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Role of Peroxisome Proliferator Activated Receptor α (PPARα) in cardiomyogenesis of mouse embryonic stem cells

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Dedicated

To

My Beloved Family

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ABBREVIATIONS

^{o}C	Degree celsius
2-ME	β-mercaptoethanol
ANF	Atrial natriuretic factor
ANP	Atrial natriuretic peptide
ASCs	Adult stem cells
bp	Base pair
ca.	Circa
CBP	CREB-binding protein
cDNA	Complementary DNA
cM	centiMorgan
CREB	cAMP response element-binding
Cy2	Carbocyanin
Cy3	Indocarbocyanin
Cy5	Indodicarbocyanin
DBD	DNA-binding domain
DCF	2′,7′-Dichlorofluorescein
D-Loop	Displacement loop
DMEM	Dulbecco′s modified Eagle′s medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPI	Diphenyleniodonium
DTT	Dithiothreitol
EBs	Embryoid bodies
EC cells	Embryonic carcinoma cells
EDTA	Ethylene diamine tetracetate
EG cells	Embryonic germ cells
EGF	Epidermal growth factor
ERK	Extracellular signal regulated kinase
ERRα	Estrogen related receptor α
ES cells	Embryonic stem cells
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
h	Hour
H_2DCF	2′,7′-Dichlorodihydrofluorescein
H_2DCF-DA	2′,7′-Dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen Peroxide
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HIF	Hypoxia-inducible factor

Hsp72	Heat shock protein 72
Hsp90	Heat shock protein 90
ICM	Inner cell mass
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IMDM	Iscoves modified Dulbecco´s medium
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
JNK	c-Jun-NH_2 terminal kinase
kDa	kiloDalton
KH_2PO_4	Potassium dihydrogen phosphate
LBD	Ligand-binding domain
LCSM	Laser confocal scanning microscope
LDL	Low-density lipoprotein
LIF	Leukemia inhibitory factor
M	Molar (mol/L)
MAPKs	Mitogen-activated protein kinases
MEF	Mouse embryonic fibroblast
MEM	Modified Eagles medium
$MgCl_2$	Magnesium chloride
min	Minute
µg	Microgram
µM	Micromolar
mM	Milimolar
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
mtTFA	Mitochondrial transcription factor-A
Na_2HPO_4	di –Sodiumhydrogenphosphate dehydrate
NaCl	Sodium chloride
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form
ND1	NADH dehydrogenase 1
nDNA	Nuclear DNA
NEA	Non-essential amino acids
NFκB	Nuclear factor kappa B
nm	Nanometer
nM	Nanomolar
NMPG	N-(2-mercaptopropionyl)glycine
NO	Nitric oxide
NOS	Nitric oxide synthase
NRF	Nuclear respiratory factor
$O_2^{\bullet -}$	Superoxide
ONOO	Peroxynitrite
p38	p38 mitogen-activated protein kinase
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Triton X-100

PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PGC-1	PPARα coactivator-1
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
*p*M	Picomolar
PPARs	Peroxisome proliferator activated receptors
PPARα	Peroxisome proliferator activated receptor α
PPARβ	Peroxisome proliferator activated receptor β
PPARγ	Peroxisome proliferator activated receptor γ
PPRE	PPAR response elements
PTP	Protein tyrosine phosphatases
qPCR	Quantitative polymerase-chain-reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
RXR	Retinoic X receptor
s	Second
SOD	Superoxide dismutase
STAT	Signal transducers and activators of transcription
Syn	Synonym
TNFα	Tumour necrosis factor α
TR	Thyroid hormone receptor
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Triton X-100	Octyl phenoxy polyethoxy ethanol
tRNA	Transfer ribonucleic acid
U	Unit
UCP	Uncoupling proteins
VEGF	Vascular endothelial growth factor
vol	Volume
VSMC	Vascular smooth muscle cell
W/O	Without

1- INTRODUCTION

Heart failure constitutes a major cause of cardiovascular morbidity and mortality in the world. Approximately 1.5 – 2% of the total population and 6 – 10% of the people over 65-years in the world suffer from symptoms of heart failure. The annual fatality due to cardiac disease despite the developments in medical treatment is approximately 5 – 10% in patients with mild symptoms, and even rises to 30 – 40% in patients with advanced heart failure (*Packer 1999, Mendez et al. 2001*).

Heart failure is caused by the loss of functional heart muscle, which is due either to ischemic heart disease or the presence of dysfunctional muscle resulting from a variety of causes, including hypertension, viruses, and idiopathic factors. Following myocardial infarction for example, functional contracting cardiomyocytes are replaced with non-functional scar tissue. This ventricular remodeling leads to ventricle dilatation and progressive heart failure, which constitute major clinical problems (*Grounds et al. 2002*). The remodeling process is characterized by the removal of necrotic cardiomyocytes accompanied by granulation tissue formation with the simultaneous induction of neovascularization in the peri-infarcted bed. The latter is a prerequisite for the survival of surrounding hypertrophied but viable cardiomyocytes and the prevention of further cardiomyocyte loss by apoptosis.

Several treatments for heart failure that reduce the symptoms and improve the quality of life of these patients are available today. They include as medical treatments, effective precutaneous and surgical revascularization, and cardiac pacing systems. Cardiac transplantation remains, however, the ultimate solution for end-stage heart failure. However, the shortage of donor hearts, the complications of immunosuppression, the failure of grafted organs, and last but not least, the advanced age of patients suffering from heart failure significantly limit the utility of cardiac transplantation.

Cell therapy (regeneration of myocardium) as a mean to repair damaged tissues unable to heal is an increasingly attractive concept in modern transplantation medicine (regenerative medicine). True regenerative medicine has many components including cell therapy and tissue engineering. However, all are linked by one common theme – to deliver safe, effective and

consistent therapies to patients whose lives are dominated by chronic illnesses or life-threatening conditions for which there are currently no cures (*Mason et al. 2008*).

For many clinical situations, i.e. congestive heart failure, Parkinson's disease, diabetes, and traumatic injuries (spinal cord), replacement of lost cells would be the ideal treatment. In most cases, however, the development of cell treatment approaches is hampered by an increasing lack of donors or by the lack of cells suitable for transplantation.

The cell-based myocardial repair technology "cellular cardiomyoplasty", attempts to regenerate functioning muscle in previously infarcted, scarred or dysfunctional myocardial tissue after transplantation of myogenic cells. The use of such a cell therapy approach to replace lost cardiomyocytes with new graftable ones would represent an invaluable, low invasive technique for treatment of heart failure as an alternative to whole heart transplantation. Replacement and regeneration of functional cardiac muscle after ischemia could be achieved either by stimulating proliferation of endogenous mature cardiomyocytes or by implanting exogenous donor-derived or allogenic cardiomyocytes. The newly formed cardiomyocytes must integrate precisely into the existing myocardial wall to augment contractile function of the residual myocardium in a synchronized manner and avoid alteration in the electrical condition and syncytial contraction of the heart (*Itescu et al. 2003*).

To date, many types of cells have been tested as a source of cell therapy for augmentation of myocardial performance in different experimental models of heart failure (*Rosenweig 2006*). These include fetal cardiomyocytes (*Li et al. 1996, Reinecke et al. 1999, Zhang et al. 2001*), skeletal myoblasts (*Murry et al. 1996, Taylor et al. 1998, Reinecke et al. 2002*), immortalized myoblasts (*Robinson et al. 1996*), fibroblasts (*Murry et al. 1996, Etzion et al. 2002*), smooth muscle cells (*Li et al. 1999*), adult cardiac-derived cells (*Li et al. 2000, Steele et al. 2005*), bone marrow-derived stem cells (*Orlic et al. 2001, Wang et al. 2006*), and embryonic stem cell-derived cardiomyocytes (*Min et al. 2002, Van Laake et al. 2008*), and the last one opens new insights for future therapeutic approaches.

It has been known today that these ES cells have potential for numerous biomedical applications, including therapeutic cell replacement to repair damaged body organs, as tools for studying genetic defects and testing drugs, and as models for studying cell differentiation and early development. Since the mid-eighties, it has been known that mouse embryonic stem (ES) cells differentiate into cardiac myocytes during *in vitro* differentiation into cystic embryoid

bodies. ES cells can be expanded *in vitro* and retain their capacity to differentiate into cardiac myocytes (*Doetschman et al. 1985*). The capacity of these cells to form all somatic cell types in the human body has captured the imagination of researchers and clinicians alike. The perspectives that they represent for cell replacement therapies in multiple chronic disorders justify the use of embryos for this purpose.

However, the main issues which limit the research and use of ES cells for cellular cardiomyoplasty include difficulties in obtaining pure and sufficient numbers of ES-derived cardiomyocytes, and for this goal, the scientists and clinicians need to have a thorough understanding of pathways involved in ES cell-derived different cell type differentiation before such high stake goals can be achieved.

The present study was undertaken to increase the amounts of ES cell-derived cardiomyocytes in culture by focusing on involvement of the peroxisome proliferator activated receptor alpha (PPARα) signaling pathway. Although PPARs are best known as transcriptional regulators of lipid and glucose metabolism, and evidence has also accumulated for their importance in cell differentiation (*Rotman et al. 2006*), little is known about the significance of PPARα in early cardiac development especially during the differentiation of cardiomyocytes. This thesis focuses on clarifying the relationship between PPARα activity and differentiation of ES cell-derived cardiomyocytes, and the mechanisms of cardiac cell differentiation control by this key nuclear receptor. Understanding PPARα signaling pathway in ES cells will help to improve the knowledge of how mass cultures of proliferating cardiac cells may be generated for cell transplantation or cellular cardiomyoplasty. During the study, the CCE mouse ES cell line was employed to examine the PPARα cascade in relation to cardiomyocyte differentiation.

1-1- STEM CELLS

There is much interest in developing stem cells and the cells derived from them for treating human disease and injury, but many biological, technological and regulatory hurdles have to be overcome before these cell therapies can be brought to commercial fruition.

Clearly, stem cells and the cells derived from them have great potential to serve medicine from therapy, to drug testing, to teaching us more about the body's biology.

Deciphering nature's secrets of heart formation might lead to new approaches to repair or regenerate damaged heart muscle. Stem cells have enormous potential in regenerative medicine,

and insights into cardiogenesis from progenitor cells during embryogenesis will form the basis of reprogramming cells for therapeutic use (*Srivastava et al. 2006*).

1-1-1- INTRODUCTION TO STEM CELLS

Research on stem cells is advancing knowledge about how an organism develops from a single cell, and how healthy cells replace damaged cells in adult organisms. This promising area of science is also leading scientists to investigate the possibility of cell-based therapies to treat disease, which is often referred to as regenerative or reparative medicine.

Stem cells are one of the most fascinating areas of biology today. However, like many expanding fields of scientific inquiry, research on stem cells raises scientific questions as rapidly as it generates new discoveries.

Scientists have also discovered ways to obtain or derive stem cells from early mouse embryos more than 20 years ago. Many years of detailed study of mouse stem cell biology led to discovery, in 1998, of how to isolate stem cells from human embryos and grow the cells *in vitro* (*Thomson et al. 1998*).

In the 3- to 5-day-old embryo, called a blastocyst, stem cells in developing tissues give rise to multiple specialized cell types that make up the heart, lung, skin, and other tissues. In some adult tissues, such as bone marrow, muscle, and brain, separate populations of adult stem cells generate replacements for cells that are lost through normal wear and tear, injury, or disease.

There are three basic kinds of stem cells. Totipotent stem cells, meaning their potential is total, have the capacity to give rise to every cell type of the body and to form an entire organism. Pluripotent stem cells, such as ES cells, are capable of generating virtually all cell types of the body but are unable to form a functioning organism. Multipotent stem cells can give rise only to a limited number of cell types. For example, adult stem cells, also called organ- or tissue-specific stem cells, refer to multipotent stem cells found in specialized organs and tissues after birth. Their primary function is to replenish cells lost from normal turnover or disease in the specific organs and tissues in which they are found.

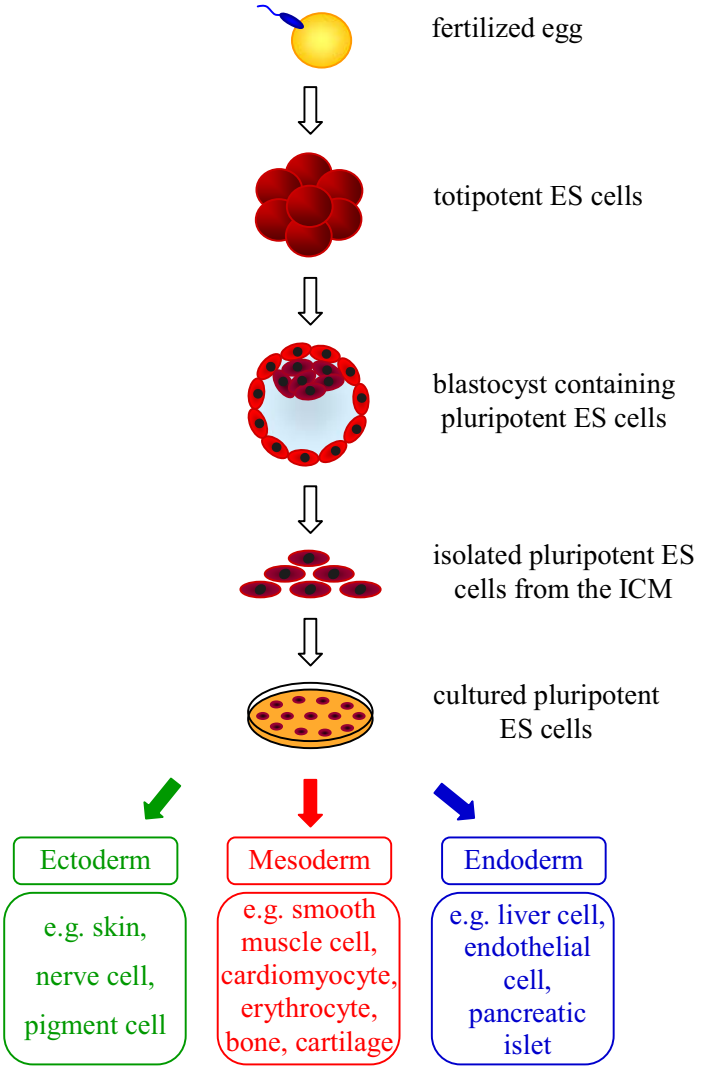


Figure 1: *Differentiation of ES cells.* The ES cells have a strong potential to differentiate into any cell type of the body, but cannot contribute to making the extra embryonic membranes.

1-1-2- PROPERTIES OF STEM CELLS

Stem cells differ from other kinds of cells in the body. All stem cells – regardless of their source – have three general properties: They are as follows:

1- **Stem cells are unspecialized:** One of the fundamental properties of a stem cell is that it does not have any tissue-specific structure that allows it to perform specialized functions.

However, unspecialized stem cells can give rise to specialized cells, including heart muscle cells, blood cells, or nerve cells.

2- **Stem cells have self-renewing capacity:** Unlike differentiated cells – which do not normally replicate themselves – stem cells may replicate many times. When cells replicate themselves over many times it is called proliferation. A starting population of stem cells that proliferates for many months in the laboratory can yield millions of cells. If the resulting cells continue to be unspecialized, like the parent stem cells, the cells are said to be capable of long-term self-renewal.

3- **Stem cells have the ability to differentiate:** when unspecialized stem cells give rise to specialized cells, the process is called differentiation.

1-1-3- TYPES OF STEM CELLS

There are three main types of stem cells under scientific study today:

- Embryonic stem (ES) cells
- Embryonic germ (EG) cells
- Adult (AS) stem cells which some scientists term as somatic stem cells.

1-1-3-1- EMBRYONIC STEM CELLS

ES cells, as their name suggests, are derived from embryos. The embryos from which ES cells are derived are typically four- or five-day-old and are a hollow microscopic sphere of cells called the blastocyst. The blastocyst includes three structures: the trophoblast, which is the outer layer of cells that surrounds the blastocyst; the blastocoele, which is the hollow cavity inside the blastocyst, and the inner cell mass (ICM), which is a group of approximately 30 cells at one end of blastocoele. Trophoblast cells form the placenta and other supporting tissues during fetal development, whereas cells of the inner cell mass go on to form all three primary germ layers: ectoderm, mesoderm, and endoderm. The three germ layers are the embryonic source of all cell types and tissues in the body.

The technique for isolating and culturing mouse ES cells from the inner cell mass of the blastocyst was first developed in 1981 (*Evans et al. 1981, Martin GR 1981*). These ES cells

derived from the ICM retain the capacity to give rise to cells of all three germ layers. However, ES cells cannot form a complete organism because they are unable to generate the entire spectrum of cells and structures required for fetal development.

ES cells are isolated by transferring the ICM into a culture dish and allowed to divide and spread over the surface of the plate. The inner surface of the culture dish is typically coated with mouse embryonic fibroblasts that have been treated with mitomycin (an inhibitor of mitosis), and is called as feeder layer. The reason for having the feeder layer at the bottom of the culture dish is to give the ICM a sticky surface to which the ES cells can attach. In addition, the feeder cells release nutrients into the culture medium, which is necessary to keep the ES cells in an undifferentiated state.

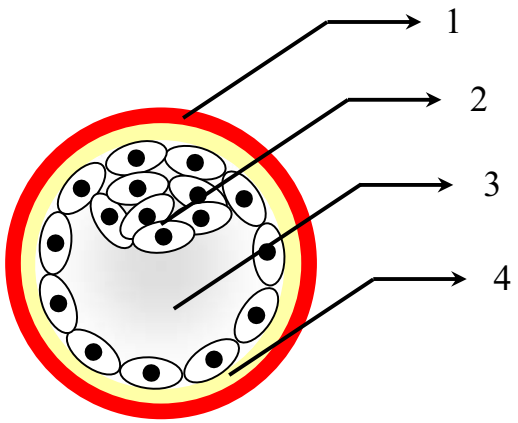


Figure 2: *Structure of Blastocyst.* The figure shows the different layers of the blastocyst. 1: The zona pellucida, 2: Inner cell mass (ICM), 3: Blastocoel, 4: Trophectoderm.

Over the course of several days, the cells of the inner cell mass spontaneously proliferate, and begin to crowd the culture dish to become confluent. When they are sufficiently confluent, these ES cells are gently removed and replated into new culture plates.

As long as the ES cells in culture are grown in presence of feeder layer cells and leukemia inhibitory factor, they can remain undifferentiated. But when feeder cells and LIF are withdrawn and cells are allowed to clump together to form embryoid bodies, they begin to differentiate spontaneously. Embryoid bodies are comprised of heterogeneous cells that are derived from all

three germ layers. These tri-dimensional cell-cell contacts allow the formation of heterogeneous cultures of differentiated cell types including cardiomyocytes, haematopoietic cells, endothelial cells, neurons, skeletal muscle cells, chondrocytes, adipocytes, liver cells, and pancreatic islets.

1-1-3-2- EMBRYONIC GERM CELLS

5 to 10 weeks after fertilization, the growing embryo, now called a fetus, develops a region known as the gonadal ridge. The gonadal ridge contains the primordial germ cells, which will eventually develop into the testes or the ovaries. Mouse embryonic germ (EG) cells were isolated and cultured from primordial germ cells for the first time by Stewart et al. on 1994 (*Stewart et al. 1994*), four years later, Gearhart et al. derived human EG cells from the gonadal ridge and mesenchymal tissue of 5- to 10-week old fetuses that were obtained from elective abortions (*Shamblott et al. 1998*).

These two groups devised methods for growing EG cells. This process requires the generation of embryoid bodies from EG cells which consist of a mix of partially differentiated cell types.

EG cells differ from ES cells in the tissue sources from which they are derived, but appear to be similar to ES cells in their pluripotency.

EG cells in culture, like cultured ES cells, form embryoid bodies, which are 3-dimensional cell aggregates consisting of partially differentiated cells. The cell lines generated from cultures of embryoid bodies can give rise to cells of all three embryonic germ layers, indicating that EG cells may be another source of pluripotent stem cells.

1-1-3-3- ADULT STEM CELLS

Adult stem cells are undifferentiated cells, which are found in tissues that have already developed in animals or humans after birth. These stem cells with their multi-differentiation potential can renew themselves.

Adult stem cells were first discovered almost 40 years ago. It was in the 1960s when researchers found that the bone marrow contains at least two kinds of stem cells. One population, called haematopoietic stem cells, give rise to all types of blood cells in the body. A second cell population, called bone marrow stromal cells, were discovered a few years later. Stromal cells are a mixed cell population that generate bone, cartilage, fat, and fibrous connective tissue. Also in

the 1960s, scientists working with rats discovered two regions of the brain that contained dividing cells, which could become nerve cells. Despite these reports, most scientists believed that new nerve cells could not be generated in the adult brain. It was not until the 1990s that scientists agreed that the adult brain does contain stem cells that are able to generate the brain's three major cell types – astrocytes and oligodendrocytes, which are non-neuronal cells, and neurons, or nerve cells.

One important point that is to be understood about adult stem cells is that there are a very small number of stem cells in each tissue. Stem cells are thought to reside in a specific area of each tissue where they may remain quiescent for many years until they are activated by disease or tissue injury.

While the ICM of the blastocyst is defined as the origin of ES cells, the origin of adult stem cells in the body is unknown. The adult tissues reported to contain stem cells include brain, heart, bone marrow, blood vessels, skeletal muscle, skin, and liver, as well as in peripheral circulation. Currently this "stem cell family" comprises of adipose-derived stem cells (ASCs), induced pluripotent stem cells derived from epithelial cells, haematopoietic stem cells, mammary stem cells, mesenchymal stem cells (MSCs), endothelial stem cells, neural stem cells, olfactory stem cells, spermatogonial progenitor cells, and testicular cells which have been found in fat, skin, blood, mammary gland, bone marrow (source of MSCs and endothelial stem cells), brain, nose, and testis, respectively.

In the past few years many scientists have been trying to find ways to culture adult stem cells and manipulate them to generate specific cell types that can be used to treat injury or disease. Some examples of potential treatments include generation of dopaminergic neurones in the brains of Parkinson's patients (*Takagi et al. 2005*), developing insulin-producing cells for type I diabetes (*Roche et al. 2007*), and repairing damaged heart muscle following a heart attack (*Van Laake wt al. 2006*).

1-2- THE NUCLEAR RECEPTOR FAMILY

Nuclear hormone receptors (NRs) are important transcription regulators involved in diverse physiological functions such as control of embryonic development, cell differentiation, and homeostasis (*Mangelsdorf et al. 1995*). In addition, these molecules are extremely important in medical research since a large number of them are implicated in diseases like cancer, diabetes,

and hormone resistance syndromes. Some of the NRs act as ligand-inducible transcription factors, while a large number of them have no defined ligand and are hence described as orphan receptors (*Tenbaum et al. 1997, Ribeiro et al. 1995*).

The nomenclature system for the nuclear receptor superfamily, divides the superfamily into six subfamilies and 26 groups of receptors, PPARs belong to subfamily 1. This subfamily, which is the largest in the entire superfamily, comprises 11 groups of receptors (TR, RAR, PPAR, REV-ERB, E78, RZR/ROR, *Caenorhabditis* CNR14, ECR, VDR, *Drosophila* DHR96 orphan receptor, and the nematode NHR1 orphan receptor from *Onchocerca volvulus*) composed of a total of 27 individual genes (*Nuclear receptors nomenclature committee 1999*).

1-2-1- PEROXISOME PROLIFERATOR ACTIVATED RECEPTORS

As mentioned above, PPARs belong to the nuclear hormone receptor superfamily. The PPARs are fatty acid and eicosanoid inducible nuclear receptors, which occur in three different isotypes:

For several years the functions of PPARs were thought to be limited to specific tissue types, having roles in lipid catabolism, peroxisome proliferation in the liver (*Issemann et al. 1990*), and adipogenesis (*Spiegelman et al. 1996, Spiegelman 1998*). Recent research has demonstrated that activated PPARs are involved in the differentiation process of several cell types, including nerve cells, pancreatic cells, cardiomyocytes, tumor cells, and also modulate the expression of various target genes implicated in several important physiological pathways (*Mueller et al. 1998, Rosen et al. 1999, Park et al. 2004, Ding et al. 2007, Finck 2007*).

1-2-1-1- HISTORY

In 1983, Lalwani et al. reported the identification of a peroxisome proliferator binding protein in rat liver, and suggested that this molecule might mediate peroxisome proliferator action (*Lalwani et al. 1983*). Several years later, Alvares and his co-workers identified this protein as Hsp72 (*Alvares et al. 1990*). After Reddy and Rao postulated that peroxisome proliferators might act in a manner similar to steroid hormones (*Reddy et al. 1986*), Issemann and Green screened a mouse liver cDNA library with a probe derived from the combined nucleotide sequences of several hormone receptors and identified four new orphan members of the nuclear hormone-like receptor family (*Issemann et al. 1990*). Because one of these receptors could be activated by a

variety of peroxisome proliferators they called it the peroxisome proliferator-activated receptor (PPAR), which is today known as PPARα. PPARα was identified as a putative member of the nuclear receptor family because it had the characteristic modular structure including the conserved DNA-binding domain (DBD) and ligand-binding domain (LBD). Two years later, the Wahli laboratory reported cloning of the *Xenopus laevis* ortholog of PPARα, and also the cloning of two closely related orphan receptors, encoded by distinct genes, which they named PPARβ/δ and PPARγ (*Dreyer et al. 1992*). The three isoforms have 90% homology in the amino acid sequences of the DBD's and 80% homology in the LBDs.

1-2-1-2- GENERAL STRUCTURE OF THE PPARs

PPARs have similar structural and functional domains like the other members of the nuclear hormone receptor superfamily. However, the amino-terminal region or A/B domain is, poorly conserved between the three PPAR isotypes, and contains a ligand-independent transactivation function called AF-1. It has been shown that its phosphorylation state contributes to the modulation of PPARα and –γ activity (*Shalev et al 1996, Hu et al. 1996, Zhang et al. 1996, Camp et al. 1997, Adams et al. 1997, Juge-Aubry et al. 1999*). The central DNA binding domain (C domain) is highly conserved, with its two zinc finger-like structures which is an α-helical DNA binding motif. when the receptor is activated the DBD or C domain bind to specific sequences of DNA known as hormone response elements. The DNA binding domain is followed by a D domain which is a flexible hinge region that allows the receptor to change conformation, and this property helps to form a suitable conformational structure at the time of dimerization with RXR and attachment to DNA strand. The E/F domain is the ligand-binding domain (LBD), and has the ligand-dependent transactivation function AF-2.

In addition to ligand binding, this region is required for nuclear localization, receptor dimerization and the interaction with proteins acting as co-activators or co-repressors. The LBD has an extensive secondary structure consisting of 13 α-helices and one β-sheet (*Zoete et al. 2007*). Various natural and synthetic components have been identified as activators of PPARs. Polyunsaturated fatty acids and some eicosanoids are defined as natural ligands whereas fibrate-derived ligands (i.e. some hypolipidemic and anti-diabetic drugs) are defined as synthetic components ligands (*Kliewer et al. 1997*).

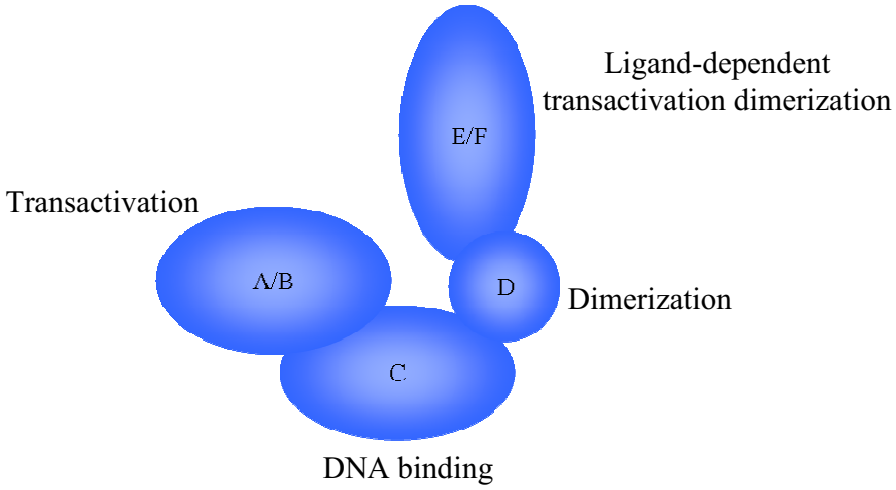


Figure 3: *Structure of the peroxisome proliferator activated receptors.* It shows four different domains of PPARs which is common to the members of nuclear receptor family.

In the inactivated state (in the absence of ligands) the PPARs are thought to be in complexes bound with co-repressor and heat shock proteins (*Pratt 1992, Smith 1993, Sumanasekera et al. 2003*). Furthermore, at this stage, in some cell types, PPARs may also have a peri-nuclear rather than the usual cytoplasmic location (*Chinetti et al. 1998*).

All three PPARs are stimulated by binding of small lipophilic compounds such as polyunsaturated fatty acids and various fatty-acid derived molecules to their ligand-binding domain (*Krey et al. 1997, Kliewer et al. 1997*). The interaction of PPARs with their ligands induces conformational changes, which then allow the recruitment of co-activators, such as steroid receptor coactivator-1 CREB-binding protein, and the release of co-repressors, such as nuclear receptor co-repressor and silencing mediator of retinoid and thyroid hormone receptor. Upon ligand activation and the following structural changes, PPARs can translocate from the cytoplasm to the nucleus. The co-activator proteins either possess histone acetyltransferase activity or recruit other proteins with this activity to the transcription start site. Acetylation of histone proteins alters chromatin structure, facilitating the binding of RNA polymerase and the initiation of transcription (*Rosen et al. 2001*). PPARs can also repress gene expression by interfering with other signaling pathways by recruiting co-repressors to un-liganded PPARs (*Xu et al. 2002*).

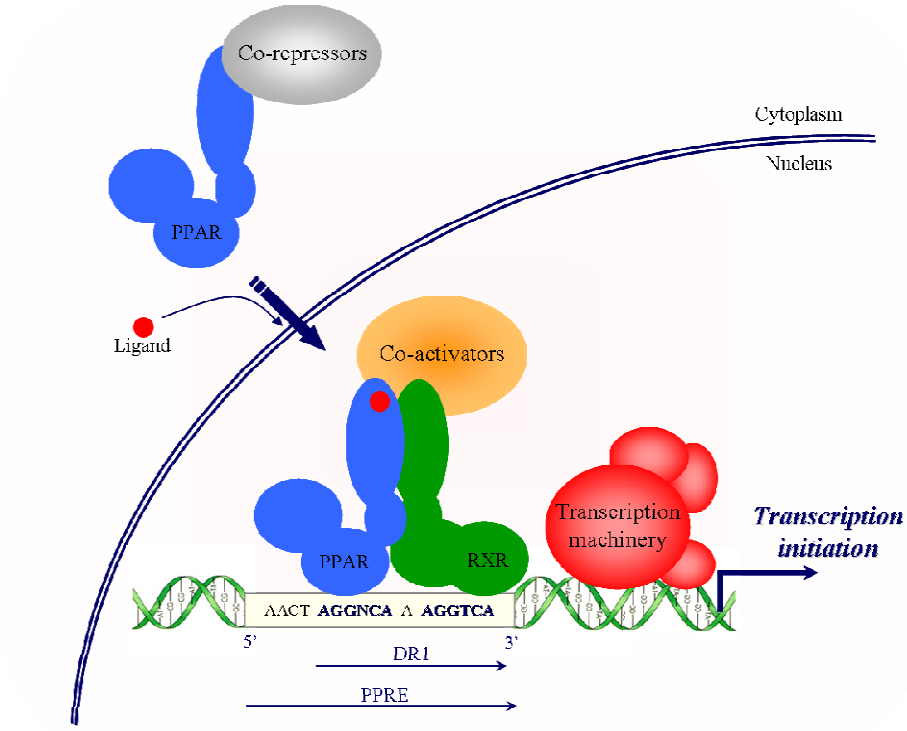


Figure 4: *Organization of the functional domains of the PPAR protein and the active transactivation PPAR:RXR complex.* The PPAR:RXR dimer recognizes specific DNA sequences on the promoter region of target genes and binds to them. This favors transcription initiation by recruitment of transcription machinery and chromatin remodeling.

Dimerization is essential for the activity of PPARs, as it is for most of the other members of the nuclear hormone receptor superfamily. In contrast to steroid hormone receptors, which act as homodimers, PPARs heterodimerize in the cytoplasm with the RXR that belongs to the same receptor superfamily, forming a complex that is able to translocate to the nucleus and bind to the PPAR response elements (PPRE) (*Kliewer et al. 1992,* Keller H et al. 1993). This response element, generally of the direct repeat 1 type (DR1), is composed of two half sites that occur as direct repetitions of the consensus sequence 5'-AGGTCA-3' with a single nucleotide spacing between the two repeats. The PPRE is usually present in one or multiple copies in the promoter region of target genes but may also be located in the proximal transcribed region of certain

PPAR-responsive genes (*Di-Poi et al. 2002, Juge-Aubry et al. 1997*). PPAR and RXR bind to the 5' half-site and 3' half-site of this element, respectively. The 5'-flanking region then mediates the selectivity of binding of the corresponding PPAR isotype (*IJpenberg et al. 1997*). Consequence of this interaction is the transcription of target genes that are key players in lipid metabolism, for example, fatty acid uptake, and peroxisomal and mitochondrial fatty acid oxidation (*Berger et al. 2002*).

