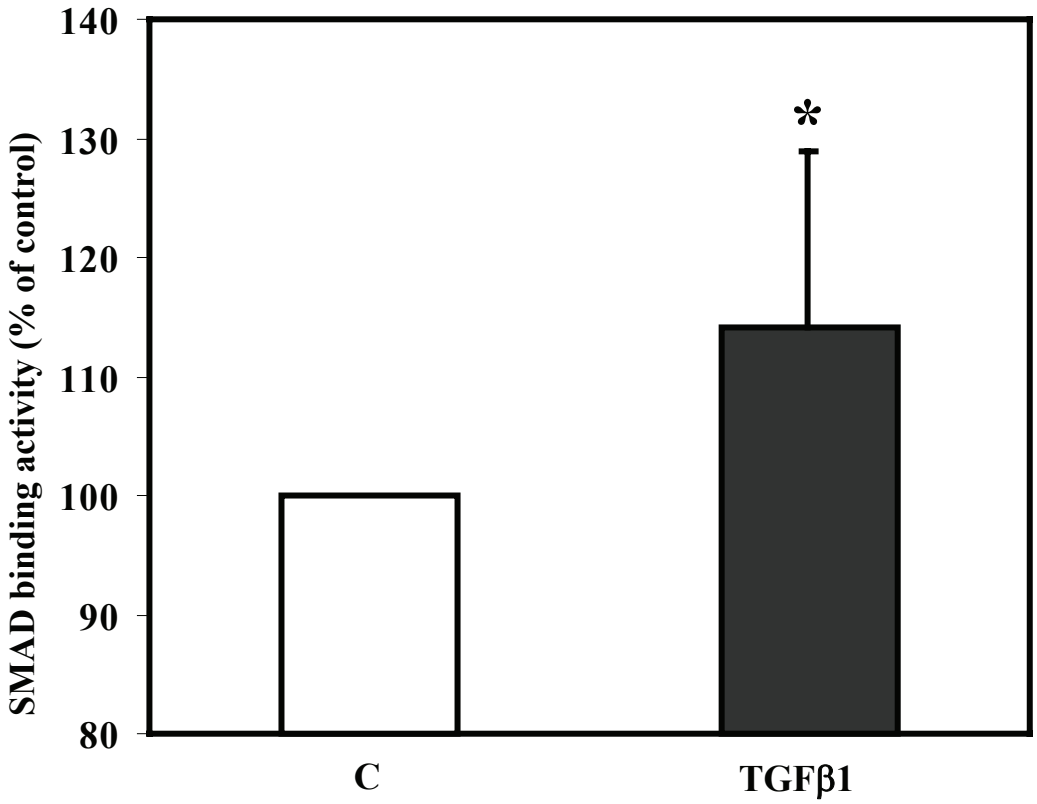

Fig: 4.1.1.B Identification of SMAD-binding activity in TGFβ1 stimulated cardiomyocytes. Cardiomyocytes were incubated in the presence of TGFβ1 (1 ng/ml) for 2 h and nuclear extracts from cells were prepared. 10 µl of nuclear extracts were tested for SMAD binding activity using fluorescence labeled SMAD-decoy oligonucleotides. Quantitative analysis of retardation assay gel. Data are mean ± SE of seven independent (n=7) culture preparations. *Differences from unstimulated control with $p<0.05$.

4.1.2. Apoptosis induction under TGFβ1 stimulation

In order to examine, if TGFβ1 also increases apoptosis, cardiomyocytes were stimulated in presence of TGFβ1 (1 ng/ml) for 20 h. Cells were analysed for apoptosis induction by chromatin condensation. TGFβ1 increased the number of apoptotic cells to 21.7 $\pm$ 4.7 % as compared to 12.7 $\pm$ 4.0 % in controls (Fig. 4.1.2).

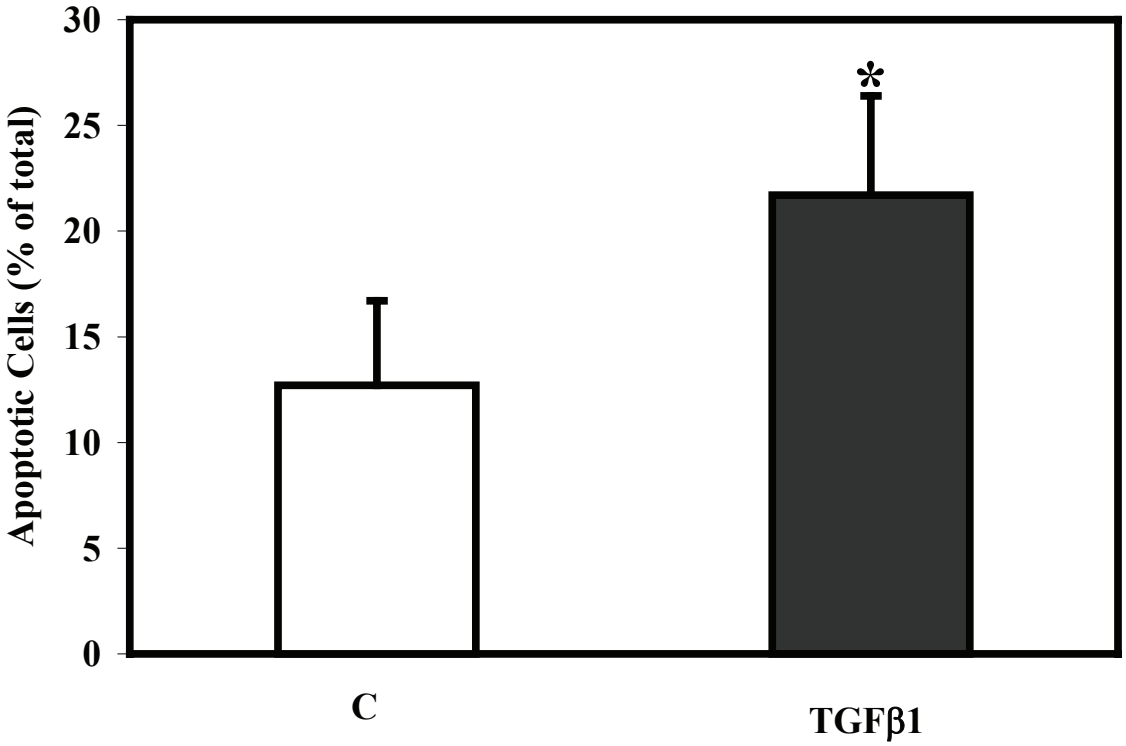

Fig: 4.1.2. Apoptosis induction in adult rat cardiomyocytes under TGFβ1 stimulation. Cardiomyocytes were stimulated in presence of TGFβ1 (1ng/ml) for 20 h. After 20 h cardiomyocytes were stained for 30 minutes with Hoechst 33258 (5 μg/ml) and propidium iodide (1 μg/ml). Cells were analysed by fluorescence microscopy. For quantification of apoptosis and necrosis 200 randomly distributed cells were counted in each experiment. Data are means ± SE of six independent (n=6) culture preparation. *Differences from unstimulated controls with $p<0.05$.

4.1.3. TGFβ1 stimulation induced DNA laddering in cardiomyocytes

In fig. 4.1.2 apoptosis induction by TGFβ1 was analysed by chromatin condensation. As an additional apoptosis parameter induction of DNA-laddering was investigated. Cardiomyocytes were stimulated with TGFβ1 (1 ng/ml) over 18-20 hours. Then DNA was extracted. 10 μg DNA per lane were separated on 1.5% agarose gels and stained with

ethidium bromide. As shown in fig 4.1.3 DNA laddering was induced under TGFβ1 in cardiomyocytes.

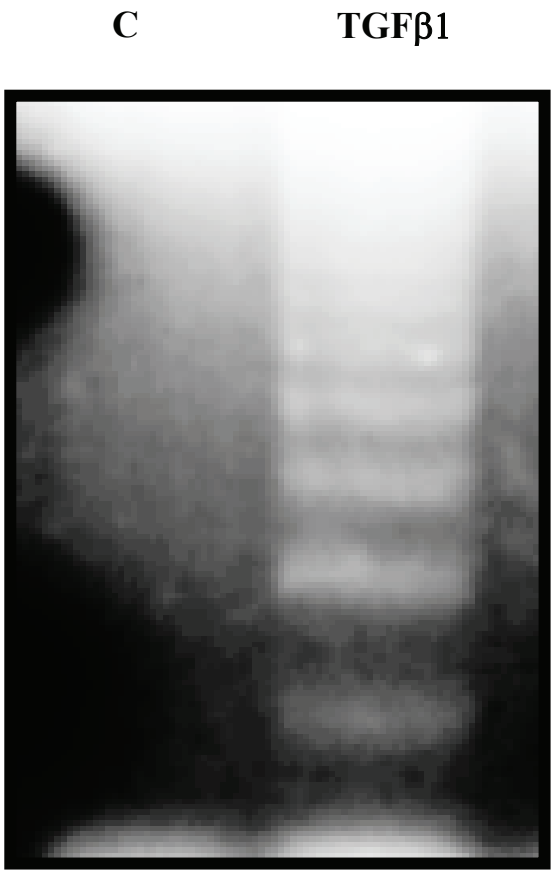

Fig: 4.1 3. TGFβ1 induces DNA laddering. Cardiomyocytes were stimulated with TGFβ1 (1 ng/ml) overnight. Genomic DNA was extracted and 10 µg DNA per lane were separated on 1.5% agarose gels and stained with ethidium bromide.

4.1.4. Inhibition of apoptosis by SMAD-decoy oligonucleotides under TGFβ1 stimulation.

For transformation with decoy nucleotides cardiomyocytes were pre-incubated with SMAD-decoy oligonucleotides (500 nM) for 5 h. The SMAD-decoy oligonucleotides contain specific SMAD binding sites and scavenge intracellular SMAD. Then cardiomyocytes were stimulated with TGFβ1 (1 ng/ml). After 20 h cardiomyocytes were

stained for 30 minutes with Hoechst 33258 (5 μg/ml) and propidium iodide (1 μg/ml). Cells were analysed by fluorescence microscopy.

TGFβ1 induced apoptosis in 11.3 ± 2.7 % of cells as compared to controls (7.47 ± 2.1 %). Pre-incubation of cardiomyocytes with SMAD-decoy oligonucleotides significantly reduced the number of apoptotic cells to 8.4 ± 2.8 %.

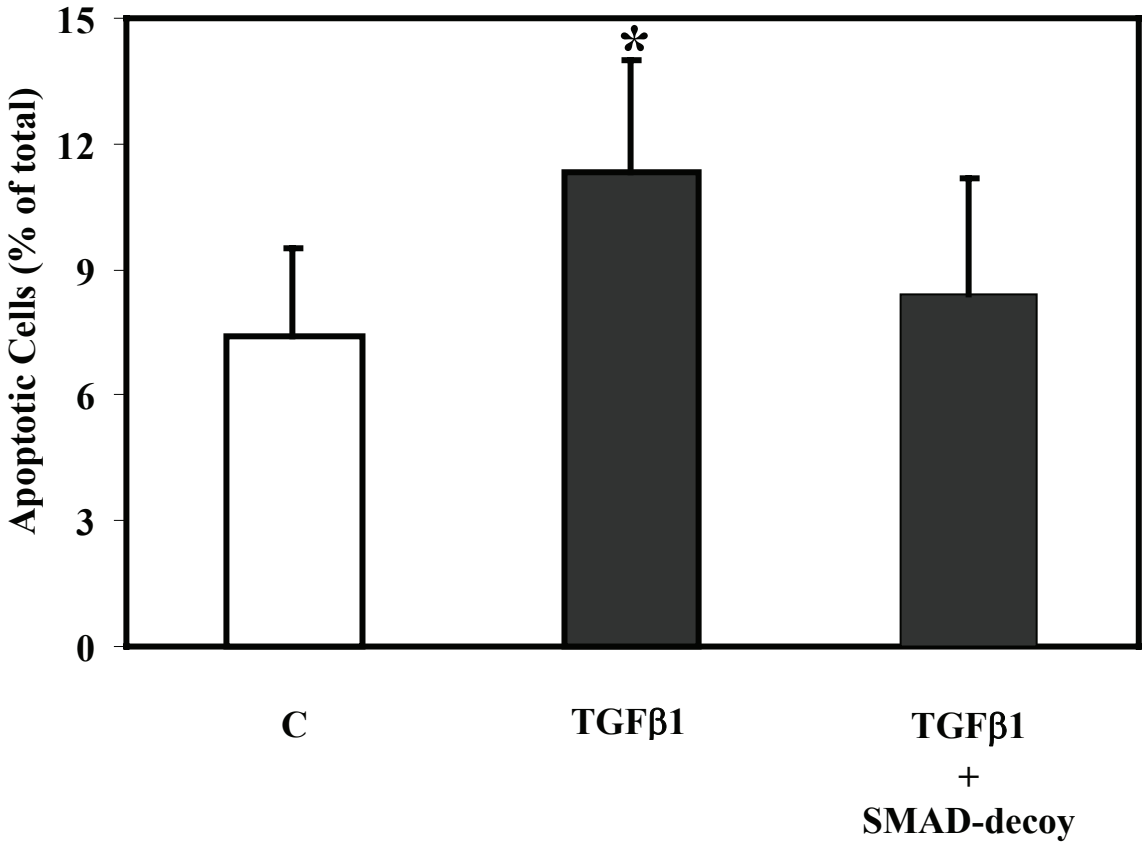

Fig: 4.1.4. Inhibition of apoptosis by SMAD-decoy oligonucleotides. Cardiomyocytes were pre-incubated with SMAD-decoy oligonucleotides (500 nM) for 5 h. Then cardiomyocytes were stimulated in the presence of TGFβ1 (1 ng/ml) for 20 h and analysed for apoptosis. Data are means ± SE of six independent (n=6) culture preparations. *Differences from unstimulated controls with $p<0.05$.

4.1.5. SMAD-decoy oligonucleotides inhibit DNA laddering in the presence of TGFβ1 stimulation

The effect of SMAD-decoy oligonucleotides was also analysed by appearance of DNA-laddering. Cardiomyocytes were pre-incubated with SMAD decoy oligonucleotides (500 nM) for 5 h and stimulated with TGFβ1 (1 ng/ml) overnight. Genomic DNA was extracted. 10 µg DNA per lane were separated on 1.5% agarose gel and stained with ethidium bromide. DNA laddering was reduced when SMAD-activity was inhibited by decoy oligos (fig.4.1.5).