It has also been demonstrated that the biological activity of each PPAR isoform is regulated not only by the availability of (natural or synthetic) ligands, but also by the recruitment of co-activators and -repressors, and the phosphorylation of PPAR.

1-2-1-3- PPAR ISOFORMS

Three types of PPARs have been identified which are named as *PPAR*α, $-\beta$ (δ) and $-\gamma$. Each is encoded by a separate gene and has a unique tissue distribution. Furthermore, their roles in mediating changes in gene expression appear to be tissue and cell-specific.

1-2-1-3-1- PPARα

PPARα (syn: Ppar, Nr1c1, and AW742785) is mostly expressed in tissues which have high energy demand, such as brown adipose tissue, liver, kidney, duodenum, brain, heart and skeletal muscle (*Kliewer et al. 1994*), demonstrating a close association between PPARα and energy turnover. PPARα is responsible for the modulation of the immune response and catabolism of fatty acids through peroxisomal and mitochondrial β-oxidation, microsomal ω-oxidation and amino acid and carbohydrate metabolism (*Kersten et al. 1999, Kersten et al. 2001*).

The *PPAR*α gene in mouse is located on chromosome 15 at the locus 15:85565994 – 85633249 and its cytogenetical location is 15 E2. The length of the *PPAR*α gene is 48.8 cM with 65618 bp consisting of eight exons. This gene can transcribe mRNA with 2063 bases which is translated into a 52.3 kDa protein with 468 amino acid residues. The zinc finger of this protein is located between residue 100 and 174 and the ligand binding domain is formed by the residues 282 to 463.

As mentioned in the last section, inactivated PPARα is located in the cytoplasm and after the process of activation translocates to the nucleus. Sumanasekera et al. described that in the

absence of a ligand, PPARα is in a cytosolic multiprotein complex that includes heat shock protein 90 (Hsp90), Hsp 70 and other cochaperone proteins. The interaction is between the center of the heat shock proteins and the hinge and the LBD domain of PPARα. Usually, the association between heat shock proteins and nuclear receptors appears to hold the receptor in a conformation that will allow high affinity binding of the ligand (*Smith 1993, Huang et al. 1994*). But, the Sumanasekera et al. have also demonstrated that Hsp90 is a regulator of PPARα and plays a repressory role for PPARα activity (*Sumanasekera et al. 2003b, Sumanasekera et al. 2003a*).

Today, it is well accepted that PPARα is expressed in cells of the cardiovascular system, including endothelial cells, vascular smooth muscle cells, monocytes/macrophages, and cardiomyocytes (*Issemann et al. 1990, Inoue et al. 1998, Staels et al. 1998, Kersten et al. 2000, Gilde et al. 2003, Baily 2000*). It also plays a key role in the transcriptional regulation of genes encoding mitochondrial fatty acid β-oxidation (FAO) enzymes during cardiac development and also in response to physiological and patho-physiological stimuli, including fasting, cardiac hypertrophy, and cellular hypoxia (*Sack et al. 1996, Sack et al. 1997, Depre et al. 1998, Leone et al. 1999*).

However, with all the increasing knowledge about PPARα, little is known about thetranduction of physiological or patho-physiological signals for modulating PPARα transcription in cardiomyocytes.

1-2-1-3-2- PPARβ

PPARβ (syn: NUC1, Nr1c2, and PPARδ) is the most ubiquitously expressed isotype, with highest expression in the gut, kidney, heart, and brain. PPARβ is found in higher amounts than α and γ in almost all tissues, except the adipose tissue (*Desvergne et al. 1999*).

The *PPARβ* gene lies on chromosome 17 of mouse at position 17:28369699 – 28438410 which cytogenetically represents the 17 A3.3 locus. The length of the *PPARβ* gene is 13.5 cM with 68712 bp consisting of 8 exons. The mRNA of this gene is about 3240 bases long, which can be translated into a protein with 440 residues weighing 49.7 kDa. The zinc finger and LBD are found between residue numbers 73 – 145 and 254 – 435, respectively.

1-2-1-3-3- PPARγ

PPARγ or Nr1c3 is expressed mainly in brown and white adipose tissues and, to a lesser extent, in the large intestine, the retina and some parts of the immune system.

In mice, the *PPARγ* gene is present on chromosome 6 with the cytogenetical location of 6 E3-F1 and between the loci 6:115372083 and 6:115440419. This gene is about 52.7 cM long with 68336 bp consisting of 7 exons. The mRNA transcript from the *PPARγ* gene is 1779 bp long and translates to a 57.6 kDa protein with 505 amino acids. In this protein, the zinc finger is found between residues 137 and 211 and the LBD is between amino acid number 319 and 500.

1-3- INTRACELLULER REDOX SIGNALING

The intracellular redox state is characterized by the balance of reactive oxygen species (ROS) production and the antioxidant capacity of the cell based on a variety of antioxidant enzymes such as superoxide dismutase (which reduces $O_2^{\bullet -}$ to H_2O_2), catalase, and glutathione peroxidase (which reduce H_2O_2 to H_2O).

In the last decade it has become increasingly evident that a wide variety of extracellular stimuli induce ROS production which is essential for downstream intracellular signal transduction. Among these stimuli are numerous ligands of tyrosine kinase and G-protein-coupled receptors including: platelet-derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), angiotensin II, insulin, tumor necrosis factor alpha (TNFα) and interleukin 1β (IL-1β) (*Nakamura et al 1998*). The enzymatic source of ligand-stimulated ROS is not completely elucidated, but cyclooxygenases, lipoxygenases and NADPH oxidase are among the most likely candidates (*Sorescu et al. 2002*). The downstream targets of ROS have remained largely unexplored. However, it has been found that oxidant bursts are often associated with an increase in tyrosine phosphorylation. Extracellular administration of H_2O_2 has been demonstrated to members of the mitogen-activated protein kinase (MAPK) pathway, consisting of extracellular-signal regulated kinase (ERK), p38 MAPK and c-Jun amino-terminal kinase (JNK) (*Tanaka et al. 2001*). It seems likely that ROS-mediated regulation of protein kinase pathways is due to alteration of the tyrosine kinase/phosphatase balance. It has been demonstrated that exogenous oxidants or oxidants generated by peptide growth factor binding can reversibly oxidize and inactivate protein tyrosine phosphatases (PTP) (*Meng et al.*

2002). PTPs contain reactive cysteine residues within their active sites. Such reactive cysteine residues are easily modified by ROS and reactive nitrogen species (RNS) providing a base for intracellular redox signaling. However, the organization of redox signaling and the use of oxygen to transmit information are proving to be complex and require further research.

1-3-1- VASCULAR REDOX SIGNALING

ROS have been shown to function as important molecules in the cardiovascular system. Cardiomyogenic cells contain several sources of ROS. In these cells, ROS mediate many patho-physiological processes such as growth, migration, apoptosis and secretion of inflammatory cytokines, as well as physiological processes, such as proliferation, and differentiation, by direct and indirect effects at multiple signaling levels. It is self-evident that ROS ideally fulfill the prerequisites for intracellular signaling molecules since they are rapidly generated, highly diffusible, easily degraded and ubiquitously present in the cells.

1-3-2- SOURCE OF ROS

In cardiac cells, ROS are generated in multiple compartments and by multiple enzymes. Important contributions include lipid metabolism within the peroxisomes, the activity of various cytosolic enzymes, such as cyclooxygenases, nitric oxide synthases (NOSs), and xanthine oxidase (*Ekelund et al. 1999*), proteins within the plasma membrane, such as the non-phagocytic of NADPH oxidase (*Sorescu et al. 2002*), as well as mitochondria (*Ide et al. 1999, Sorescu et al. 2002*). The latter two are described herein.

1-3-2-1- NADPH OXIDASE

NADPH oxidase has been studied extensively in phagocytes (neutrophilic and eosinophilic granulocytes, monocytes, and macrophages), and non-phagocytic cells. The phagocytic leukocytes utilize self-generated ROS to kill invading pathogens. However, ROS generated by the putative non-phagocyte NADPH oxidase is involved in various physiological and patho-physiological responses of different cell types such as mitosis, migration, differentiation, apoptosis, and modification of the extracellular matrix. Various studies suggest distinct differences in amino acid sequence and antibody reactivity between the central subunit (gp91phox) of the phagocyte and non-phagocyte NADPH oxidase and evidence distinct

genetically and structurally differences between the gp91phox subunit of both NADPH oxidase types (*Ushio-fukai et al 96, De Keulenaer et al 98, Li et al 2003*). Meanwhile, it is known that unlike the phagocytic NADPH oxidase, the enzyme in non-phagocytic cells continuously generates intracellular ROS at a low level even in the absence of cell stimulation, and this activity can be significantly enhanced by several different stimuli in physiological and pathophyiological conditions (*Ushio-fukai et al 96, De Keulenaer et al 98, Li et al 2003*). NADPH oxidase activation by these stimuli involves both transcriptional upregulation of component oxidase subunits and acute activation through post-translational modification of oxidase regulatory subunits.

1-3-2-1-1- STRUCTURE OF NADPH OXIDASE

At least five proteins are required for the formation of an active oxidase complex. The membrane-bound cytochrome b_{558}, consisting of two subunits, gp91phox and p22phox that need each other to form a stable catalytic core which is named flavocytochrome b_{558}. Cytochrome b_{558} is a flavoprotein containing both flavin adenine dinucleotide (FAD)- and NADPH-binding sites, based only on the amino acid homology between gp91phox and the putative $NADP^+$- and FAD-binding sites of the ferredoxin reductase family. The cytosolic proteins, p47phox, p67phox and a small GTP-binding protein, Rac-1 or Rac-2 (*Bokoch 1994*). Some scientists have identified two additional components, these being the cytosolic protein, p40phox, that appears to be associated with p67phox (*Wientjes et al. 1993, Tsunawaki et al. 1994*), and the membrane associated small GTP-binding protein Rap1a (*Gabig et al. 1995*). Under resting conditions the cytosolic components exist as a 240-300 kDa oligomer (*Park et al. 1992*). As mentioned, these recent subunits are localized in the cytoplasm of cells. Upon stimulation of the cells by various agents, these components translocate, guided by the GTPase Rac, to the flavocytochrome to form the active enzymatic NADPH oxidase complex (*Leusen et al. 1996, Lassegue et al. 2003, Segal et al. 1993, Shatwell et al. 1996, Puceat et al. 2003*). The assembly process of the active NADPH oxidase complex is thought to be mediated by a mechanism involving both protein binding through Src homology 3 (SH3) domains and phosphorylation of p47phox (*Rotrosen et al. 1990, McPhail 1994, Park et al. 1995*).

To date, several isoforms of the gp91phox catalytic subunit of NADPH oxidase have been described, each one encoded by separate genes. These isoforms are now termed NOXs, and

comprise Nox1 – 5. Nox2 is used as a new name for gp91phox (*Lambeth 2004*). All these isoforms show homology to gp91phox/Nox2 ranging from approximately 30 to 60% (*Goerlach et al. 2002*).

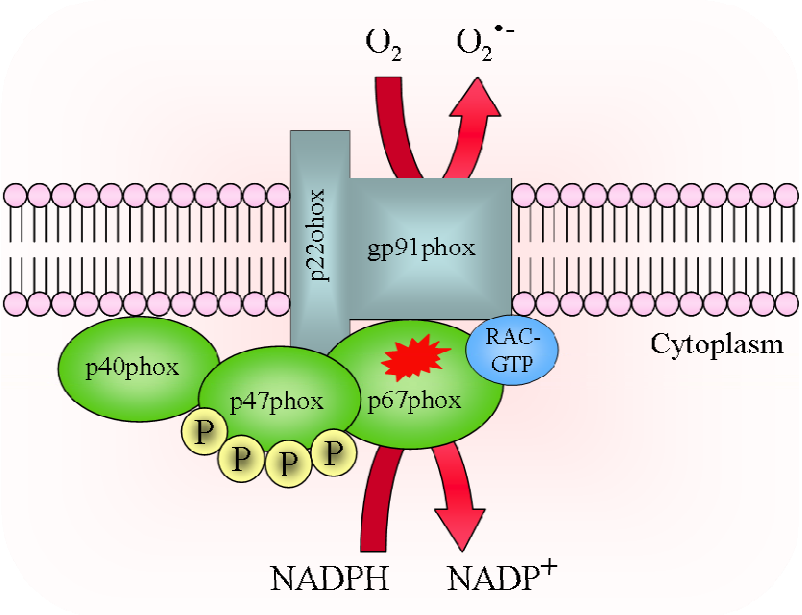


Figure 5: *Structure of NADPH oxidase.* Schematic diagram showing the structure of the classical NADPH oxidase. The cytosolic subunits p47phox, p67phox, p40phox, and Rac translocate to the gp91phox-p22phox complex to activate the enzyme.

The first gp91phox isoform is Nox1 (syn: Mox1 and NOH1) (*Lambeth 2004*). Interestingly, ROS produced by Mox1 seem to be generated intracellularly. Furthermore, overexpression of Mox1 resulted in increased cell growth, suggesting a possible role for Mox1 in growth regulation. The second isoform is Nox2 (gp91phox) which is a 91 kDa glycosylated protein with six hydrophobic, probably membrane spanning, segments in its N-terminal half. Four histidine residues in this transmembrane cluster have been shown to participate in the ligation of two hemes. The cytosolic C-terminal half of the protein contains FAD and NADPH binding sites homologous to those found in some other flavoproteins; it also encompasses yet poorly defined regions of interaction with the cytosolic oxidase components p47phox and

p67phox (*Goerlach et al. 2002*). The other isoform is Nox3 about which to date, nothing is known about this homologue except for a seemingly exclusive expression in fetal kidney tissue. Another gp91phox isoform, Nox4 (renox, Kox1), has been detected in the renal cortex, and predominantly in the proximal convoluted tubule epithelial cells (*Lambeth 2004*). Based on the expression pattern, it has been suggested that renox participates in the oxygen sensing mechanism that regulates the production of erythropoietin. Nox5 is the other isoform of gp91phox which builds on the basic structure of gp91phox, adding an amino-terminal calmodulin-like domain that contains four binding sites for calcium (*Lambeth 2004*).

1-3-2-1-2- NADPH OXIDASE ROS PRODUCTION

Upon activation, NADPH oxidase catalyses the reduction of one electron of oxygen using NADPH as the electron donor on the cytosolic side of the membrane, and reduces oxygen across the membrane to generate superoxide, a powerful oxidising and reducing agent (*Griendling et al. 2000*):

$$NADPH + 2O_2 \longrightarrow NADP^+ + 2O_2^{\bullet -} + H^+$$

In the presence of superoxide dismutase, the superoxide anion radicals ($O_2^{\bullet -}$) generated in this way can be dismutated to hydrogen peroxide (H_2O_2), either spontaneously or by the antioxidant enzyme superoxide dismutase. This H_2O_2 produced may subsequently be converted into a variety of active oxygen species, such as singlet oxygen and hydroxyl radicals (*Griendling et al. 2000*), and also, into hypobromous acid in the presence of peroxidase and bromide (*Parini et al. 1986*). Alternatively, in the presence of ferrous ions, $O_2^{\bullet -}$ and H_2O_2 interact to form the membrane-perturbing hydroxyl radical ($OH^{\bullet}$), one of the most unstable oxidising species known. Other pathways of free radical formation have also been described including the reaction of $O_2^{\bullet -}$ with nitric oxide to form peroxynitrite which provides an additional, iron-independent route of $OH^{\bullet}$ formation together with nitrogen dioxide radicals. Likewise, the produced hypobromous acid is able to interact with H_2O_2 to form singlet oxygen, too.

1-3-2-2- THE MITOCHONDRION

Mitochondria are organelles found within most eukaryotic cells. They are observed as small (0.5 – 1.0 μM), typically rod shaped bodies, with two distinct membrane bi-layers surrounding them. Active cells, such as heart and skeletal muscle, have a large number of mitochondria. They are responsible for the generation of cellular ATP through oxidative phosphorylation. A proton gradient across the mitochondrial inner membrane, maintained by the oxidative phosphorylation enzyme complexes, is utilized to drive the production of ATP from ADP and phosphate within the mitochondrial inner matrix.

The synthesis or hydrolysis of ATP by intact mitochondria occurs in the matrix space, because the catalytic sites are on the F_1 head of the ATP synthase lollipop, which projects on the matrix side (N-phase) of the membrane. Nevertheless, mitochondria are able to rapidly hydrolyze externally added ATP, and to synthesize ATP outside from added ADP and phosphate. This is possible because of two transport systems which allow these metabolites to cross the membrane: a) The adenine nucleotide transporter, b) The phosphate transporter. The net effect of these two transport processes is that the proton gradient drives the concentration of ADP and phosphate inside the mitochondrion, and the export of ATP, at the expense of $1H^+$/ATP synthesized.

Since mitochondria are a major source of cellular ATP, we should not be surprised to learn that the number of them per cell, as well as their intracellular location, varies with the type of cell and with its metabolic state.

1-3-2-2-1- STRUCTURE OF MITOCHONDRIA

In the mitochondrion, there are four major structural regions. These are the outer and inner membranes and the two spatial regions delineated by these membranes, namely the inter-membrane space and the matrix. The inner membrane is impermeable, whereas the outer membrane of mitochondria is permeable to ions and small molecules.

Mitochondria have their own genome. Typically, the mtDNA is a small self-replicating circular DNA molecule has in the mitochondrial matrix. The mtDNA is characteristically very small and compact, 15-17 kb in size, with very few non-coding nucleotides in the D-Loop region (*Wolstenholme 1992*).

All mammalian mitochondrial genes involved in the transcription of the mt-genome are encoded within the nuclear genome, and are imported into the mitochondria.

The standard mitochondrial genome encodes all the necessary tRNA and rRNA and thirteen proteins (*Anderson et al. 1981, Nesti et al. 2007*) of the approximately hundred proteins that comprise the machinery of the electron transport system, which is located at the inner membrane of mitochondria. These proteins which are encoded by mtDNA include NADH dehydrogenase subunit 1 (ND1), ND2, ND3, ND4, ND4L, ND5 and ND6 from complex I (NADH-ubiquinol oxidoreductase) of OXPHOS, Cytochrome B (Cyt-b) from complex III (ubiquinone-cytochrome C oxidoreductase), Cytochrome C oxidase subunit 1 (COX1), COX2, and COX3 from complex IV (Cytochrome C oxidase), and ATP synthase F_0 subunit 6 (atp6) and atp8 from complex V (ATP synthase). The rest of the necessary protein for an intact functional mitochondrion is encoded by the nuclear genome.

1-3-2-2-2- FUNCTION OF MITOCHONDRIA

The mitochondrial structural regions are broadly associated with specific and different functions. The most important of them are ATP production, electron transfer by oxidative phosphorylation (OXPHOS), glycolysis, fatty acid oxidation, Ca^{2+} ion transport, protection against oxidative stress, and involvement in apoptosis processes (*McBride et al. 2006*).

ATP production is the main role of the mitochondrion that is performed by the electron transport chain system, and this electron transport machinery is located at the mitochondrial inner membrane. The machinery operates by electron transport, but the machinery is prone to leakage. A small percentage of electrons that leak through the complex I (*Kushnareva et al. 2002*) and complex III (*Chen et al. 2003*) of electron transport machinery, are trapped by oxygen, which then becomes $O_2^{\bullet -}$ (*Boveris et al. 1973, Takeshige et al. 1979*). As mentioned before, $O_2^{\bullet -}$ is a powerful oxidizing and reducing agent, which leads to generation of various forms of active oxygen species.

In mitochondria, manganese superoxide dismutase (MnSOD) converts the $O_2^{\bullet -}$ produced by the electron transport system to H_2O_2 which is more stable that $O_2^{\bullet -}$ and can diffuse out of mitochondria and into the cytosol, and can be converted to the various types of ROS (*Weisiger et al. 1973, St. Clair et al. 2005*). Mitochondria are normally protected from oxidative damage by a multilayer network of mitochondrial antioxidant systems (*Andreyev et al. 2005*).

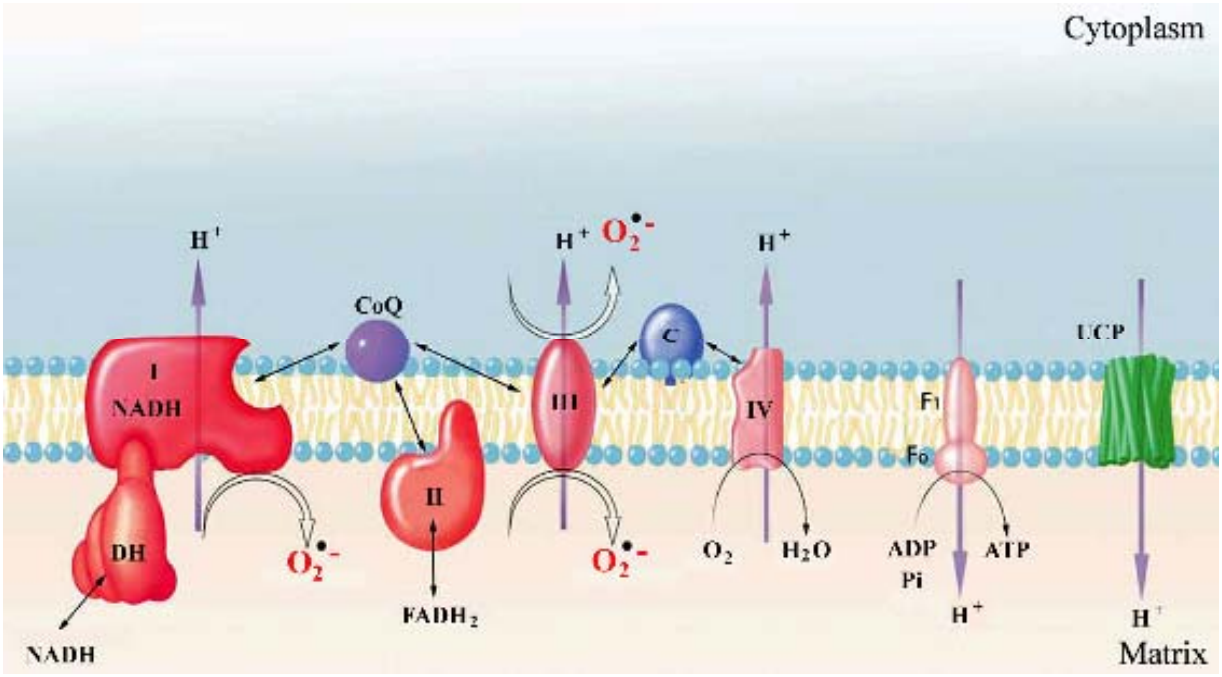


Figure 6: *The sites of ROS production in the mitochondrial electron transport chain.* Two ROS-forming sites in the mitochondrial ETC are known, namely the ubiquinone site in complexIII and a flavin mononucleotide group in complexI (*Balaban et al. 2005*).

H_2O_2 can be readily converted to water by mitochondrial glutathione peroxidase, which oxidizes reduced glutathione (GSH) to oxidized glutathione (GSSG). Glutathione reductase then converts GSSG back to GSH. GSH is synthesized in the cytosol and must be imported into mitochondria by a transporter. In addition to GSH, mitochondria have two other small thiol-disulfide oxidoreductases (i.e. thioredoxin and glutaredoxin) that play important roles in thiol redox control. Another detoxification enzyme, catalase, is present only in heart mitochondria. In addition to these antioxidant enzymes, mitochondria possess several low-molecular-weight antioxidants, including α-tocopherol and ubiquinol. These molecules are particularly effective in scavenging lipid peroxyl radicals and preventing the free radical chain reaction of lipid peroxidation (*Andreyev et al. 2005*).

Mitochondria undergo oxidative damage when ROS production exceeds the antioxidant capacity of mitochondria. Oxidative damage to mitochondria has been shown to impair mitochondrial function and leads to cell death via apoptosis and necrosis. Because dysfunctional mitochondria will produce more ROS, a feed-forward loop is set up whereby ROS-mediated oxidative damage to mitochondria favors more ROS generation, resulting in a vicious cycle.

1-3-2-2-3- BIOGENESIS OF MITOCHONDRIA

Mitochondrial biogenesis is one of the striking responses observed in a variety of physiological conditions and during cell proliferation and differentiation (*Alcolea et al. 2006*). Recapitulation and differentiation of the myogenic program requires energy production for the execution of a number of regulatory and biosynthesis events. Accordingly, mitochondrial biogenesis accompanies the *in vitro* differentiation of myoblasts into myotubes. In addition, muscle cell differentiation appears to depend on mitochondrial function. Indeed, respiration-deficient myoblasts devoid of mitochondrial DNA fail to differentiate.

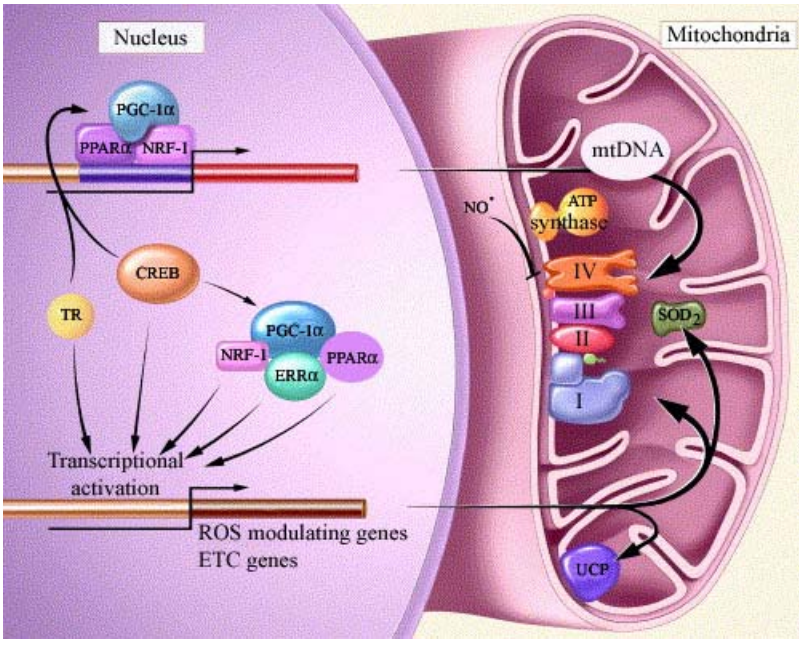


Figure 7: *Schematic illustration of the intergenomic regulation of mitochondrial biogenesis* (*McLeod et al. 2005*).

Similarly, inhibition of mitochondrial protein synthesis with chloramphenicol prevents the differentiation of myoblasts into myotubes (*Rochard et al. 2000, Korohoda et al. 1993*). These studies illustrate the importance of the biosynthesis of functional mitochondria during in vitro myogenesis.

The synthesis of mitochondrial proteins, which is necessary for mitochondrial biogenesis, involves the expression of genes originating from both nuclear and mitochondrial genomes (*Hood et al. 2000*). This dual genomic organization is coordinated by a set of transcription factors, including PPARα, PPARα coactivator-1 (PGC-1), nuclear respiratory factor-1 (NRF-1), and NRF-2, among others. PGC-1 stimulates the expression of NRF-1 and NRF-2, whose regulatory elements are shared by many genes encoding mitochondrial proteins (*Koulmann et al. 2006*).

NRFs are transcription factors that act on nuclear genes and regulate the transcription and replication of mitochondrial DNA. PPARα, PGC-1, and NRF-1 also co-activate the expression of mitochondrial transcription factor a (mtTFA) (*Koulmann et al. 2006*), the only known regulator of mitochondrial replication and transcription. Thus the transcription factors involved in mitochondrial biogenesis are key contributors in the nuclear control of mitochondrial phenotypic alteration and energy production in skeletal muscles.