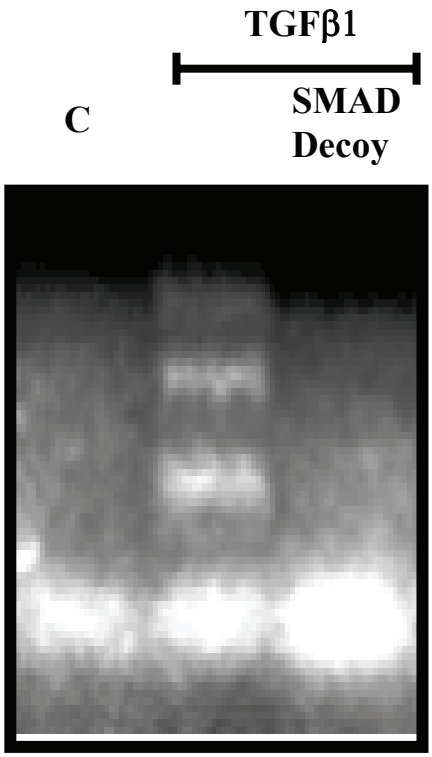

Fig: 4.1.5 SMAD-decoy oligonucleotides reduce DNA laddering. Cardiomyocytes were pre-incubated with SMAD-decoy oligonucleotides (500 nM) for 5 h and stimulated with TGFβ1 (1 ng/ml) overnight. Genomic DNA was extracted. 10 µg DNA per lane were separated on 1.5% agarose gels and stained with ethidium bromide. DNA laddering was reduced when cells were transformed with SMAD-decoy oligonucleotides.

4.1.6. AP-1 binding activity under TGFβ1 stimulation

It is shown that NO induces apoptosis with participation of SMAD and AP-1 (activator protein 1). It was analysed if TGFβ1 also activates AP-1. Cardiomycytes were stimulated with TGFβ1 (1 ng/ml) for 2 h. Nuclear extracts were isolated and retardation assays were performed to investigate AP-1-binding activity. Nuclear extracts from TGFβ1 induced cells showed a strong band shift in retardation assay as compared to the control. This indicates (fig. 4.1.6 A) that TGFβ1 induces AP-1 binding activity in cardiomyocytes.

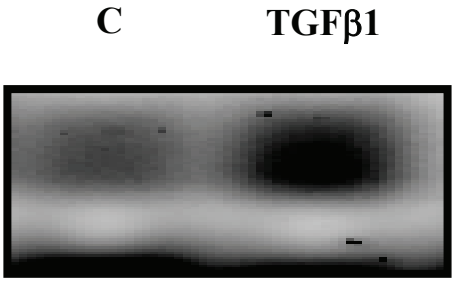

Fig: 4.1.6.A. Identification of AP-1-binding activity after TGFβ1-stimulation of cardiomyocytes. Cardiomyocytes were incubated in the presence of TGFβ1 (1 ng/ml) for 2 h and nuclear extracts from cells were prepared. 10 µl of nuclear extracts were tested for AP-1 binding activity using fluorescence labeled AP-1-oligonucleotides with specific binding sequences. This is a representative band shift from retardation assay.

Quantitative analysis of AP-1 binding activity in retardation assays revealed an increase in AP-1 binding activity to 124.8 % $\pm$ 6.9 % due to stimulation of cardiomyocytes with TGFβ1 (1 ng/ml) for 2 h.

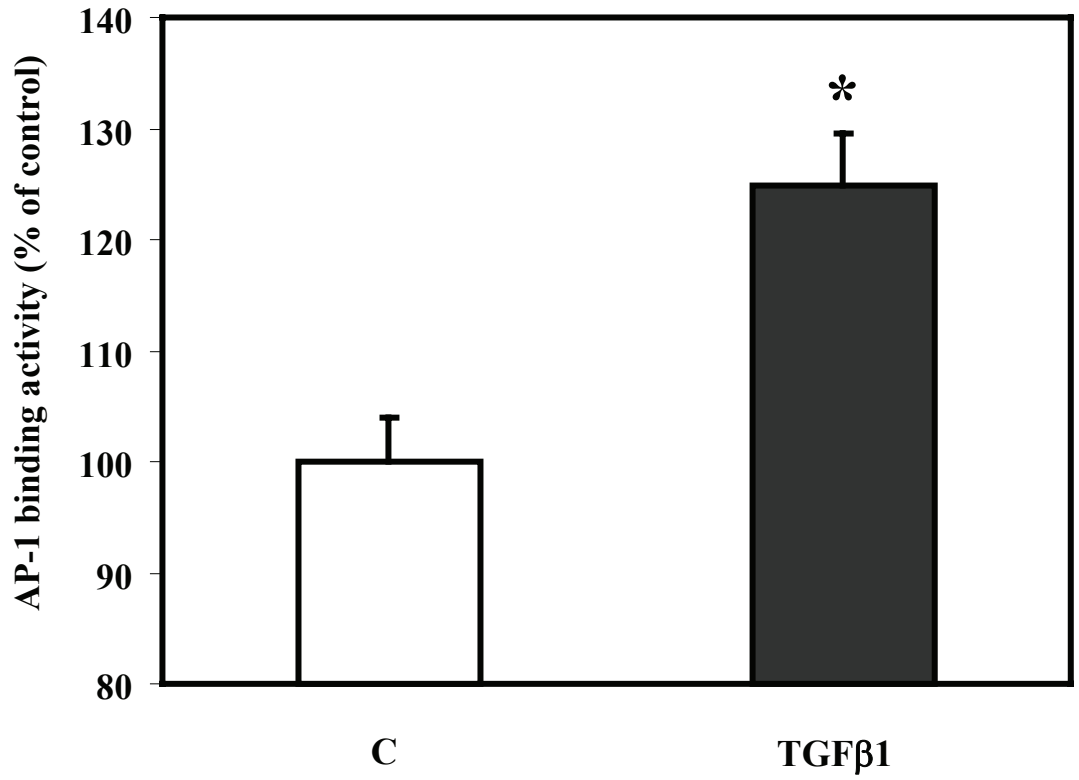

Fig: 4.1.6.B. Identification of AP-1-binding activity after TGFβ1-stimulation of cardiomyocytes. Cardiomyocytes were incubated in presence of TGFβ1 (1 ng/ml) for 2 h and nuclear extract from cells was prepared. 10 µl of nuclear extract were tested for AP-1 binding activity using fluorescence labeled AP-1 oligonucleotides with specific binding sequences. Quantitative analysis of retardation assay gels. Data are mean ± SE of six independent (n=6) culture preparations. *Differences from unstimulated controls with $p<0.05$.

4.1.7. Inhibition of apoptosis by AP-1 decoy oligonucleotides under TGFβ1 stimulation

To analyse if AP-1 is involved in TGFβ1-induced apoptosis cardiomyocytes were transformed with AP-1 decoy oligo nucleotides (500 nM) for 5 h. AP-1 decoy oligo nucleotides contain specific binding sites for AP-1 and scavenge the intracellular AP-1. Then cardiomyocytes were stimulated with TGFβ1 (1 ng/ml) for 20 h. Then

cardiomyocytes were stained for 30 minutes with Hoechst 33258 (5 μg/ml) and propidium iodide (1μg/ml). Cells were analysed by fluorescence microscopy.

TGFβ1 induced apoptosis in 14.0 ± 2.7 % cells as compared to 11.1 ± 1.7 % in controls. The pre-incubation of cardiomyocytes with AP-1-decoy oligonucleotides significantly reduced apoptosis to 9.8 ± 2.3 %.

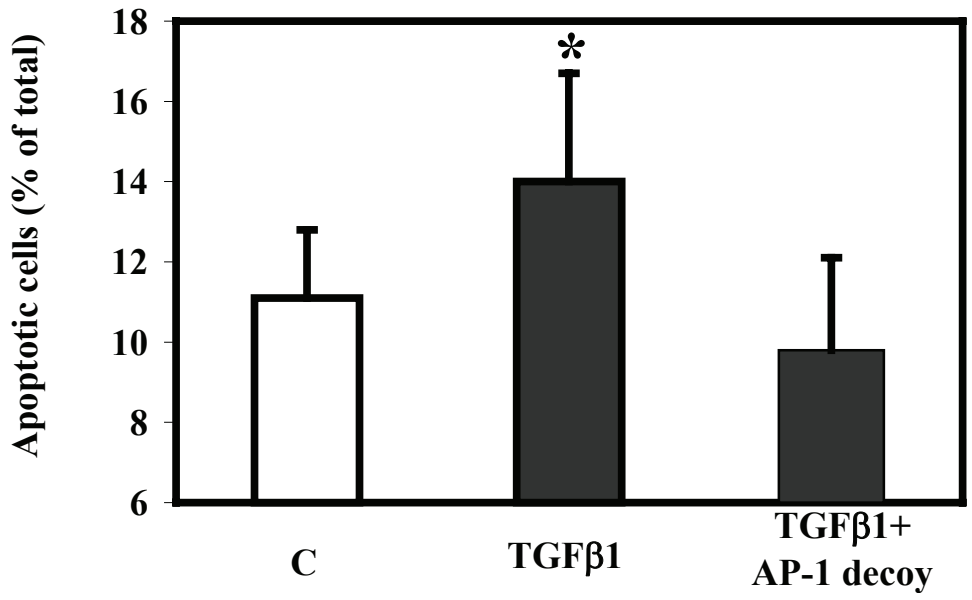

Fig: 4.1.7. Inhibition of apoptosis by AP-1 decoy oligo nucleotides. Cardiomyocytes were pre-incubated with AP-1 decoy oligonucleotides (500 nM) for 5 h. Then cardiomyocytes were stimulated in the presence of TGFβ1 (1 ng/ml). After 20 h cardiomyocytes were stained for 30 minutes with Hoechst 33258 (5 μg/ml) and propidium iodide (1 μg/ml). Cells were analysed by fluorescence microscopy. Data are means ± SE of six independent (n=6) culture preparation. *Differences from unstimulated control with $p<0.05$.

4.1.8. AP-1-decoy oligonucleotides inhibit DNA laddering in the presence of TGFβ1 stimulation

The effect of AP-1 decoy oligonucleotides determined by chromatin condensation should be confirmed by detection of DNA-laddering. Therefore cardiomyocytes were pre-incubated with AP-1 decoy oligonucleotides (500 nM) for 5 h and stimulated with TGFβ1 (1 ng/ml) overnight. Genomic DNA was extracted and run on 1.5% agarose gels and stained with ethidium bromide. As can be seen in figure 4.1.8 TGFβ1 induced DNA laddering could be decreased by AP-1 decoy transformed cardiomyocytes.

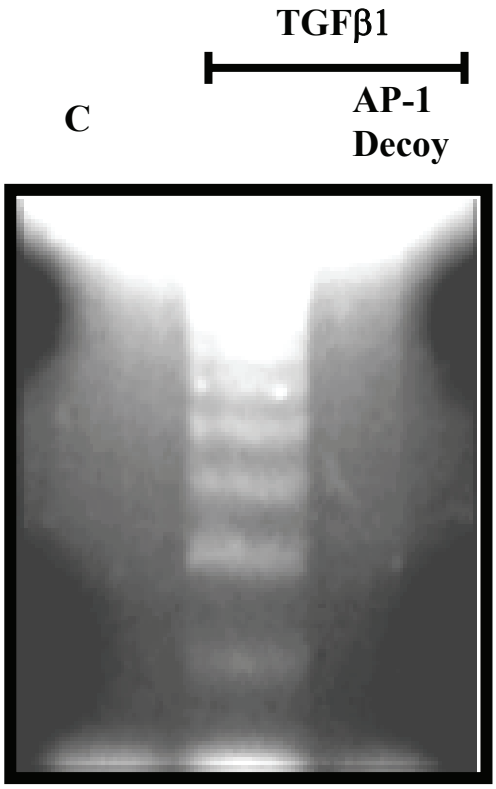

Fig: 4.1.8. AP-1 decoy oligonucleotides reduced DNA laddering. Cardiomyocytes were transformed with AP-1 decoy oligonucleotides (500 nM) for 5 h and stimulated with TGFβ1 (1 ng/ml) 18 hours. Genomic DNA was extracted and 10 µg DNA per lane were separated on 1.5% agarose gels and stained with ethidium bromide. AP-1 decoy oligo nuleotides inhibited apoptotic effects of TGFβ1.

4.1.9. Activation of SMAD isoforms under TGFβ1 stimulation

In the last paragraph it was shown that TGF-β1 induces apoptosis via SMADs. Now it was further analysed which SMAD isoforms are activated under TGF-β1 stimulation. Since phosphorylation of SMAD proteins is a sign of SMAD activity, activation of SMAD isoforms were determined in western blots using phospho specific SMAD antibodies.

4.1.9.1. Phosphorylation of SMAD-2 in TGFβ1 stimulated cardiomyocytes

Cardiomyocytes were stimulated with TGFβ1 (1 ng/ml) and total protein extracts were prepared at different time points. Samples were run on 10 % SDS gels and blotted on nitro cellulose membranes. Activation of SMAD-2 was analysed using phospho specific SMAD-2 antibodies. After 15 minutes phosphorylation of SMAD-2 started and reaching a maximum after 2 h.

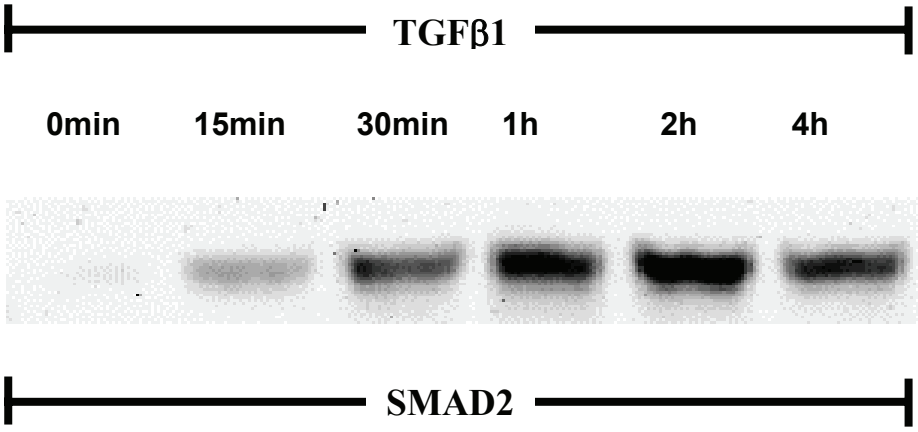

Fig: 4.1.9.1. SMAD-2 phosphorylation. Cardiomyocytes were stimulated with TGFβ1 (1 ng/ml). Proteins were extracted at different time points and were analysed in western blots using phospho specific SMAD-2 antibodies.

4.1.9.2. Phosphorylation of SMAD-3 in TGFβ1 stimulated cardiomyocytes

Cardiomyocytes were stimulated with TGFβ1 (1 ng/ml). Total proteins were extracted and analysed in western blots using phospho specific SMAD-3 antibodies. Phosphorylation after different time points were analysed. Within 30 minutes phosphorylation of SMAD3 started to increase and was still present after 60 minutes. Actin expression was used as internal control.