1-4- AIM AND OBJECTIVES

The present study was performed to assess the cascade that is leading to activation of PPARα during cardiomyogenesis and enhancement of cardiomyogenic differentiation of ES cells, and on the other hand, to investigate downstream signal transduction pathways of PPARα activation. In this project, the ES cells were used as a cell culture model, because differentiating ES cells within embryoid bodies mimic cardiomyogenic differentiation (*Desbaillets et al. 2000, Itskovitz-Eldor et al. 2000, Kehat et al. 2001, Hescheler et al. 1997*). ES cell-derived cardiomyocytes are an excellent *in vitro* model to study developmental aspects of cardiomyogenesis including gene regulatory mechanisms during cardiac cell differentiation. The aim of this thesis was to investigate whether:

i) Cardiomyogenesis is stimulated by PPARα receptor activation
ii) PPARα-mediated cardiomyocyte differentiation involves a ROS-dependent mechanism

In order to perform this, the following objectives were drawn:

- Evaluation of the effect of PPARα receptor activation on cardiomyogenic differentiation

- Analysis of the effect of PPARα activation on cardiac gene- and cardiogenic transcription factor expression during the differentiation of ES cells

- Evaluation of the effect of PPARα on mitochondrial content

- Assessment of the involvement of ROS generation in PPARα-induced cardiomyogenesis

- Analysis of the site of PPARα-induced ROS generation by evaluation the sources of ROS production (NADPH oxidase and mitochondrion)

2- MATERIALS & METHODS

2-1- MATERIALS

2-1-1- SUPPLIERS OF LABWARES AND CONSUMABLES

1. Abcam Limited, Cambridge CB4 0TP, Cambridgeshire, UK
2. Alexis, 35305 Gruenberg, Germany
3. Ambion Europe Ltd., Huntington Cambridgeshire, UK
4. Amersham Biosciences Europe GmbH, 79111 Freiburg, Germany
5. Biochrom, 12247 Berlin, Germany
6. Biorad GmbH, 80901 Munich, Germany
7. Biozym Scientific GmbH, 31833 Olendorf, Germany
8. Buhler, 72379 Hechingen, Germany
9. Calbiochem-Novabiochem GmbH, 65796 Bad Soden, Germany
10. Cell Signaling, 159J Cummings Center Beverly, MA 01915, USA
11. Chemicon International, Hampshire SO53 4NF, UK
12. Dianova, 20354 Hamburg, Germany
13. Dunn Labortechnik, 53567 Asbach, Germany
14. Eppendorf, 22339 Hamburg, Germany
15. Gilson International B.V., 65520 Bad Camberg, Germany
16. Greiner Bio-one GmbH, 72636 Frickenhausen, Germany
17. Heidolph Elektro GmbH, 93309 Kehlheim, Germany
18. Heraeus Instruments, 63452 Hanau, Germany
19. Hirschmann Laborgerate, 74246 Eberstadt, Germany
20. Integra Biosciences, 35463 Fernwald, Germany
21. Invitrogen, 76131 Karlsruhe, Germany
22. Kendro Laboratory Products, 63505 Langenselbold, Germany
23. Labnet International Inc., Windsor, Berkshire, UK
24. Langenbrick, 79312 Emmendingen, Germany

25. Leica, 64625 Bensheim, Germany
26. Menzel-Glass, 38116 Braunschweig, Germany
27. Merck, 64293 Darmstadt, Germany
28. Molecular Probes, Eugene, Oregon, USA
29. New England Biolabs, Beverly, MA 01915-5599, USA
30. Paesel-Lorei Vertriebs & Marketing GmbH, 63452 Hanau, Germany
31. Roche Diagnostics GmbH, 68305 Mannheim, Germany
32. Roth, 76231 Karlsruhe, Germany
33. PAA, 35091 Coelbe, Germany
34. Promega, 68199 Mannheim, Germany
35. Santa Cruz Biotechnology Inc., California, USA
36. Sartorius, 37075 Goettingen, Germany
37. Schuelke-Mayr-GmbH, 22851 Norderstadt, Germany
38. Serva, 69115 Heidelberg, Germany
39. Sigma (-Aldrich), 82024 Taufkirchen, Germany
40. Techne Progene, 97877 Wertheim-Bestenheid, Germany
41. Zeiss, 07745 Jena, Germany

2-1-2- MEDIUMS AND CHEMICALS

Product	***Company***	***Cat. No.***
100 bp DNA ladder	New England Biolabs	N3231L
β-Mercaptoethanol	Sigma	M7522
Apocynin	Sigma	A10809
Bromophenol blue	Sigma	B5525
Chloroform	Merck	2445
Ciprofibrate	Sigma	C0330
Collagenase B	Roche	1088807
Detergent 7x	Serva	34205.01
DMEM high glucose	Sigma	D5671

DMSO	Calbiochem	317275
DNase I	Invitrogen	18068-015
dNTP's	Invitrogen	18427-013
DPI	Sigma	D-2926
DTT	Invitrogen	y00147
Dulbecco's PBS (1x)	PAA	H15-002
ESGRO (LIF)	Chemicon	ESG1106
Ethanol	Sigma	32205
FCS	Sigma	F7524
Glacial acetic acid	Sigma	33206
Glycerol	Sigma	49781
GW1929	Sigma	G5668
GW7647	Sigma	G6793
H_2DCF-DA	Molecular Probes	D399
IMDM	Biochrom	F0465
Immersion oil	Roth	X899.1
Isopropanol	Fluka	33539
KH_2PO_4	Roth	3094.2
L-165,041	Sigma	L2167
L-Glutamine (100x)	PAA	M11-004
Methanol	Merck	6009
$MgCl_2$	Invitrogen	y02010
Mitomycin C	Sigma	M4287
MK886	Sigma	M2692
MMLV RT	Invitrogen	10297-0117
N-(2-mercaptopropionyl)glycine	Sigma	M6635
Na_2EDTA	Roth	8043.2
NaH_2PO_4 x $2H_2O$	Roth	4984.2
Non-Essential Amino Acids (100x)	Biochrom	K0293
Penicillin/Streptomycin	PAA	P11-010

Primers	Invitrogen	See table 8
Random hexamer primer	Invitrogen	48190-011
RNA*later*-ICE	Ambion	7030
RNAsin	Promega	N261B
Rotenone	Sigma	R8875
RT-buffer	Invitrogen	y00146
Phenol solution	Sigma	P4557
Sigmacote	Sigma	SL-2
Sodium acetate	Sigma	32319
Sodium chloride	Roth	3957.1
Sodium hydroxide	Roth	6771.2
Sodium pyruvate	Biochrom	L0473
Strillium	Bode Chemie	975512
Superscript II RTase	Invitrogen	18064-014
SYBR Green Fluorescein Mix	ABgene	AB-1219
SYBR safe	Fluka	86205
Tris	Sigma	T0625
Triton X-100	Sigma	8787
Trizol reagent	Invitrogen	15596-018
Trolox	Sigma	238813
Trypsin/EDTA	Invitrogen	25300-062
Water, DEPC treated, sterile	Applichem	A2864.0500
WY14643	Sigma	C7081
Xylene cyanol	Sigma	X4126

Table 1: *List of media and chemicals used.*

2-1-3- EQUIPMENT

	Device	***Company***
Electrophoresis	Power supply	Biotech

	Gel electrophoresis chamber	Biotech
Microscopy	Microscope TMS-F	Zeiss
	Leica LCSM TCS-SP2	Leica
PCR cycler	iCycler	Biorad
Centrifugation	Temperature Centrifuge (Labofuge 300)	Heraeus
	Centrifuge (Biofuge 15R)	Heraeus
	Table top centrifuge 5417C	Eppendorf
Cell culture	Incubator (HERAcell 240)	Kendro
	Laminar air hood (sterile bank)	Heraeus
	Cell spin system	Integra
	Spinner flask (CELLspin 240)	Integra
	Waterbath	Hirshmann
	Culture dishes	Greiner
	Petri perm plates	Greiner
	Cell scraper	Becton Dickinson
	Glass pipettes	Roth
	Plastic pipettes	Becton Dickinson
Other devices	System of distilled water production	Millipore
	Vortex	Heidolph
	pH-meter digital	Sartorius
	Spectrophotometer (Nano drop ND-1000)	Peqlab
	Pipettors	Hirshmann/Eppendorf
	Pipette tips	Eppendorf
	Reaction tube (15 & 50 ml)	Greiner
	Reaction tubes	Eppendorf
	Glass ware	Roth
	Gloves	Noba

Table 2: *List of devices used in this study.*

2-1-4- MEDIA, BUFFERS AND SOLUTIONS

10x PBS	2 g KCL 2 g KH_2PO_4 80 g NaCl 12.5 g Na_2HPO_4 Dissolved in 1 l ddH_2O, set pH to 7.4 with HCl

1x PBS	100 ml 10x PBS + 900 ml ddH_2O
1% PBST	100 ml 1x PBS + 1 ml Triton X-100
0.01% PBST	100 ml 1x PBS + 100 µl Triton X-100
Blocking solution	5 ml FCS + 45 ml 0.01% PBST
IK buffer	0.4 g KCl 7 g NaCl 1.23 g MgSO4 3.96 g Glucose 2.5 g Taurin 2.38 g HEPES 50 ml Na-Pyruvate Dissolved in 1 l ddH_2O, set pH to 6.9 with NaOH
EMFI medium	500 ml DMEM medium 2.5 ml Penicillin/Streptomycin 6 ml L-Glutamine 6 ml NE-Aminoacids 60 ml Heat-inactivated FCS
LIF medium (1000 U/ml)	50 ml cultivation medium + 50 µl LIF solution
Cultivation medium	500 ml IMDM medium 2.5 ml Penicillin/Streptomycin 6.25ml β-mercaptoethanol 6.25ml L-Glutamine 6.25ml NE-Aminoacids 6.25ml Penicillin/Streptomycin 6.25ml Na-Pyruvate 50 ml FCS 47.5ml Heat-inactivated FCS

Table 3: *Composition of cell culture media and buffers.*

2-2- METHODS

2-2-1- CELL CULTURE

2-2-1-1- EMBRYONIC STEM CELL CULTURE

In the early 1980s, pluripotent cells called ES cells were isolated from the ICM of the developing murine blastocyst (*Evans et al. 1981, Martin GR 1981*). ES cells are able to proliferate in vitro and generate different type of cells. To maintain them in the undifferentiated and pluripotent state, they were grown in culture medium supplemented with LIF and on feeder cell layers of mitotically inactivated murine embryonic fibroblasts. When grown in the absence of feeder cells and LIF, ES cells are able to differentiate spontaneously. Because of their ability to form spheroid aggregates mimicking post-implantation embryonic tissues, they were termed embryoid bodies.

During the study, the CCE cell line was used as embryonic stem cell model. CCE is a mouse ES cell line derived from the 129/Sv mouse strain which is a result of separate investigations of Dr. Elizabeth Robertson and Dr. Gordon Keller (*Robertson et al. 1986, Keller G, et al. 1993*).

The frozen stem cells were carefully taken from the liquid nitrogen tank (-196^{o}C) and immediately thawed at 37^{o}C in a water-bath. The thawed cells were then centrifuged at 450 *g* for 5 min and the resulted pellet was re-suspended in 2 ml normal culture medium. Iscoves modified Dulbecco medium (IMDM) has been used in the present study as normal culture medium. 500 ml of this medium was supplemented by sterile additives: 2.5 ml Penicillin/Streptomycin (100x), 6.25 ml β-Mercaptoethanol (10 μM in PBS), 6.25 ml L-glutamine (100x, 2mM), 6.25 ml non-essential amino acids (100x), 6.25 ml sodium pyruvat (100x, 1mM), and 15% (v/v) fetal calf serum containing 50 ml normal FCS and 47.5 ml heat-inactivated FCS.

The prepared CCE cells were cultivated in an undifferentiated state by culturing on relatively high density mitotically inactivated feeder layer cells in normal culture medium supplemented with 1% LIF (1000U/ml) which is necessary to cultivate cells in undifferentiating state and incubated in a 95% humidified 5% CO_2 atmosphere at 37^{o}C.

LIF belongs to the family of interleukin 6 (IL-6) type cytokines. LIF engages a heterodimeric receptor complex consisting of gp130 and LIF receptor α-chain. This complex

leads to activation of the Janus family of tyrosine kinases (JAKs). STAT proteins signal transducers and transcription activators, are key substrates for JAKs. STAT3 (*Ernst et al. 1999, Niwa et al. 1998*) together with Src-family kinases (*Anneren et al.2004*) and myc are essential and sufficient to maintain ES cell pluripotency and self-renewal properties. Wnt3A is also important in the maintenance of ES cell pluripotency (*Singla et al. 2006*). It was found that its presence in the media can maintain the pluripotent nature of ES cells, but it appears that this action occurs synergistically with LIF (*Ogawa et al. 2006*).

Under the above conditions, CCE cells spontaneously proliferate and form clumps on the feeder layer. After 2 days of cultivation, when they attained ≥ 70% confluency, the CCE cells were trypsinized and passaged onto new inactivated feeder layer plates and/or transferred into siliconised spinner flasks to commence differentiation.

All culture processes were performed under a sterile laminar flow hood class-II in order to prevent any type of contamination.

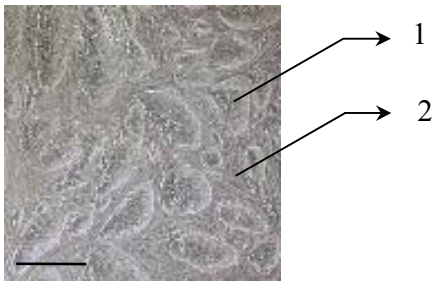


Figure 8: *Undifferentiated ES cells.* The image shows confluent mouse ES cells on feeder layer cells in cell culture. 1: Undifferentiated ES cells, 2: mouse fibroblast or feeder layer cells. The scale bar represents 100 μM.

2-2-1-2- CULTIVATION OF FEEDER LAYER CELLS

For maintaining the growth potential and pluripotency of ES cells, mouse ES cells can be grown on a feeder layer, usually consisting of mouse embryonic fibroblast cells. The feeder cells support ES cells by offering a sticky surface for easy attachment and -most importantly- secreting LIF into the culture medium. Some research groups including us add extra purified LIF into the culture medium.

In the present investigation, the feeder layer were prepared as previously described from 12-day-old post coitus of mouse pregnant females, strain C57BL/6J, under defined instruction by Dr. David Conner (*Conner 2000*) and stored in a liquid nitrogen tank.

To prepare feeder layer plates, the frozen feeder cells were quickly thawed in a water-bath at 37°C. The feeder cells were then seeded at a density of $9x10^4$ cells per plate into sterile 60 mm tissue culture dishes and fed with normal culture medium. When the plated feeder cells were confluent, their proliferation was stopped by treating with Mitomycin C (10 μg/ml).

Mitomycin C is a potent DNA cross-linker that can block DNA replication by crosslink formation and leads to cell death. In cell culture, mitomycin C mitotically arrests cells by suppressing the synthesis of DNA in the late G1 and early S phase of the cell cycle.

After 2-3 days, when the feeder cells were about ≥ 70 % confluent, the culture medium was aspirated and cells were washed once with pre-warmed sterile 1x PBS. For the inactivation procedure, the feeder cells were incubated with 10 μg/ml mitomycin C for 2.5h at 37°C and 5% CO_2. To remove mitomycin C after inactivation, the feeder layer plates were extensively washed three times with excess pre-warmed sterile EMFI medium. These mitotically inactivated feeder layer plates were stored at 37°C in a CO_2 incubator for not more than one week, and medium exchange was performed every second day using pre-warmed sterile EMFI medium.

EMFI medium is prepared using 500 ml DMEM (Dulbecco's modified Eagle's minimal essential medium) medium was supplemented by following sterile additives: 2.5 ml Penicillin/Streptomycin (100x), 6 ml L-glutamine (100x), 6 ml non-essential amino acids (100x), and 60 ml heat-inactivated FCS.

2-2-1-3- CELL TRYPSINATION

Passaging of adherent cells was performed using trypsin/EDTA (normal culture medium containing 0.2% trypsin + 0.05% EDTA in PBS) solution at 37°C. Before trypsination, the cells were carefully washed twice with 1 ml pre-warmed trypsin/EDTA solution to remove all LIF medium from the culture dish. After 2 min treatment with 2 ml trypsin solution, trypsination was quickly stopped by adding 10 ml normal culture medium. If possible, cell aggregates should be minimized by a repeated passage of the cell suspension through pipetting up and down 5-6 times with a 1ml filter tip. The cells were finally seeded onto a freshly prepared inactivated feeder layer plate containing LIF medium to reach approximately ≥ 70 % confluence.

2-2-1-4- SILICON COATING OF SPINNER FLASKS

A clean and dry spinner flask was rinsed with silicon solution and baked for 1 h at 60^{o}C. The siliconised spinner flask was then washed with deionized water and finally autoclaved for sterilization. The siliconising spinner flask step is very important and necessary to prevent the cells from adhering to the spinner flask walls.

2-2-1-5- THREE-DIMENSIONAL CULTIVATION OF EMBRYOID BODIES

Upon withdrawal of feeders and LIF, ES cells differentiate. Usually the methodology for ES cell differentiation involves the formation of spheroidal structures termed embryoid bodies. There are several techniques available for the generation of embryoid bodies. The most common is embryoid body formation in suspension (*Doetschman et al 1988*), by cultivation in hanging drops (*Wobus et al 1997*), by culture in methyl-cellulose (*Wiles et al 1991*), or by cultivation in siliconised spinner flasks (*Wartenberg et al. 1998*) of which the last method -spinner flask cultivation- was used during the present study.

For ES cell differentiation, feeder layer cells and LIF were omitted. The confluent ES cells on the feeder layer were washed twice with pre-warmed trypsin/EDTA solution and were then incubated for 2 min with 2 ml trypsin/EDTA solution in the incubator for cell dissociation. These dissociated ES cells were seeded at a density of $1x10^{7}$ cells/ml in siliconised spinner flasks with 125 ml pre-warmed normal culture medium. The spinner flask was placed in the 37^{o}C incubator with 5% CO_2 on a magnetic stirrer with 20 rpm/min; the rotating direction is changed after every four rotations. 24 h after seeding ES cells, the spinner flasks were filled with pre-warmed normal culture medium up to 250 ml. Daily 150 ml of the normal culture medium from the spinner flask was exchanged with fresh one. Under this condition, the ES cells aggregate to form spherical embryoid bodies.

It is noticeable that the day of ES cell seeding into siliconised spinner flasks after trypsination was counted as day 0 throughout the study.

2-2-2- TREATMENT PROCEDURES

All PPARs drugs were dissolved in DMSO, and depending on company's instruction were stored at RT, 4°C, or -20°C. Final concentration for all these drugs used was 10 μM (Table 4).

Product name	*Function*	*Company*	*Cat. No.*	*Final concentration*
WY14643	PPARα agonist	Sigma	C7081	10 μM
GW7647	PPARα agonist	Sigma	G6793	10 μM
Ciprofibrate	PPARα agonist	Sigma	C0330	10 μM
MK886	PPARα antagonist	Sigma	M2692	10 μM
L-165,041	PPARβ agonist	Sigma	L2167	10 μM
GW1929	PPARγ agonist	Sigma	G5668	10 μM

Table 4: *Drugs used with their final concentrations.*

In treatment schedule with PPARs drugs, 4-day-old embryoid bodies were transferred from spinner flasks to suitable kind of culture dishes (depends on the respective experiment) and treated in normal culture medium supplemented with drugs every day, and kept in incubator at 37°C, 5% CO_2, with 95% relative humidity.

For treatments with substances such as free radical scavenger reagents, NADPH oxidase inhibitors, the mitochondrial complex I of respiratory chain inhibitor rotenone. The final concentration used is mentioned in Table 5. The samples were pre-incubated with the substances for 2 h and then defined amounts of PPARs drugs were added.

Product name	*Function*	*Company*	*Cat. No.*	*Final concentration*
Trolox	Free radical scavenger	Sigma	238813	100 μM
NMPG	Free radical scavenger	Sigma	M6635	100 μM

DPI	NADPH oxidase inhibitor	Sigma	D2926	100 nM
Apocynin	NADPH oxidase inhibitor	Sigma	A10809	10 μM
Rotenone	Inhibitor of mitochondrial complex1	Sigma	R8875	2 μM

Table 5: *List of substances with their final concentrations.*

2-2-3- ASSESSING THE NUMBER OF CONTRACTING FOCI

In this experiment, 4-day-old embryoid bodies were removed from spinner flasks, plated into Petri perm dishes, and kept under defined conditions as above in the incubator. The cell culture medium supplemented with 10 μM of PPARs drugs was exchanged every day.

Between day 7 and 8, the plated embryoid bodies usually start to display spontaneous contracting cell clusters, which indicate cardiomyocyte differentiation. Therefore, the cultivated embryoid bodies on Petri perm dishes were daily monitored under light microscope from day 6 till day 10, and number of beating cardiomyocyte area per plate recorded everyday. It should be noted that the number of adherent embryoid bodies per plate was counted and recorded at day 5. This was necessary for calculating areas of contracting foci of cardiomyocytes per plate after treatment with PPAR drugs in comparison to untreated group.

2-2-4- ISOLATION OF ES CELL-DERIVED CARDIOMYOCYTES

The cell culture medium of 4-day-old embryoid bodies cultivated on Petri perm plates was exchanged with normal culture medium every day till day 8 when they start to differentiate to cardiomyocytes which can be visualized by assessing spontaneous contracting areas under a light microscope. Beating areas of 8-day-old embryoid bodies containing differentiated cardiomyocytes were carefully cut out/isolated using sterile needles and a micro-scalpel under a light microscope, and collected in a fresh Eppendorf tube with 200 μl pre-warmed normal culture medium. For obtaining single cell cardiomyocytes, the dissociation procedure was used where the isolated beating areas were washed once by pre-warmed IK buffer and incubated with 1mg/ml

collagenase B in IK buffer for 15 min on the shaker in the CO_2 incubator. The supernatant was then transferred in a new 15 ml reaction tube containing 6 ml pre-warmed normal culture medium for stopping digestion. This dissociation step was repeated 4-5 times, the supernatant was collected and the reaction stopped by adding pre-warmed normal culture medium. Finally, the dissociated cells were centrifuged at 450 *g* for 5 min at RT. After centrifugation, the supernatant was discarded very carefully, the pellet was slowly resuspended using pre-warmed normal culture medium, and divided to prepared sterile coverslips on a 24-well plate. These prepared coverslips were kept in the incubator under defined conditions, and their culture medium was daily exchanged with fresh one.

2-2-5- IMAGING ASSAYS

2-2-5-1- IMMUNOHISTOCHEMISTRY

Immunohistochemistry is the localization of antigens or proteins in biological samples by the use of labeled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, or colloidal gold.

Dr. Albert H. Coons et al. were the first group to label antibodies with a fluorescent dye, and use it to identify antigens in tissue sections (*Coons 1954*). To date, different kinds of labels such as peroxidase, alkaline phosphatase, colloidal gold, etc. exist and numerous immunohistochemistry methods depending on different parameters of samples of investigation have been introduced.

Since immunohistochemistry involves a specific antigen-antibody reaction, it has apparent advantage over traditionally used special enzyme staining techniques that identify only a limited number of proteins, enzymes and tissue structures.

In this study, whole mount embryoid bodies, differentiated cultivated embryoid bodies on Petri perm dishes or cultivated isolated cardiomyocytes on coverslips were fixed either for 20 min at -20^{o}C with ice-cold 100% methanol or by incubation for 1 h on ice with ice-cold 4% paraformaldehyde (PFA). In the next step, the fixed samples were washed 3 – 4 times with 0.01% PBST to remove the excess fixative and were permeabilized by incubating for 10 min at 1% PBST when fixed by 4% PFA. The samples were then washed twice with 0.01% PBST and incubated with blocking buffer 45 – 60 min at RT to reduce unspecific binding of the antibodies. The blocking buffer which contained 10% FCS in 0.01% PBST was used for diluting primary

and secondary antibodies. After the blocking step, samples were washed three times with 0.01% PBST and then incubated with diluted primary antibody in blocking buffer for 1 h. In the following, they were washed 3 – 4 times with 0.01% PBST to remove the unbound antibodies and then incubated for 1 h with diluted secondary antibody in the dark. Finally, the stained samples were washed 3 – 4 times with 0.01% PBST and stored in the same buffer at $4^{o}C$ till their immunofluorescence was analyzed using the Leica confocal laser scanning microscope. It should be noted that all above explained steps except for fixation were performed at room temperature.

The samples on coverslip or Petri perm plate were mounted on a glass slide with one drop of fluoromount-G. The stained samples were analyzed by a laser confocal scanning microscope (model Leica TCS SP2). All primary and secondary antibodies used in this study with the corresponding dilution used are listed in following.

Antibody	Produced in	Dilution
α-actinin	Mouse	1:100
Phosphor-PPARα	Rabbit	1:100
Cy5 anti-mouse	Sheep	1:100
Cy5 anti-rabbit	Goat	1:100
FITC anti-mouse	Goat	1:100
FITC anti-rabbit	Sheep	1:100

Table 6: *Dilutions of primary and secondary antibodies.*

2-2-5-2- LASER CONFOCAL SCANNING MICROSCOPY

Confocal scanning microscopy, invented by Dr. Minsky (*Minsky 1988*), is a widely used technique in many fields of science particularly in life and biosciences (*Amos et al. 1987, Amos et al. 2003*). Unlike conventional microscopy, CSM illuminates and images only one small spot at a time in the focal plane of the objective. The sequences of points of light from the specimen are detected through a pinhole and the output from the detector is built into an image and displayed by a computer. Because a confocal image is built up pixel by pixel by scanning the illuminated spot over the specimen, it is intrinsically time-consuming if a large area needs to be investigated.

Lasers are the most common light sources for confocal scanning microscopes as they provide ideal types of excitation light for fluorescence microscopy applications. In laser confocal scanning microscope (LCSM) a laser beam is focused by an objective onto a fluorescent specimen through an X-Y deflection mechanism. The mixture of reflected light and emitted fluorescent light is captured by the same objective and (after conversion into a static beam by the X-Y scanner device) is focused onto a photomultiplier via a dichroic beam splitter.

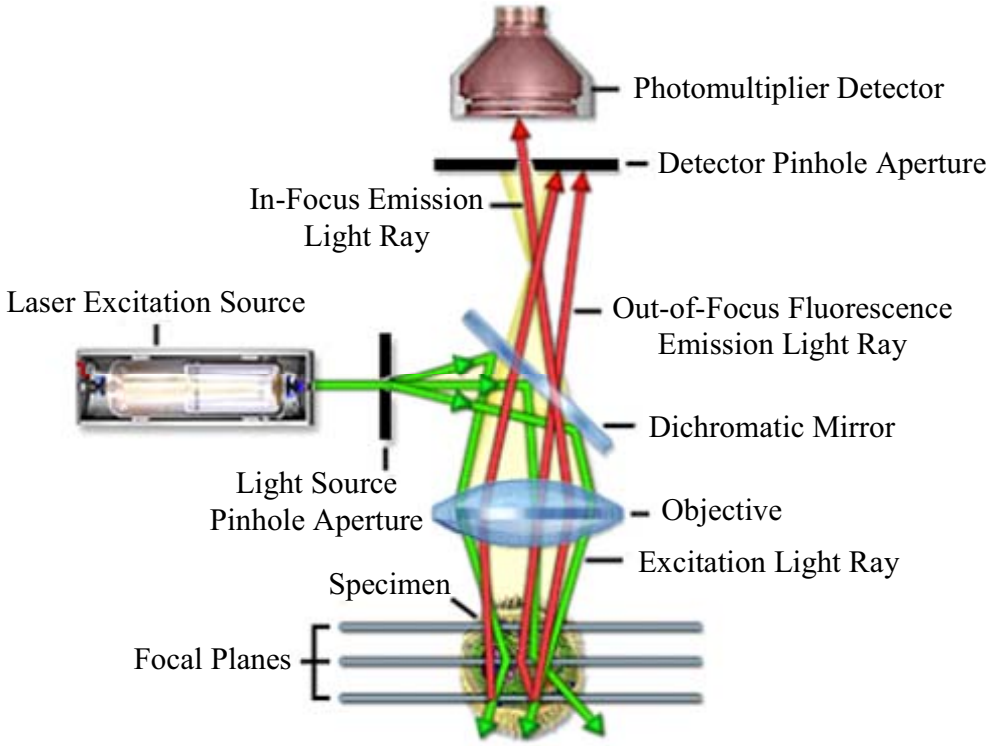


Figure 9: *A simplified schematic illustration of the beam path in the LCSM.* The prism splits the beam into its spectral components. The enlarged details show the detector system with the motorized slit plates, which can be opened and closed (*http://www.olympusfluoview.com*).

Commonly, a raster scan is generated by reflection from two moving mirrors, aligned at 90^{o} with the motion of each mirror being driven by a linear saw-tooth control signal. This

scanning unit is elaborately designed so that the laser location is changing linearly without image distortion.

Current LCSMs collect images with a scan speed of up to 5 frames/s with 512×512 pixels, such as the Leica TCS SP2 in which the scanner consists of two independent galvanometric scanning mirrors. By varying the distance between the objective and the specimen, users can generate a Z-series that dissect through the specimen with a Z-scan interval down to 50 –100 nm. Object features in the order of 0.2 μm can be resolved, and height differences of less than 0.1 μm are made visible.

Three types of confocal microscopes are commercially available: Laser confocal scanning microscopes, spinning-disk (Nipkow disk) confocal microscopes and programmable array microscopes (PAM). During this study, the confocal laser scanning model was used. In the Leica TCS SP2 which was used in this study, three different lasers i.e. argon laser for 458/488 nm wavelength, helium/neon laser for 543 nm wavelength, and helium/neon laser for 633 nm wavelength exist.

Flurochrome	***Laser***	***Spectral data (nm)***	
		Absorption	***Emission***
Cy5	red helium-neon	649	666
FITC	argon	490	520
H_2DCF-DA	argon	505	535

Table 7: *Spectral information of fluorochromes.*

2-2-5-3- MEASURING THE SIZE OF CONTRACTING CARDIOMYOCYTE AREAS

4-day-old embryoid bodies were plated onto Petri perm dishes and medium exchange with normal culture medium supplemented with mentioned concentrations of PPARs drugs was performed every day till day 10. In some experiments in which other substances plus PPARs agonists/antagonist were used (see section 2-2-2), a 2 h pre-incubation with those substances was performed, and then defined amount of PPAR drugs were added.

The embryoid bodies cultivated and treated on Petri perm dishes were fixed at day 10 using 20 min incubation with ice-cold 100% methanol at -20^{o}C. These fixed plates were then stained with an antibody against α-actinin which is a cardiomyocyte-specific marker and assessed by laser confocal scanning microscopy. The recorded images of α-actinin immunofluorescence by microscope were analyzed using the "area measure" option of the Leica image analysis software. The size of α-actinin-positive cell areas was measured after correction for background fluorescence.

2-2-5-4- ROS GENERATION ASSAY

A number of intracellular probes have been used to study ROS activities in different cell types, but 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) has been most widely used as a relatively nonspecific intracellular probe for ROS (*Zuo et al. 2002*).

H_2DCF-DA is a nonpolar compound that is converted into a non-fluorescent polar derivative (H_2DCF) by cellular esterases after incorporation into cells. H_2DCF is membrane impermeable and rapidly oxidizes to the highly fluorescent 2', 7'-dichlorofluorescein (DCF) in the presence of intracellular ROS.

For the experiments, embryoid bodies treated with substances and drugs as indicated were incubated in serum-free medium and 50 μM H_2DCF-DA dissolved in DMSO was then added. After 30 min, they were washed once with pre-warmed serum-free medium and intracellular DCF fluorescence was evaluated by LCSM. For fluorescence excitation, the 488 nm band of the argon ion laser of the confocal setup was used and its emission was recorded at 535/540 nm. The intensity of recorded fluorescence was analyzed by assessing grey level values using the Leica software which indicated levels of ROS production.

2-2-6- EXPERIMENTS WITH RNA

2-2-6-1- RNA ISOLATION

Total RNA was purified using *TRIzol* Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, embryoid bodies cultured on bacteriological or tissue culture plates were gently mechanically detached with the sterile cell scraper and collected into

1.5 ml Eppendorf tubes. After washing once with 1x PBS, 300 µl RNA*later* was added to each tube and kept at -20^{o}C till extraction.

For performing RNA isolation, the samples stored at -20^{o}C were subjected to 30 s centrifugation at 300 *g* at RT. RNA*later* was removed from the samples and 1 ml *TRIzol* reagent added. Following 5 min incubation at RT, samples were homogenized by pipetting at least 10 times and vortexing. 200 µl chloroform was then added to the homogenate and mixed well by vortexing and incubated at RT till phase separation became visible. After 20 min centrifugation at 3600 *g* at 4^{o}C in a pre-cooled centrifuge, the aqueous upper colorless phase was transferred into a new Eppendorf tube and 200 µl chloroform added to each one. Following a 20 min centrifugation at 3600 *g* at 4^{o}C, the upper phase was transferred to a fresh Eppendorf tube. Then 500 µl ice-cold isopropanol was added and incubated for 1 h at -20^{o}C. The RNA was precipitated. The precipitated RNA was pelleted by centrifugation with 3600 *g* at 4^{o}C for 20 min. In continuation, the supernatants were carefully discarded, and the pellets were washed once with 1 ml ice-cold 70% ethanol (EtOH) by centrifugation at 2400 *g* for 5 min at 4^{o}C. Finally, the resultant pellets were air dried for about 10 min in an RNase-free place at RT, and re-suspended in 33 µl nuclease-free water (DEPC-water), and kept at -20^{o}C.

2-2-6-2- DNase DIGESTION

Prior to the implementation of a cDNA synthesis, a DNase treatment was performed for removing DNA contamination if any from the RNA samples.

For DNase digestion, the RNA solution was mixed with 10 unit of RNase-free DNase I in the presence of 50 mM Tris pH 7.5, 1 mM $MgCl_2$, 5 mM DTT, and 1 U/µl RNAsin and incubated at 37^{o}C for 1 h. Then, the volume was made up to 200 µl with nuclease-free water, and the RNA was purified by phenol-chloroform procedure.

For the RNA purification process, 200 µl phenol-chloroform in the ratio of 1:1 was added to 200 µl of the resultant RNA solution after the DNase-treatment step. This suspension was centrifuged at 3900 *g* for 10 min in a pre-cooled centrifuge at 4^{o}C and the upper phase transferred into a fresh tube. Following this, 200 µl chloroform was added to each tube and centrifuged under the same conditions as above. Again, the upper phase was separated and transferred to a new Eppendorf tube, and the RNA was precipitated from this aqueous phase by adding 20 µl of 3M

sodium acetate and 500 µl absolute ethanol. After 20 min centrifugation at 3900 *g* at 4°C, the resultant pellets were washed once with ice-cold 70% ethanol and centrifuged at 3900 *g* for 15 min at 4°C. Finally, the RNA pellet was air dried for about 10 min at RT and re-dissolved in 20-30 µl of nuclease-free water, and the RNA concentration was measured with a spectrophotometer.

2-2-6-3- ESTIMATION OF RNA PURITY AND QUANTITATION

Quantitation of RNA was performed on a Beckmann spectrophotometer. Both RNA and DNA absorb light of wavelength 260 nm. However, proteins (aromatic side chains) have an absorption maximum at 280 nm. Therefore, the ratio A260/280 gives an estimation of the degree of contamination of RNA samples with protein. For pure RNA, the ratio A260/280 is between 1.9 and 2.1. A ratio lesser than this range means that the prepared RNA is contaminated with proteins and aromatic substances (e.g. phenol). In this case, it is recommended to purify the RNA again before using it in any subsequent application.

For RNA quantitation, an approximate A260 value of 1.0 was used corresponding to approximately 40 µg/ml single-stranded RNA. RNA concentration was calculated using the following formula:

RNA concentration (µg/ml) = A260 x 40 µg/ml x dilution factor

2-2-6-4- cDNA SYNTHESIS

As RNA cannot serve as a template for PCR, the first step after RNA isolation in a RT-PCR assay is the reverse transcription of the RNA template into cDNA, which is then followed by its exponential amplification in a PCR reaction.

For cDNA synthesis, a specific enzyme i.e. RNA-dependent DNA polymerase was used, which is also called reverse transcriptase. There are two commonly used reverse transcriptases. They are Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT) and Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT). MMLV-RT was used in this study. Each RNA sample was reverse transcribed by oligo-dT primers to synthesize the cDNA.

For preparing cDNA synthesis reactions 2 µg of DNase-treated RNA in a total volume of 10 µl DEPC water was taken and incubated at 60^{o}C for 15 min for denaturating the RNA. After this incubation, the denatured RNA samples were quickly chilled on ice for 1 min and centrifuged for 10 s at 3600 *g*. Following this, a mix containing 4 µl of 5x reverse transcription buffer (RT-buffer), 2 µl oligo-dT primer, 1 µl of 0.1 M DTT, 0.4 µl RNAsin, 0.6 µl of 200 U/µl MMLV-RT enzyme, and 2 µl of 40 mM dNTP mix were added into each tube of 10 µl denatured RNA. Then, these mixes were incubated at 37^{o}C for 1 h, and then they were immediately incubated for 5 min at 95^{o}C to inactivate the MMLV-RT enzyme in the reaction. Finally, the samples were centrifuged very shortly at 3600 *g* and kept on ice for cooling down, and then stored at -20^{o}C for future experiments.

2-2-6-5- REAL-TIME REVERSE TRANSCRIPTASE-PCR ASSAY

2-2-6-5-1- PRINCIPLE OF THE TECHNIQUE

The reverse transcription polymerase chain reaction (RT-PCR) is an *in vitro* method for enzymatically amplifying defined sequences of RNA (*Rappolee et al 1989*), and permits the analysis of different samples from as little as one cell in the same experiment. It is the most sensitive and the most flexible of the quantification methods (*Wang et al. 1999*), and can be used to compare the levels of mRNAs in different sample populations to characterize patterns of mRNA expression.