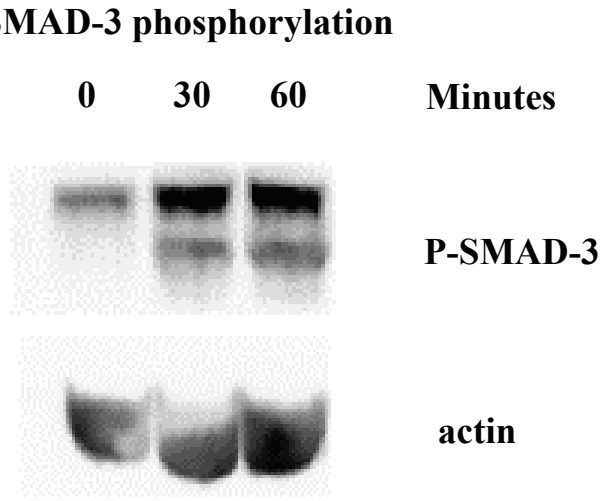

Fig: 4.1.9.2 SMAD-3 phosphorylation. Cardiomyocytes were stimulated with TGFβ1 (1 ng/ml). Western blots were performed to analyse SMAD-3 phosphorylation.

4.1.9.3. Activation of SMAD-4 by TGFβ1 stimulation

It is known that SMAD-4 is present in the cytoplasm in non-activated forms. After activation it translocates into the nucleus and participates in transactivation. Therefore, as a sign of SMAD-4 activation, its translocation to the nucleus was studied. Cardiomyocytes were stimulated with TGFβ1 (1 ng/ml). Proteins were extracted from cytoplasm and nucleus. Proteins were analysed in western blot using specific SMAD-4 antibodies. Activation SMAD-4 in the nucleus was analysed at different time points. After 2 h SMAD-4 has activation in the nucleus.

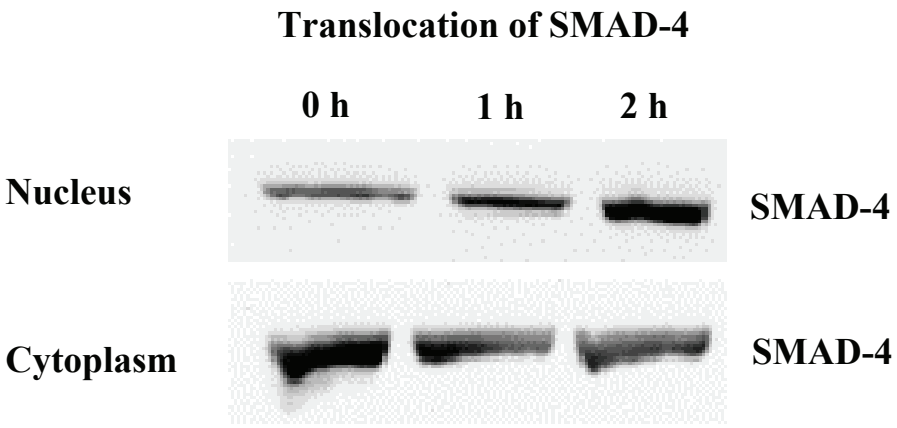

Fig: 4.1.9.3. Transcolation of SMAD-4. Cardiomyocytes were stimulated with TGFβ1 (1 ng/ml). Proteins were isolated from cytoplasm and nucleus. Western blots were performed using SMAD-4 antibodies.

4.2. Involvement of myostatin in apoptosis induction in adult rat cardiomyocytes

4.2.1. SMAD-binding activity under myostatin stimulation of adult rat cardiomyocytes

To investigate the involvement of myostatin in SMAD activation cardiomyocytes were stimulated with myostatin (10 ng/ml) for 2 h. Nuclear extracts were isolated, incubated with fluorescence labeled SMAD-decoy oligonucleotides and retardation assays were performed. The extract from myostatin induced cells showed a strong band shift as compared to the control. This demonstrates that myostatin induces SMAD binding activity in cardiomyocytes.

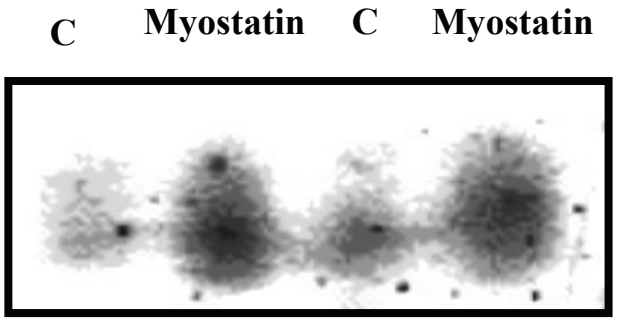

Fig: 4.2.1.A. SMAD-binding activity in myostatin stimulated cardiomyocytes. Cardiomyocytes were stimulated in the presence of myostatin (10 ng/ml) for 2 h. Retardation assay gel showing SMAD-activation in myostatin induced cardiomyocytes.

Stimulation of cardiomyocytes with myostatin (10 ng/ml) for 2 h showed a significant increase in SMAD-binding activity to 119.6 % $\pm$ 5.1 % as compared to control values.

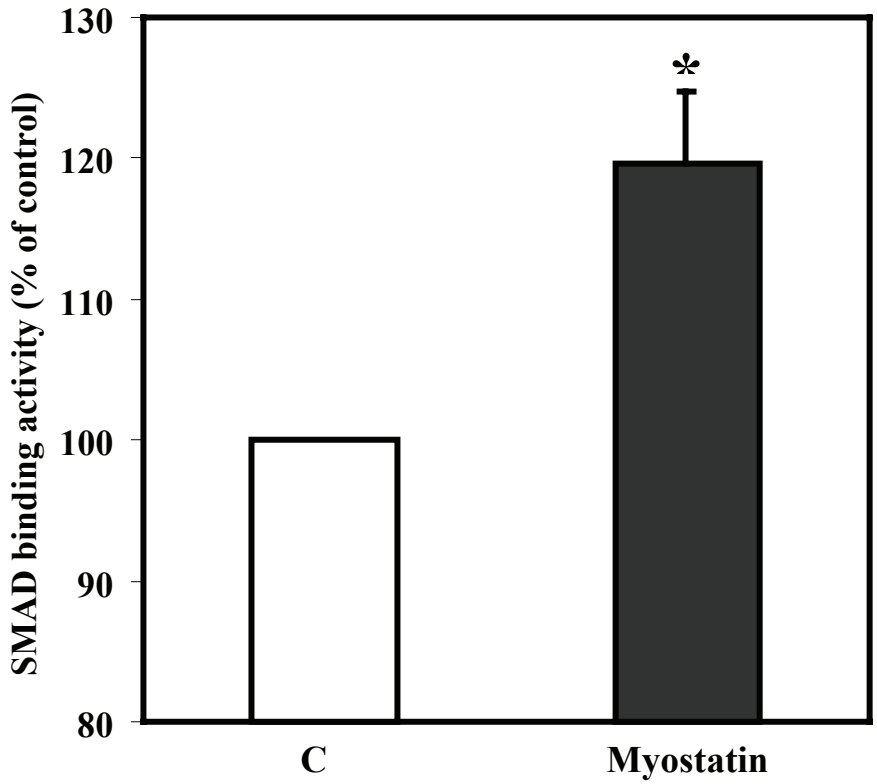

Fig: 4.2.1.B. SMAD-binding activity in myostatin stimulated cardiomyocytes. Cardiomyocytes were stimulated in the presence of myostatin (10 ng/ml) for 2 h and nuclear extracts from cells were isolated. The nuclear extracts were tested for SMAD binding activity in retardation assays. Quantitative analysis of retardation assay gels. Data are means ± SE of six independent (n=6) culture preparation. *Differences from unstimulated control with $p<0.05$.

4.2.2. Apoptosis induction under myostatin stimulation

The effect of myostatin on apoptosis induction was analysed by stimulating cardiomyocytes with myostatin (10 ng/ml). After 20 h cardiomyocytes were analysed for apoptosis induction. Myostatin increased the number of apoptotic cells to 15.9 $\pm$ 1.09 % as compared to 8.5 $\pm$ 0.9 % apoptotic cells in controls.

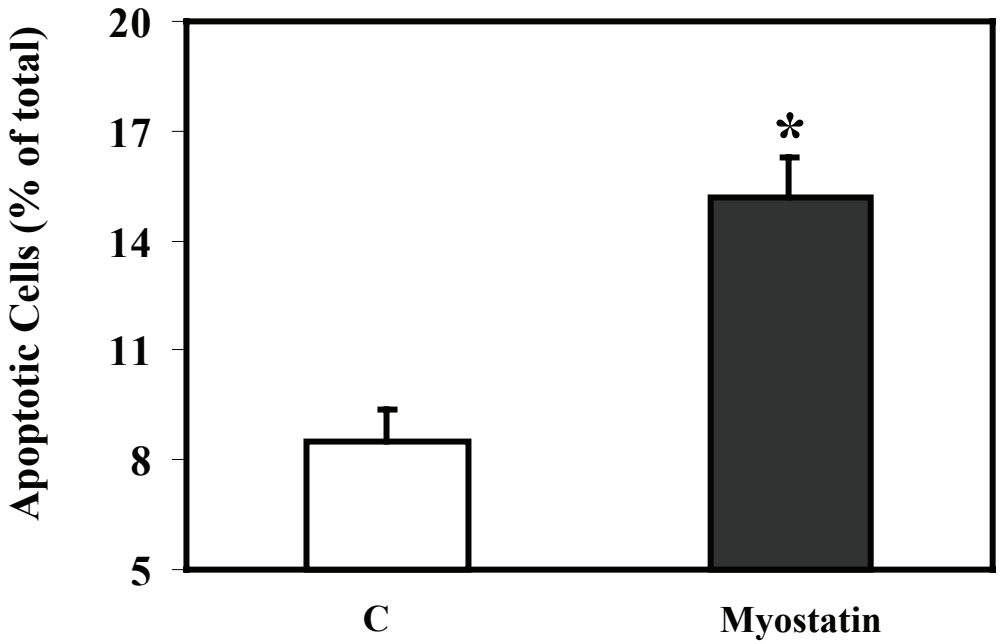

Fig: 4.2.2. Apoptosis induction in adult rat cardiomyocytes under myostatin stimulation. Cardiomyocytes were stimulated in presence of myostatin (10 ng/ml). After 20 h cardiomyocytes were stained for 30 minutes with Hoechst 33258 (5 µg/ml) and propidium iodide (1 µg/ml). Cells were analysed by fluorescence microscopy. For quantification of apoptosis and necrosis 200 randomly distributed cells were counted in each experiment. Data are means ± SE of five independent (n=5) culture preparation. *Differences from unstimulated control with $p<0.05$

4.2.3. DNA laddering induced under myostatin stimulation in cardiomyocytes

In fig.4.2.3 apoptosis induction under myostatin stimulation was analysed by chromatin condensation. Additionally apoptosis was analysed by DNA laddering. Cardiomyocytes were stimulated with myostatin (10 ng/ml) over night for 18 hours. Genomic DNA was extracted. 10 µg DNA per lane were separated on 1.5% agarose gels and stained with ethidium bromide. DNA laddering was induced under myostatin in cardiomyocytes (fig. 4.2.3.).

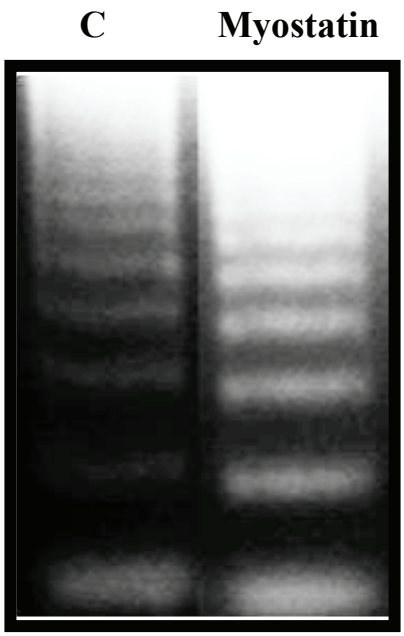

Fig: 4.2.3. Myostatin induces DNA laddering. Cardiomyocytes were stimulated with myostatin (10 ng/ml) overnight. Genomic DNA was extracted and 10 µg DNA per lane were separated on 1.5% agarose gels and stained with ethidium bromide.

4.2.4. Inhibition of apoptosis by SMAD-decoy oligonucleotides under myostatin stimulation.

Cardiomyocytes were pre-incubated with SMAD-decoy oligonucleotides (500 nM) for 5 h. SMAD-decoy oligonucleotides contain specific binding sites for SMAD and can therefore scavenge the intracellular SMAD. After transformation with decoy oliog nucleotides cardiomyocytes were stimulated with myostatin for 20 h (10 ng/ml). Cardiomyocytes were stained for 30 minutes with Hoechst 33258 (5 µg/ml) and propidium iodide (1 µg/ml). Cells were analysed by fluorescence microscopy.

Myostatin induced apoptotsis in 18.0 $\pm$ 0.7 % cells as compared to 10.5 $\pm$ 0.7 % apoptotic cells in controls. Pre-incubation of cardiomyocytes with SMAD-decoy oligonucleotides significantly reduced the number of apoptotic cells to 13.2 $\pm$ 0.9 %.

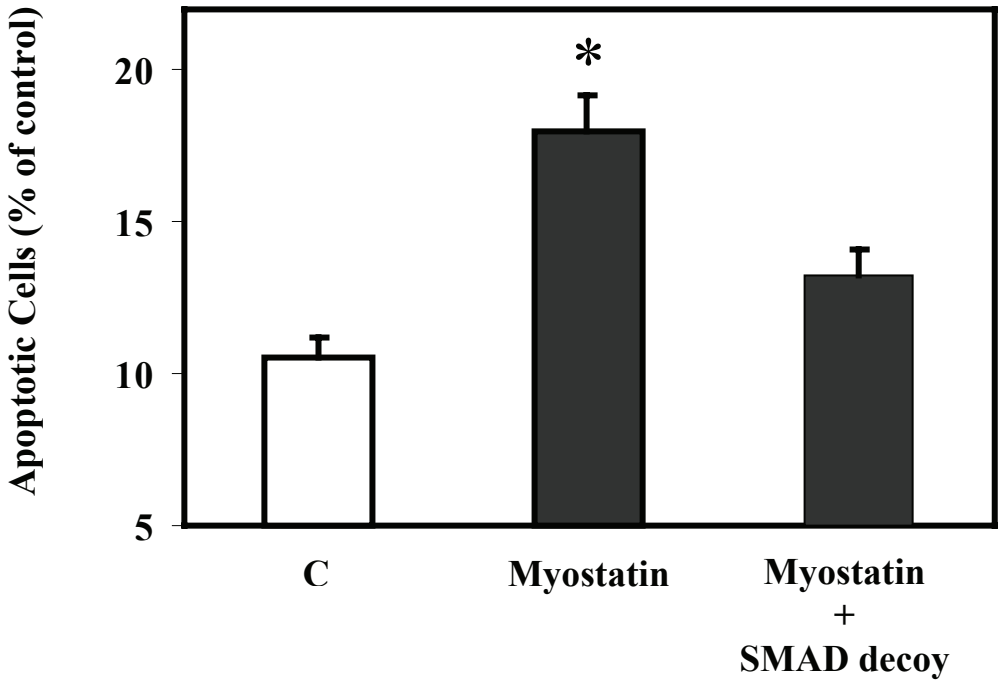

Fig: 4.2.4. Inhibition of apoptosis by SMAD-decoy oligonucleotides. Cardiomyocytes were pre-incubated with SMAD-decoy oligonucleotides (500 nM) for 5 h. Then cardiomyocytes were stimulated in the presence of myostatin (10 ng/ml) for 20 h and analysed for apoptosis. Data are means ± SE of fifteen independent (n=15) culture preparations. *Differences from unstimulated control with $p<0.05$.