Real-time reverse-transcriptase (RT) PCR quantifies the initial amount of the template most specifically, sensitively and reproducibly, and is a preferable alternative to other forms of quantitative RT-PCR that detect the amount of final amplified product at the end-point (*Freeman et al 1999, Raeymaekers 2000, Nolan et al. 2006*). The real-time PCR system is based on the detection and quantitation of a fluorescent reporter (Lee *et al 1993, Livak et al. 1995*). This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

At the end of each reaction, the recorded fluorescence intensity is used for the following calculations by the software of the system. There are different approaches for quantitation the amount of template (*Livak et al 2001*):

1. **Absolute standard curve method**: This method of data analysis is similar to Northern blot protocols as it quantifies the fold changes in the target and reference genes. This method needs a good dilution curve required for both standard and reference genes on every plate. If a problem exists with either dilution curve, the data either cannot be analyzed or a suboptimal curve must be used. The formula which was applied in this method is as follows:

$$\text{ratio} = \frac{(E_{ref.})^{\text{CT Sample}}}{(E_{target})^{\text{CT Sample}}} \div \frac{(E_{ref.})^{\text{CT Calibrator}}}{(E_{target})^{\text{CT Calibrator}}}$$

(E = experiment, ref. = reference, CT = crossing point)

2. **Relative standard method**: In this method, one of the experimental samples is the calibrator, or 1x sample. Each of the normalized target values is divided by the calibrator normalized target value to generate the relative expression levels. Target quantity is determined from the standard curve and divided by the target quantity of the calibrator.

3. **Pfaffl method:** This method is a new mathematical model for relative quantification in real-time RT-PCR. In this method, a calculation was made without doing an internal dilution curve each time as is needed in the absolute standard curve method. There is an universal formula for this calculation:

$$\text{ratio} = \frac{(E_{target})^{\Delta CT \text{ target (control – treated)}}}{(E_{ref.})^{\Delta CT \text{ ref. (control – treated)}}}$$

4. **The comparative C_T method ($\Delta\Delta C_T$):** Unlike either the standard curve or Pfaffl methods, the Delta-Delta CT method is an approximation of transcription based on the change in threshold values for control vs. target cells (the cycle number at which fluorescent signaling crosses the "threshold" of logarithmic increases in cDNA concentration).

   $\Delta\Delta C_T$ method enables relative quantitation of template and increases sample throughput by eliminating the need for standard curves when looking at expression levels relative to an active reference control (endogenous control). Running the target and endogenous control amplifications in separate tubes and using the standard curve method requires the least amount of optimization and validation. The universal formula of this method is as follows:

$$\text{ratio} = 2^{-[\Delta CT\ \text{Sample} - \Delta CT\ \text{Control}]} \longrightarrow \text{ratio} = 2^{-\Delta\Delta CT}$$

   ΔCT Sample = The Ct value for any sample normalized to the endogenous housekeeping gene

   ΔCT Control = The Ct value for the calibrator/control normalized to the endogenous housekeeping gene

   * This method ($\Delta\Delta C_T$ method) was used for analyzing data in the present study.

2-2-6-5-2- EXPERIMENT DESIGN

In the present study, the iCycler optical module (Biorad) was used for PCR amplification, and the data analyzed by the Biorad iCycler iQ software.

Singleplex reactions were used in this project in triplicates. Singleplex is when the expression of only one gene is analyzed in each tube. PCR reaction was prepared in 20 µl volume per tube which contained 10 µl of SYBR Green PCR master mix, 3 µl cDNA which was diluted to 1:10 ratio from stock cDNA prepared in cDNA synthesis step, and 10 *p*M of each forward and reverse primers. The primer sets which were used during this study are listed in the following table.

Gene	*Strand*	*Sequence*
ANP	forward	5'-CGTGCCCCGACCCACGCCAGCATGGGCTCC-3'
	reverse	5'-GGCTCCGAGGGCCAGCGAGCAGAGCCCTCA-3'
Cardiac α-actin	forward	5'-CTCGATTCTGGCGATGGTGTA-3'
	reverse	5'-CGGACAATTTCACGTTCAGCA-3'
DTEF1	forward	5'-CCCGAACGCTTTCTTCCTTGTC-3'
	reverse	5'-ACCTTGGTGGAGACGCTGATG-3'
GAPDH	forward	5'-TCCATGCCATGACTGCCACTC-3'
	reverse	5'-TGACCTTGCCCACAGCCTTG-3'
GATA4	forward	5'-TCAAACCAGAAAACGGAAGC-3'
	reverse	5'-GTGGCATTGCTGGAGTTACC-3'
MEF2c	forward	5'-AGCCGGACAAACTCAGACAT-3'
	reverse	5'-TATTCCTCTGCAGAGACGGG-3'
MHCα	forward	5'-TGAAAACGGAAAGACGGTGA-3'
	reverse	5'-TCCTTGAGGTTGTACAGCACA-3'
MHCβ	forward	5'-CTACAGGCCTGGGCTTACCT-3'
	reverse	5'-TCTCCTTCTCAGACTTCCGC-3'
MLC2a	forward	5'-TCAGCTGCATTGACCAGAAC-3'
	reverse	5'-AAGACGGTGAAGTTGATGGG-3'
MLC2v	forward	5'-AAAGAGGCTCCAGGTCCAAT-3'
	reverse	5'-CCTCTCTGCTTGTGTGGTCA-3'
ND1	forward	5'- GTCAAATAAGTCACTGGTCC-3'
	reverse	5'- TGATGGAAGAGTGGGTATGA-3'
Nkx2.5	forward	5'-CCACTCTCTGCTACCCACCT-3'
	reverse	5'-CCAGGTTCAGGATGTCTTTGA-3'
PPARα	forward	5'-GTGGCTGCTATAATTTGCTGTG-3'
	reverse	5'-GAAGGTGTCATCTGGATGGGT-3'
PPARβ	forward	5'-TTGAGCCCAAGTTCGAGTTTG-3'
	reverse	5'-CGGTCTCCACACAGAATGATG-3'
PPARγ	forward	5'-AATCAGCTCTGTGGACCTCTC-3'
	reverse	5'-CTCCAAGAATACCAAAGTGCGA-3'

Table 8: *Synthetic oligonucleotides used.* The nucleotide sequence starts at the 5'-end.

Amplifications were performed at 95°C for 15 min, 45 cycles of 95°C for 30 s, and the specific annealing temperatures for 30 s, then 72°C for 30 s, and a final extension at 50°C for 10 min. Increase in fluorescence of SYBR Green was automatically measured after each extension

step. SYBR Green which has been used to prepare the PCR reaction mix is a fluorescent dye that binds to the minor groove of double-stranded DNA.

The PCR amplification products for first experiments were resolved by electrophoresis on 2% agarose gels to verify that the primer pairs amplified a single product of the predicted size. All PCR products were checked by melting curve analysis to exclude the possibility of multiple products or primer dimers.

2-2-6-5-3- ENDOGENOUS CONTROL

As an endogenous control a housekeeping gene must be used to correct for loading errors and deviations in concentration measurements. The ideal endogenous control should be expressed at a constant level among different tissues of an organism at all stages of development, and should be unaffected by the experimental treatment. In addition, an endogenous control should also be expressed at roughly the same level as the RNA under study.

Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as housekeeping gene in this study, since expression of GAPDH was unchanged after treatment with PPARα agonists. Its primer sequence was 5'-TCCATGCCATGACTGCCACTC-3' as forward and 5'-TGACCTTGCCCACAGCCTTG-3' as reverse. It should be pointed out that this primer yielded good results between 57^{o}C and 60^{o}C annealing temperatures. Therefore, it was used as reference gene for all set of primers in this study.

2-2-6-6- GEL ELECTROPHORESIS

For setting up the annealing temperature of primers, the PCR products were run on agarose gels and analyzed.

At neutral pH, the negatively charged nucleic acid molecules migrate under the influence of an electrical field from the cathode to the anode. The distance migrated depends on fragment size and is fairly independent of base or sequence composition. The bands are visible under ultraviolet light of 302 nm when stained with SYBR safe solution (0.5 µg / ml).

1.5 g agarose powder was added to 100 ml of 1x TAE and heated in a microwave oven until the agarose had dissolved, then 5 µl SYBR safe solution was added to the liquid and mixed well. The liquid was then allowed to cool to ~50°C, and the gel was casted. To increase the density of the applied sample and to allow monitoring of the progress of the electrophoresis, 0.2

volume loading buffer was added to the samples (PCR products). All gels were run in 1x TAE at ~75 V. Documentation was performed with the aid of a CCD camera.

2-2-7- STATISTICAL ANALYSIS

Data are presented as mean ± standard deviation of the mean, and *"n"* represents the number of experimental replicates unless otherwise indicated. All data were normalized with control group of each experiment set to 100%. Statistical significance was assessed using student's *t*-test and one-way ANOVA. Comparisons were deemed statistically significant where $p < 0.05$.

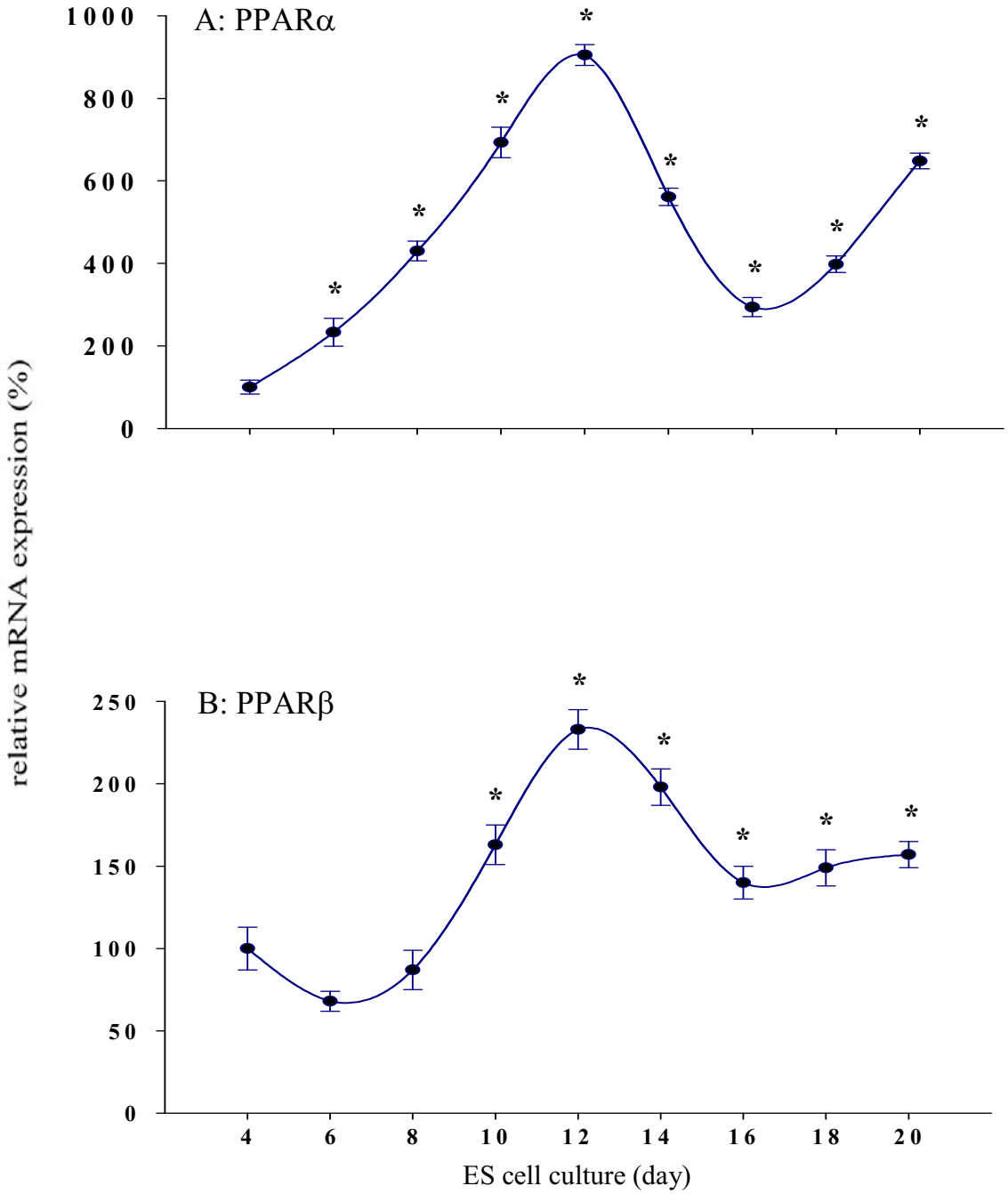
3- RESULTS

3-1- EXPRESSION OF PPARα, -β, -γ mRNA DURING THE DIFFERENTIATION OF MOUSE ES CELLS

According to the literature, PPARs are expressed during fetal life and at different levels. They also play a role in the differentiation and development of organs.

To investigate expression levels of PPARs, their mRNA levels were analyzed during the time course of embryoid body differentiation (from day 4 to day 20 of cell culture) by real time RT-PCR. Figure 10 shows mRNA expression of each PPAR isoform during ES cell differentiation. Interestingly, as shown in Figure 10A, PPARα expression significantly increases on day 6 of embryoid bodies differentiation which is also the time period of cardiomyogenesis (*Hescheler et al. 1999, Boheler et al. 2002, Wei et al. 2005*). The PPARα mRNA level then decreased from day 12, and an upregulation was seen again from day 14 of cell culture. It is also notable that the level of PPARα mRNA expression was higher than that of two other isoforms of PPAR during the time course measurement.

Figures 10B and 10C show the mRNA level of two other PPAR isoforms, PPARβ and –γ, respectively. A significant increase in expression of PPARβ in comparison to day 4 starts at day 10, and reaches maximum values on day 12. After day 12, the level of PPARβ mRNA starts to decrease until day 16, and reaches a plateau in late stages of differentiation. With PPARγ, two peaks of expression were observed which occur at day 8 and 18, respectively (Fig. 10C).


A: PPARα
1000
800
600
400
200
0
B: PPARβ
250
200
150
100
50
0
4
6
8
10
12
14
16
18
20
ES cell culture (day)
relative mRNA expression (%)

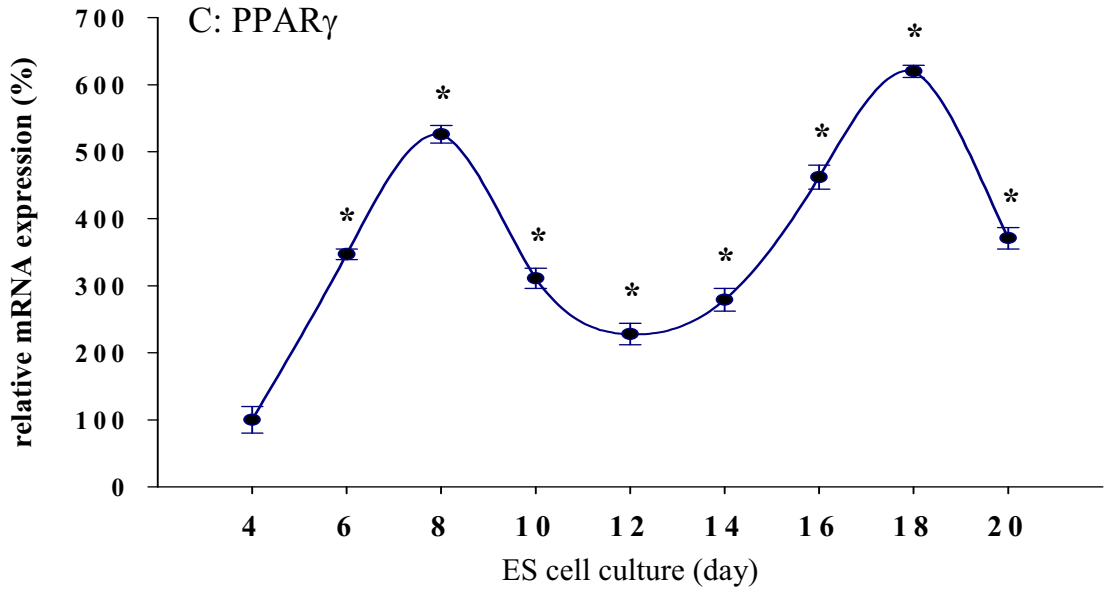


Figure 10: *mRNA expression of PPARα, -β, and -γ during ES cell differentiation.* Measurement was performed with the 3D tissue of embryoid bodies by real time RT-PCR. Note that the mRNA level of *PPARα* was higher as compared to the two other isoform of *PPARs*. A: *PPARα*, B: *PPARβ*, C: *PPARγ*. * $p < 0.05$, statistically significant as compared to PPAR mRNA expression on day 4 of cell culture ($n = 4$).

3-2- STIMULATION OF CARDIOMYOGENESIS IN MOUSE ES CELLS BY PPARα, BUT NOT BY –β or –γ AGONIST

The culture was established by plating 4-day-old embryoid bodies onto Petri perm plates and allowing continuous cell proliferation and differentiation. Within this multi-cellular three dimensional embryoid body, cardiomyocytes grow as spontaneously contracting, round cell clusters as reported previously by different research groups (*Kehat et al. 2001, Snir et al. 2003*). Each embryoid body contained one or more beating foci. An increase in size, strength of contraction, and beating frequency (data not shown) was observed during further differentiation.

3-2-1- ASSESSMENT OF NUMBERS OF SPONTANEOUSLY CONTRACTING FOCI OF CARDIOMYOCYTES UPON TREATMENT WITH PPAR AGONISTS

Differentiating embryoid bodies were treated from day 4 through day 10 of cell culture with either 10 μM of the PPARα agonists WY14643, GW7647 and ciprofibrate, the PPARβ agonist L165,041 or the PPARγ agonist GW1929. In parallel, cell cultures were treated with the PPARα antagonist MK886. The number of spontaneously contracting foci were carefully analyzed by microscopic inspection, the numbers of foci recorded per plate till day 10 of cell culture.

A significant increase in numbers of contracting cardiomyocyte foci was observed in all PPARα agonist-treated plates in comparison to untreated control embryoid bodies (see Fig. 11). Unlike PPARα agonists, plates treated with PPARβ agonist showed no difference as compared to untreated controls. It was also observed that the number of contracting foci was decreased in the samples treated with PPARγ agonist. No significant difference was seen in the plates treated with the PPARα antagonist MK886 when compared with the untreated control.

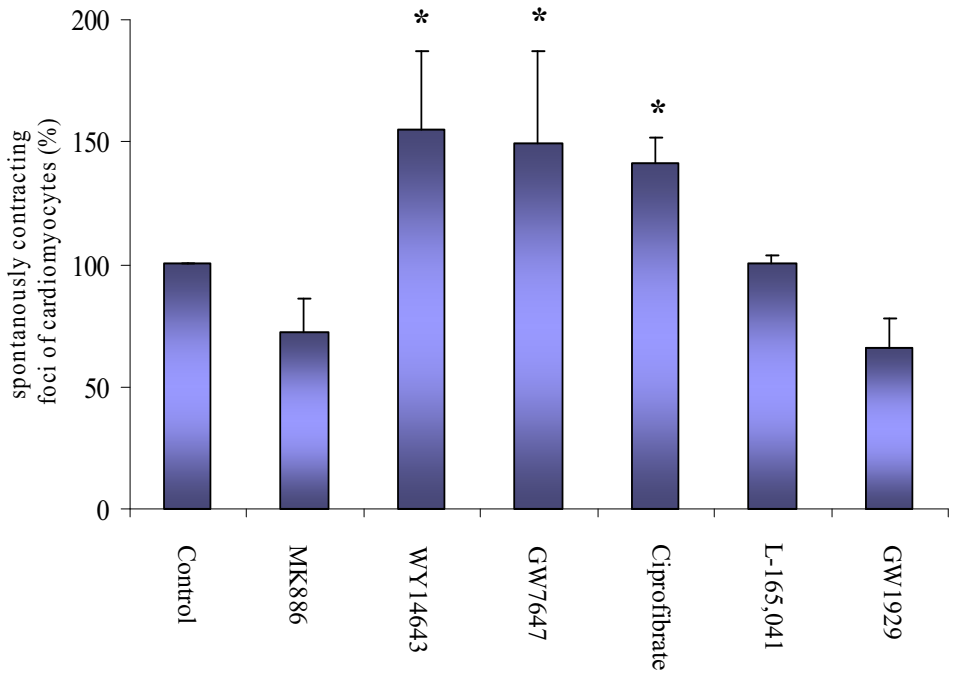


Figure 11: *Increase of spontaneously contracting foci number upon treatment with PPARα agonists (10 μM).* Note that only PPARα agonists increased the number of contracting beating foci. * $p < 0.05$, statistically significant as compared to the untreated control ($n = 6$).

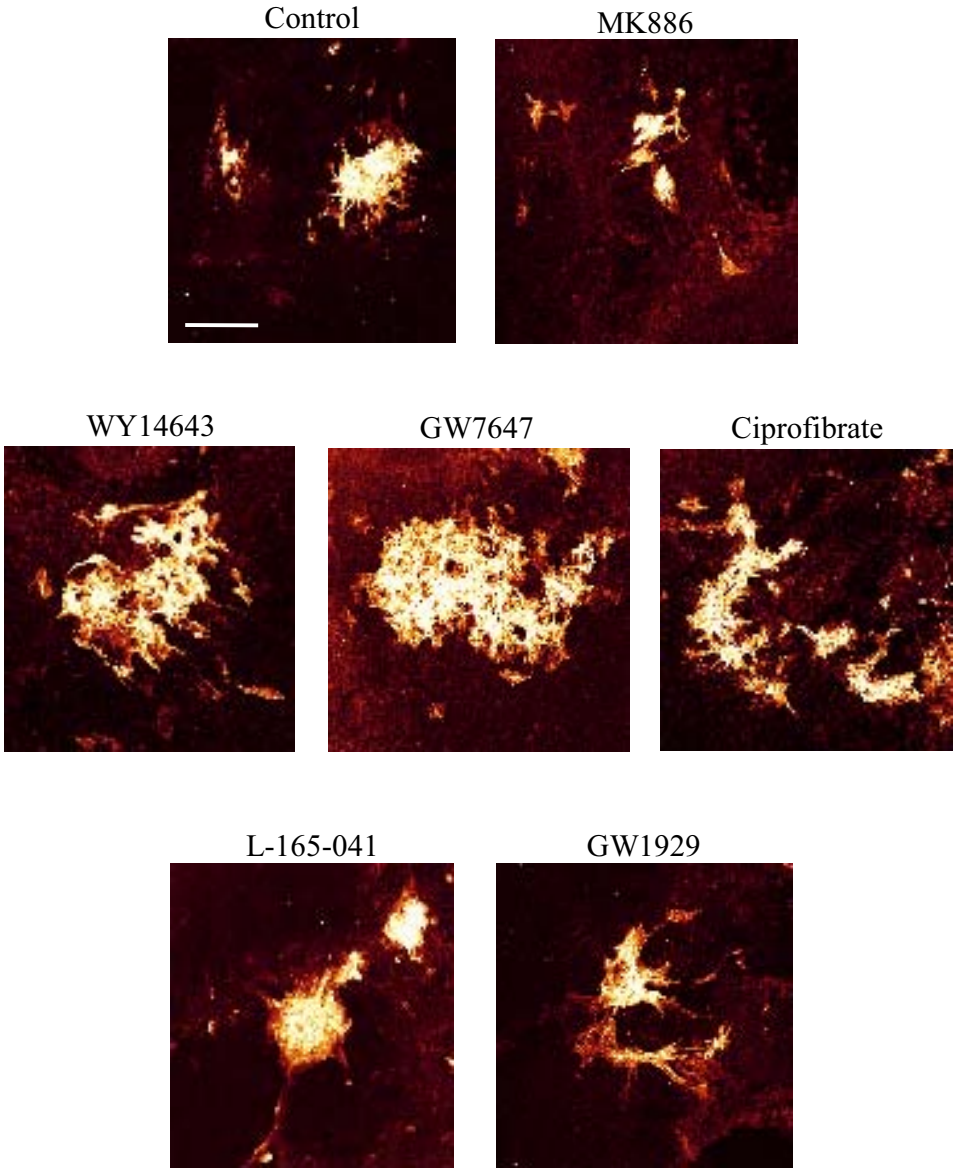
3-2-2- EVALUATION OF THE SIZE OF THE CONTRACTING AREA UPON TREATMENT WITH PPAR AGONISTS

Differentiated cardiomyocytes from the mouse CCE ES cell line treated with PPARs agonists were fixed and stained using an antibody directed against α-actinin, which is a cardiomyocyte specific marker. The plates were then analyzed by laser confocal scanning microscopy and the bio-images assessed using Leica software.

Figure 12B depicts the increase in the area of ES cell-derived cardiomyocytes after treatment with PPARα agonists. The augmentation was significant in comparison to untreated control. The decrease in the size of contracting foci upon treatment with the PPARα antagonist MK886 was evident, and this effect was significantly different from the untreated control group. In contrast, the size of the beating areas in samples treated with PPARβ and PPARγ agonists was not significantly different from the untreated control. The images from the untreated control and each treated group in figure 12A are representatives for the differences which were observed under different experimental conditions as indicated.

The data interestingly indicate that only PPARα effectors exerted significant effects on differentiation of ES cell-derived cardiomyocytes. These findings strongly support the notion that PPARα is involved in the differentiation of ES cell-derived cardiomyocytes.

A:


Control
MK886
WY14643
GW7647
Ciprofibrate
L-165-041
GW1929

B:

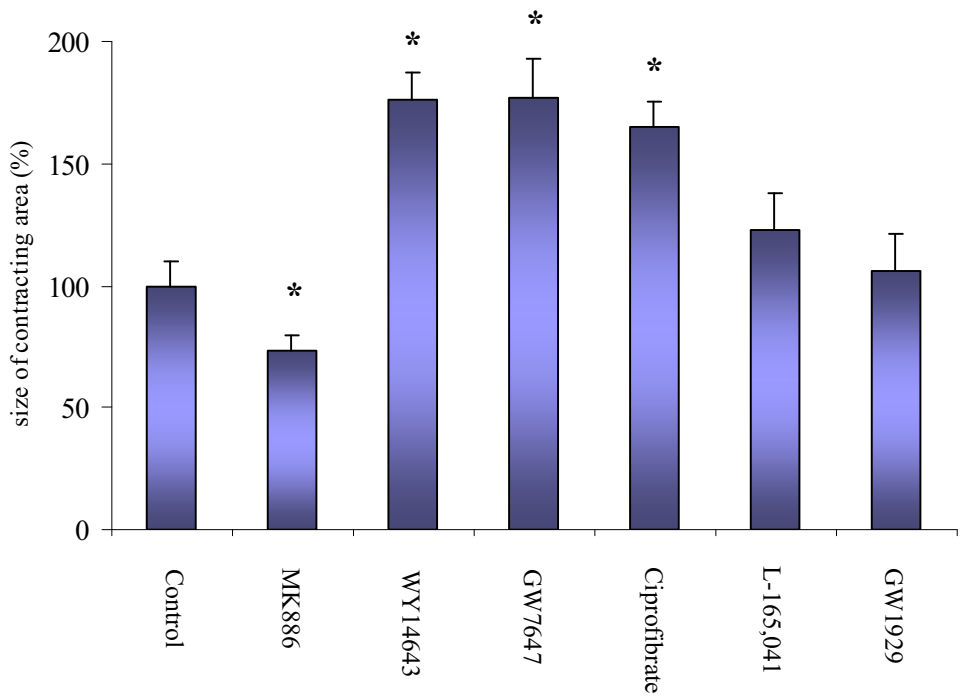


Figure 12: *Effect of PPARα agonists (10 μM) on the size of ES cells-derived contracting foci of cardiomyocytes.* It is interesting to note that only PPARα effectors had effect on the extension of the contracting area (B). The size of CCE ES cell-derived beating foci was assessed by computer-assisted image analysis after staining with α-actinin antibody. Representative images showing ES cell-derived contracting foci stained with α-actinin (A). The bar in the images represents 200 μm. * $p < 0.05$, statistically significant as compared to the untreated control ($n = 4$).

3-3- UN-EFFECTIVENESS OF PPARα AGONIST TREATMENT ON SIZE OF EMBRYOID BODIES

The data above suggest that PPARα agonists modulate cardiomyogenesis, and enhance the extension of contracting cardiomyocyte foci. To investigate whether the observed results on extension of contracting foci are a consequence of PPARα agonist treatment and not caused by a mere increase in embryoid body size, 4-day-old embryoid bodies were treated with 10 μM WY14643. Size of the embryoid bodies in treated and untreated control group were daily analyzed by Leica laser confocal scanning microscope from day 4 to day 9 of cell culture.

As shown in figure 13, PPARα agonist treatment made no significant changes on the size of embryoid bodies in comparison to the untreated control. Therefore, this finding supports our observed results that PPARα agonists apparently increased the size of contracting cardiomyocyte foci in comparison to the untreated control (Fig. 12).

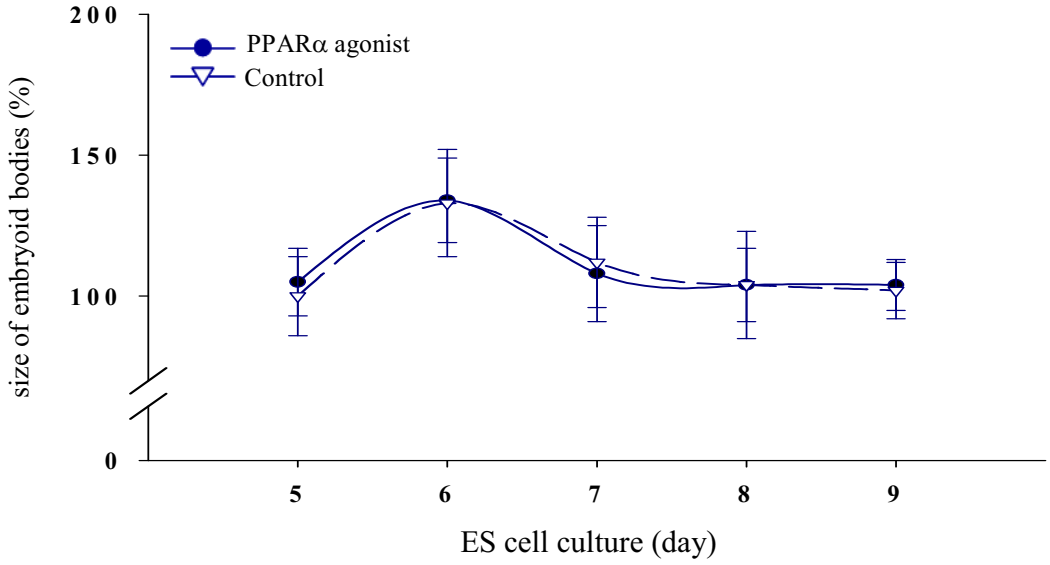


Figure 13: *Effect of PPARα agonist (10 μM) on the size of embryoid bodies.* It is interesting to note that PPARα agonist had no effect on the size of embryoid bodies in comparison to the untreated control (n = 3).

3-4- AUGMENTATION OF PPARα mRNA EXPRESSION UPON TREATMENT WITH PPARα AGONIST

To investigate whether the observed results upon treatment of ES cells with the PPARα agonist WY14643 are a consequence of the molecular involvement of PPARα ligand in the cells, mRNA levels of *PPARα* gene were evaluated by quantitative real time RT-PCR. For the analysis, 4-day-old embryoid bodies were plated onto tissue culture dishes and treated from day 4 to day 8 of cell culture with 10 µM WY14643 after which mRNA was extracted every 24 h.

Our data illustrated that *PPARα* expression was increased after treatment with PPARα agonist. This finding is in agreement with a report from Gebel et al. (*1992*) who have demonstrated that PPARα agonist treatment increased *PPARα* mRNA expression in the liver cells of rat.

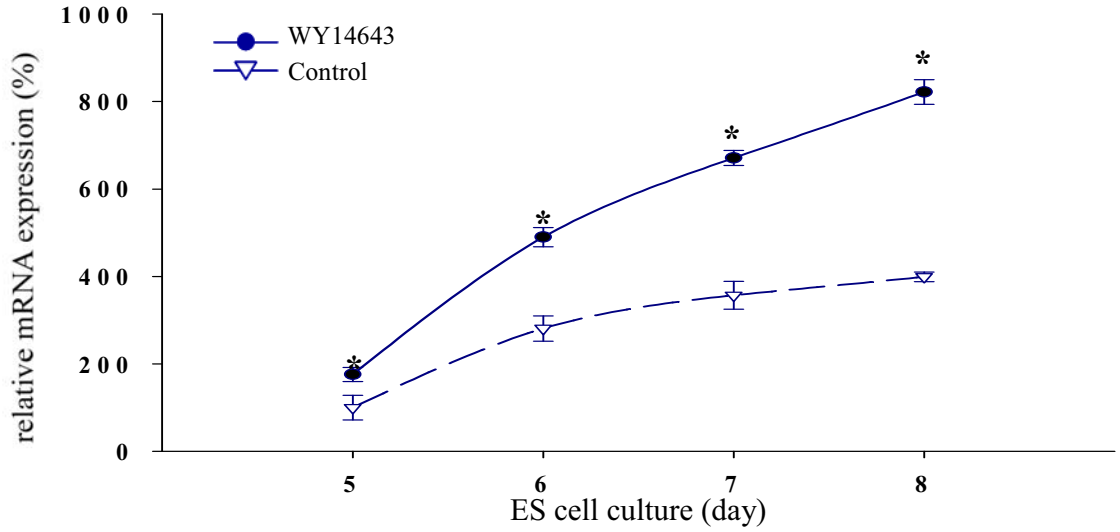


Figure 14: *Effect of the PPARα agonist WY14643 on mRNA expression of PPARα.* The data are presented as relative change in mRNA expression as compared to the untreated control on day 5 (set to 100 %). Note the gradually increase of *PPARα* mRNA expression from day 5 to day 8 of cell culture upon treatment with PPARα agonist (10 µM) in comparison to the untreated control group. * $p < 0.05$, statistically significant as compared to the mRNA expression of untreated control on day 5 of cell culture ($n = 3$).

3-5- ENHANCEMENT OF PPARα ACTIVITY UPON TREATMENT WITH THE PPARα AGONIST

It was previously reported that the activity of PPARα increases on administration of PPARα agonist (Gebel et al. 1992, Pineda et al. 2002). To address this assumption, 4-day-old embryoid bodies were treated with 10 μM PPARα agonist WY14643. The embryoid bodies were then fixed with 4% PFA after 0, 5, 10, 15, 30, 45, and 60 min treatment with WY14643, where 0 min represents the untreated control. The fixed embryoid bodies were stained with phospho-PPARα antibody, and then analyzed by Leica LCSM.

Interestingly, the phosphorylation level of PPARα significantly increased upon 5 min treatment with PPARα agonist, and approximately stayed on a steady state level for at least 69 min (Fig. 15). Our present data clearly illustrate that the PPARα agonist enhances the activation level of PPARα, which is in agreement with the literature (Gebel et al. 1992, Pineda et al. 2002).

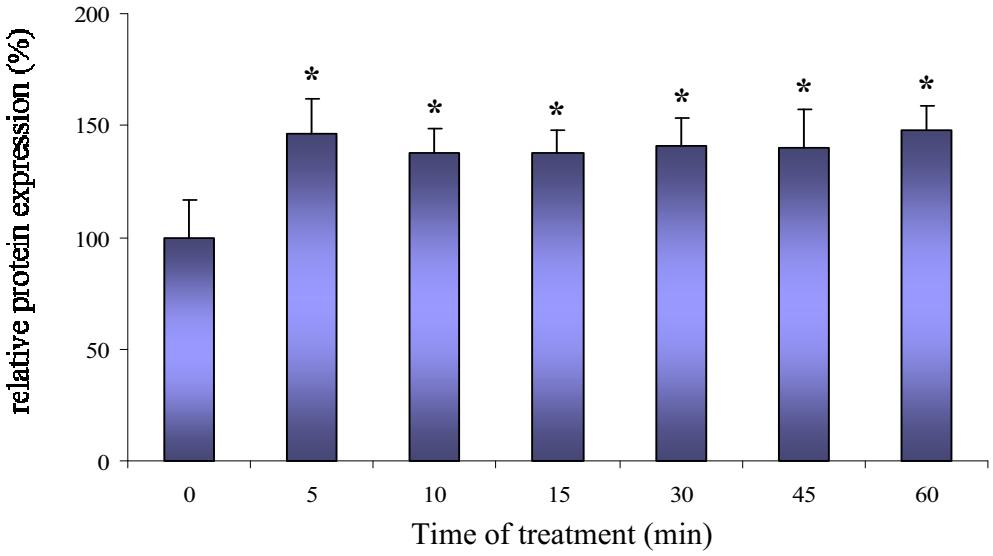


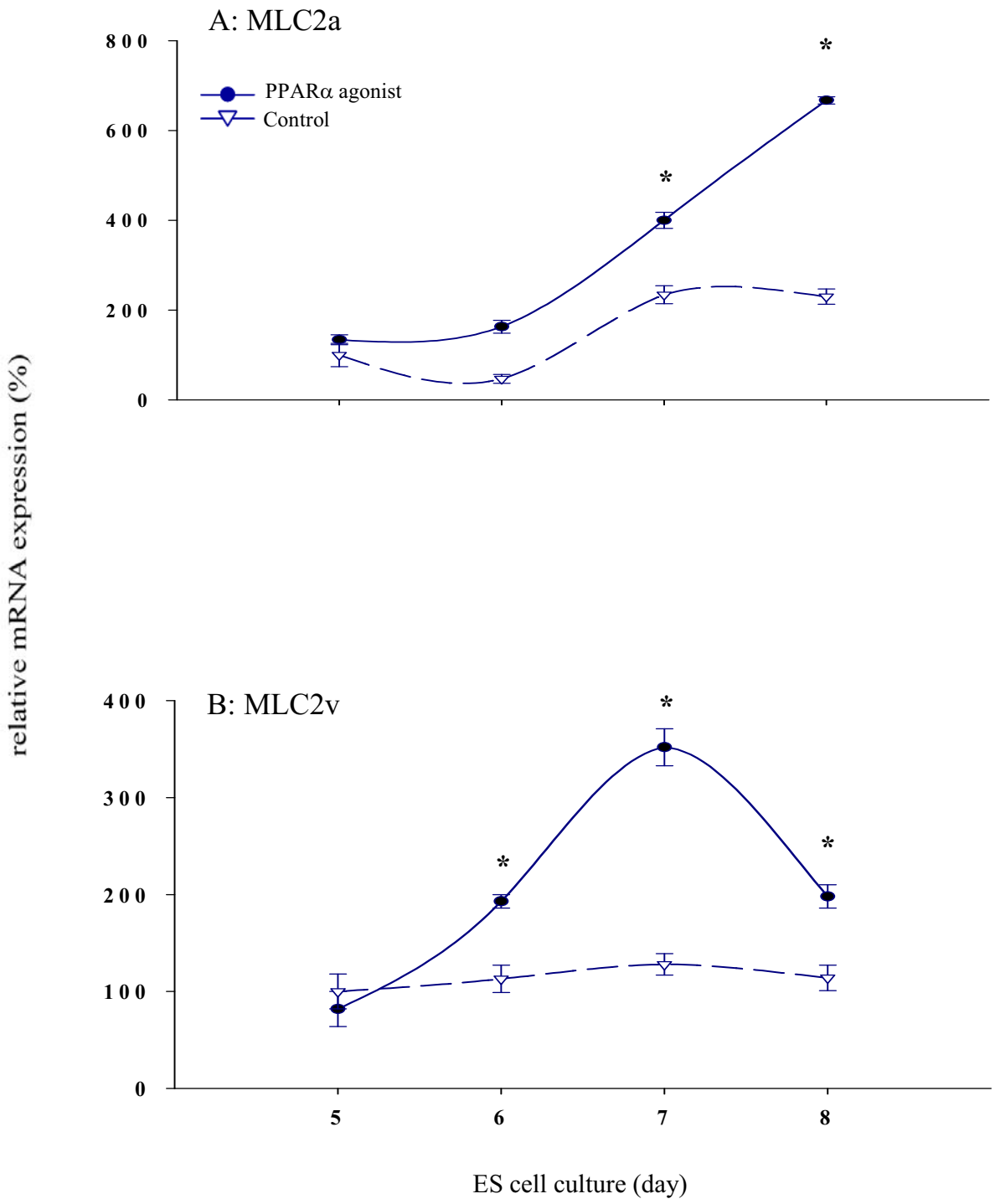
Figure 15: *Effect of PPARα agonist (10 μM) on the activity (phosphorylation) of PPARα in embryoid bodies.* It is interesting to note that PPARα agonist significantly increased phosphorylation of PPARα after 5 min and kept it on an elevated plateau in comparison to the untreated control. 0 min treatment represents the untreated control group (n = 3).

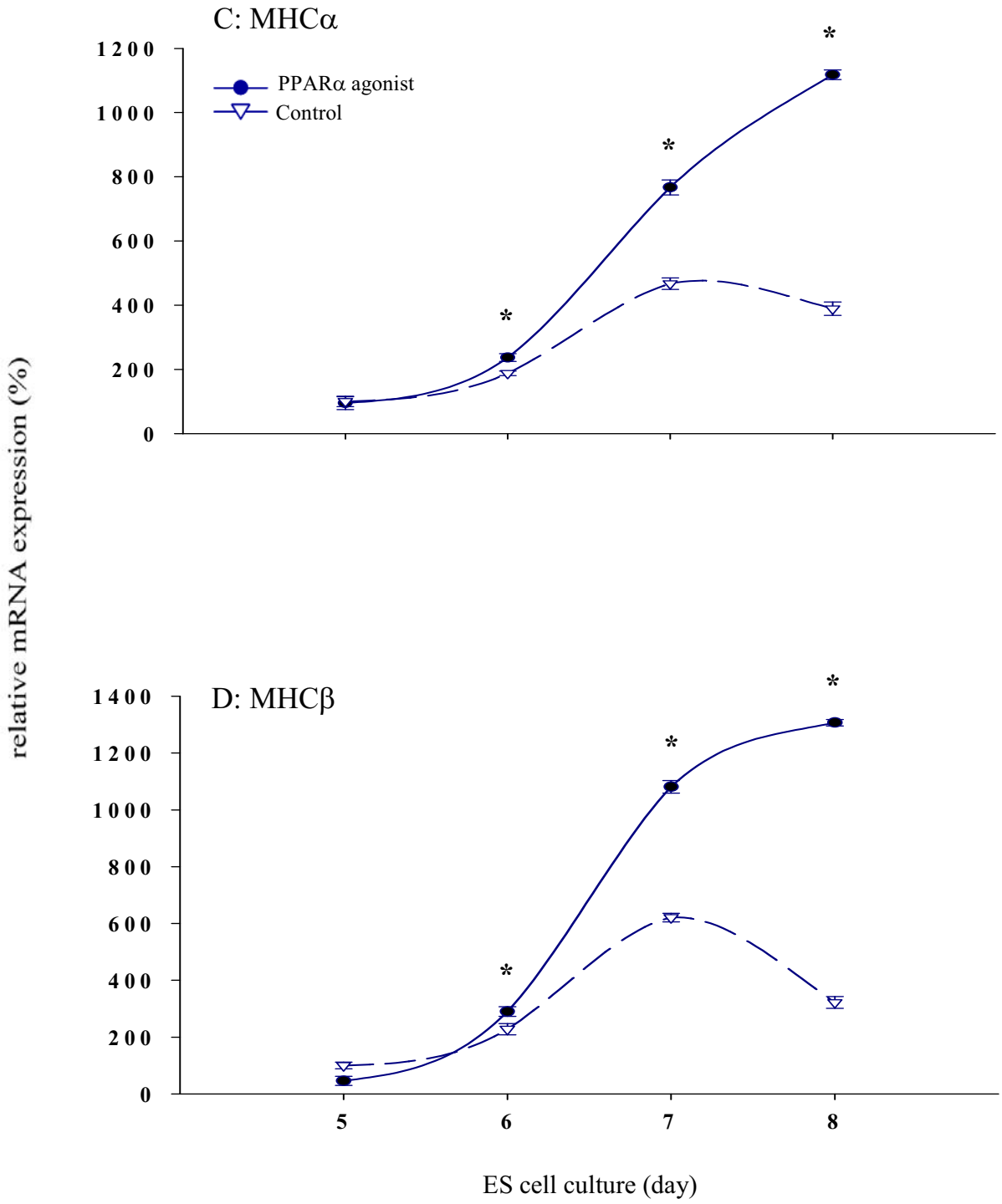
3-6- ACTIVATION OF CARDIAC SPECIFIC mRNA EXPRESSION UPON TREATMENT WITH WY14643

A criterion for successful cardiac myocyte differentiation of ES cells *in vitro* is the expression of genes that are normally involved in cardiac cell development *in vivo*. To examine if the stimulation of cardiomyogenesis of mouse ES cells by PPARα is paralleled by an increase in the expression of cardiac-specific genes, the expression of these cardiac marker genes was assessed during the time course of treatment with PPARα agonist. The mRNA expression analyses of the following genes as specific markers for cardiomyocyte differentiation were performed: *ANP*, *MLC2a*, *MLC2v*, *MHCα*, *MHCβ*, and *cardiac α-actin*.

As mentioned above, mRNA levels of cardiac specific markers during treatment with WY14643 from day 4 to day 8 was evaluated by quantitative real time RT-PCR as described in materials and methods (see 2-2-6). Figure 16E shows a transient increase in basal *ANP* gene expression reaching maximum values on day 7 of differentiation. Treatment with WY14643 (10 μM) significantly enhanced ANP expression compared to the untreated control on day 6 to 8 of ES cell differentiation. The mRNA expression of *MLC2a* was significantly increased after PPARα agonist treatment in comparison to the untreated control from day 6 of cell culture (Fig. 16A). Figure 16B depicts the mRNA expression pattern of *MLC2v*. As shown in this graph, the mRNA level of *MLC2v* in untreated control group showed a basal maximum expression on day 7, which displayed a 2.5 fold increase upon treatment with PPARα agonist on the same day.

Comparably the pattern of mRNA expression of *MHCα*, *MHCβ*, and *cardiac α-actin* genes was significantly increased as indicated in the PPARα agonist-treated group in comparison to untreated control (Fig. 16C, D, and F, respectively). Days 6-8 of differentiation have been previously reported by others as the critical period of cardiac commitment in ES cells (*Hescheler et al. 1999, Boheler et al. 2002, Wei et al. 2005*).


A: MLC2a
800
600
400
200
0
PPARα agonist
Control
*
*
*
B: MLC2v
400
300
200
100
0
*
*
*
5
6
7
8
relative mRNA expression (%)
ES cell culture (day)


C: MHCα
PPARα agonist
Control
1200
1000
800
600
400
200
0
*
*
*
D: MHCβ
1400
1200
1000
800
600
400
200
0
*
*
*
5
6
7
8
relative mRNA expression (%)
ES cell culture (day)

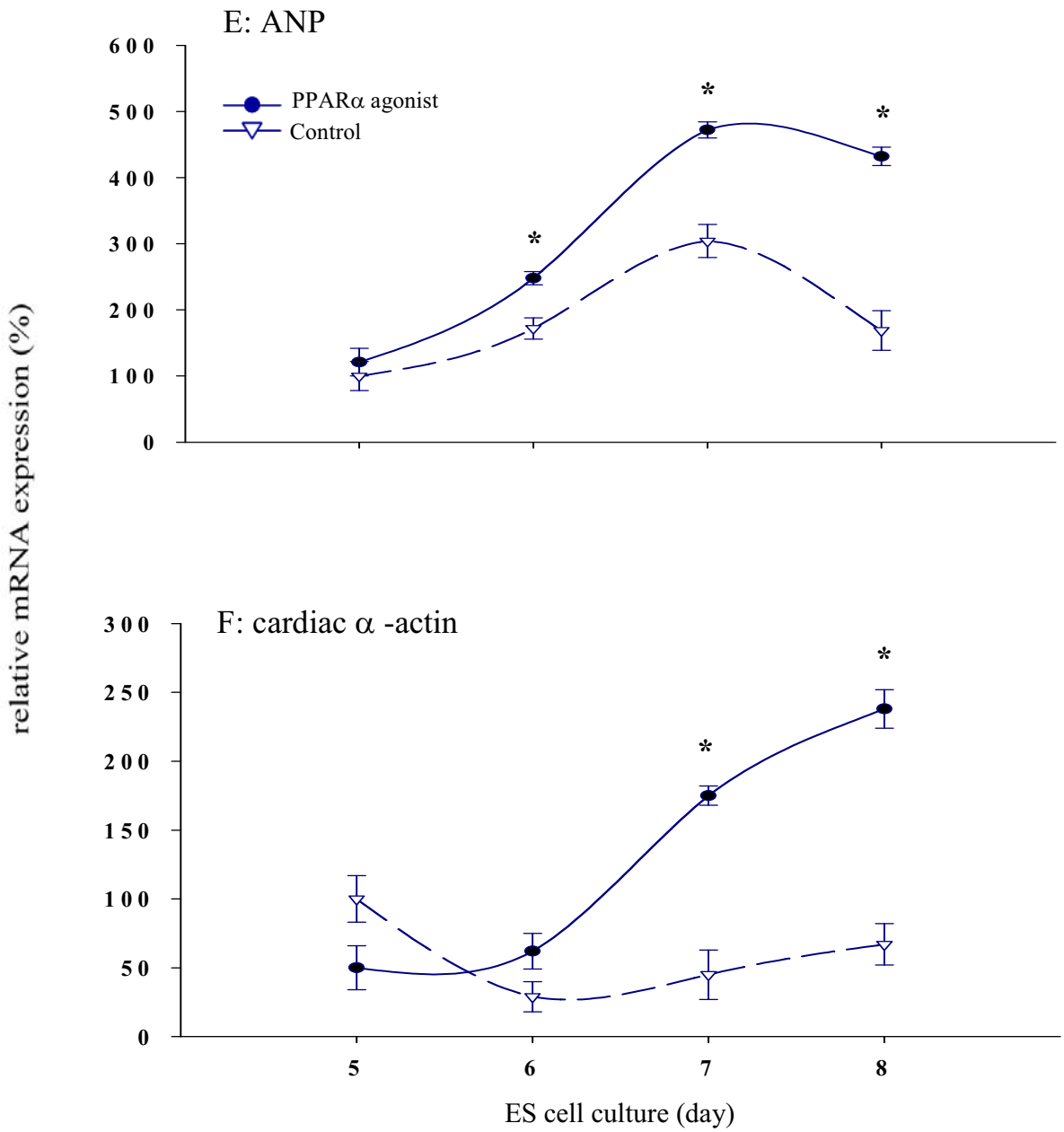


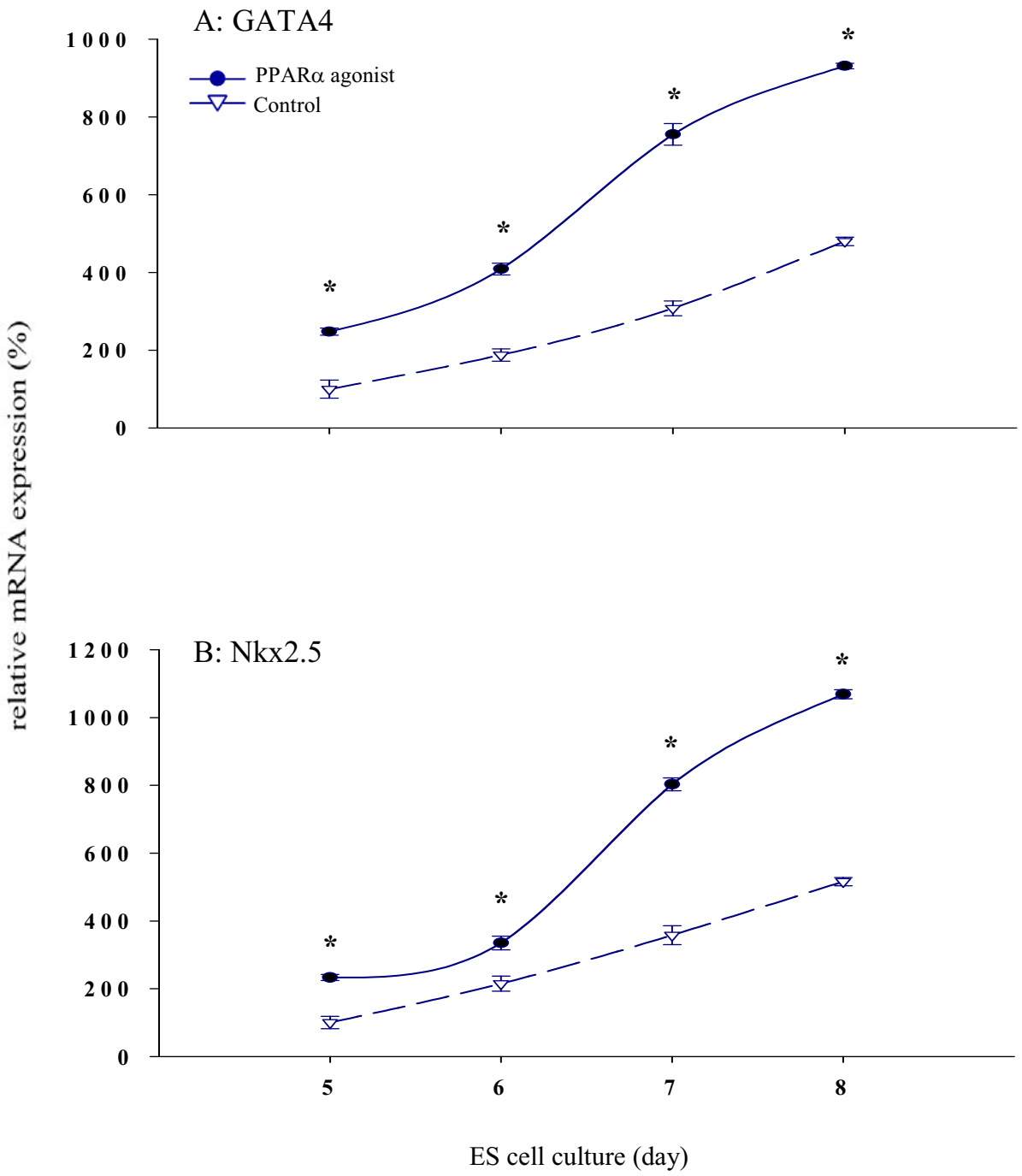
Figure 16: *Effect of PPARα agonist on the transcription of cardiac specific genes.* Note that PPARα agonist (10 µM) changed the mRNA expression of all examined cardiogenic specific markers during the time course of cardiomyocyte differentiation. A: *MLC2a*, B: *MLC2v*, C: *MHCα*, D: *MHCβ*, E: *ANP*, F: *cardiac α-actin*. * $p < 0.05$, statistically significant as compared to mRNA expression on day 5 of untreated control group ($n = 3$).

3-7- INCREASE IN mRNA EXPRESSION OF CARDIOGENIC TRANSCRIPTION FACTORS FOLLOWING PPARα AGONIST TREATMENT

If PPARα activation indeed stimulates cardiomyogenesis of mouse ES cells rather than enhancing cardiomyocyte survival it should be assumed that PPARα agonists increase the expression of cardiogenic transcription factors. The transcription factors most often cited as markers of the cardiac cell lineage are GATA4, Nkx2.5, MEF2c, and DTEF1 (*Parmacek 2006*).

To investigate the mRNA expression of cardiogenic transcription factors upon treatment with WY14643, quantitative real time RT-PCR was performed (see 2-2-6). Total RNA was extracted every 24 h from mouse CCE embryoid bodies which were plated into tissue culture dishes at day 4, and daily treated using the PPARα agonist WY14643 (10 μM) till day 8 of cell culture.

As shown in figure 17A-D, mRNA expression of the cardiac transcription factors *GATA4*, *Nkx2.5*, *Mef2c*, *DTEF1* increased during the time course of ES cell differentiation. With *GATA4* and *Nkx2.5* a steady mRNA increase was observed until day 8, whereas maximum expression on day 7 was found for *Mef2c* and *DTEF1* followed by down regulation on day 8. Treatment with WY14643 resulted in significant enhancement of the expression of all cardiac transcription factors, thus indicating that PPARα activation resulted in cardiac commitment rather than inhibition of apoptosis of cardiac cells or induction of cardiac cell proliferation.


A: GATA4
PPARα agonist
Control
1000
800
600
400
200
0
*
B: Nkx2.5
1200
1000
800
600
400
200
0
5
6
7
8
relative mRNA expression (%)
ES cell culture (day)

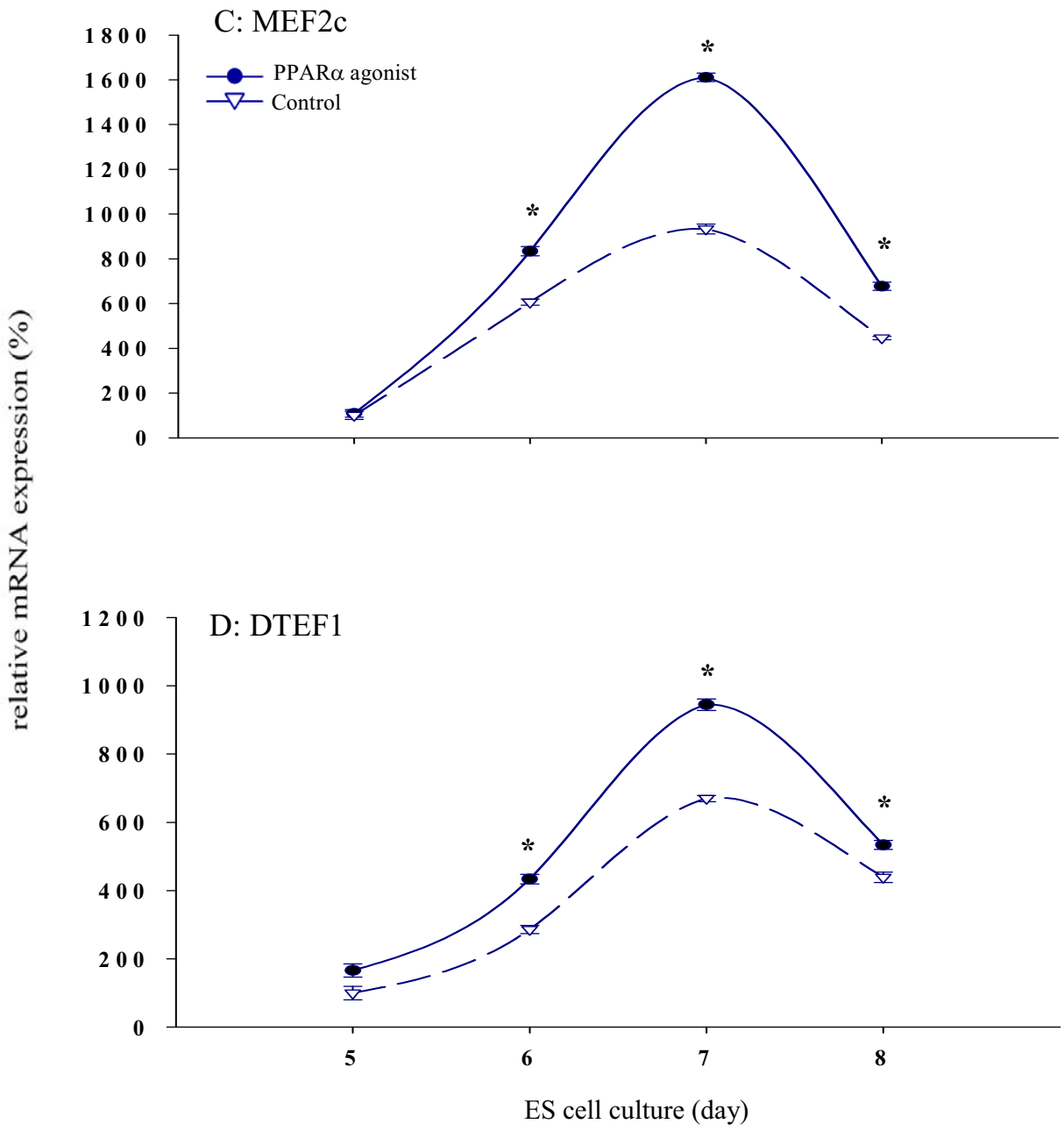


Figure 17: *Effect of PPARα agonist (10 μM) on mRNA expression of cardiogenic transcription factors.* Note that maximum expression of mRNA was achieved on day 7 of cell culture which is the time point at which cardiomyogenesis begins. A: *GATA4*, B: *Nkx2.5*, C: *Mef2c*, D: *DTEF1*. * $p < 0.05$, statistically significant as compared to mRNA expression on day 5 of the untreated control group ($n = 3$).

3-8- ENHANCEMENT OF MITOCHONDRIAL BIOGENESIS IN RESPONSE TO PPARα RECEPTOR ACTIVATION

The increased demand for energy during differentiation and in the functional myocardium has significant consequences for mitochondria, the sub-cellular organelles that furnish energy in a complex set of bioenergetics pathways by generating ATP. Upon cardiomyogenic induction, the copy number of mitochondrial DNA increases which indicates the up-regulation of mitochondrial biogenesis (*Rochard et al. 2000, Korohoda et al. 1993*). This then means that, when cardiomyogenesis is induced by PPARα, the mitochondrial content should be also increased.

The ratio of mtDNA to nDNA reflects the tissue concentration of mitochondria per cell (*Bogacka et al. 2005a*). The *ND1* gene lies in mitochondrial circular genome and is transcribed in the mitochondrial matrix.

Therefore, increase in the amount of *ND1* expression indicates augmentation of mitochondrial DNA and consequently the increase of mitochondrial biogenesis. Analysis of the expression of *ND1* has been successfully used by several groups for assessing the mitochondrial content (*Bogacka et al. 2005a, Bogacka et al. 2005b, He et al. 2002*). For this reason, the mRNA expression of *ND1* was evaluated by quantitative real time RT-PCR as described in materials and methods (see 2-2-6). Using *ND1* for calculation of the mitochondrial content was performed as previously described (*Bogacka et al. 2005a, Bogacka et al. 2005b, He et al. 2002*).

Figure 18 depicts the mRNA level of the *ND1* gene during the differentiation of ES cells in the presence and absence of WY14643. This graph shows that mRNA expression of *ND1* was increased upon treatment with WY14643 and follows a similar trend as compared to the untreated group. Following 24 h treatment with PPARα agonist, the amount of *ND1* mRNA expression was 2 fold more as compared to the untreated control, and this enhancement progressively increased till day 8 of cell culture.

This finding indicated that activation of PPARα receptor by its ligand WY14643 augmented the mitochondrial content, which consequently should enhance mitochondrial biogenesis.

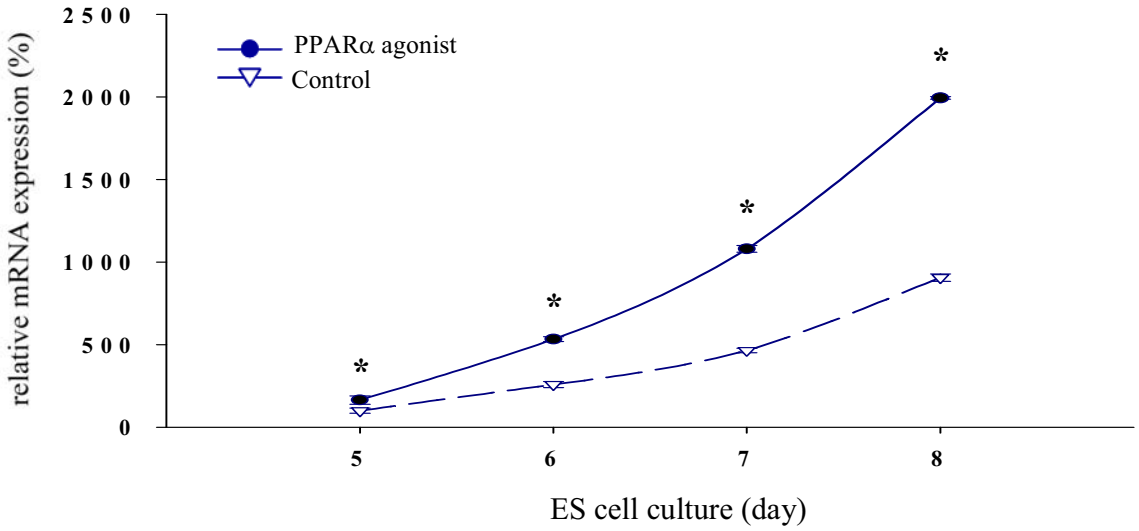


Figure 18: *Enhancement of mitochondrial content upon PPARα receptor activation.* Note that *ND1* mRNA expression continuously increased during the time course of ES cell differentiation. Treatment of ES cells with PPARα agonist (10 μM) resulted in a significant enhancement of *ND1* expression as compared to the untreated control. * $p < 0.05$, statistically significant as compared to mRNA expression on day 5 of untreated control group ($n = 3$).

3-9- GENERATION OF ROS UPON TREATMENT WITH PPARα AGONISTS

Recently, it has been demonstrated that ES cells endogenously generate ROS during differentiation toward cardiac cells (*Buggisch et al. 2007*). Furthermore, it was previously shown by our group and others that exogenous treatment of embryoid bodied with ROS induced cardiac differentiation (*Sauer et al. 2000, Li et al. 2006, Schmelter et al. 2006, Ateghang et al. 2006*). We wanted to test, if a comparable mechanism may be the reason for the effects of PPARα agonists on cardiomyogenesis of ES cells.