4.2.5. Inhibition of DNA laddering by SMAD-decoy oligonucleotides under myostatin stimulation

The effect of SMAD-decoy oligonucleotides was further investigated by genomic DNA analysis. Cardiomyocytes were pre-incubated with SMAD-decoy oligonucleotides (500 nM) for 5 h and stimulated with myostatin (10 ng/ml) overnight. 10 µg DNA per lane were separated on 1.5% agarose gels and stained with ethidium bromide. DNA laddering was reduced by the use of SMAD-decoy oligonucleotides in myostatin (10 ng/ml) stimulated cardiomyocytes. This result showed that apoptosis induction is mediated by SMAD proteins.

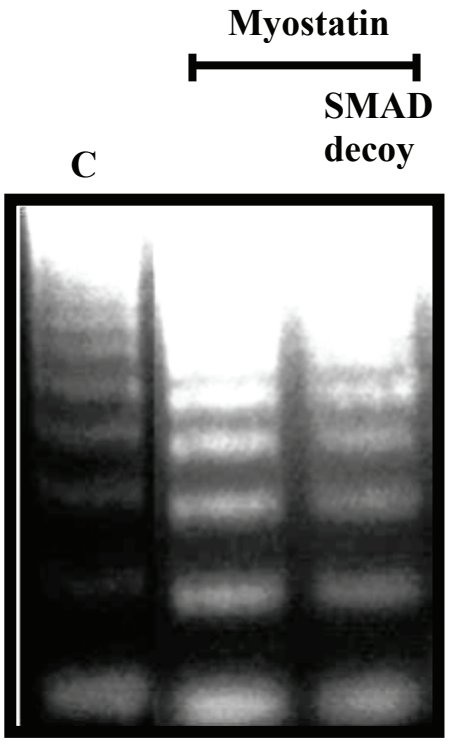

Fig: 4.2.5. SMAD-decoy oligonucleotides reduce DNA laddering. Cardiomyocytes were pre-incubated with SMAD-decoy oligonucleotides (500 nM) for 5 h and stimulated with myostatin (10 ng/ml) overnight for 18 hours. 10 µg DNA per lane were separated on 1.5% agarose gels and stained with ethidium bromide.

4.2.6. Inhibition of apoptosis by AP-1-decoy oligonucleotides under myostatin stimulation

Cardiomyocytes were preincubated with AP-1 decoy oligonucleotides (500 nM) for 5 h. The AP-1 decoy oligonucleotides contain specific binding sites for AP-1 and scavenge AP-1 intracellularly. Transformed cardiomyocytes were stimulated in presence of myostatin (10 ng/ml) for 20 h. Cells were analysed for apoptosis induction.

Myostatin induced apoptosis in 17.8 $\pm$ 0.7 % of cells compared to 10.8 $\pm$ 1.2 % apoptotic cells in controls. Pre-incubation of cardiomyocytes with AP-1-decoy oligonucleotides significantly reduced the number of apoptotic cells due to myostatin stimulation to 13.8 $\pm$ 1.3 %.

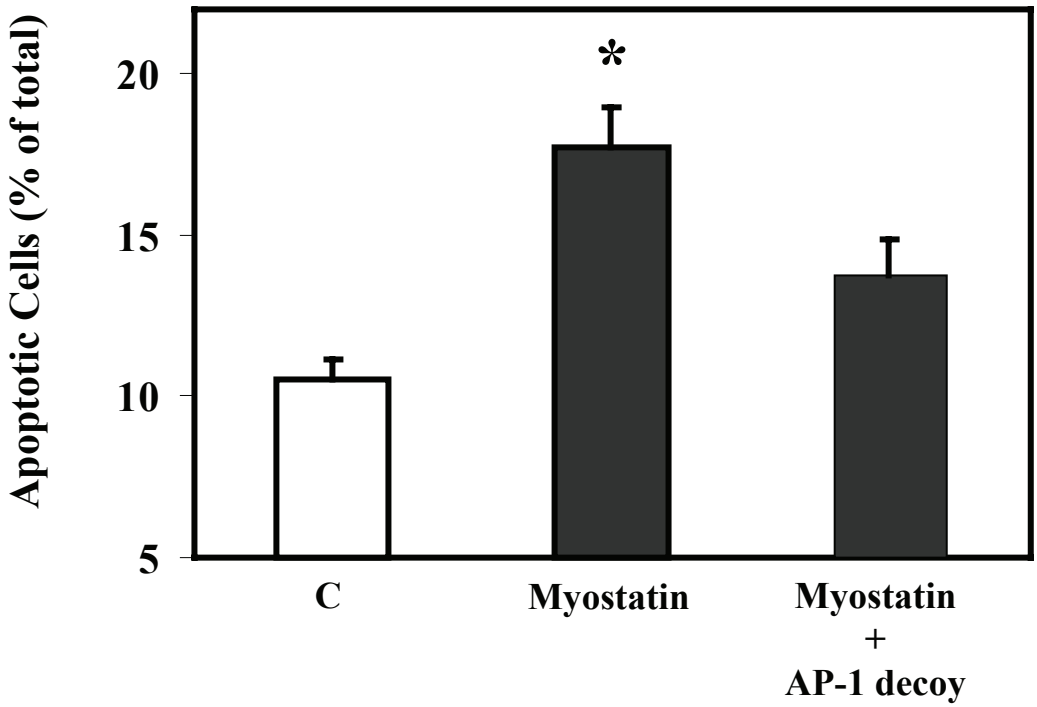

Fig: 4.2.6. Inhibition of apoptosis by AP-1-decoy oligonucleotides. Cardiomyocytes were pre-incubated with AP-1-decoy oligonucleotides (500 nM) for 5 h. Then cardiomyocytes were stimulated in presence of myostatin (10 ng/ml). After 20 h cardiomyocytes were stained for 30 minutes with Hoechst 33258 (5 μg/ml) and propidium iodide (1 μg/ml). Cells were analysed by fluorescence microscopy. Data are means ± SE of fifteen independent (n=15) culture preparation. *Differences from unstimulated control with $p<0.05$.

4.2.7. Inhibition of DNA laddering by AP-1-decoy oligonucleotides under myostatin stimulation

The effect of AP-1-decoy oligonucleotides was further analysed by genomic DNA laddering. Cardiomyocytes were pre-incubated with AP-1-decoy oligonucleotides (500 nM) for 5 h and stimulated with myostatin (1 ng/ml) overnight. 10 μg of DNA per lane were separated on 1.5% agarose gels and stained with ethidium bromide. DNA laddering was reduced by use of AP-1-decoy oligonucleotides in myostatin stimulated

cardiomyocytes. This result confirmed that activation of AP-1 is necessary for apoptosis induction by myostatin in cardiomyocytes.

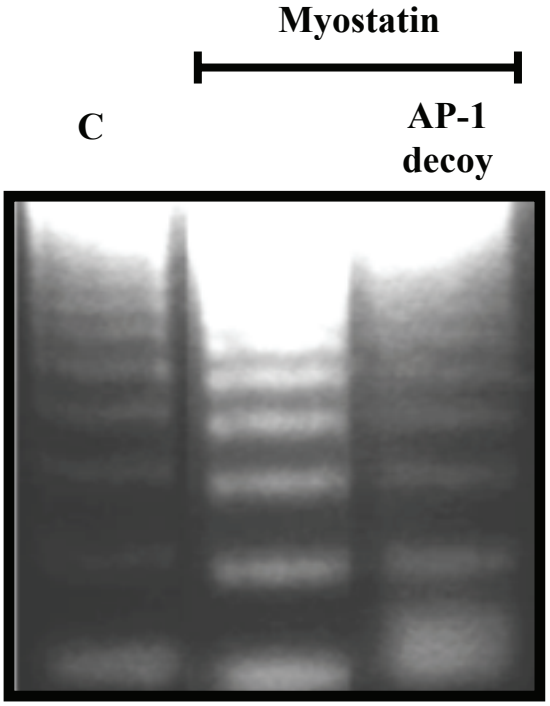

Fig: 4.2.7. AP-1-decoy oligonucleotides reduce DNA laddering. Cardiomyocytes were preincubated with AP-1-decoy oligonucleotides (500 nM) for 5 h and stimulated with myostatin (10 ng/ml) overnight for 18 hours. 10 µg DNA per lane were separated on 1.5% agarose gel and stained with ethidium bromide.

4.3. BMP-2 and apoptosis induction in cardiomyocytes

4.3.1. SMAD-Binding activity under BMP2 stimulation

Cardiomyocytes were stimulated with BMP-2 (bone morphogenetic protein-2) (80 ng/ml) for 2 h. Nuclear extracts were analysed for SMAD-binding activity by retardation assays. Extracts from BMP-2 induced cells showed a strong band shift as compared to control.

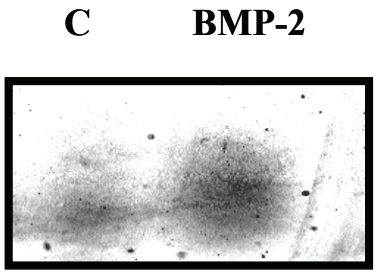

Fig: 4.3.1.A. BMP-2 stimulates SMAD activation. Cardiomyocytes were stimulated by BMP-2 (80 ng/ml) for 2 h. Nuclear extracts were prepared. 10 µl of nuclear extracts were tested for SMAD binding activity using fluorescence labeled SMAD-decoy oligonucleotides with specific SMAD binding sequences. This is a representative band shift from retardation assay gel.

BMP-2 (80 ng/ml) stimulated cardiomyocytes showed a significant increase of SMAD-binding activity to 121.1 ± 16.4 % as compared to control values.

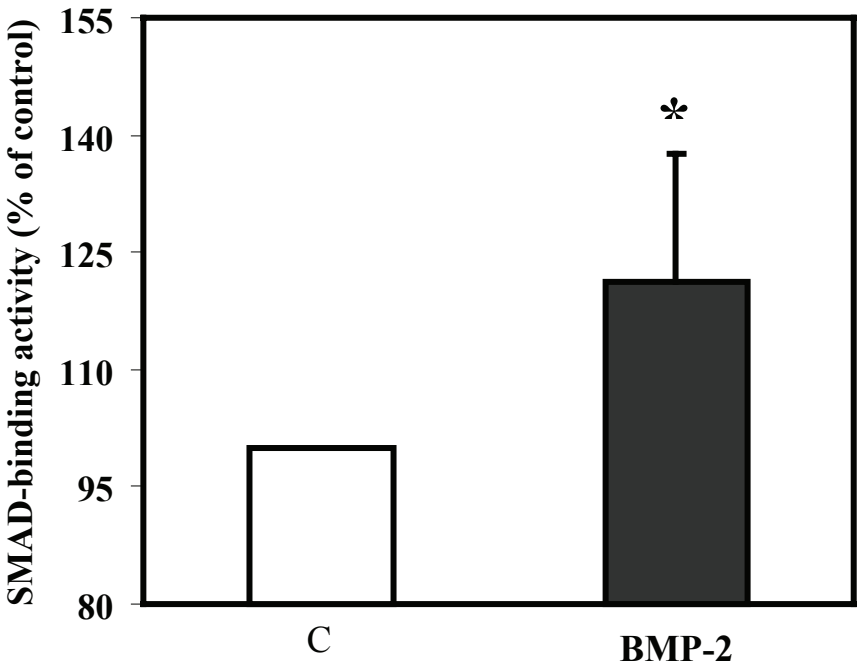

Fig: 4.3.1.B. Quantitative analysis of retardation gels. BMP-2 stimulates SMAD activation. Cardiomyocytes were stimulated with BMP-2 (80 ng/ml) for 2 h. Retardation assays showed a significant increase of SMAD-binding activity in BMP-2 stimulated cardiomyocytes. Data are means ± SE of five independent (n=5) culture preparation. *Differences from unstimulated control with $p<0.05$.

4.3.2. BMP-2 activates apoptosis induction

The effect of BMP-2 was analysed by cell morphology. Cardiomyocytes were stimulated by BMP-2 (80 ng/ml) over night. Cells were stained with Hoechst 33258 (5 µg/ml) and propidium iodide (1 µg/ml). Chromatin condensation of cells were analysed by fluorescence microscopy. About 200 randomly distributed cells were examined for apoptosis in each experiment. BMP-2 induced 11.2 ± 1.2 % apoptotic cells as compared to 7.3 ± 0.4 % apoptotic cells in controls.