In order to verify the hypothesis that PPARα receptor activation-induced cardiomyogenic differentiation involved redox-dependent mechanisms, ROS measurements after PPARα treatment were undertaken. Therefore, 4-day-old embryoid bodies were incubated for 24 h with the PPARα agonists WY14643 (10 μM), GW7647 (10 μM), ciprofibrate (10 μM), and the PPARα antagonist MK886 (10 μM). Subsequently the embryoid bodies were stained with the redox-sensitive ROS indicator H_2DCFDA. Microfluorometric images of DCF stained embryoid bodies were obtained using Leica LCSM as described in materials and methods (see 2-2-5-4).

While PPARα agonists significantly increased ROS generation, a significant downregulation of ROS generation was observed in the presence of the PPARα antagonist MK886 (Fig. 19). This finding clarified that PPARα activation caused an induction of intracellular ROS production in embryoid bodies.

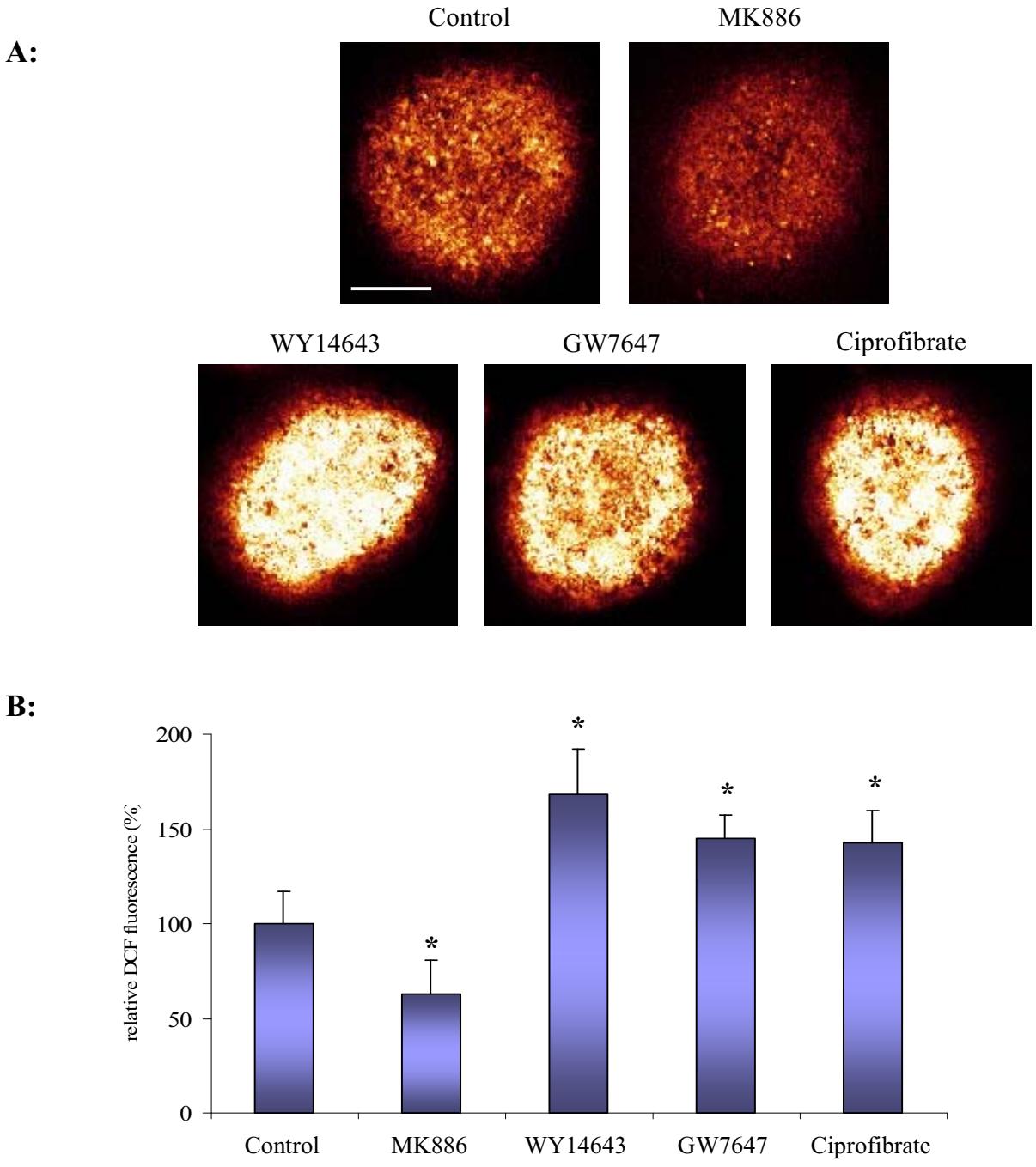


Figure 19: *Effect of PPARα agonists on ROS generation.* Note that PPARα activation resulted in increased ROS generation as compared to the untreated control. A: Representative images showing embryoid bodies stained with H_2DCF-DA and displaying DCF fluorescence. The scale bar in the images represent 300 µm. B: Semiquantitative evaluation of DCF-fluorescence in embryoid bodies under different conditions as indicated. * $p < 0.05$, statistically significant as compared to untreated control group ($n = 6$).

3-10- UN-EFFECTIVENESS OF PPARβ AND –γ AGONIST TREATMENT ON ROS GENERATION

Since stimulation of PPARβ and PPARγ failed to enhance cardiomyogenesis as compared to treatment with PPARα agonists, it was hypothesized that PPARβ and PPARγ agonists should not increase ROS generation. Therefore, the embryoid bodies at day 4 of cell culture were incubated with 10 μM of the PPARβ L-165,041 and the PPARγ agonist GW1929 and then stained by H_2DCFDA dye as described in 2-2-5-4.

As represented in figure 20, in contrast to the effects of PPARα agonist on ROS production (see Fig. 19B), neither PPARβ nor PPARγ induced any considerable change in the amount of ROS generation in comparison to the untreated control.

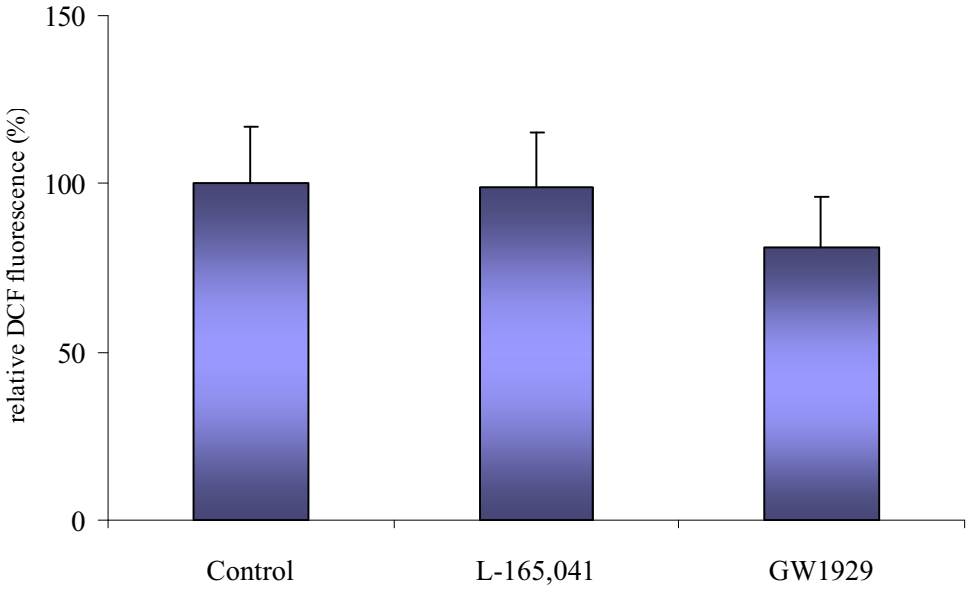


Figure 20: *ROS generation upon treatment with PPARβ and –γ agonists (10 μM).* Note that neither the PPARβ nor the PPARγ agonist caused significant differences in the amount of ROS generation in comparison to untreated embryoid bodies. * $p < 0.05$, statistically significant as compared to the untreated control group ($n = 6$).

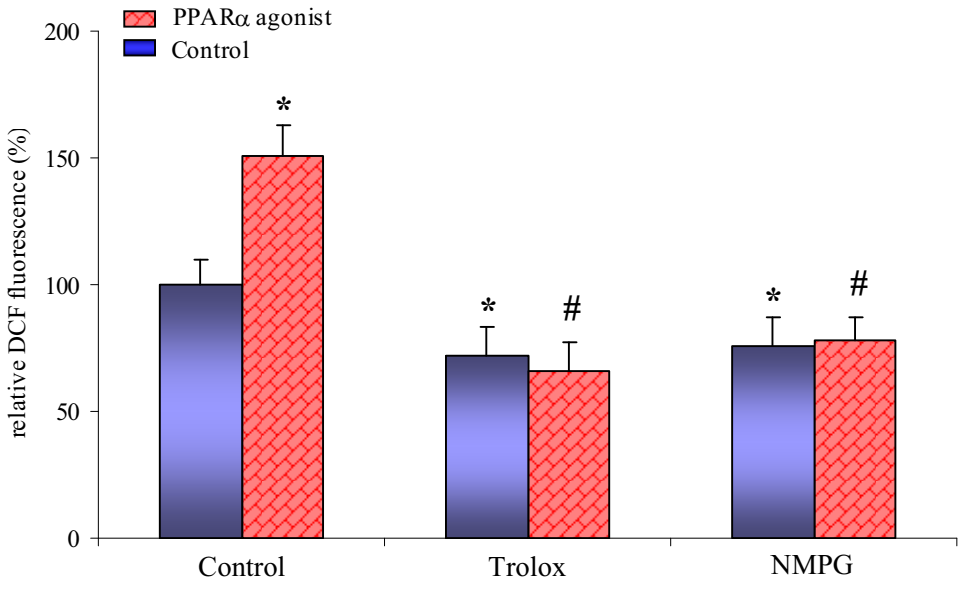
3-11- INHIBITION OF PPARα-INDUCED ROS PRODUCTION UPON FREE RADICAL SCAVENGER TREATMENT

The hypothesis of involvement of ROS in PPARα-mediated cardiomyogenesis mentioned above was further strengthened by experiments where ROS generation following PPARα agonist treatment was assessed in the presence of the free radical scavengers vitamin E (trolox) and NMPG. For this experiment, embryoid bodies were pre-incubated for 2 h with 100 µM of trolox or NMPG on day 4 of cell culture and then treated with 10 µM of WY14643. ROS generation was monitored 24 h thereafter by microfluorometry of DCF fluorescence.

As shown in figure 21A, it was found that the co-incubation of trolox or NMPG with the PPARα agonist resulted in a strong attenuation in the amount of ROS production which was induced by PPARα agonist alone.

This observation indicated that free radical scavengers suppress intracellular ROS generation produced by administration of PPARα agonists and therefore, verified the involvement of ROS in PPARα-mediated cardiomyocyte differentiation of ES cells.

A:



B:

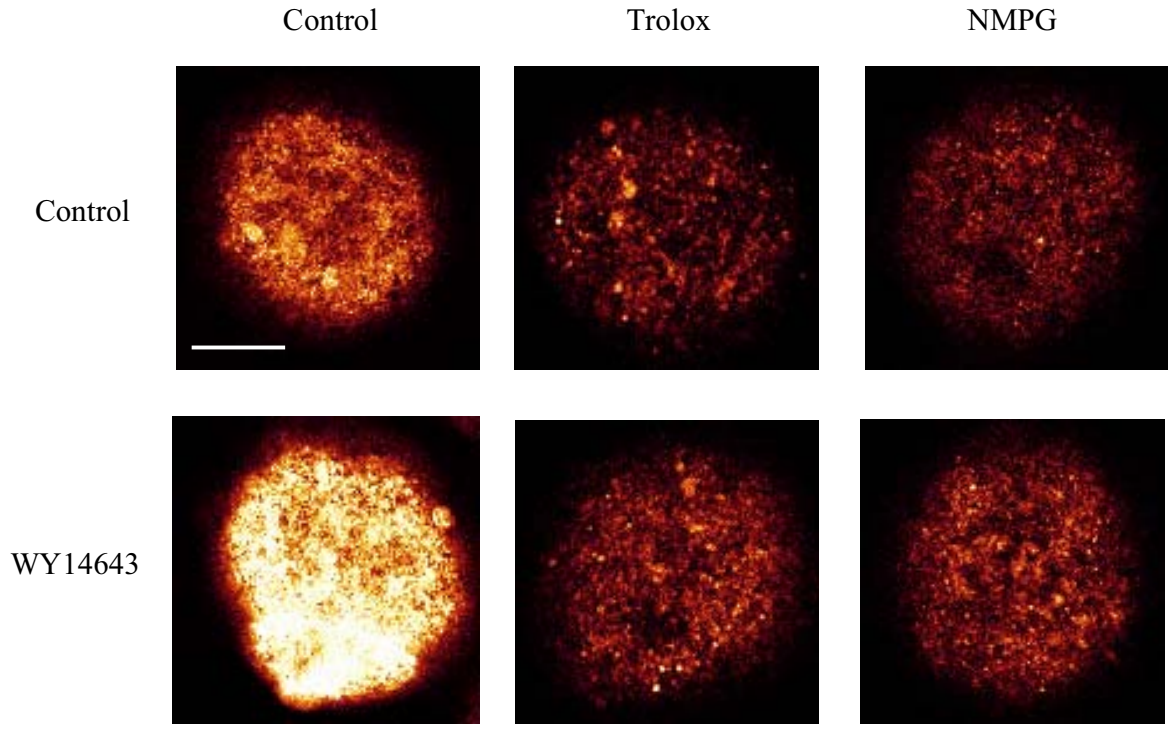


Figure 21: *Role of free radical scavengers trolox (100 μM) and NMPG (100 μM) on PPARα-induced ROS production.* Note the inhibition of PPARα agonist induced ROS generation upon free radical scavenger treatment (A). Representative images showing embryoid bodies stained with H_2DCF-DA and displaying DCF fluorescence. The scale bar in the images represent 300 μm. * and # $p < 0.05$, statistically significant as compared to untreated control and PPARα agonist treatment, respectively ($n = 6$).

3-12- ATTENUATION OF PPARα-INDUCED ROS GENERATION BY NADPH OXIDASE INHIBITORS

To ascertain whether the ROS production observed under the effect of PPARα agonist was generated from NADPH oxidase, the effect of two different specific inhibitors of NADPH oxidase were tested.

In order to perform this experiment, 4-day-old embryoid bodies were pre-incubated for 2 h with 100 nM of DPI or 10 μM of apocynin and then treated by 10 μM of either the PPARα agonists WY14643, GW7647 or ciprofibrate. After 24 h co-treatment with above mentioned reagents, ROS generation was analyzed.

It was observed that the amount of ROS induced by PPARα agonists was completely abolished after co-treatment with DPI (Fig. 22A). Comparably apocynin treatment significantly decreased PPARα agonist-induced ROS generation in embryoid bodies (Fig. 22B).

These finding apparently corroborate the hypothesis that the increased ROS observed upon PPARα agonist treatment were generated by NADPH oxidase activity.

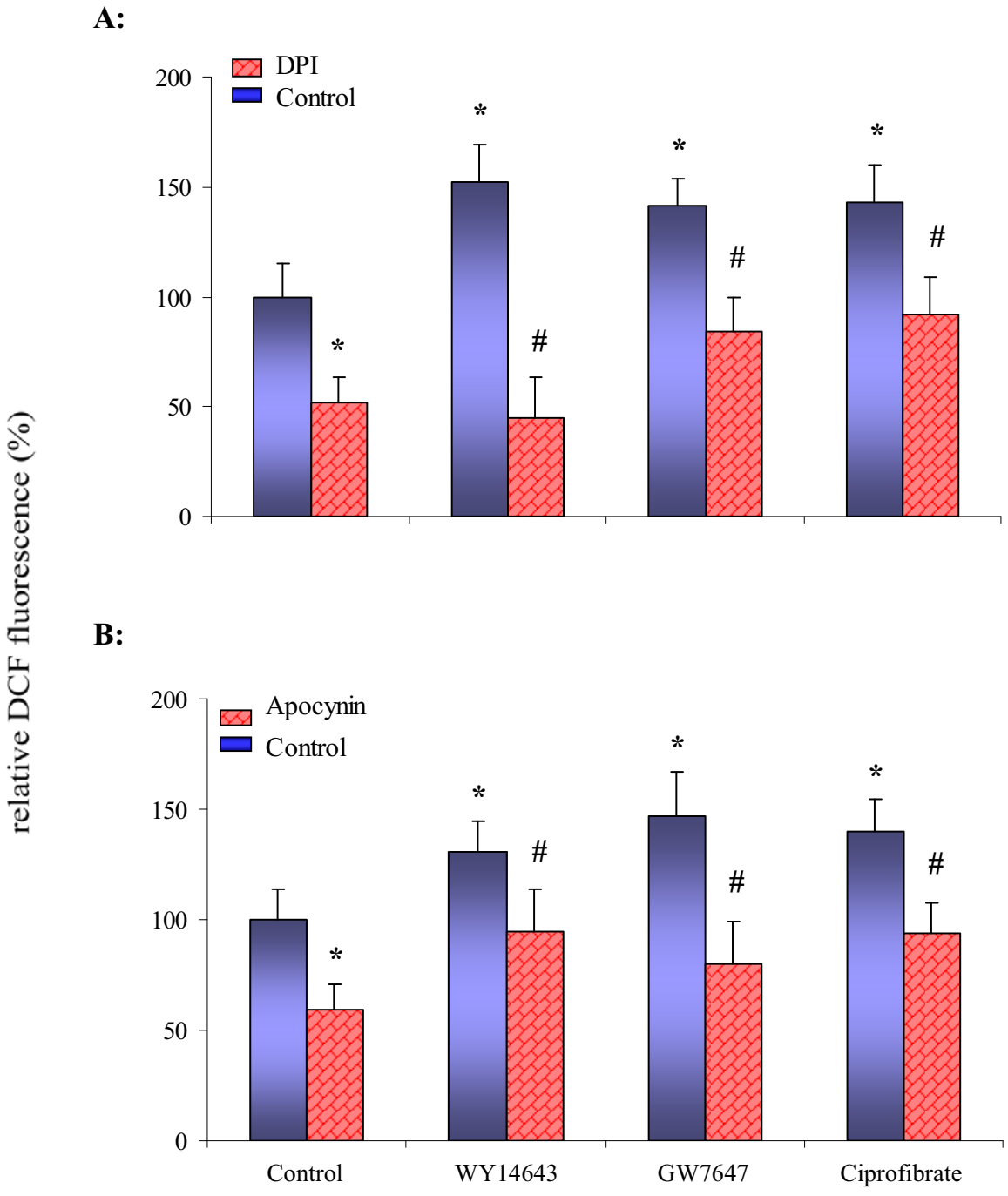


Figure 22: *Effect of NADPH oxidase inhibition on PPARα-induced ROS generation.* Note that DPI (100 nM) and apocynin (10 µM) treatment significantly inhibited PPARα agonist-induced ROS generation (21A and 21B, respectively). * and # $p < 0.05$, statistically significant as compared to untreated control and PPARα agonist treatment, respectively ($n = 6$).

3-13- EFFECT OF ROTENONE ON PPARα-INDUCED ROS GENERATION

It has already been demonstrated in this study that PPARα agonists increase mitochondrial ND1 mRNA expression suggesting induction of mitochondrial biogenesis. On the other hand, it was recently reported that increase in mitochondrial mass is accompanied by augmentation of ATP production, and consequently, by a higher production of ROS (*Nesti et al. 2007*). This finding raised the assumption that a portion of ROS produced upon treatment with PPARα agonists may be generated within the mitochondrial respiratory chain. To test this notion, 4-day-old embryoid bodies were pre-incubated with 2 μM of rotenone as inhibitor of complex I of the mitochondrial respiratory chain and then treated with 10 μM of PPARα agonist. Following, this ROS generation was tested after 24 h treatment. It was apparent that the inactivation of complex I of the mitochondrial respiratory chain with rotenone made no significant changes in the amount of PPARα-induced ROS production in comparison to embryoid bodyies treated with only PPARα agonist (Fig. 23). This result confirms that the increased ROS generation observed in PPARα treated embryoid bodies is independent of mitochondria and involves the NADPH oxidase.

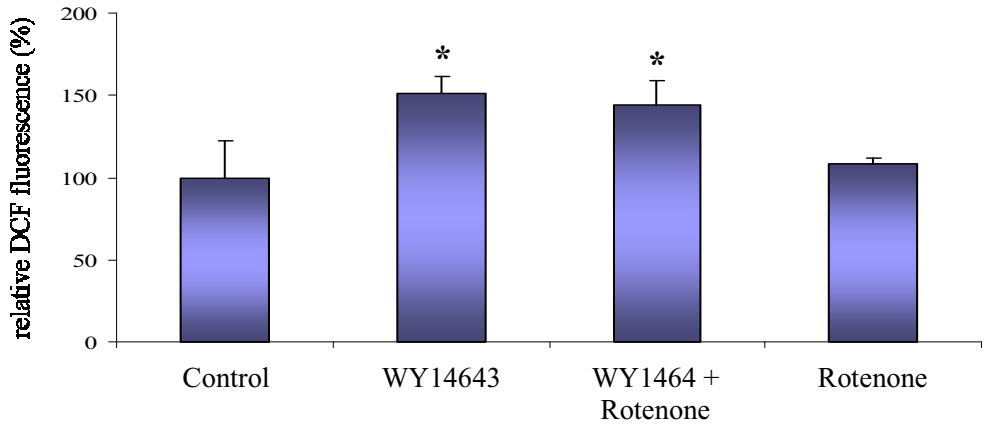


Figure 23: *Role of mitochondrial complex I inhibition by rotenone (2 μM) on PPARα-induced ROS generation.* Note that inhibition of the respiratory chain by rotenone failed to blunt the increase of ROS generation achieved with WY14643. * $p < 0.05$, statistically significant as compared to untreated control ($n = 3$).

3-14- INHIBITORY EFFECT OF FREE RADICAL SCAVENGERS ON PPARα-INDUCED CARDIOMYOGENESIS

According to our working hypothesis PPARα promotes cardiomyogenesis by utilizing a ROS-dependent mechanism. Hence free radical scavenger treatment should lead to the suppression of cardiomyogenesis. To test this notion, 4-day-old differentiating embryoid bodies were plated into Petri perm dishes and after 2 h pre-incubation with 100 μM trolox and 100 μM NMPG treated with PPARα agonist. This treatment schedule was repeated daily till day 10 of cell culture followed by daily recording of the number of spontaneously contracting foci of cardiomyocytes. The differentiated embryoid bodies on Petri perm dishes were fixed at day 10 of cell culture and stained by α-actinin for analyzing the extension of contracting area under the different experimental conditions.

As seen in figure 24, treatment with free radical scavengers (trolox and NMPG) totally abolished the effect of PPARα agonist on cardiomyocyte differentiation in comparison to untreated control. A remarkable decrease in number (Fig. 24A) and area (Fig. 24B) of contracting foci was observed after co-incubation of the embryoid bodies with free radical scavengers and PPARα agonist.

This finding confirmed the notion that PPARα-mediated ROS are involved in the process of cardiomyocyte differentiation and is caused by PPARα activation with its ligands.

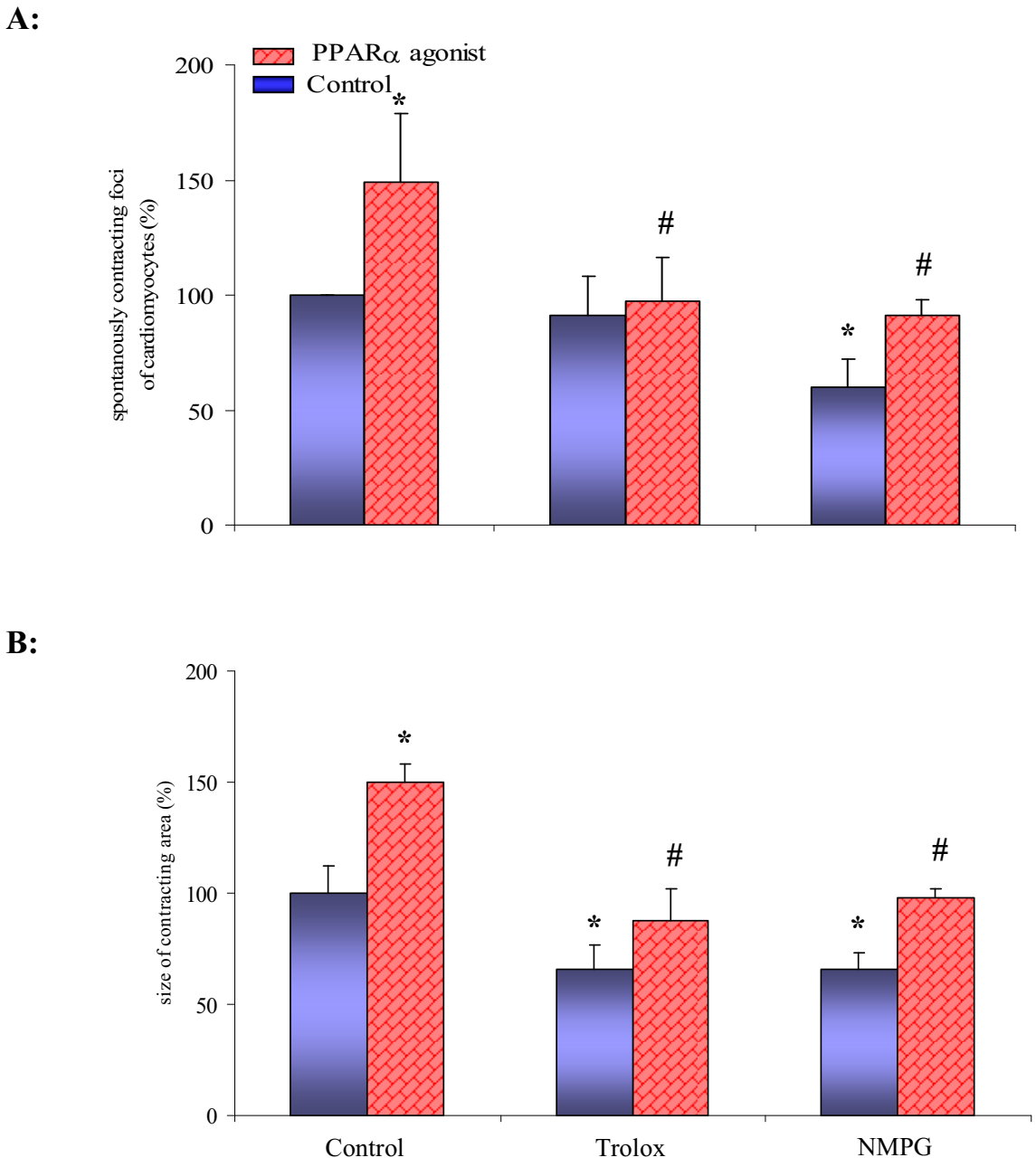


Figure 24: *Inhibitory effect of free radical scavengers (100 μM) on PPARα-induced cardiomyogenesis.* Note the reduction in number (A) and area (B) of contracting foci following pre-incubation with free radical scavengers in comparison to the sample treated with WY14643 (10 μM) alone. * and # $p < 0.05$, statistically significant as compared to untreated control and PPARα agonist treatment, respectively ($n = 4$).

3-15- DOWN-REGULATION OF PPARα-INDUCED CARDIOMYOGENESIS BY INHIBITION OF NADPH OXIDASE ACTIVATION

In order to assess the involvement of NADPH oxidase in PPARα-induced cardiomyogenesis of mouse ES cells, 4-day-old embryoid bodies were plated in Petri perm dishes, and after 2 h pre-incubation with NADPH oxidase inhibitors (100 nM DPI and 10 μM apocynin) embryoid bodies were treated with WY14643. This treatment was repeated daily till day 10, and monitored on a day to day basis using a light microscope followed by recording the status of contracting beating foci per plate.

Treatment with NADPH oxidase inhibitors strongly abolished the effect of PPARα agonists on the number of differentiated contracting foci of mouse ES cells (Fig. 25). This means that NADPH oxidase is involved in the pathway of PPARα-mediated cardiomyocyte differentiation.

Taken together, our observations on the effect of free radical scavengers and NADPH oxidase inhibitors on PPARα-induced cardiomyogenesis suggest that PPARα agonists stimulate cardiomyocyte differentiation of ES cells via a ROS-dependent mechanism involving NADPH oxidase.

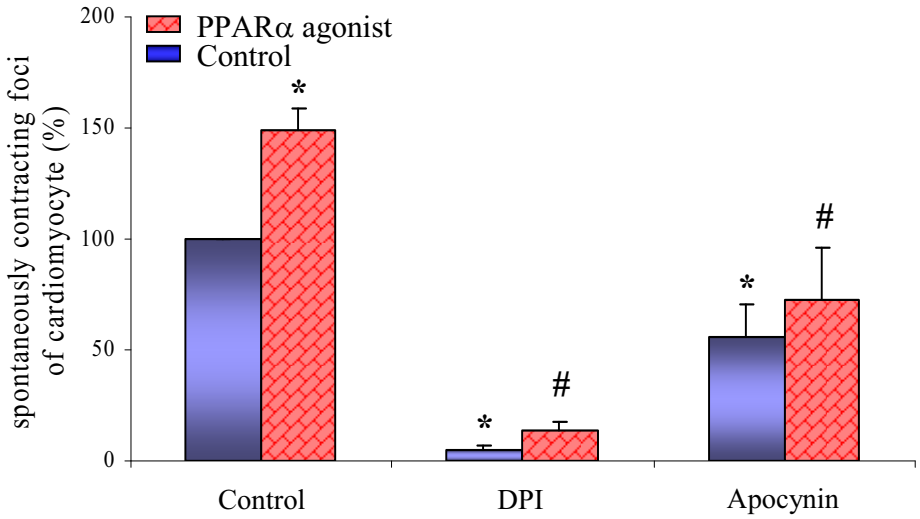


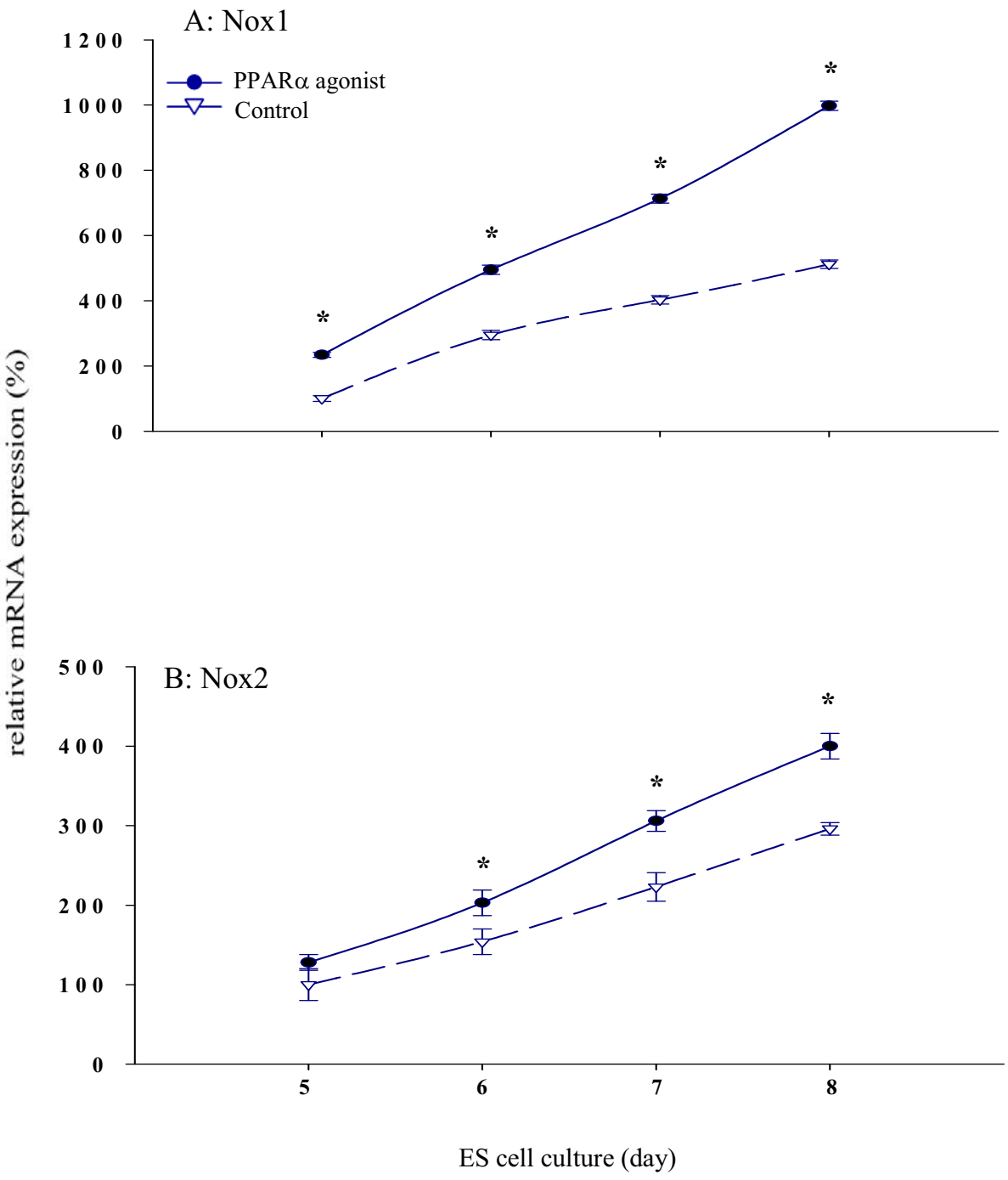
Figure 25: *Inhibition of PPARα-mediated enhancement of cardiomyogenesis upon NADPH oxidase inhibitor treatment.* Treatment with the NADPH oxidase inhibitors DPI (100 nM) and apocynin (10 µM) totally abolished the enhancement of cardiomyogenesis achieved with WY14643 (10 µM). * and # $p < 0.05$, statistically significant as compared to untreated control and PPARα agonist treatment, respectively ($n = 5$).

3-16- UP-REGULATION OF NADPH OXIDASE ISOFORMS UPON TREATMENT WITH WY14643

According to the findings of the present study, PPARα agonists stimulate cardiomyocyte differentiation in mouse ES cells by utilization of a NADPH oxidase-dependent ROS signaling pathway. Previously, it has been established that the mechanism of activation of NADPH oxidase-dependent ROS production depends on the nature of particular Nox isoforms involved in the formation of the active holoenzyme (*Byrne et al. 2003, Ago et al. 2004, Clempus et al. 2007*). Therefore it was imperative to test the mRNA expression of the most common defined isoforms of the gp91phox subunit. The mRNA expression of *Nox1*, *Nox2*, and *Nox4* isoforms was evaluated in the presence and absence of WY14643 treatment by quantitative real time RT-PCR. 4-day-old embryoid bodies were treated daily with 10 μM of PPARα agonist till day 8 of cell culture.

Day series observation indicated a progressive increase in mRNA levels of all three examined Nox isoforms upon PPARα treatment as compared to the untreated control. Figure 25 depicts the results of these day series experiments which were normalized with the mRNA levels for each gene in the untreated control at day 5 of cell culture. Comparing the mRNA levels of these isoforms, it became apparent that the PPARα agonist increased significantly mRNA expression of *Nox1* (Fig. 26A), *Nox2* (Fig. 26B), and *Nox4* (Fig. 26C) as compared to the untreated control group.

Taken together, this finding suggests that activation of PPARα induces NADPH oxidase expression presumably resulting in a long term ROS generation and induction of cardiomyogenesis.


A: Nox1
PPARα agonist
Control
1200
1000
800
600
400
200
0
B: Nox2
500
400
300
200
100
0
relative mRNA expression (%)
5
6
7
8
ES cell culture (day)

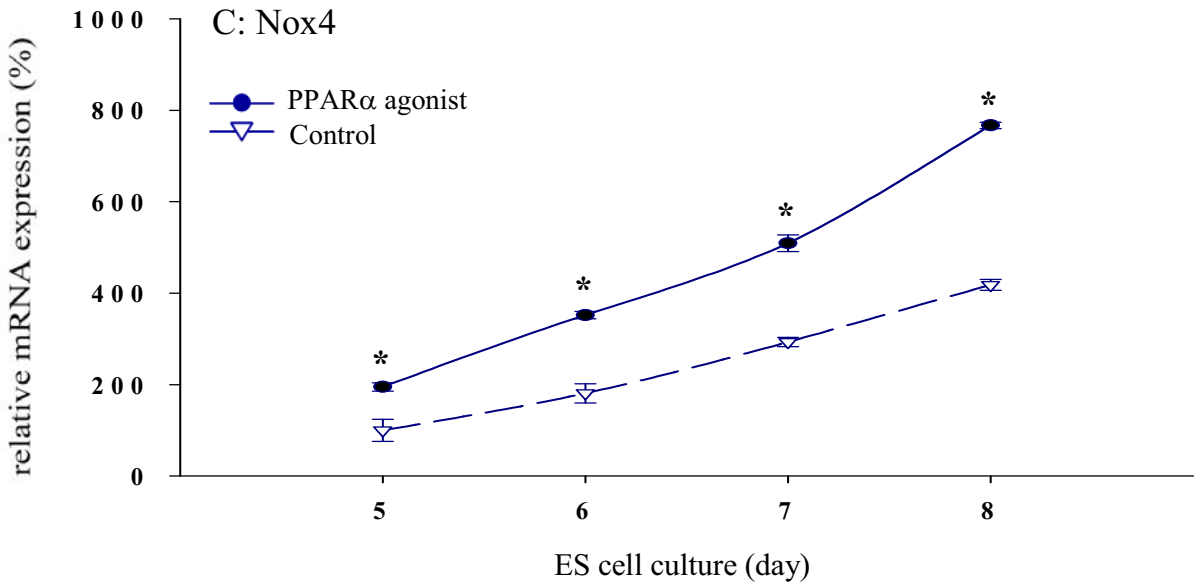


Figure 26: *Increase in mRNA expression of different isoforms of NADPH oxidase upon treatment with the PPARα agonist WY14643 (10 μM).* Note that the enhancement of *Nox1* and *Nox4* mRNA was increased as compared to *Nox2*. A: *Nox1*, B: *Nox2*, C: *Nox4*. * $p < 0.05$, statistically significant as compared to the untreated control ($n = 3$).

4- DISCUSSION

In the world, heart failure is the main causative disease for mortality. Therefore, scientists and clinicians today mainly focus on finding a solution to effectively treat these diseases. To date, various treatments exist to attenuate symptoms and ameliorate life quality of these patients. One of many therapies which give a promising treatment to these patients is cellular cardiomyoplasty (related to regenerative medicine). This novel remedy is a myocardial repairing technology that is based on cell (replacement) therapy. Many diverse types of cells have been examined as a source of this cell treatment. Among all tested, stem cells are a powerful source which gives new hope for future therapeutic approaches. ES cells, for reason of their strong potential to differentiate to different cell types and their unlimited self-renewal capacity are amongst the most promising stem cell types that may be used in future clinical applications.

In the past few years scientists have been trying to find ways to grow stem cells in cell culture and manipulate them to generate specific cell types so they can then be used to treat injury or disease. Some examples of potential treatments include replacing dopamine-producing cells in the brains of Parkinson's patients (*Takagi et al. 2005*), developing insulin-producing cells for type I diabetes (*Roche et al. 2007*), and repairing damaged heart muscle following a heart attack with cardiac muscle cells (*Van Laake wt al. 2006*).

The biggest hurdle in the way of cellular cardiomyoplasty using ES cells as a basis, is to obtain pure and sufficient amounts of ES cell-derived cardiomyocytes. To fulfill this goal, improvement of the knowledge and understanding of the pathways involved in differentiation of ES cell-derived cardiomyocytes is necessary and helpful.

The goal of the present study was to unravel signaling pathways of cardiomyogenesis from ES cells. The energy demands of the heart are satisfied through ATP generated by oxidative phosphorylation in the mitochondria, and the major energy substrate within the mitochondria is fatty acids which are catabolised via the β-oxidation pathway (*Lehman et al. 2000*). On the other hand, it is demonstrated that PPARα plays key roles in fatty acid metabolism and controls the expression of genes encoding nearly every step of the cellular fatty acid utilization pathway (*Desvergne et al. 1999, Fink 2006*). Consequently, over-production of ATP in contracting

cardiomyocytes and/or during differentiation is due to high-level mitochondrial fatty acid oxidation which is mediated by the activation of the PPARα system. In this respect, it has been proven that in the heart, PPARα shows a higher expression than its other two isoforms (*Bailey 2000*). Therefore, a hypothesis was raised that PPARα may be involved in the process of cardiomyocyte differentiation. To attain the goal of understanding how PPARα pathways are involved in ES cell-derived cardiomyogenesis, the mouse ES cell line CCE was employed and treated with WY14643, GW7647, or ciprofibrate to activate PPARα receptor and/or MK886 to inhibit the activity of PPARα.

It has been shown that the three isoforms of PPAR receptors are differentially expressed in numerous tissues originating from all three embryonic layers during rat development (*Braissant et al. 1998, Steinmetz et al. 2005*) as well as in the adult rat (*Braissant et al. 1996*). Also, Kliewer et al. have reported a similar observation that differential expression of PPARs occurs in different tissues during embryonic development of mice (*Kliewer et al. 1994*). No sufficient information is available about the expression level of the PPAR family during differentiation of ES cell-derived embryoid bodies in the literature. Recently Ding et al. reported expression levels of one member of the PPAR family, PPARα, on limited time points during differentiation of mouse ES cells (i.e. day 5, -8, -10, and -12 of cell culture). They showed that PPARα expression attains a maximum on day 10 of cell culture and then a decrease on day12 (*Ding et al. 2007*). In the current study, the mRNA expression level of all members of the PPAR family was assessed in mouse ES cells during a long time course of differentiation (from day 4 until day 20 of cell culture) which gives a good overview of the expression of PPARs during prolonged cell culture. Interestingly, PPARα expression significantly increased on day 6 of mouse ES cell-derived embryoid bodies which is also the starting time point for cardiomyogenesis (*Hescheler et al. 1999, Boheler et al. 2002, Wei et al. 2005*). The expression of PPARα abates after day 12 of cell culture and then increases again in the late stages of differentiation (day 18 and -20 of cell culture). This observation is in agreement with the findings of Ding et al. (*2007*). An increasing expression of PPARα during the time period of cardiomyogenesis in ES cells strongly suggests that a period of PPARα exposure may be critical for the development of ES cell-derived cardiomyogenesis. Previously, it has been shown that the expression of PPARα in developing cardiomyocytes at the starting point of their differentiation

induce the peroxisomal β-oxidation-encoding genes that are expressed in the progenitors of these cells before they differentiate (*Keller JM, et al. 1993*).

The present study showed that PPARβ follows a relatively similar expression pattern as PPARα during the period of measurement. Comparably to PPARα, PPARβ reaches a maximum expression on day 12 of cell culture, but the expression level is lower. Although, PPARγ expression levels significantly increase at day 6 in parallel with PPARα, it followed a different expression pattern as compared with PPARα during late stages of differentiation thus suggesting that PPARγ may be involved in signaling pathways different from cardiomyogenesis, e.g. in adipogenesis or macrophage differentiation (Tontonoz et al. 1998, Spiegelman 1997).

As shown in this study, treatment of embryoid bodies with PPARα agonist produced a noteworthy increase in the number and size of contracting foci in comparison to untreated embryoid bodies. In contrast the PPARα antagonist used (MK886) decreased the number and size of the beating foci in embryoid bodies. Furthermore, no difference in the effect of the PPARβ and -γ agonist (L-165,041 and GW1929, respectively) on cardiomyocyte differentiation from murine ES cells was observed in the present study. The stimulating effect of PPARα agonists on cardiomyogenesis of ES cells that was observed in the present study was comparable to a recent study of Ding et al. (*2007*). Ding and his colleagues employed another kind of murine ES cell line (D3 stem cell line) and differentiated them by the hanging drop method. Our findings, in parallel of the reports of Ding et al., strongly support the possibility that PPARα agonist treatment of ES cell-derived embryoid body results in stimulation of cardiomyogenesis.

Since cardiomyocyte differentiation is a consequence of the expression of cardiac genes, the mRNA expression of *ANP*, *MLC2a*, *MLC2v*, *MHCα*, *MHCβ*, and *cardiac α-actin* was analyzed by real time RT-PCR. It was shown that PPARα agonist increased the expression level of these genes during cardiomyocyte differentiation when compared with the untreated control. This finding is in agreement with a recent publication, which showed a similar result in augmenting the mRNA expression level of *MHCα* and *MLC2v* upon PPARα activation (*Ding et al. 2007*). Furthermore, it has been reported by Metzger et al. that *MHCβ* is predominantly expressed in ES cell-derived cardiomyocytes, and after prolonged contraction, *MHCα* expression levels increase, and override *MHCβ* expression (*Metzger et al. 1995*). Metzger et al. suggested that ES cell cardiac myocyte differentiation follows the normal developmental program of murine

cardiomyogenesis. Surprisingly, in the current project *MHCβ* displayed a higher expression level than *MHCα* upon treatment with PPARα agonist on day 8 of cell culture, which is within the period of spontaneous contractile activity in the ES cells. Generally, this finding confirms that there is a strong correlation between PPARα activation and fetal cardiac gene expression and that PPARα-induced cardiomyogenesis follows the normal developmental program of mouse cardiomyocyte differentiation.

It has been clearly demonstrated that transcription of cardiac embryonic genes during cardiomyogenesis follows the activation of cardiogenic transcription factors (Skerjanc et al. 1998, Watt et al. 2004). In the present study, assessment of the expression profiles of transcription factors which are involved in differentiation of cardiomyocytes *in vivo* (i.e. *GATA4*, *Nkx2.5*, *Mef2c*, and *DTEF1*), showed that activation of PPARα apparently induces the cardiogenic transcriptional program in mouse ES cell-derived embryoid bodies.

A survey of literature is indicative of the important role of Mef2c in early cardiovascular development (*Lin et al. 1997, Lin et al. 1998*), and that it also plays a pivotal role in morphogenesis and myogenesis of skeletal, cardiac, and smooth muscle cells (*Zheng et al. 2002*). This crucial cardiogenic transcription factor is responsible for the activation of several cardiac specific embryonic genes (*Harvey 1999*). Likewise, it has been shown that functional Nkx2.5 is essential for *Mef2c* expression in mammalian cardiogenesis (*Skerjanc et al. 1998*). On the other hand, it has been previously reported that Nkx2.5 appears to play a unique function in cardiac development and in the regulation of the expression of some cardiac genes (*Zheng et al. 2003*), but for its optimal activity requires physical interaction with GATA4 (*Lien et al. 1999*). Nkx2.5 as a co-activator of GATA4 acts via association with the c-terminal zinc finger of GATA4, and thus elicits the transcription of cardiac-restricted genes (*Lee et al. 1998, Durocher et al. 1997*). It has been shown that GATA factors are important regulators in the heart, and GATA4 promotes cardiac muscle development and regulates the expression of several cardiac specific genes including *MHCα*, *cardiac troponin C* and *troponin1*, *ANF*, and *ANP* (*Ventura et al. 2000, Molkentin et al. 1997*). Furthermore, *GATA4* is presented as an early marker of the cardiac cell lineage at various stages of cardiogenesis (*Watt et al. 2004, Molkentin et al. 1997*). In parallel, Grepin et al. previously indicated a requirement for *GATA4* in the differentiation of cardiac restricted cells to beating cardiomyocytes in embryonic carcinoma cells (*Grepin et al. 1997*). Moreover, it has been shown that GATA activity is required for differentiation of the

myocardium rather than its induction in embryos (*Peterkin et al. 2007*). Finally, it has been demonstrated that *GATA4* and *Nkx2.5* are essential for cardiomyogenesis, and that over-expression of either one alone cannot initiate cardiogenesis, but enhances recruitment and differentiation of committed precursors, indicating that these proteins may be mutual cofactors (*Phiel et al. 2001*). This information gives more meaning to the apparent observation in the present study, that *GATA4* and *Nkx2.5* showed higher expression levels than the other cardiogenic transcription factors upon treatment with the PPARα agonist WY14643. In summary, increased spontaneous beating activity of PPARα-expressing embryoid bodies and elevated expression of cardiogenic transcription factors and cardiac genes which were observed in the present study confirm an inductive effect of PPARα-mediated signaling pathways on ES cell-derived cardiomyogenesis.

It has been previously reported that ligand-activated PPARα could drive its own transcription (*Pineda et al. 2002, Gebel et al. 1992*). A similar effect was seen in the present study where increased mRNA expression of *PPARα* after treatment of mouse embryoid bodies with PPARα agonist was observed, confirming that ligand-activated PPARα enhances its own expression. Furthermore, it was demonstrated that ligand-activated PPARα increases its own protein activity (*Pineda et al. 2002, Gebel et al. 1992*). Our present data clearly indicate augmentation of the PPARα phosphorylation on administration of PPARα agonist which indicates enhancement of the activation of PPARα in mouse embryoid bodies.

ROS are generally thought of as being broadly reactive, mutagenic and cytotoxic entities that are produced largely as an accidental byproduct of aerobic metabolism. While such unintentional production of ROS clearly occurs, it is also known that ROS can be produced in a regulated manner and serve useful biological purposes. Recently published reviews of Sauer et al. give a new perspective on specific roles of ROS during cell differentiation (*Sauer et al. 2005, Sauer et al. 2001*). It has been previously reported by different research groups that low concentrations of ROS could function as intracellular second messengers and be utilized within signaling cascades that result in the transcription of genes directing differentiation toward the cardiomyogenic as well as endothelial cell lineage (*Sauer et al. 2001, Sauer et al. 2004, Sauer et al. 2000, Li et al. 2006, Laloi et al. 2004, Griendling et al. 2000*). Furthermore, it has been shown that ES cells endogenously generate ROS during differentiation toward cardiac cells (*Buggisch et*

al. 2007), and that exogenous treatment of embryoid bodies with low concentrations of ROS stimulates cardiomyogenesis (*Sauer et al. 1999, Sauer et al. 2000, Li et al. 2006, Buggisch et al. 2007, Ateghang et al. 2006*). In another study, it has been reported that the initiation of the cardiovascular differentiation program of ES cells is largely dependent on ROS sensitive MAPK signaling cascades (*Schmelter et al. 2006*), which implies that ROS generated at a suitable concentration exert a role as signaling molecules in cardiogenic cell commitment. In addition, it was earlier demonstrated that ROS are involved in the expression of several cardiac genes (*Morel et al. 1999, Puceat et al. 2003*). These articles motivated us to evaluate the involvement of ROS in PPARα-induced cardiomyocyte differentiation. Our data demonstrate that all three different PPARα agonists used in the study strongly augmented ROS generation in embryoid bodies whereas PPARα antagonist treatment significantly reduced ROS production in comparison to untreated control. Notably, ROS generation in embryoid bodies was induced at times when the cells were still undifferentiated (i.e. at day 4 of cell culture) while differentiation of the cardiovascular cell lineage occurs between day 6 and day 9 of cell culture (*Hescheler et al., 1999, Boheler et al. 2002*). This observation suggests that PPARα-induced cardiomyocyte differentiation of mouse ES cells is dependent on ROS generation. These findings are consistent with other reports in the literature implicating ROS involvement in cardiomyogenesis of ES cells (*Sauer et al. 2004, Li et al. 2006, Buggisch et al. 2007*). In the current study the effects of two known PPARβ and -γ agonist on ROS generation were also evaluated. Cultures treated with L-165,041 and GW1929 showed no enhanced ROS production when compared with cultures treated with PPARα agonist.

In contrast to the data of the present study, Cabrero et al. have reported that enhanced generation of ROS down-regulates activation of PPARα and in the following, transcription of target genes of PPARα in the skeletal muscle cells (*Cabrero et al 2002*). The interesting point in this article is that the effect of ROS on PPARα expression level was dose-dependent. In this respect PPARα expression is down-regulated by high concentrations of ROS generated in skeletal muscle cells, but a lesser amount of ROS production leads to augmentation of the PPARα expression level in those cells. Although, this article is contrary to the observed results in the present study, it demonstrates that the concentration of ROS generated in cells plays a differential role during physiological and patho-physiological conditions.

The effect of PPARα agonist on ROS generation was abolished in the presence of free radical scavengers (Trolox and NMPG). Moreover, both Trolox and NMPG significantly reduced PPARα-induced cardiomyogenesis of mouse ES cells. These findings confirm the involvement of ROS in the PPARα pathway of cardiomyogenesis of murine ES cells. Having investigated ROS generation upon PPARα activation an assessment of the sources of ROS generation was undertaken. This could be either NADPH oxidase or mitochondria which are the two main sources of ROS in the cells, and hence these were assessed in the present study.

The mechanism of the receptor-mediated generation of ROS has been studied extensively in phagocytic cells, wherein $O_2^{\bullet -}$ (and thus H_2O_2) is produced via the reduction of O_2 by an enzyme system named NADPH oxidase (Rada et al. 2004). The mechanism of ROS generation in non-phagocytic cells is mediated by the non-phagocytic NADPH oxidase, which is specific to these cells. However, the physiological function of ROS in non-phagocytic cells remains largely unclear.

Increasing scientific reports have demonstrated that embryoid bodies grown from ES cells actively generate ROS presumably through the activity of ROS-generating NADPH oxidase (*Li et al. 2006*). It also has been reported that a non-phagocytic NADPH oxidase is involved in the development of left ventricular hypertrophy and heart failure progression (*Li et al. 2002*), supporting the notion that activation of the non-phagocytic NADPH oxidase in response to neurohormones mediates cardiac myocyte hypertrophy. During cardiac hypertrophy parts of the genetic program of fetal life are re-expressed, which implies that fetal signaling pathways are re-activated. This may also hold true for signaling pathways involving ROS in the regulation of cardiac growth and differentiation. Furthermore, it has been demonstrated by us that NADPH oxidase-generated ROS are involved in cardiomyogenesis and angiogenesis of ES cells (*Sauer et al. 2004, Schmelter et al. 2006, Ateghang et al. 2006, Buggisch et al. 2007*). Since NADPH oxidase inhibitors (DPI and Apocynin) strongly decreased PPARα-induced ROS generation in embryoid bodies in the presence of PPARα agonists, the results of the present project are also consistent with the literature. Interestingly, both NADPH oxidase inhibitor used in this study significantly abolished the PPARα-induced cardiomyocyte differentiation of mouse ES cells observed in the presence of PPARα agonist. Moreover, these data are in agreement with a report from Teissier et al. who found that synthetic PPAR agonists induce the production of ROS in a

PPARα-dependent manner by inducing NADPH oxidase, a key enzyme in oxidative stress (*Teissier et al. 2004*). Previously, they also offered an intriguing view that ROS interact with LDL to activate PPARα and subsequently limit inflammation, as indicated by PPAR-dependent repression of inducible nitric oxide synthase (iNOS) gene transcription, which was earlier reported by another research group (*Cai et al. 2003*).

It has been demonstrated that *Nox1* and *Nox4* are up-regulated during differentiation of vascular smooth muscle cells (*Clempus et al. 2007*). In the heart of the human fetus, *Nox4* has higher expression than *Nox1* (*Cheng et al. 2001*). Furthermore, it has been shown that *Nox4* was highly expressed in ES cells whereas *Nox1* was absent (*Li et al 2006*). On the other hand, it was previously demonstrated by others that *Nox2* and *Nox4* have higher expression level in cardiomyocytes (*Murdoch et al. 2006*). In addition, it has been recently published that both *Nox1* and *Nox4* were up-regulated under different conditions during cardiomyogenesis of mouse ES cells (*Ateghang et al. 2006, Schmelter et al. 2006*). Increase in ROS generation either by mechanical stress and/or exogenous addition of H_2O_2 resulted in upregulation of *Nox4* and *Nox1*, which were presumably involved in ES cell-derived cardiomyocyte differentiation (*Ateghang et al. 2006, Schmelter et al. 2006*). The findings of the current thesis revealed that the gp91phox isoforms, *Nox1* and *Nox4*, were up-regulated during PPARα-induced cardiomyocyte differentiation from ES cells, but with a higher level of expression of *Nox1*. It has been reported in the literature that *Nox2* and *Nox4* are specifically expressed in adult cardiomyocytes (*Cheng et al. 2001, Byrne et al. 2003, Ago et al. 2004*). Furthermore, the Nox2:Nox4 mRNA expression ratio by real time PCR was reported to be about 1:2 in cardiomyocytes (*Byrne et al. 2003*), which was comparable to the ES cell-derived cardiomyocytes upon treatment with PPARα agonist observed in the present study. Usually the isoforms of gp91phox are differentially expressed during differentiation into different tissues and may also depend on the cell type (*Cave et al. 2005*). The data of the present study suggest that the type of stimulation for induction of cardiomyogenesis may influence the expression level of specific isoform of gp91phox. For instance, it was reported that *Nox4* had higher expression than *Nox1* under the effect of mechanical stress during ES cell-derived cardiomyogenesis (*Schmelter et al. 2006*), while the opposite, *Nox1* higher than *Nox4*, was observed under the effect of exogenous ROS treatment of embryoid bodies (*Ateghang et al. 2006, Li et al. 2006*), and also under the effect of PPARα agonist treatment in the present study. Although increasing evidence indicates a potential role of

NOX enzymes and ROS in differentiation processes, the downstream pathways mediating this differentiation have not yet been elucidated. It may be assumed that cardiovascular differentiation of ES cells with the onset of beating activity causes the energy demand of the growing tissue to increase, thus initiating a switch from glycolytic ATP generation towards fatty acid oxidation. This switch in energy metabolism consequently requires increased expression and function of PPARs which may - besides their function in stimulating fatty acid metabolism - increase ROS generation thereby inducing cardiomyogenesis and cardiac cell proliferation of ES cells.

Mitochondria are the other main source of intracellular reactive oxygen species (ROS), and are particularly vulnerable to oxidative stress. Mitochondrial number and functional capacity are dynamically regulated in accordance with cardiac energy demands during developmental stages and in response to diverse physiological conditions (*Attardi et al 1988*). Since cardiomyocyte differentiation of ES cells *in vitro* faithfully replicates the process *in vivo*, and these cardiomyocytes display properties similar to those observed *in vivo* or in primary cultures, there must be an increase in mitochondrial number with the emergence of beating clusters. In addition to the activation of genes encoding contractile proteins, cardiac differentiation is accompanied by a balancing of metabolism which is related to enhancement of ATP production. It has been demonstrated that PPARα, PGC-1, and NRF1 co-activate the expression of mitochondrial transcription factor-A (*Koulmann et al. 2006, Kraft et al. 2006*), which is the only known regulator of mitochondrial replication and transcription. Therefore, it was postulated that PPARα activation stimulates expression of *mtTFA* during cardiomyogenesis of mouse ES cells thus leading to enhancement of the mitochondrial content, which is necessary in the differentiation period in order to increase ATP production. The mitochondrial content was evaluated according to reported methods in the literature (*Bogacka et al. 2005a, Bogacka et al. 2005b, He et al. 2002*). The result showed that the mitochondrial DNA copy number of PPARα-treated embryoid bodies was significantly higher than in untreated embryoid bodies. A positive relationship was observed between mitochondrial content and the time point of ES cell-derived cardiomyogenesis upon treatment with PPARα agonists. Thus, our data suggest that PPARα activation enhances the number of mitochondria in embryoid bodies, which it is essential for the increased production of ATP during cardiomyocyte differentiation of mouse ES cells.

According to numerous articles, the mitochondrion is one of the major ROS producers within cells (*Balaban et al. 2005, Richter et al. 1995*). Hence, it was proposed that a portion of

PPARα-induced ROS generated could be derived from mitochondria. Surprisingly, the mitochondrial complex I inhibitor Rotenone had no effect on the PPARα-induced ROS generation in the presence of PPARα agonist. This suggests that the mitochondrial respiratory chain is approximately not a contributor to the ROS produced upon PPARα activation. Furthermore, this finding in parallel with the recent report of Spitkovsky et al. suggests that ES cells need normal mitochondrial function for differentiation toward beating cardiomyocytes. Spitkovsky et al. have used Antimycin A (a mitochondrial complex III inhibitor) to inhibit the function of the mitochondrial respiratory chain in ES cell. They did not analyze the amount of ROS generation after treatment, but have shown that ES cell-derived cardiomyogenesis is totally abolished upon treatment with Antimycin A. Therefore, they have suggested that normal mitochondrial function is a prerequisite for ES cell-derived cardiac differentiation (*Spitkovsky et al. 2004*).

In conclusion, the present study uncovers the underlying mechanisms that result in the inducible effect of PPARα agonists on cardiomyocyte differentiation and has deciphered why PPARα increased cardiomyogenesis. During the initiative stage, PPARα ligands activate PPARα, which then induces and increases mRNA expression of NADPH oxidase with the consequence of intracellular ROS generation. These generated ROS could be a potential inducer of PPARα through a second signal transduction pathway, which has yet to be elucidated. Furthermore, this study clarifies a biological role for NOX-generated ROS in mediating PPARα agonist-induced cardiomyocyte differentiation in mouse ES cells. In parallel, the study also showed that the PPARα signaling cascade triggers cardiomyogenesis and hand in hand increases mitochondrial content to supplement the increased energy needs generated due to cardiomyogenesis. The findings of the present study provide a new insight into PPARα induced cardiac differentiation of ES cells and may ultimately contribute to the future therapeutic use of ES cells.

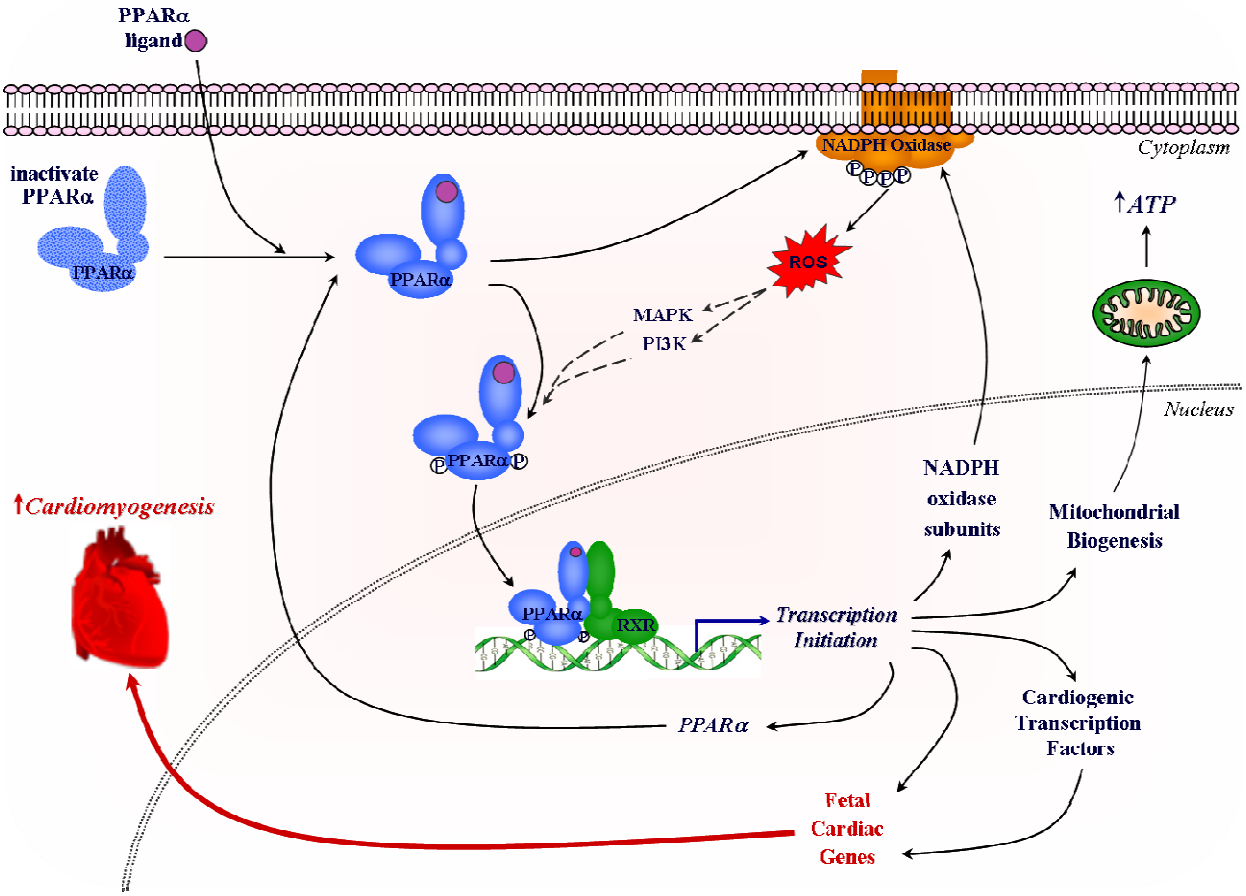


Figure 27: *A pictorial representation of the findings from this study.* PPARα ligand on attachment to PPARα results in the activation of NADPH oxidase and subsequent ROS production via a non-genomic pathway. These PPARα-induced ROS in turn cause activation of PPARα in a feed forward cycle. Upon this ROS mediated-activation, the genome-mediated action of PPARα comes into play. Activated PPARα then translocates to the nucleus and induces transcription of target genes. NADPH oxidase subunits are one of the PPARα target genes that are transcribed which then leads to increased activity of NADPH oxidase and ROS production. In parallel, activated PPARα increases self-expression giving a positive feedback. Furthermore, cardiogenic transcription factors and fetal cardiac genes are transcribed with activated PPARα, which finally results in cardiomyogenesis. Moreover, activated PPARα in a cascade of events induces mitochondrial biogenesis, thus aiding the cells to cope with the increasing energy demands during the process of cardiomyocyte differentiation.