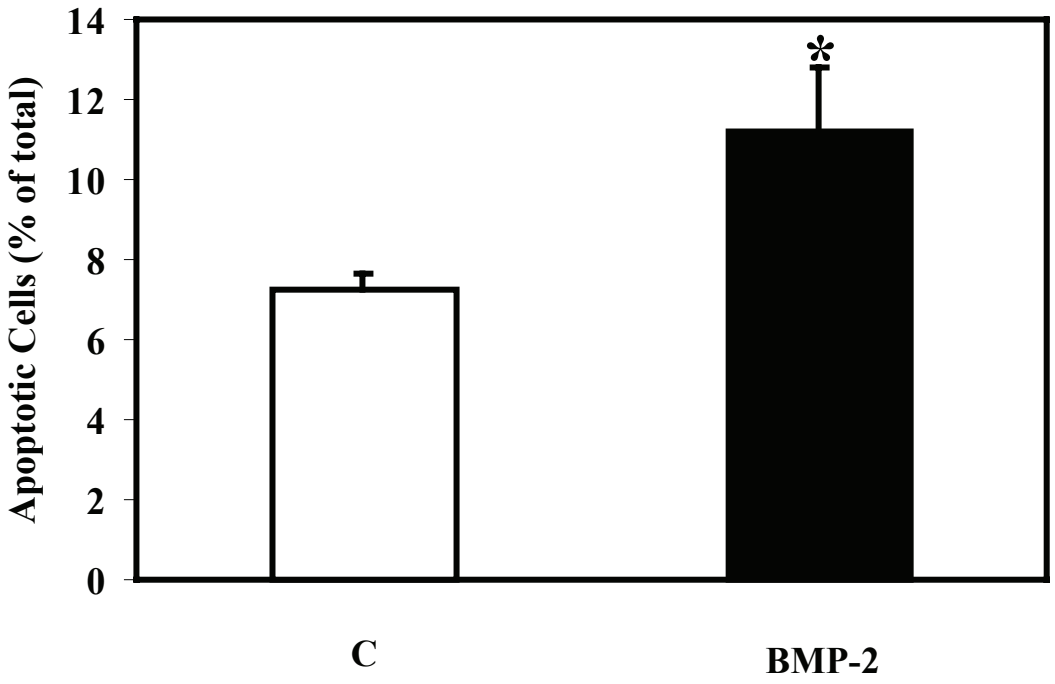

Fig: 4.3.2. BMP-2 stimulates apoptosis in cardiomyocytes. 20 h after BMP-2 (80 ng/ml) stimulation cardiomyocytes were stained with Hoechst 33258 (5 μg/ml) and propidium iodide (1 μg/ml). Cells were analysed by fluorescence microscopy. For quantification of apoptotsis and necrosis 200 randomly distributed cells were counted in each experiment. Data are means ± SE of five independent (n=5) culture preparation. *Differences from unstimulated control with $p<0.05$

4.3.3. BMP-2 stimulation increases DNA laddering

Apoptotic effect of BMP-2 was confirmed by DNA laddering. Cardiomyocytes were stimulated with BMP2 (80 ng/ml) overnight for 18 hours. Genomic DNA were analysed on 1.5% agarose gels. DNA laddering was induced in BMP-2 stimulated cardiomyocytes (fig. 4.3.2).

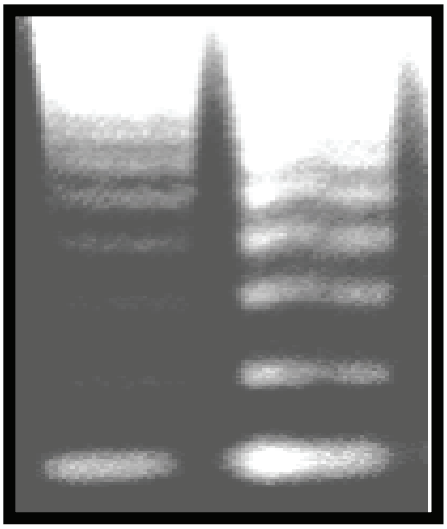

Fig: 4.3.3. BMP-2 induces DNA laddering. Cardiomyocytes were stimulated with BMP2 (80 ng/ml). Genomic DNA was extracted and 10 µg DNA per lane were analysed on 1.5 % agarose gels.

4.3.4. SMAD-decoy oligonucleotides abolish BMP-2 apoptotic effect

Cardiomyocytes were pre-incubated with SMAD-decoy oligos (500 nM) for 5 h and then stimulated with BMP-2 (80 ng/ml) overnight. Cardiomyocytes were stained with Hoechst 33258 (5 µg/ml) and propidium iodide (1 µg/ml). Cells were analysed by fluorescence microscopy. BMP-2 increased the number of apoptotic cells to18.98 $\pm$ 0.91 % as compared to controls (13.4 $\pm$ 0.9 %). The pre-incubation of cardiomyocytes with SMAD-decoy oligonucleotides significantly reduced the number of apoptotic cells (15.0 $\pm$ 1.0 %).

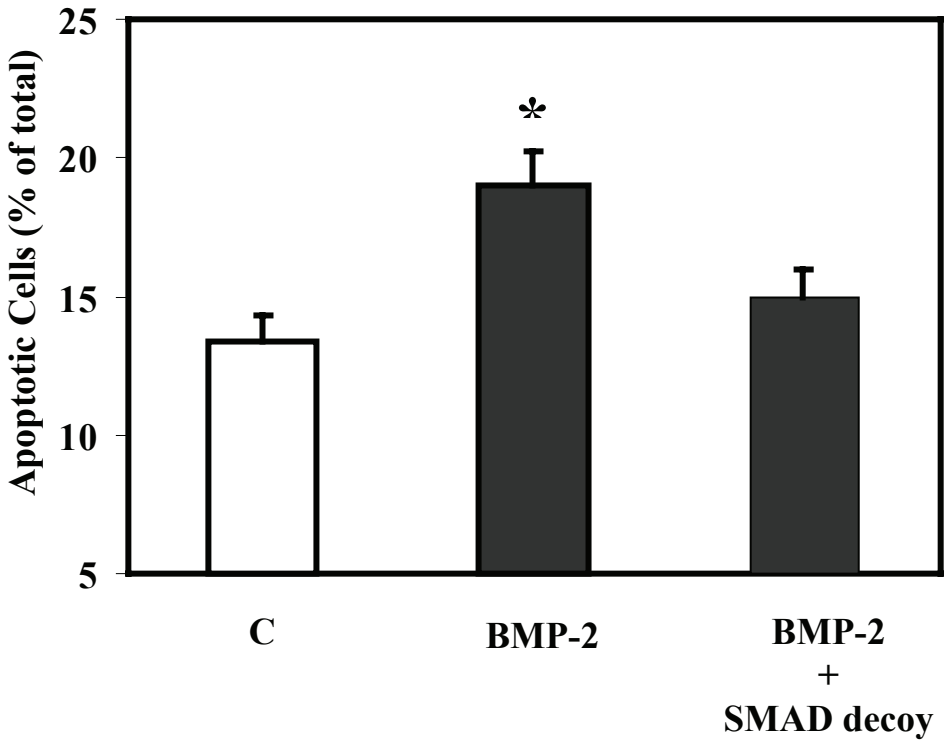

Fig: 4.3.4. Apoptosis inhibition by SMAD decoy oligonucleotides. Cardiomyocytes were pre-incubated with SMAD-decoy oligonucleotides (500 nM) for 5 h. Then cardiomyocytes were stimulated with BMP-2 (80 ng/ml). After 20 h cardiomyocytes were stained for 30 minutes with Hoechst 33258 (5 μg/ml) and propidium iodide (1 μg/ml). Cells were analysed by fluorescence microscopy. Data are means ± SE of twenty-one independent (n=21) culture preparation. *Differences from unstimulated control with $p<0.05$.

4.3.5. Inhibition of DNA laddering by SMAD-decoy oligonucleotides under BMP-2 stimulation

The effect of SMAD-decoy oligonucleotides was further investigated genomic DNA analysis. Cardiomyocytes were pre-incubated with SMAD-decoy oligonucleotides (500 nM) for 5 h and stimulated with BMP-2 (80 ng/ml) overnight. Genomic DNA was analysed on agarose gels. DNA laddering was reduced by use of SMAD-decoy oligonucleotides in BMP-2 stimulated cardiomyocytes.

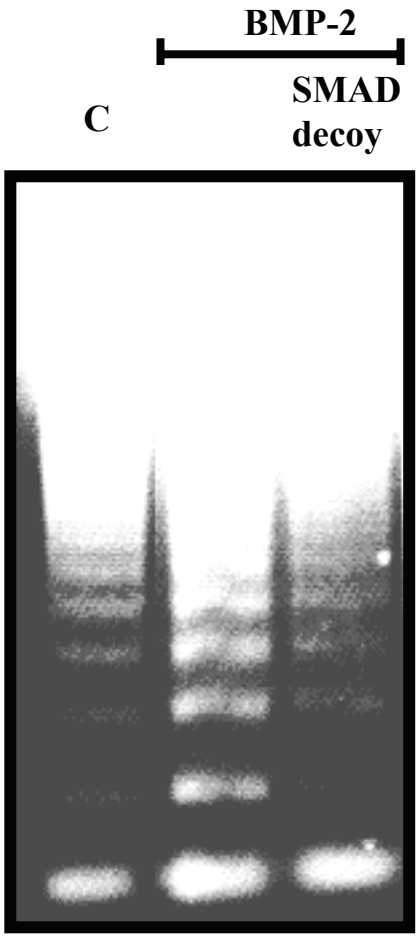

Fig: 4.3.5. SMAD-decoy oligonucleotides reduced DNA laddering. Cardiomyocytes were pre-incubated with SMAD-decoy oligonucleotides (500 nM) for 5 h and stimulated with BMP-2 (80 ng/ml) overnight. 10 µg of genomic DNA were separated on 1.5% agarose gels.

4.3.6. AP-1 decoy oligonucleotides impaired BMP-2 induced apoptosis

Cardiomyocytes were pre-incubated with AP-1 decoy oligonucleotides (500 nM) for 5 h. The AP-1-decoy oligonucleotides contain specific AP-1 binding sites and scavenge intracellular AP-1 binding activity. Then the cardiomyocytes were stimulated with BMP-2 (80 ng/ml). After 20 h cardiomyocytes were stained with Hoechst and propidium iodide. Cells were analysed by apoptosis assays. BMP-2 induced number of apoptotic

cells 19.0 ± 0.9 % as compared to controls 13.4 ± 0.9 %. Pre-incubation of cardiomyocytes by AP-1 decoy oligonucleotides significantly reduced the number of apoptotic cells to 14.1 ± 0.8 %.

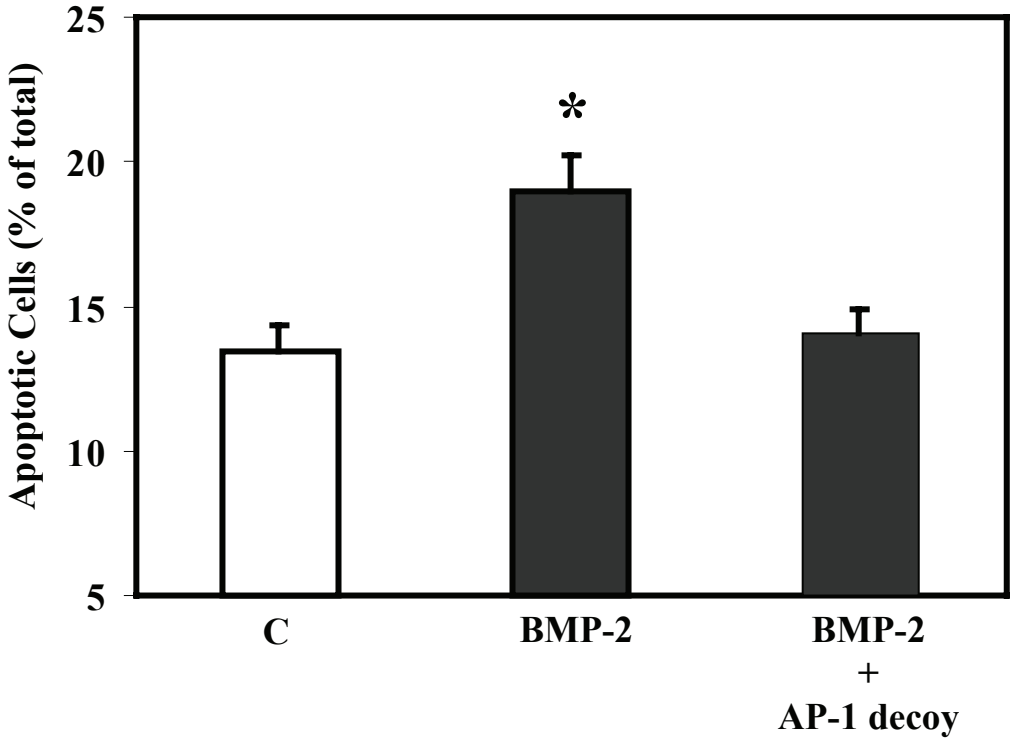

Fig: 4.3.6. Inhibition of apoptosis by AP-1-decoy oligonucleotides. Cardiomyocytes were pre-incubated with AP-1-decoy oligonucleotides (500 nM) for 5 h. Then the cardiomyocytes were stimulated in the presence of BMP-2 (80 ng/ml). After 20 h induction cardiomyocytes were analysed by apoptosis assays. Data are means ± SE of twenty-one independent (n=21) culture preparation. *Differences from unstimulated control with $p<0.05$.

4.3.7. Inhibition of DNA laddering by AP-1-decoy oligonucleotides under BMP-2 stimulation

The effect of AP-1-decoy oligonucleotides was further studied by genomic DNA analysis. Cardiomyocytes were pre-incubated with AP-1-decoy oligonucleotides (500 nM) for 5 h and stimulated with BMP-2 (80 ng/ml) overnight for 18 hours. Analysis of genomic DNA confirmed that AP-1 decoy oligo nucleotides inhibit apoptotic effects of BMP-2.

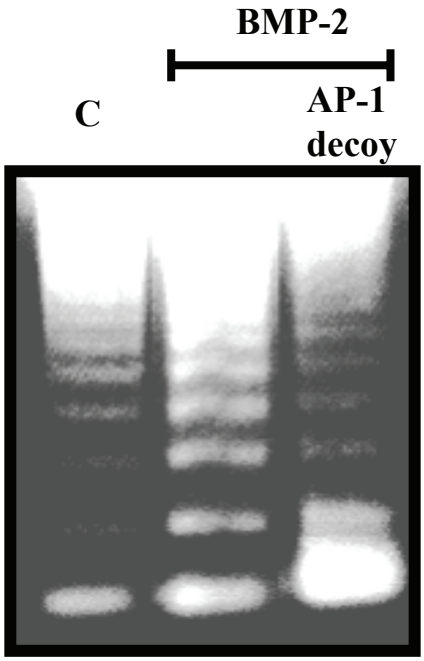

Fig: 4.3.7. AP-1-decoy oligonucleotides reduced DNA laddering. Cardiomyocytes were pre-incubated with AP-1-decoy oligonucleotides (500 nM) for 5 h and stimulated with BMP-2 (80 ng/ml) overnight. Genomic DNA was analysed on agarose gel for DNA-laddering.

4.4. Involvement of activin A in apoptosis induction

4.4.1. Activin A stimulates apoptosis induction in cardiomyocytes

Cardiomyocytes were stimulated with activin A (100 ng/ml). After 20 h induction cardiomyocytes were stained for 30 minutes with Hoechst 33258 (5 μg/ml) and propidium iodide (1 μg/ml). Cells were analysed by fluorescence microscopy. Activin A increased the number of apoptotic cells (18.1 ± 1.6 %) as compared to controls (12.8 ± 0.9 %).

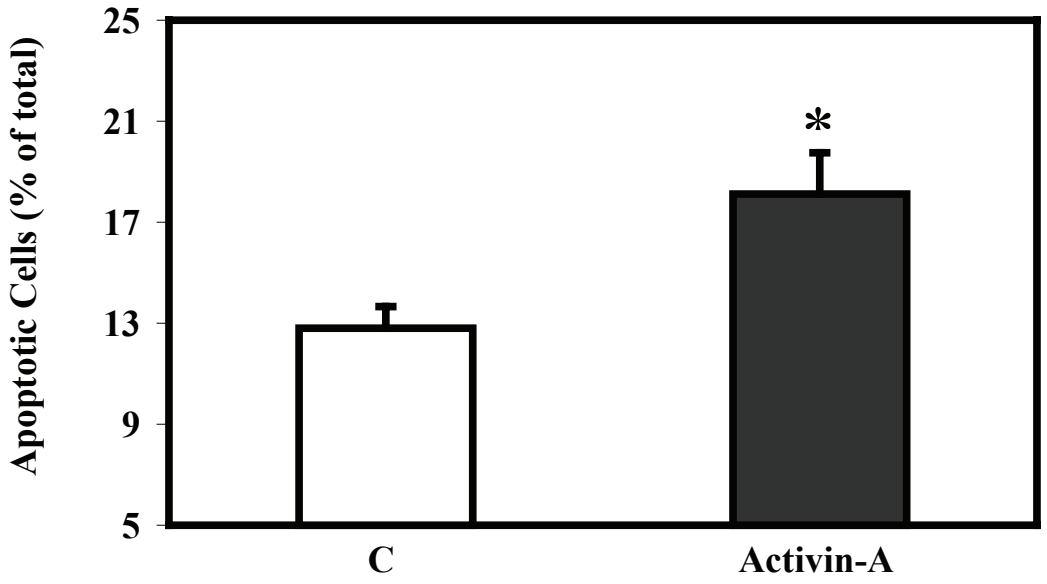

Fig: 4.4.1. Apoptosis induction in adult rat cardiomyocytes under activin A stimulation. Cardiomyocytes were stimulated with activin A (100 ng/ml). After 20 h cardiomyocytes were analysed with Hoechst 33258 (5 μg/ml) and propidium iodide (1 μg/ml) staining. Data are means ± SE of seven independent (n=7) culture preparations. *Differences from unstimulated control with $p<0.05$

4.4.2. Activin A increases DNA laddering in cardiomyocytes

In fig.4.4.1. apoptosis induction by activin A was shown by analysis of chromatin condensation. Apoptotic effects were also investigated by genomic DNA analysis. Therefore, cardiomyocytes were stimulated with activin A (100 ng/ml) overnight for 20 hours. Genomic DNA was isolated. 10 µg DNA per lane were separated on 1.5% agarose gel and stained with ethidium bromide. DNA laddering was induced in activin A stimulated cardiomyocytes (fig.4.4.2).

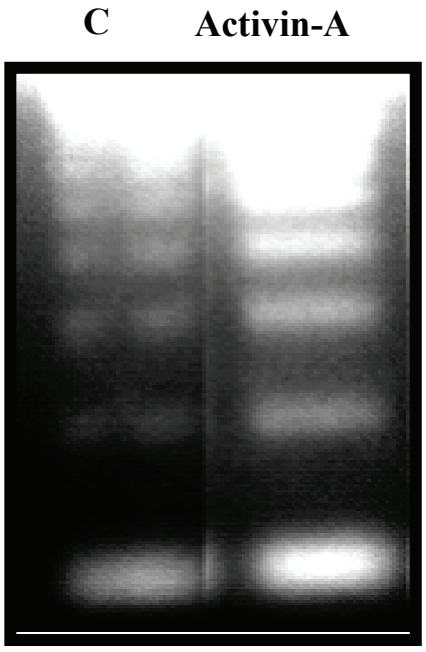

Fig: 4.4.2. Activin A induces DNA laddering. Cardiomyocytes were stimulated with activin A (100 ng/ml) overnight for 20 hours. Genomic DNA were extracted. 10 µg DNA per lane were separated on 1.5% agarose. Figure demonstrates activin A induced cardiomyocytes have strong laddering.

4.4.3. SMAD-decoy oligonucleotides inhibited apoptotic effects of activin A

Cardiomyocytes were preincubated with SMAD decoy oligonucleotides (500 nM) for 5 h to scavenge SMAD intracellularly. Then cardiomyocytes were stimulated with activin A (100 ng/ml). After 20 h cardiomyocytes were stained for 30 minutes with Hoechst 33258

(5 μg/ml) and propidium iodide (1 μg/ml). Cells were analysed by fluorescence microscopy.

Activin A increased the number of apoptotic cells to 10.5 ± 0.7 % as compared to 7.4 ± 0.4 % apoptotic cells in controls. Preincubation of cardiomyocytes with SMAD-decoy oligonucleotides significantly reduced the number of apoptotic cells to 8.0 ± 0.4 %.

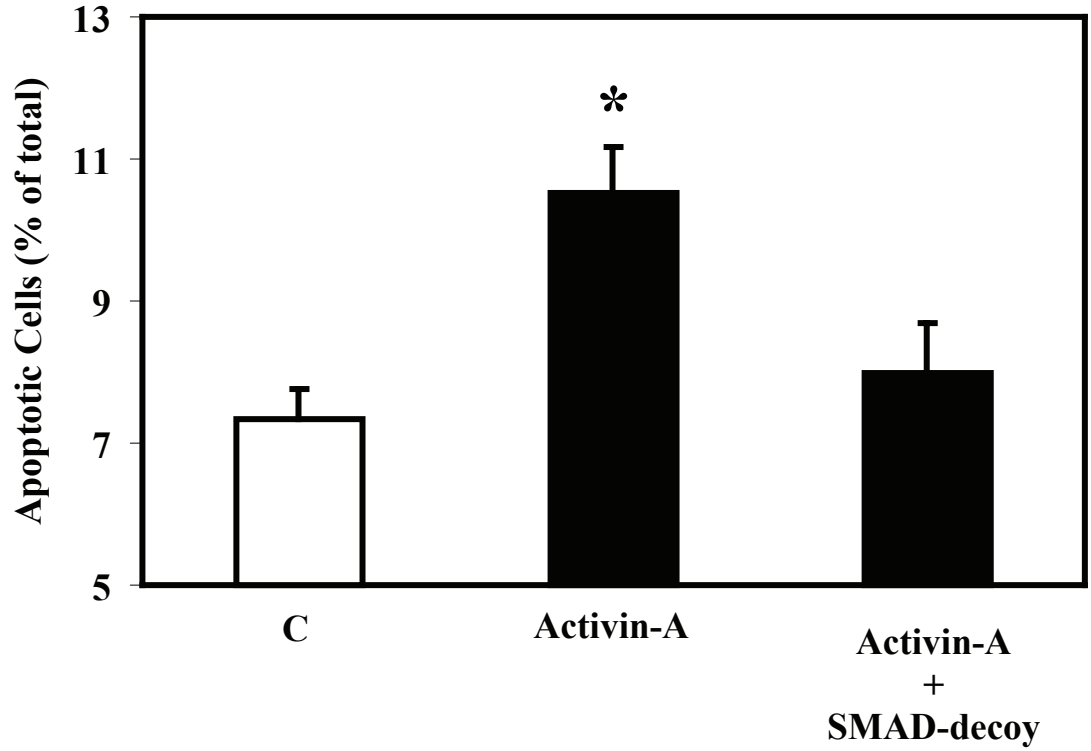

Fig: 4.4.3. Inhibition of apoptosis by SMAD-decoy oligonucleotides. Cardiomyocytes were preincubated with SMAD-decoy oligos (500 nM), and after 5 h cardiomyocytes were stimulated with activin A (100 ng/ml). After 20 h cells were analysed for apoptosis induction. Data are means ± SE independent (n=18) culture preparations. *Differences from unstimulated control with $p<0.05$.

4.4.4. Inhibition of DNA laddering by SMAD-decoy oligonucleotides under activin A stimulation

Cardiomyocytes were preincubated with SMAD-decoy oligo nucleotides (500 nM) for 5 h and stimulated with activin A (100 ng/ml) overnight. Analysis of genomic DNA confirmed the results of chromatin condensation by apoptosis analysis. DNA laddering was reduced by SMAD-decoy oligonucleotides in activin A stimulated cardiomyocytes.

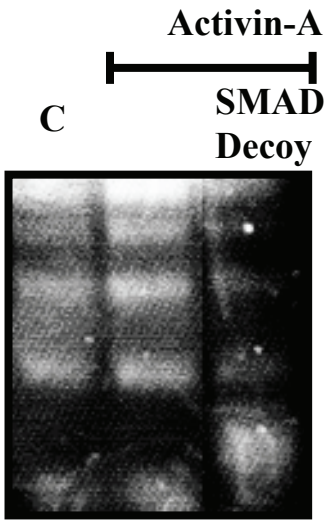

Fig: 4.4 4. SMAD-decoy oligonucleotides reduced DNA laddering. Cardiomyocytes were pre-incubated with SMAD-decoy oligonucleotides (500 nM). After 5 h cells were stimulated with activin A (100 ng/ml) overnight. Genomic DNA was extracted. 10 µg DNA per lane were separated on 1.5% agarose gels and stained with ethidium bromide.

4.4.5. AP-1 is involved in activin A induced apoptosis

It was further analyzed if AP-1 is also involved in activin A induced apoptosis. This was investigated by transforming cardiomyocytes with AP-1 decoy oligonucleotides (500 nM) for 5 hours.Then cardiomyocytes were treated with activin A (100 ng/ml) overnight for 20 hours. Activin A increased the number of apoptotic cells to 10.5 $\pm$ 0.7 % as compared to controls 7.4 $\pm$ 0.4 %. When cardiomyocytes are preincubated with AP-1 decoy oligo nucleotides (7.3 $\pm$ 0.8 % apoptotic cells). These results demonstrate that apoptotic effects of activin A are mediated via AP-1 activation.

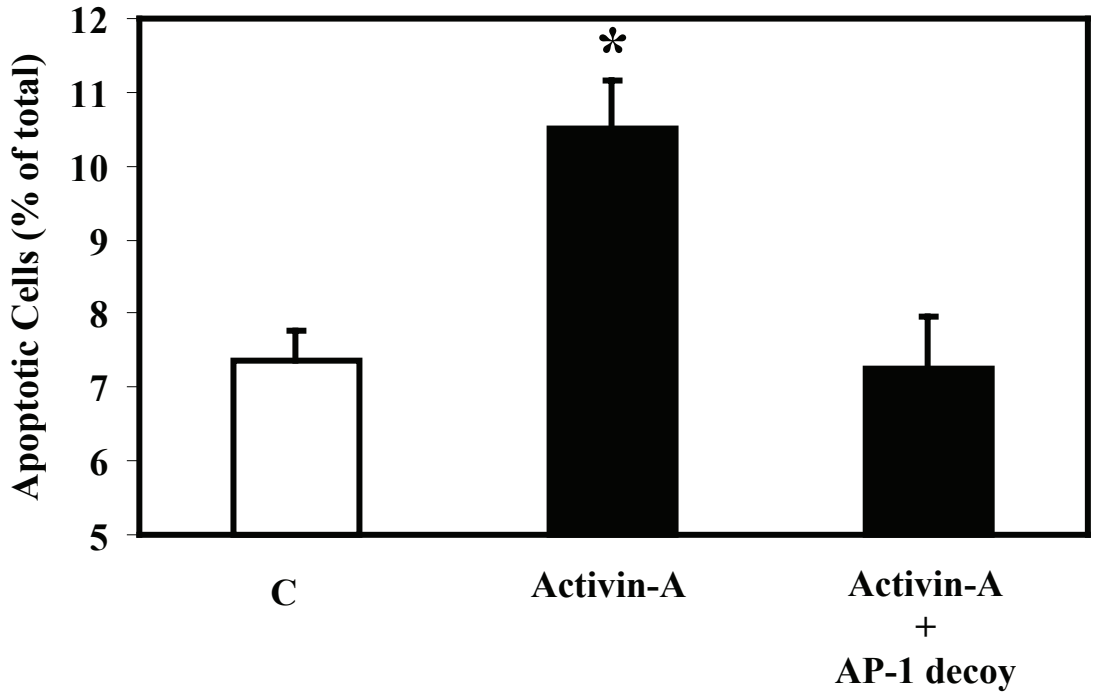

Fig: 4.4.5. Inhibition of apoptosis by AP-1 decoy oligonucleotides. Cardiomyocytes were preincubated with AP-1 decoy oligonucleotides (500 nM) for 5 h. Then cardiomyocytes were stimulated in presence of Activin A (100 ng/ml). After 20 h cardiomyocytes were stained for 30 minutes with Hoechst 33258 (5 μg/ml) and propidium iodide (1 μg/ml). Cells were analysed by fluorescence microscopy. Data are meanss ± SE n=18 culture preparation. *Differences from unstimulated control with $p<0.05$.

4.4.6. Inhibition of DNA laddering by AP-1 decoy oligo nucleotides under activin A stimulation

In fig.4.4.5. apoptosis was analysed by detection of chromatin condensation in presence of AP-1 decoy oligos. The effect of AP-1 decoy oligonucleotides was further studied by genomic DNA analysis. Cardiomyocytes were preincubated with AP-1 decoy oligonucleotides (500 nM) for 5 h and stimulated with activin A (100 ng/ml) overnight. Genomic DNA was extracted and 10 µg DNA per lane were separated on 1.5% agarose gel and stained with ethidium bromide. DNA laddering was reduced by AP-1 decoy oligonucleotides in activin A stimulated cardiomyocytes.

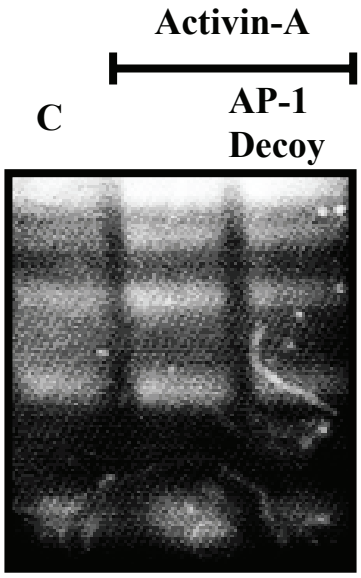

Fig: 4.4.6. AP-1 decoy oligonucleotides reduced DNA laddering. Cardiomyocytes were preincubated with AP-1-decoy oligonucleotides (500 nM). After 5 h cells were stimulated with activin A (100 ng/ml) overnight. Analysis of genomic DNA confirmed that AP-1 decoy oligos inhibit apoptotic effect of acvtivin A.

4.5. Anti-apoptotic effect of GDF15 in isolated adult rat cardiomyocytes

4.5.1. SMAD binding activity under GDF15 stimulation of adult rat cardiomyocytes

To investigate the involvement of GDF15 (growth differentiation factor 15) SMAD activation cardiomyocytes were stimulated with GDF15 (3 ng/ml) for 2 h. Nuclear extracts were isolated, incubated with fluorescence labeled SMAD-decoy oligonucleotides and retardation assays were performed. The extract from GDF15 induced cells showed a band shift as compared to the control. This demonstrates that GDF15 induces SMAD binding activity in cardiomyocytes. TGFβ (1 ng/ml) was used as control stimules for SMAD activation.

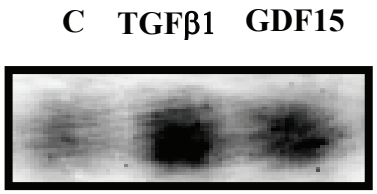

Fig: 4.5.1.A. SMAD-binding activity in GDF15 stimulated cardiomyocytes. Cardiomyocytes were stimulated in the presence of GDF15 (3 ng/ml) or TGFβ1. (1 ng/ml) for 2 h. Representative band shift of retardation assay gel is shown.

Stimulation of cardiomyocytes with GDF15 (3 ng/ml) for 2 h showed a significant increase in SMAD-binding activity to 122.9 % $\pm$ 6.1 % as compared to control values.

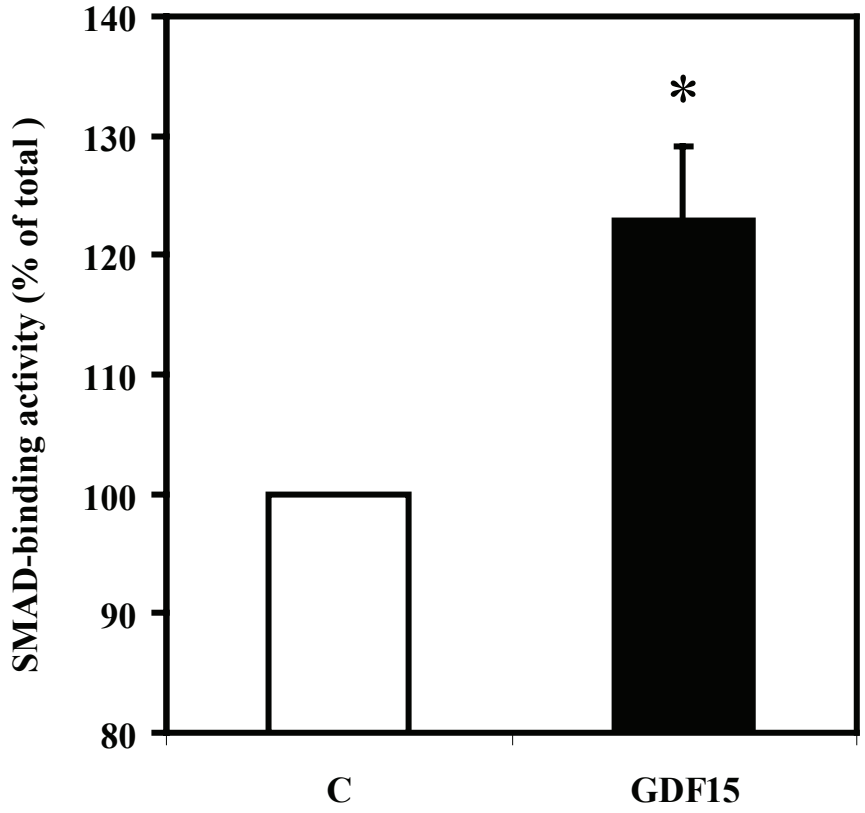

Fig:.4.5.1.B. SMAD-binding activity in GDF15 stimulated cardiomyocytes. Cardiomyocytes were stimulated in the presence of GDF15 (3 ng/ml) for 2 h and nuclear extracts from cells were isolated. The nuclear extracts were tested for SMAD binding activity in retardation assays. Quantitative analysis of retardation assay gels. Data are means ± SE of six independent (n=12) culture preparation. *Differences from unstimulated controls with $p<0.05$.

4.5.2. GDF15 did not induce apoptosis in isolated adult cardiomyocytes

Isolated adult rat cardiomyocytes were stimulated with GDF15 (3 ng/ml). After 20 h cardiomyocytes were stained for 30 minutes with Hoechst 33258 (5 μg/ml) and propidium iodide (1 μg/ml). Cells were analysed by fluorescence microscopy. GDF15 did

not induce apoptosis (8.1 ± 0.8 %) as compared to controls (7.2 ± 0.5 %). It was further investigated, if higher concentrations of GDF15 have any effect on apoptosis induction. Cardiomyocytes were stimulated with GDF15 (6 ng/ml) overnight. Also this concentration had no effect on apoptosis induction (8.2 ± 0.7 %).

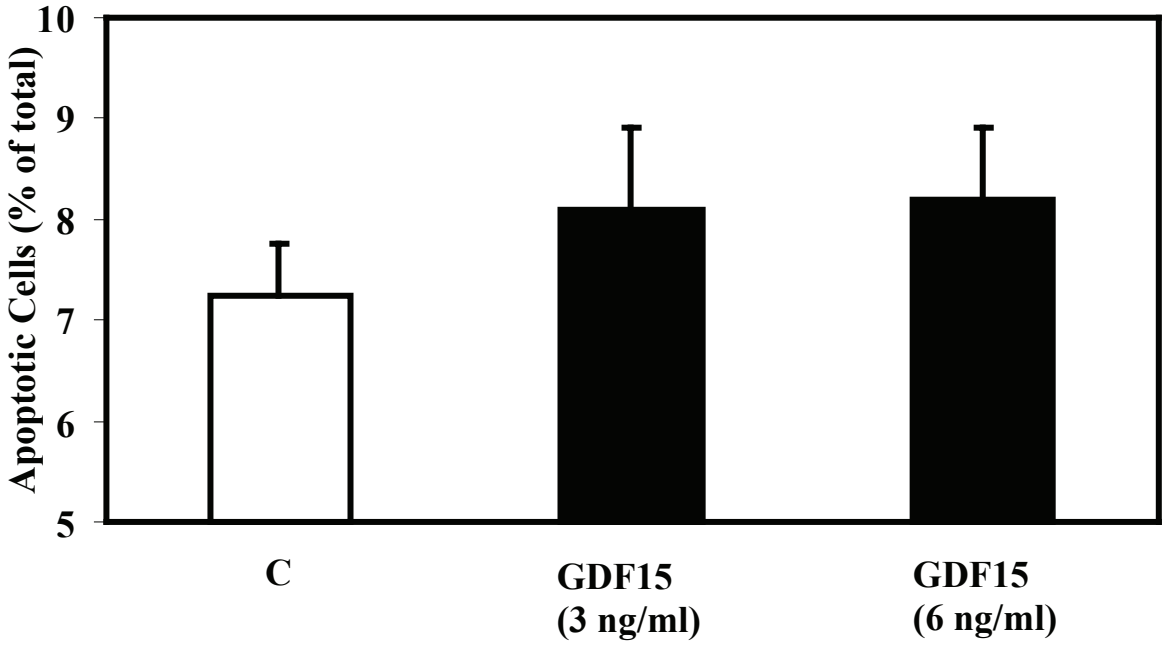

Fig: 4.5.2. GDF15 did not induce apoptosis. Cardiomyocytes were stimulated with GDF15 (3 ng/ml) and (6 ng/ml). After 20 h cardiomyocytes were stained for 30 minutes with Hoechst 33258 (5 μg/ml) and propidium iodide (1 μg/ml). For quantification 200 randomly distributed cells were counted in each experiment. Data are means ± SE of n=12 culture preparation.

4.5.3. GDF15 has anti-apoptotic effects in adult rat cardiomyocytes

Fig.4.5.2. showed that GDF15 did not induce apoptosis. When cells were stimulated with TGFβ1 (1 ng/ml) and GDF15 (3 ng/ml) at the same time, GDF15 reduced apoptotic

effect induced by TGFβ1 (1 ng/ml) from 11.9 ± 0.7 % to 9.5 ± 0.7 % apoptotic cells. These results indicate that GDF15 has anti-apoptotic effects in adult cardiomyocytes.

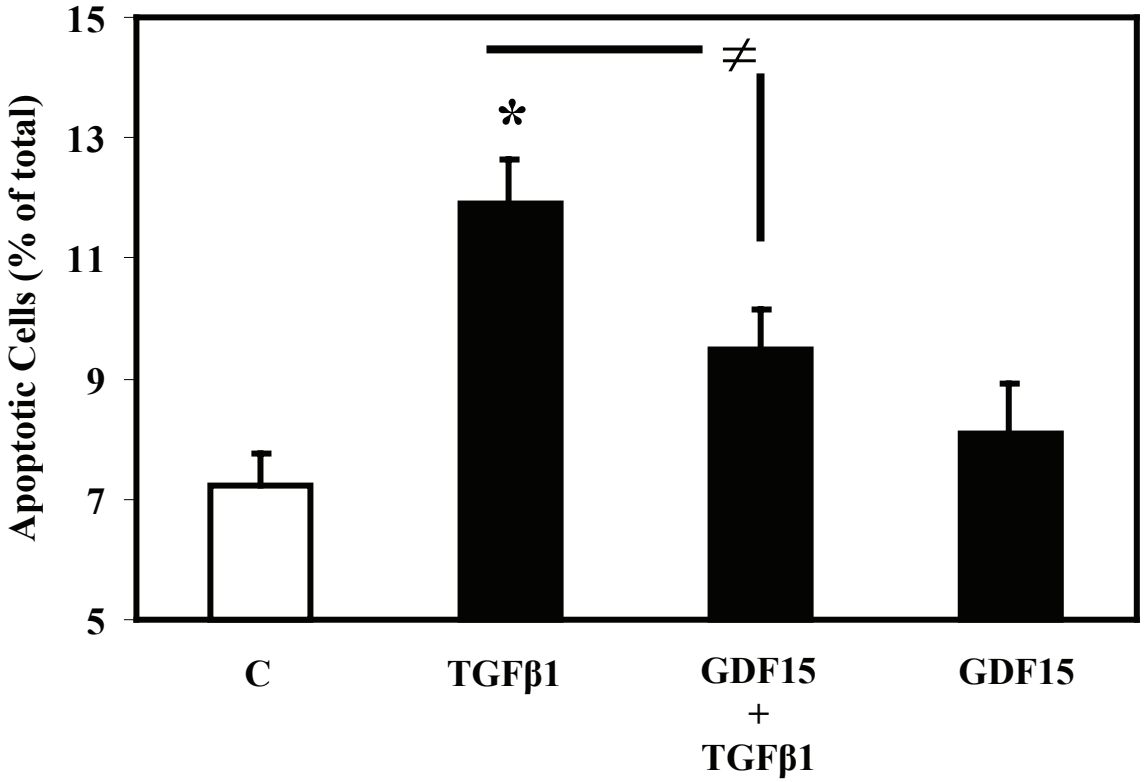

Fig:.4.5.3. Anti-apoptotic effect of GDF15. Cardiomyocytes were stimulated in presence of TGFβ1 (1 ng/ml) and GDF15 (3 ng/ml). After 20 h cardiomyocytes were stained for 30 minutes with Hoechst 33258 (5 μg/ml) and propidium iodide (1 μg/ml). Cells were analysed by fluorescence microscopy. Data are means ± SE of six independent (n=12) culture preparations. *Differences from unstimulated controls with $p<0.05$. ≠ Difference from TGFβ1 induced cardiomyocytes with $p<0.005$.

4.5.4. GDF15 inhibited DNA laddering in TGFβ1 induced cardiomyocytes

Anti-apoptotic effects of GDF15 were confirmed by analysis of genomic DNA analysis. When cardiomyocytes were stimulated with GDF15 (3 ng/ml) and TGFβ1 (1 ng/ml) DNA-laddering induced by TGFβ1 was reduced in presence of GDF15.

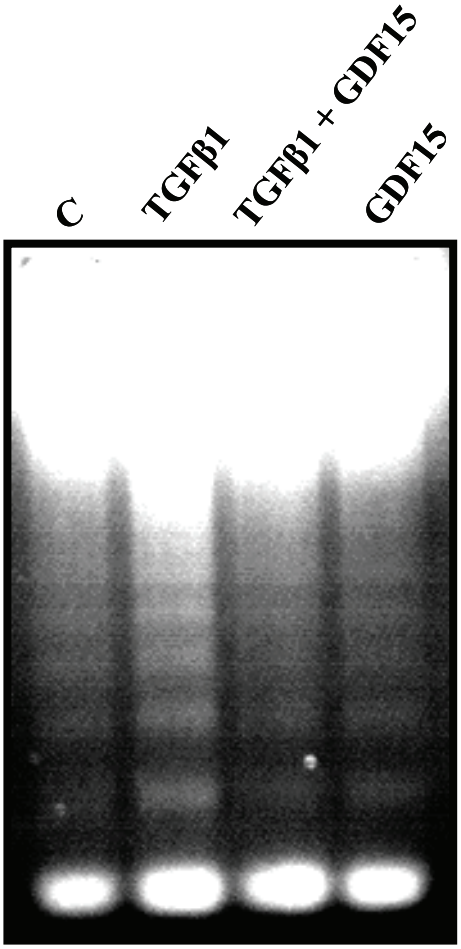

Fig: 4.5.4. Anti-apoptotic effects of GDF15. Cardiomyocytes were stimulated with TGFβ1 (1 ng/ml) and/or GDF15 (3 ng/ml). After 24 h genomic DNA was extracted and separated at 1.5 % agarose gels. GDF15 inhibited DNA laddering of TGFβ1. This result shows that apoptotic effects of TGFβ1 are reduced by GDF15.

5. Discussion

Main findings of this study are that the TGF-β family members, TGF-β1, myostatin, activin A, and BMP-2 induce apoptosis in isolated adult rat cardiomyocytes by activation of SMAD/AP-1 signaling. In contrast to those family members, GDF15 acts anti-apoptotic and reduces apoptosis induction by TGF-β1.

5.1. TGF-β1 induces apoptosis via AP-1/SMAD in adult rat cardiomyocytes

In my study I have demonstrated that TGF-β1 (transforming growth factor β1) induces apoptosis. Since SMAD (small mother against decapentaplegic) proteins are the central molecules in TGF-β signaling, activation and involvement of SMADs in apoptosis induction was analysed. In retardation assays stimulation of SMAD binding activity by TGF-β1 was shown. To investigate the involvement of SMAD proteins in apoptosis induction SMAD-decoy oligos were used. SMAD decoy oligos are double stranded DNA fragments with consensus binding sequences for SMADs. Inhibition of SMADs after transformation of cardiomyocytes with these oligos is very specific, since only proteins with binding affinity to this site will attach to the oligos. These SMAD proteins are then no more available for transcriptional activation in the cell. Using SMAD decoy oligos apoptosis induction by TGF-β1 was inhibited. These findings demonstrate that TGF-β1 induces apoptosis via SMAD proteins in cardiomyocytes.

TGF-β members elicit their cellular responses through formation of heteromeric complexes of specific type I and type II serine/theronine kinase receptors which activate SMAD proteins (Caestecker et al., 2000: Derynck et al., 1997). Up to now eight distinct SMAD proteins are known. Diverse isoforms of SMADs have been shown to exert different function, i.e. SMAD2/3 is known to stimulate collagen synthesis in the heart (Wang et al., 1996) whereas SMAD1 induces differentiation of embryonic stem cells into cardiomyocytes (Monzen et al., 2001). Therefore, it was necessary to analyse which SMAD isoforms are induced under apoptotic conditions, which was analysed in western blots. My study shows that in TGF-β1 stimulated adult cardiomyocytes SMAD 2 and 3 isoforms are phosphorylated and SMAD4 transcolates into the nucleus. This indicates that SMAD isoforms2/3 are activated by phosphorylation. They may undergo

dimerization and thereafter form heterotrimers with SMAD4, which translocates into the nucleus and can regulate transcription.

The recruitment of SMADs to DNA can be regulated by cooperation with other transcription factors. These factors facilitate binding of SMADs to DNA. The activator protein AP-1 is a transcription factor, which interacts with SMAD proteins (Ross. 2004). Physical interaction of SMAD with AP-1 is responsible for attraction of SMADs into the transcription complex (Frantz et al., 2003; Kon et al., 1999). Therefore, in the presence of AP-1 binding of SMADs is enhanced and results in elevated transcriptional activity.

In the present study I have shown that TGF-β1 activates AP-1 in addition to SMAD proteins. AP-1 decoy oligonucleotides inhibited apoptosis induction by TGF-β1. This indicates that an interaction between AP-1 and SMAD is necessary for TGF-β1 induced apoptosis. In line with these data are findings demonstrating that the NO donor SNAP induces apoptosis in adult cardiomyocytes via simultaneous activation of the transcription factors AP-1 and SMADs (Schneiders et al., 2005). Therefore, interaction of AP-1 with SMAD proteins may be a common pathway in cardiomyocyte apoptosis.

5.2. Expression of TGF-β1, SMAD and AP-1 in the heart

Importance of the finding that TGF-β1 induces apoptosis in cardiomyocytes is emphasised by studies which demonstrate elevated levels of TGF-β under pathophysiological conditions in the heart: TGF-β1 and its family members are increased at the transition from compensated hypertrophy to failing hearts **(**Boluyt et al., 1995; Wang et al., 2005) and after myocardial infarction (Hao et al., 2000; Sharma et al., 1999; Yndestad et al., 2004). It contributes to heart failure progression due to hypertrophy and fibrosis. TGF-β stimulates proliferation of fibroblasts and increases expression of genes like collagen, c-Jun, (Lopez-Rovira et al, 2000) endothelin (Sanchez-Elsner et al. 2001) or peroxisome proliferator-activated receptor gamma (PPARγ) (Sanchez-Elsner et al, 2001). These genes play important roles in ventricular remodelling due to cardiac fibrosis. TGF-β also has indirect hypertrophic effects, since it causes induction of hypertrophic responsiveness to β-adrenoceptor stimulation (Schlüter et al., 1995).

Now, in addition to these fibrotic and hypertrophic effects apoptosis induction by TGF-β1 is shown. This may additionally contribute to a bad ventricular remodelling. That the pro-apoptotic AP-1/SMAD signalling pathway is indeed activated by TGFβ in vivo is indicated by findings showing activation of these transcription factors under the same pathological conditions as TGFβ1: Activation of AP-1 in correlation with apoptosis induction has been noticed in ischemia/reperfusion injury models (Janknecht et al., 1998; Bannister et al., 1996; Ghosh et al., 2001). The expression and activation of SMAD 2, 3 and 4 proteins are increased after ischemia/reperfusion (Hao et al., 2000; Poncelet et al., 2001). Since elevated level of TGFβ and its family members are found also in human after myocardial infarction, these findings of my study in rats may be applicable to humans.

Therefore, activation of SMAD and AP-1 after myocardial infarction may induce apoptosis in the heart of rat.

5.3. Activin A induces apoptosis via AP-1/SMAD in adult rat cardiomyocytes

Another member of the TGFβ-family which was analysed in this study is activin A. Activin A has been recognized as a multifunctional cytokine expressed in a wide range of tissues and cells, regulating apoptosis induction (Chen et al., 2002), carcinogenesis and fibrosis (Phillips et al., 2001; Gribi et al., 2001). Also in the heart activin A may exert such effects, because elevated levels of activin A are found in failing hearts. Patients with heart failure display significantly elevated levels of activin A in the serum compared to healthy control patients (Yndestad et al., 2004). Similarly, elevated levels of activin A are found in ischemic cardiomyocytes of neonatal rat. This indicates that the failing myocardium itself contributes to enhanced activin A levels, with cardiomyocytes as a primary cellular source. Therefore, activin A mediated responses may involve autocrince action on cardiomyocytes.

Since activin A was already characterised as mediator of apoptosis in hepatosoma cells (Chen et al., 2000) and in human prostate cancer cell lines (Wang et al., 1996) I have hypothesized that activin A might also be involved in the apoptosis of adult rat cardiomyocytes. My findings show that activin A induces apoptosis in adult cardiomyocytes. By using SMAD decoy oligos I have now shown that SMAD proteins

are also involved in activin A induced apoptosis in cardiomyocytes. Since TGFβ1 induced apoptosis via SMAD2/3 in cardiomyocytes and activin A has been shown to induce apoptosis in liver cells via the same isoforms (Kanamaru et al, 2002) it can be assumed that in cardiomyocytes apoptosis induction by activin A could also be mediated via SMAD2/3.

Interestingly activin A, similarly to TGFβ1, needs AP-1 in addition to SMAD for apoptosis induction. This was again demonstrated by use of AP-1 decoy oligo nucleotides which inhibited apoptosis induction by activin A. So both, AP-1 and SMAD, are necessary for apoptosis induction by activin A. This again stresses that AP-1/SMAD signalling is a central pathway of apoptosis induction in cardiomyocytes.

5.4. Myostatin as inducer of apoptosis

Myostatin is another TGFβ family member. It is known to suppress proliferation and differentiation in muscle cell lines. Myostatin has negative effects on muscle growth. Mutations in myostatin leads to heavy muscle conditions due to hypertrophy in cattle breeds of Belgian Blue (Forbes et al., 2006) because myostatin, as endogenous suppressor of muscle growth, is defect**.** The loss of skeletal muscle mass that occurs during spaceflight is associated with increased myostatin mRNA and protein levels in the skeletal muscle indicating importance of myostatin for reduction of muscle growth (Lalani et al., 2000). If loss of muscle mass is also due to apoptosis has not been investigated yet. Although myostatin was first characterized in skeletal muscle, it has been also identified in the heart (McPherron et al., 1997; Lee et al., 2001; Cook et al., 2002). The expression of myostatin was previously documented in fetal and adult heart. Its expression is upregulated in cardiomyocytes after infarction (Sharma et al., 1999). The upregulation of myostatin in cardiomyocytes in ischemic conditions attracted my intention to investigate its possible involvement in apoptosis induction of cardiomyocytes. I have shown that myostatin is an inducer of apoptosis in cardiomyocytes.

It is already known that in skeletal muscle, myostatin activates SMAD proteins (Forbes et al, 2006). Due to these findings I have hypothesized that SMAD proteins may be involved in myostatin induced apoptosis in adult rat cardiomyocytes. I have shown that

SMAD proteins are stimulated by myostatin and this activation is needed for apoptosis, which was documented by use SMAD-decoy oligos. The involvement of AP-1 was investigated using AP-1 decoy oligos. My findings indicate that AP-1 is also involved in apoptosis induction by myostatin. These findings demonstrate that AP-1/SMAD signaling is also necessary for myostatin induced apoptosis.

5.5. BMP-2 induced apoptosis via SMAD/AP-1 signaling

Further investigations on TGFβ family members revealed that BMP-2 activates SMAD proteins and induces apoptosis in cardiomyocytes. This apoptosis induction is impaired when SMAD signaling is blocked by SMAD-decoy oligos. Beside SMADs, activation of AP-1 transcription factor is necessary for stimulation of apoptosis. This is demonstrated by inhibition of apoptosis with AP-1 decoy oligos. These findings are in agreement with results in other cells types, as BMP-2 induces apoptosis in mouse hybridoma MH60 cells (Kimura et al., 200) and also in human myeloma cells (Kawamura et al., 2000).

The effects of BMP-2 on heart cells have been analysed predominantly in the developing heart. BMP-2 is involved in heart development and plays an important role in differentiation of embryonic cells into cardiomyocytes (Chen et al., 1998). Surprisingly, in neonatal cardiomyocytes BMP-2 showed anti-apoptotic effects. In contrast to adult cardiomyocytes neonatal cardiomyocytes are not terminally differentiated and are therefore in a completely different situation of cell cycle and cell signalling. The different findings on apoptosis induction in neonatal and adult cardiomyocytes may be explained by these different cell cycle states of the cells. This may result in activation of different signalling molecules upon BMP-2 stimulation. The anti-apoptotic effect of BMP on neonatal cardiomyocytes is mediated by SMAD1, whereas pro-apoptotic effects of TGFβ are mediated via SMAD2/3. So, BMP may activate different SMAD isoforms in neonatal vs. adult cardiomyocytes. Up to now activation of SMAD isoforms 1, 5 and 8 are known for BMP signalling. Which isoform is induced in adult cardiomyocytes has to be determined in future experiments. Besides the classical signaling via SMAD proteins, BMP may also activate other signaling molecules with pro-apoptotic action i.e. BMP activates the Ras-ERK pathway in osteoblast (Suzawa et al., 2002). ERKs have been shown to be involved in NO induced apoptosis in cardiomyocytes (Taimor et al., 2001)

and may also influence pro-apoptotic BMP signalling. However, the exact pro-apoptotic pathway of BMP-2 in cardiomyocytes has still to be determined.

5.6. GDF15 has anti-apoptotic effects in isolated adult rat cardiomyocytes

The only TGFβ1 family member that does not induce apoptosis in adult cardiomyocytes is GDF15 (growth differentiation factor 15). Even different concentrations of GDF15 did not induce apoptosis.

Growth differentiation factor-15 (GDF15), also called macrophages inhibitory cytokine-1 (MIC-1), is a new member of the transforming growth factor beta (TGF-β) superfamily, which was first detected in activated macrophages (Bootcov et al., 1997). GDF15 has inflammatory functions (Schlittenhard et al., 2004) and exerts anti-apoptotic actions in neurons (Schlittenhard et al., 2004). The latter finding in neurons is in agreement with my findings in cardiomyocytes.

Up to now knowledge about cardiovascular effects of GDF15 are low. Elevated levels of GDF15 in plasma are a marker for the risk to develop cardiovascular disorders in women (Brown et al., 2002). In patients at increased risk for development of future myocardial infarction, thrombosis or stroke the concentrations of GDF15 were raised to more than 856 pg/ml (Brown et al., 2002). It is documented that women with highest concentrations of GDF15 had a risk for future myocardial death due to atherosclerosis nearly three times higher than women with lower concentrations (Ridker et al., 2000). In my study I have shown anti-apoptotic effects of GDF15 in adult rat cardiomyocytes. Apoptotic effects of TGF-β1 are abolished by GDF15. Thus GDF 15 has adverse effects in the cardiovascular system. In vessels GDF15 is associated with damage due to atherosclerosis but it protects cardiomyocytes against apoptosis.

So these findings indicate that for therapeutic treatment to protect cardiomyocytes from apoptosis, GDF15 should be locally enhanced. A systemic therapy would rather damage the vessels and increase the risk for cardiac disease.

So far I have shown activation of SMAD proteins under GDF15 stimulation. But involvement of SMADs in anti-apoptotic effects of GDF15 are still to be investigated. Also involvement of AP-1 has not been shown yet. But since AP-1/SMAD signalling was shown to mediate apoptosis induced by diverse TGFβ family members it seems unlikely that this pathway will also have anti-apoptotic features. Therefore, it can be assumed that

GDF15 acts via totally different signaling molecules or different SMAD isoforms. Since GDF15 is an endogenous anti-apoptotic molecule it will be an interesting question to analyse its signaling mechanisms in future studies.

Conclusion. Several studies have shown upregulation of TGF-β family members, AP-1 and SMAD in cardiac diseases. Now it is shown that most family members have pro-apoptotic effects that are mediated via AP-1 and SMAD. Thus upregulation of TGF-β1, myostatin, activin A and BMP-2 may contribute to the ventricular remodelling process and are predictors for reduction of heart function due to cardiomyocytes loss via apoptosis. Besides these apoptosis inducing members GDF15 has anti-apoptotic effects in cardiomyocytes and may therefore be involved in cell survival mechanisms in the heart. The protective mechanisms of GDF15 are still not clear and future work is necessary to investigate the pathways and mechanisms of protection for therapeutic use.

6. Summary

Several TGFβ1 family members are upregulated in the heart by increased work load after myocardial infarction. Therefore, their expressions correlate with cardiac apoptosis induction in vivo. I have now tested in this study, if TGFβ superfamily members induce apoptosis in ventricular cardiomyocytes of rat.

Cardiomyocytes were stimulated with TGFβ1, myostatin, BMP-2, activin A or GDF15. Apoptosis was determined by detection of chromatin condensation and DNA laddering. Apoptosis was induced by TGFβ1, myostatin, activin A and BMP-2. A classical signaling molecule of TGFβ family members is the transcription factor SMAD, which is able to interact with AP-1, a factor known to mediate apoptosis in cardiomyocytes after NO stimulation. In retardation assays I have now demonstrated that AP-1 and SMAD binding were activated by TGFβ1, myostatin, BMP-2 or activin A. Intracellular scavenging of SMAD or AP-1 binding activity by transformation of cardiomyocytes with SMAD-decoy oligos or AP-1 decoy oligos inhibited apoptosis induction by TGFβ1, myostatin, BMP-2 or activin A.

GDF15 stimulates SMAD binding activity similar to the other family members. But in contrast to them GDF15 showed anti-apoptotic effects. It inhibited apoptosis induction by TGFβ1.

Conclusions: Most of the investigated TGFβ-superfamily members activate the transcription factors AP-1 and SMAD and induce apoptosis in cardiomyocytes. For TGFβ1, myostatin, BMP-2 and activin-A SMAD/AP-1 is a common pathway for apoptosis induction in cardiomyocytes. GDF 15 has anti-apoptotic effects and inhibits TGFβ1 induced apoptosis. Therefore, further investigations on GDF15 signaling may have potential to discover now anti-apoptotic therapies in heart.

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