4-1- STATEMENT ON THE IMPACT OF THE STUDY

In the world, heart failure is the main cause of mortality. Therefore, scientists and clinicians today mainly focus on finding a solution to effectively treat these diseases. To attenuate the symptoms and ameliorate the life quality of the patients, various treatments are available. One of the numerous therapies, which seem to be promising, is cellular cardiomyoplasty. This novel remedy is a myocardium repairing technology that is based on cell replacement. Diverse cell types have been examined as a source for this cell treatment. ES cells, due to their strong potential to differentiate into various cell types therefore are of major interest.

The biggest hurdle in cellular cardiomyoplasty using embryonic stem cells, is to obtain pure and sufficient amounts of ES-derived cardiomyocytes. To fulfill this goal, a thorough understanding of the pathways involved in the differentiation of ES-derived cardiomyocytes is a pre-requisite.

This article deals with a signaling cascade to increase the amounts of ES cell-derived cardiomyocytes in culture by focussing on the involvement of the PPARα pathway. Although the PPARs are best known as transcriptional regulators of lipid and glucose metabolism, little is known about the significance of PPARα in early cardiac development especially during the differentiation of cardiomyocytes. The present article focusses on linking PPARα activity to the differentiation of ES cell-derived cardiomyocytes by elucidating the mechanisms of cardiac cell differentiation controlled by this key nuclear receptor. This could provide scientists with a potent tool for the production of mass cultures of proliferating cardiac cells, which could then be utilized for cardiac regeneration therapy.

5- SUMMARY

5-1- SUMMARY

Peroxisome proliferator-activated receptors (PPARα, -β and -γ) are nuclear receptors involved in transcriptional regulation of lipid and energy metabolism. Since the energy demand increases when cardiac progenitor cells develop rhythmic contractile activity, we hypothesized that PPAR activation may play a critical role during cardiomyogenesis of embryonic stem (ES) cells.

We show that ES cells express *PPARα*, *-β*, and *-γ* mRNA during differentiation of ES cells towards cardiac cells. Treatment of embryoid bodies with PPARα agonists (WY14643, GW7647 and ciprofibrate) significantly increased cardiomyogenesis and expression of the cardiac genes *ANP*, *MHCα*, *MHCβ*, *MLC2a*, *MLC2v*, and *cardiac α-actin*. Furthermore, WY14643 increased *PPARα* gene expression and the expression of the cardiogenic transcription factors *GATA4*, *Nkx2.5*, *DTEF1* and *MEF2c*. In contrast, the PPARα antagonist MK886 decreased cardiomyogenesis, whereas the PPARβ agonist L-165,041 as well as the PPARγ agonist GW1929 were without effects. Treatment with PPARα- but not PPARβ, and PPARγ agonists and MK886 resulted in generation of reactive oxygen species (ROS), which was inhibited in the presence of the NADPH oxidase inhibitors diphenylen iodonium (DPI) and apocynin and the free radical scavengers vitamin E and N-(2-mercapto-propionyl)-glycine (NMPG), whereas the mitochondrial complex I inhibitor rotenone was without effects. The effect of PPARα agonists on cardiomyogenesis of ES cells was abolished upon preincubation with free radical scavengers and NADPH oxidase inhibitors, indicating involvement of ROS in PPARα-mediated cardiac differentiation.

In summary our data indicate, that stimulation of PPARα but not PPARβ and -γ enhances cardiomyogenesis in ES cells using a pathway that involves ROS and NADPH oxidase activity.

5-2- ZUSAMMENFASSUNG

Peroxisomen-Proliferator-aktivierte Rezeptoren (PPARα, -β, und -γ) sind nukleäre Rezeptoren, die an der Regulation des Fettstoffwechsels und des Energiehaushalts beteiligt sind. Da die rhythmische kontraktile Aktivität der Herz-Vorläuferzellen im Laufe der Entwicklung einen gesteigerten Energieverbrauch voraussetzt, liegt die Vermutung nahe, dass PPAR eine entscheidende Rolle in der Kardiomyogenese spielen.

In dieser Arbeit konnte gezeigt werden, dass während des Differenzierungsprozesses embryonaler Stammzellen *PPARα*, *-β*, und *-γ* mRNA nachweisbar ist. Eine Behandlung der aus Stammzellen gewonnenen embryoid bodies mit den PPAR Agonisten WY14643, GW7647 und Ciprofibrat führte zu einer signifikant gesteigerten Kardiomyogenese, sowie zu einem signifikanten Anstieg der Genexpression kardialer Marker wie *ANP*, *MHCα*, *MHCβ*, *MLC2a*, *MLC2v* und *kardiales α-Aktin*. Desweiteren erhöht WY14643 die PPARα Genexpression, sowie die Expression der kardialen Transkriptionsfaktoren *GATA4*, *Nkx2.5*, *DTEF1* und *MEF2c*. Im Gegensatz dazu reduzierte der PPARα Antagonist MK886 die Differenzierung von Kardiomyozyten, während sowohl der PPARβ Agonist L-165041 als auch der PPARγ Agonist GW1929 ohne Effekt blieben. PPARα Agonisten und der Antagonist MK886, jedoch nicht die PPARβ und -γ Agonisten, führten zur Entstehung von reaktiven Sauerstoffspezies (ROS), die in Anwesenheit von NADPH Oxidase Inhibitoren Diphenylen iodonium (DPI) und Apocynin, sowie der freien Radikalfänger Vitamin E und N-(2-mercapto-propionyl)-Glycerin (NMPG) in ihrer Bildung gehemmt wurden. Rotenon, ein Inhibitor des mitochondrialen Komplexes I, zeigte dagegen keine Beeinflussung der ROS. Das Aufheben des Effektes der PPARα Agonisten auf die Kardiomyogenese mittels freier Radikalfänger und NADPH Oxidase Inhibitoren lässt auf eine Beteiligung der ROS in der PPARα-vermittelten kardialen Differenzierung schließen.

Zusammenfassend kann gesagt werden, dass die Aktivierung von PPARα, jedoch nicht PPARβ und PPARγ, eine gesteigerte Kardiomyogenese zur Folge hat, welche über einen ROS und NADPH Oxidase abhängigen Signalweg induziert wird.

6- LITERATURE

1- Adams M, Reginato MJ, Shao D, et al. (1997). "Transcriptional activation by peroxisome proliferator-activated receptor gamma is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site". J. Biol. Chem.; 272: 5128 - 5132.

2- Ago T, Kitazono T, Ooboshi H, et al. (2004). "Nox4 as the major catalytic component of an endothelial NADPH oxidase". Circulation; 109: 227 – 233.

3- Alcolea M.P, Colom B, Llado I, et al. (2006). "Mitochondrial transcription factor A is increased in rat embryo during planetation and associated with mitochondrial differentiation". Cell. Physiol. Biochem.; 17: 79 - 88.

4- Alvares K, Carrillo A, Yuan PM, et al. (1990). "Protein identification of cytosolic peroxisome proliferator binding protein as a member of the heat shock protein HSP70 family". Proc. Natl. Acad. Sci. USA; 87: 5293 - 5297.

5- Amos WB, White JG (2003). "How the Confocal Laser Scanning Microscope entered Biological Research". Biol. Cell; 95: 335 - 342.

6- Amos WB, White JG, Fordham M (1987). "Use of Confocal Imaging in the Study of Biological Structures". Appl. Optics; 26: 3239 - 3243.

7- Anderson S, Bankier At, Barell BG, et al. (1981). "Sequence and organization of the human mitochondrial genome". Nature; 290: 457 - 465.

8- Andreyev AY, Kushnareva YE, Starkov AA (2005). "Mitochondrial metabolism of reactive oxygen species". Biochemistry; 70: 200 – 214.

9- Anneren C, Cowan CA, Melton DA (2004). "The Src family of tyrosine kinases is important for embryonic stem cell self-renewal". J. Biol. Chem.; 279: 31590 - 31598.

10- Ateghang B, Wartenberg M, Gassmann M, Sauer H (2006). “Regulation of cardiotrophin1 expression in mouse embryonic stem cells by HIF1a and intracellular reactive oxygen species”. J. Cell Sci.; 199: 1043 - 1052.

11- Attardi G, Schatz G (1988). “Biogenesis of mitochondria”. Annu. Rev. Cell Biol.; 4: 289 - 333.

12- Bailey DB (2000). “Peroxisome proliferator activated receptors in the cardiovascular system”. Br. J. Pharma.; 129: 823 - 834.

13- Balaban R.S, Nemoto S, Finkel T (2005). “Mitochondria, oxidants, and aging”. Cell; 120: 483 - 495.

14- Berger J, Moller DE (2002). “The mechanisms of action of PPARs”. Annu. Rev. Med.; 53: 409 - 435.

15- Bogacka I, Ukropcova B, McNeil M, et al. (2005a). “Structural and functional consequences of mitochondrial biogenesis in human adipocytes in vitro”. J. Clin. Endocr. Metab.; 90: 6650 - 6656.

16- Bogacka I, Xie H, Bray GA, Smith SR (2005b). “Pioglitazone induces mitochondrial biogenesis in human suncutaneous adipose tissue in vivo”. Diabetes; 54: 1392 - 1399.

17- Boheler KR, Czyz J, Tweedie D, et al. (2002). “Differentiation of pluripotent embryonic stem cells into cardiomyocytes”. Circ. Res.; 91: 189 - 201.

18- Bokoch GM (1994). “Regulation of the human neutrophil NADPH oxidase by the Rac GTP-binding proteins”. Curr. Opin. Cell. Biol.; 6: 212 - 218.

19- Boveris A, Chance B (1973). “The mitochondrial generation of hydrogen peroxide”. Biochem. J.; 134: 707 - 716.

20- Braissant O, Foufelle F, Scotto C, et al. (1996). “Differential expression of Peroxisome proliferator activated receptors: Tissue distribution of PPARα, -β, and –γ in the adult rat”. Endocrinology; 137: 354 - 366.

21- Braissant O, Wahli W (1998). “Differential expression of Peroxisome proliferator activated recetor-alpha, -beta, and –gamma during rat embryonic development”. Endocrinology; 139: 2748 - 2754.

22- Buggisch M, Ateghang B, Ruhe C, et al. (2007). “Stimulation of ES-cell-derived cardiomyogenesis and neonatal cardiac cell proliferation by reactive oxygen species and NADPH oxidase”. J. Cell. Sci.; 120: 885 - 894.

23- Byrne JA, Grieve DJ, Bendall JK, et al. (2003). “Contrasting roles of NADPH oxidase isoforms in pressure overload versus AngII-induced cardiac hypertrophy”. Circ. Res.; 93: 802 - 805.

24- Cabrero A, Alegret M, Sanchez RM, et al. (2002). “Increased reactive oxygen species production down-regulates Peroxisome proliferator activated recetor-α pathway in C2C12 skeletal muscle cells”. J. Biol. Chem.; 277: 10100 - 10107.

25- Cai H, Griendling KK, Harrison DG (2003). “The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases”. Trends Pharmacol. Sci.; 24: 471- 478.

26- Camp HS, Tafuri SR (1997). “Regulation of peroxisome proliferatoractivated receptor gamma activity by mitogen-activated protein kinase”. J. Biol. Chem.; 272: 10811 - 10816.

27- Cave A, Grieve D, Johar S, et al. (2005). “NADPH oxidase-derived reactive oxygen species in cardiac pathophysiology”. Phil. Trans. R. Soc. B.; 360: 2327 - 2334.

28- Chen Q, Vazquez E.J, Moghadda S, et al. (2003). “Production of reactive oxygen species by mitochondria: Central role of comlexIII”. J. Biol. Chem.; 278: 36027 - 36031.

29- Cheng G, Cao Z, Xu X, et al. (2001). “Homologs of gp91phox: Cloning and tissue expression of Nox2, Nox4, and Nox5”. Gene; 269: 131 - 140.

30- Chinetti G, Griglio S, Antonucci M, et al. (1998). “Activation of Peroxisome proliferator activated receptor-α and -γ induces apoptosis of human monocyte-derived macrophage”. J. Biol. Chem.; 273: 25573 - 25580.

31- Clempus RE, Sorescu D, Dikalova AE, et al. (2007). “Nox4 is required for maintenance of the differentiated vascular smooth muscle cell phenotype”. Arterioscler. Thromb. Vasc. Biol.; 27: 42 - 48.

32- Conner DA (2000). “Mouse embryo fibroblast (MEF) feeder cell preparation”. Curr. Protocol Mol. Biol.; suppl. 51: 23.2.1 – 23.2.7.

33- Coons AH (1954). “Labelled antigen and antibodies”. Annu. Rev. Microbiol.; 8: 333 - 352.

34- Depre C, Shipley GL, Chen W, et al (1998). “Unloaded heart in vivo replicates fetal gene expression of cardiac hypertrophy”. Nat. Med.; 4: 1269 - 1275.

35- Desbaillets I, Ziegler U, Groscurth P, Gassmann M (2000). “Embryoid bodies: An in vitro model of mouse embryogenesis”. Exp. Physiol.; 85: 645 - 651.

36- Desvergne B, Wahli W (1999). “Peroxisome proliferators activated receptor: Nuclear control of metabolism”. Endocrinol. Rev.; 20: 649 - 688.

37- Ding L, Liang X, Zhu D, Lou Y (2007). “Peroxisome proliferator activated receptor-α is involved in cardiomyocyte differentiation of murine embryonic stem cells in vitro”. Cell Biol. Int.; 31: 1002 - 1009.

38- Di-Poi N, Tan NS, Michalik L, et al. (2002). “Anti-apoptotic role of PPARβ in keratinocytes via transcriptional control of the Akt1 signaling pathway”. Mol. Cell.; 10: 721 - 733.

39- Doetschman TC, Eistetter H, Katz M, et al. (1985). “The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium”. J. Embryol. Exp. Morphol.; 87: 27 - 45.

40- Doetschman TC, Williams P, Maeda N (1988). “Establishment of hamster blastocyst-derived embryonic stem cells”. Develop. Biol.; 127: 224 - 227.

41- Dreyer C, Krey G, Keller H, et al. (1992). “Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors”. Cell; 68: 879 - 887.

42- Durocher D, Charron F, Warren R, et al. (1997). “The cardiac transcription factors Nkx2.5 and GATA4 are mutual cofactors”. EMBO J.; 16: 5687 - 5696.

43- Ekelund UE, Harrison RW, Shokek O, et al. (1999). “Intravenous allopurinol decreases myocardial oxygen consumption and increases mechanical efficiency in dogs with pacing-induced heart failure”. Circ. Res.; 85: 437 - 445.

44- Ernst M, Novak U, Nicholson SE, et al. (1999). “The carboxyl-terminal domains of gp130-related cytokine receptors are necessary for suppressing embryonic stem cell differentiation. Involvement of STAT3”. J. Biol. Chem.; 274: 9729 - 9737.

45- Etzion S, Barbash M, Feinberg M.S, et al. (2002). “Cellular cardiomyoplasty of cardiac fibroblasts by adenoviral delivery of MyoD ex vivo: An unlimited source of cells for myocardial repair”. Circulation; 106: I–125 - I–130.

46- Evans MJ, Kaufman MH (1981). “Establishment in culture of pluripotential cells from mouse embryos”. Nature; 292: 154 - 156.

47- Fijnvandraat AC, Ginneken AV, Schumacher CA, et al. (2003). “Cardiomyocytes purified from differentiated embryonic stem cells exhibit characteristics of early chamber myocardium”. J. Mol. Cell. Cardiol.; 35: 1461 - 1472.

48- Finck BN (2006). “Effects of PPARα on cardiac glucose metabolism: A transcriptional equivalent of the glucose-fatty acid cycle?”. Expert Rev. Cardiovasc. Ther.; 4: 161 - 171.

49- Freeman WM, Walker SJ, Vrana KE (1999). “Quantitative RT-PCR: pitfalls and potential”. Biotechniques; 26: 112 - 122.

50- Gabig TG, Crean CD, Mantel PL, Rosli R (1995). “Function of wild-type or mutant Rac2 and Rap1a GTPases in differentiated HL60 cell NADPH oxidase activation”. Blood; 85: 804 - 811.

51- Gebel T, Arand M, Oesch F (1992). „Induction of the peroxisome proliferators activated receptor by fenofibrate in rat liver”. FEBS Lett.; 309: 37 - 40.

52- Gilde AJ, van der Lee KA, Willemsen PH, et al. (2003). “Peroxisome proliferator-activated receptor (PPAR) alpha and PPAR beta/delta, but not PPAR gamma, modulate the expression of genes involved in cardiac lipid metabolism”. Circ. Res.; 92: 518 - 524.

53- Goerlach A, Kietzmann T, Hess J (2002). “Redox signaling through NADPH oxidases: Involvement in vascular proliferation and coagulation”. Ann. NY Acad. Sci.; 973: 505 - 507.

54- Grepin C, Nemer G, Nemer M (1997). “Enhanced cardiogenesis in embryonic stem cells over-expressing the GATA4 transcription factor”. Development; 124: 2387 - 2395.

55- Griendling KK, Sorescu D, Ushio-Fukai M (2000). “NADPH oxidase: Role in cardiovascular biology and disease”. Circ. Res.; 86: 494 - 501.

56- Grounds MD, White JD, Rosenthal N, Bogoyevitch MA (2002). “The role of stem cells in skeletal and cardiac muscle repair”. J. Histochem. Cytochem.; 50: 589 - 610.

57- Harvey RP (1999). “Seeking a regulatory roadmap for heart morphogenesis”. Semin. Cell Dev. Biol.; 10: 99 - 107.

58- He L, Chinnery PF, Durham SE, et al. (2002). “Detection and quantification of mitochondrial DNA deletions in individual cells by real time PCR”. Nucleic Acids Res.; 30: E68.

59- Hescheler J, Fleischman BK, Wartenberg M, et al. (1999). “Establishment of ionic channels and signaling cascades in the embryonic stem cell-derived primitive endoderm and cardiovascular system”. Cell. Tiss. Organs; 165: 153 - 164.

60- Hescheler J, Fleischmann BK, Lentini S, et al. (1997). “Embryonic stem cells: A model to study structural and functional properties in cardiomyogenesis”. Cardiovasc. Res.; 36: 149 - 162.

61- Hood DA, Takahashi M, Connor MK, Freyessent D (2000). “Assembly of the cellular powerhouse: Current issues in muscle mitochondrial biogenesis”. Exerc. Sport Sci. Rev.; 28: 68 - 73.

62- Hu E, Kim JB, Sarraf P, Spiegelman BM (1996). “Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPAR gamma”. Science; 274: 2100 - 2103.

63- Huang Q, Alvares K, Chu R, et al. (1994). “Association of proxisome proliferator activated receptor and heat shock protein-72”. J. Biol. Chem.; 269: 8493 - 8497.

64- Ide T, Tsutsui H, Kinugawa S, et al. (1999). “Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium”. Circ. Res.; 85: 357 - 363.

65- IJpenberg A, Jeannin E, Wahli W, Desvergne B (1997). “Polarity and specific sequence requirements of peroxisome proliferator-activated receptor (PPAR)/retinoid X receptor (RXR) heterodimer binding to DNA”. J. Biol. Chem.; 272: 20108 - 20117.

66- Inoue I, Shino K, Noji S, et al. (1998). “Expression of Peroxisome proliferator-activated receptor-α (PPARα) in primary cultures of human vascular endothelial cells”. Biochem. Biophys. Res. Commun.; 246: 370 - 374.

67- Issemann I, Green S (1990). “Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators”. Nature; 347: 645 - 650.

68- Itescu S, Schuster MD, Kocher AA (2003). “New directions in strategies using cell therapy for heart disease”. J. Mol. Med.; 81: 288 - 296.

69- Itskovitz-Eldor J, Schuldiner M, Karsenti DK, et al. (2000). “Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers”. Mol. Med.; 6: 88 - 95.

70- Juge-Aubry C, Pernin A, Favez T, et al. (1997). “DNA binding properties of Peroxisome proliferator activated receptor subtypes on various natural Peroxisome proliferator response element”. J. Biol. Chem.; 272: 25252 - 25259.

71- Juge-Aubry CE, Hammar E, Siegrist-Kaiser C, et al (1999). “Regulation of the transcriptional activity of the peroxisome proliferator-activated receptor alpha by phosphorylation of a ligand-independent trans-activating domain”. J. Biol. Chem.; 274: 10505 - 10510.

72- Kehat I, Kenyagin-Karsenti D, Snir M, et al. (2001). “Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes”. J. Clin. Inves.; 108: 407 - 414.

73- Keller G, Kennedy M, Papayannopoulou T, Wiles MV (1993). “Hematopoietic commitment during embryonic stem cells differentiation in culture”. Mol. Cell. Biol.; 13: 473 - 486.

74- Keller H, Dreyer C, Medin J, et al. (1993). “Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers”. Proc. Natl. Acad. Sci. USA; 90: 2160 - 2164.

75- Keller JM, Cable S, Heusser S, et al. (1993). “Peroxisome through cell differentiation and neoplasia”. Biol. Cell.; 77: 77 - 88.

76- Kersten S, Desvergne B, Wahli W (2000). “Roles of PPARs in health and disease”. Nature; 405: 421 - 424.

77- Kersten S, Mandard S, Escher P, et al. (2001). “The Peroxisome proliferator activated recetpr α regulates amino acid metabolism”. FASEB J.; 15: 1971 - 1978.

78- Kersten S, Seydoux J, Peters J, et al. (1999). “Peroxisome proliferator activated receptor α mediates the adaptive response to fasting”. J. Clin. Invest.; 103: 1489 - 1498.

79- Kliewer SA, Forman BM, Blumberg B, et al. (1994). “Differential expression and activation of a family of murine peroxisome proliferator-activated receptors”. Proc. Natl. Acad. Sci. USA; 91: 7355 - 7359.

80- Kliewer SA, Sundseth SS, Jones SA, et al. (1997). Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. Proc. Natl. Acad. Sci. USA; 94: 4318 - 4323.

81- Kliewer SA, Umesono K, Noonan DJ, et al. (1992). "Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors". Nature; 358: 771 - 774.

82- Korohoda W, Pietrzkowski Z, Reiss K (1993). "Chloeamphenicol, an inhibitor of mitochondrial protein synthesis, inhibits myoblast fusion and myotube differentiation". Histochem. Cytobio.; 31: 9 - 13.

83- Koulmann N, Bigard AX (2006). "Interaction between signaling pathways involved in skeletal muscle responses to endurance exercise". Pflugers Arch. Eur. J. Physiol.; 452: 125 - 139.

84- Kraft CS, LeMoine CM, Lyons CN, et al. (2006). "Control of mitochondrial biogenesis during myogenesis". Am. J. Physiol. Cell. Physiol.; 290: C1119 - 1127.

85- Krey G, Braissant O, L'Horset F, et al. (1997). "Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay". Mol. Endocrinol.; 11: 779 - 791.

86- Kushnareva Y, Murphy A.N, Andreyev A (2002). "Complex I-mediated reactive oxygen species generation: Modulation by cytochrome c and NADP+ oxidation-reduction state". Biochem. J.; 368: 545 - 553.

87- Laloi C, Apel K, Danon A (2004). "Reactive oxygen signaling: the latest news". Curr. Opin. Plant Biol.; 7: 323 - 328.

88- Lalwani ND, Fahl WE, Reddy J (1983). “Detection of a nafenopin binding protein in rat liver cystosol associated with the induction of peroxisome proliferation by hypolipidemic compounds”. Biochem. Biophys. Res. Commun.; 116: 388 - 393.

89- Lambeth JD (2004). “NOX enzymes and the biology of reactive oxygen”. Nat. Rev. Immunol.; 4: 181 - 189.

90- Lassegue B, Clempus RE (2003). “Vascular NADPH oxidases: Specific features, expression, and regulation”. Am. J. Physiol. Regul. Integr. Comp. Physio.; 285: R277 - 297.

91- Lee LG, Connell CR, Bloch W (1993). “Allelic discrimination by nick-translation PCR with fluorogenic probes”. Nucleic acid Res.; 21: 3761 - 3766.

92- Lee Y, Shioi T, Kasahara H, et al. (1998). “The cardiac tissue-restricted homeobox protein Csx/Nkx2.5 physically associates with the zinc finger protein GATA4 and cooperatively activates atrial nautriuretic factor gene expression”. Mol. Cell Biol.; 18: 3120 - 3129.

93- Lehman JJ, Barger PM, Kovacs A, et al. (2000). “Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis”. J. Clin. Invest.; 106: 847 - 856.

94- Leone TC, Weinheimer CJ, Kelly DP (1999). “A critical role for the peroxisome proliferator-activated receptor alpha (PPARα) in the cellular fasting response: the PPAR alpha-null mouse as a model of fatty acid oxidation disorders”. Proc. Natl. Acad. Sci. USA; 96: 7473 - 7478.

95- Leusen JH, de Klein A, Hilarius PM, et al. (1996). “Disturbed interaction of p21-rac with mutated p67phox causes chronic granulomatous disease”. J. Exp. Med.; 184: 1243 - 1249.

96- Li J, Stouffs M, Serrander L, et al. (2006). “The NADPH oxidase NOX-4 drives cardiac differentiation: Role in regulating cardiac transcription factors and MAP kinase activation”. Mol. Biol. Cell.; 17: 3978 - 3988.

97- Li JM, Gall NP, Grieve DJ, et al. (2002). “Activation of NADPH oxidase during progression of cardiac hypertrophy to failure”. Hypertension; 40: 477 - 484.

98- Li RK, Jia ZQ, Weisel RD, et al. (1996). “Cardiomyocyte transplantation improves heart function”. Ann. Thorac. Surg.; 62: 654 - 661.

99- Li RK, Jia ZQ, Weisel RD, et al. (1999). “Survival and function of bioengineered cardiac grafts”. Circulation; 100: II63 - II69.

100- Li RK, Weisel RD, Mickle DA, et al. (2000). “Autologous porcine heart cell transplantation improved heart function after a myocardial infarction”. J. Thorac. Cardiovasc. Surg.; 119: 62 - 68.

101- Lien CL, Wu C, Mercer B, et al. (1999). “Control of early cardiac-specific transcription of Nkx2.5 by a GATA-dependent enhancer”. Development; 126: 75 - 84.

102- Lin Q, Lu J, Yanagisawa H, et al. (1998). “Requirement of the MADS-box transcription factor Mef2c for vascular development”. Development; 125: 4565 - 4574.

103- Lin Q, Schwarz J, Bucana C, Olson EN (1997). “Control of mouse cardiac morphogenesis and myogenesis by transcription factor Mef2c”.Science; 276: 1404 - 1407.

104- Livak KJ, Flood SJ, Marmaro J, et al. (1995). “Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization”. PCR Methods Appl.; 4: 357 - 362.

105- Livak KJ, Schmittgen TD (2001). “Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method”. Methods; 25: 402 - 408.

106- Mangelsdorf DJ, Thummel C, Beato M, et al. (1995). “The nuclear receptor superfamily: The second decade”. Cell; 83: 835 - 839.

107- Martin GR (1981). “Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells”. Proc. Natl. Acad. Sci. USA; 78: 7634 - 7638.

108- Mason C, Dunnill P (2008). “A brief definition of regenerative medicine”. Regen. Med.; 3: 1 - 5.

109- McBride HM, Neuspiel M, Wasial S (2006). “Mitochondria: More than just a powerhouse”. Curr. Biol.; 16: R551 - R560.

110- McPhail LC (1994). “SH3-dependent assembly of the phagocyte NADPH oxidase”. J. Exp. Med.; 180: 2011 - 2015.

111- Mendez G.F, Cowie M.R (2001). “The epidemiological features of heart failure in developing countries”. Int. J. Cardiol.; 80: 213 - 219.

112- Meng TC, Fukada T, Tonks NK (2002). “Reversible oxidation and inactivation of protein tyrosine phosphatase in vivo”. Mol. Cell; 9: 387 - 399.

113- Metzger JM, Lin WI, Johnston RA, et al. (1995). “Myosin heavy chain expression in contracting myocytes isolated during embryonic stem cell cardiogenesis”. Circ. Res.; 76: 710 - 719.

114- Min JY, Yang Y, Converso KL, et al. (2002). “Transplantation of embryonic stem cells improves cardiac function in postinfarcted rats”. J. Appl. Physiol.; 92: 288 - 296.

115- Minsky M (1988). “Memoir on inventing the confocal scanning microscope”. Scanning; 10: 128 - 138.

116- Molkentin J.D, Lin Q, Duncan S.A, Olson E.N (1997). “Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis”. Genes Dev.; 11: 1061 - 1072.

117- Morel Y, Barouki R (1999). “Repression of gene expression by oxidative stress”. Biochem. J.; 342: 481 - 496.

118- Mueller E, Sarraf P, Tontonoz P, et al. (1998). “Terminal differentiation of human breast cancer through PPARγ”. Mol. Cell.; 1: 465 - 470.

119- Murdoch CE, Grieve DJ, Cave AC, et al. (2006). “NADPH oxidase and heart failure”. Curr. Opin. Pharmacol.; 6: 148 - 153.

120- Murry CE, Wiseman RW, Schwartz SM, Hauschka SD (1996). “Skeletal myoblast transplantation for repair of myocardial necrosis”. J. Clin. Invest.; 98: 2512 - 2523.

121- Nakamura K, Fushimi K, Kouchi H, et al. (1998). “Inhibitory effects of antioxidants on neonatal rat cardiac myocyte hypertrophy induced by tumor necrosis factor-α and angiotensin II”. Circulation; 98: 794 - 799.

122- A C, Pasquali L, Vaglini F, et al. (2007). “The role of mitochondria in stem cell biology”. Biosci. Rep.; 27: 165 - 171.

123- Niwa H, Burdon T, Chambers I, Smith A (1998). “Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3”. Genes. Dev.; 12: 2048 - 2060.

124- Nolan T, Hands RE, Bustin SA (2006). “Quantification of mRNA using real-time RT-PCR”. Nat. Proto.; 1: 1559.

125- Nuclear receptors nomenclature committee (1999). “A unified nomenclature system for the nuclear receptor superfamily”. Cell; 97: 161 - 163.

126- Ogawa K, Nishinakamura R, Iwamatsu Y, et al. (2006). “Synergistic action of Wnt and LIF inmaintaining pluripotency of mouse ES cells”. Biochem. Biophys. Res. Comm.; 343: 159 - 166.

127- Orlic D, Kajstura J, Chimenti S et al. (2001). “Bone marrow cells regenerate infracted myocardium”. Nature; 410: 701 - 705.

128- Packer M (1999). “Implication of the ATLAS study for clinical practice”. Int. J. Clin. Pract.; Suppl. 100: 21 - 23.

129- Parini IM, Tringale SM, Tauber AI (1986). “Identification of distinct activation of the human neutrophil NADPH oxidase”. J. Immunol.; 137: 2925 - 2929.

130- Park JW, Ahn SM (1995). “Translocation of recombinant p47phox cytosolic component of the phagocyte oxidase by in vitro phosphorylation”. Biochem. Biophys. Res. Commun.; 211: 410 - 416.

131- Park JW, Babior BM (1992). “The translocation of respiratory burst oxidase components from cytosol to plasma membrane is regulated by guanine nucleotides and diacylglycerol”. J. Biol. Chem.; 267: 19901 - 19906.

132- Park KS, Lee RD, Kang SK, et al. (2004). “Neuronal differentiation of embryonic midbrain cells by upregulation of peroxisome proliferator-activated receptor-γ via the JNK-dependent pathway”. Exp. Cell. Res.; 297: 424 - 433.

133- Parmacek MS (2006). “Cardiac stem cells and progenitors: Developmental biology and therapeutic challenges”. Transact. Am. Clin. Climatol. Asso.; 117: 239 - 256.

134- Peterkin T, Gibson A, Patient R (2007). “Redundancy and evolution of GATA factor reqruitments in development of the myocardium”. Dev. Biol.; 311: 623 - 635.

135- Phiel CJ, Gabbeta V, Parsons LM, et al. (2001). “Differential binding of an SRF/NK2/Mefs transcription factor complex in normal vrsus neoplastic smooth muscle tissues”. J. Biol. Chem.; 276: 34637 - 34650.

136- Pineda TI, Jamshidi Y, Flavell DM, et al. (2002). “Characterization of the human PPARα promoter: Identification of a functional nuclear receptor response element”. Mol. Endocrinol.; 16: 1013 - 1028.

137- Pratt WB (1992). “Control of steroid receptor function and cytoplasmic-nuclear transport by heat shock proteins”. Bioessays; 14: 841 - 848.

138- Puceat M, Travo P, Quinn MT, Fort P (2003). “A dual role of the GTPase Rac in cardiac differentiation of stem cells”. Mol. Biol. Cell; 14: 2781 - 2792.

139- Rada BK, Geiszt M, Kaldi K, et al. (2004). “Dual role of phagocytic NADPH oxidase in bacterial killing”. Blood; 104: 2947 - 2953.

140- Raeymaekers L (2000). “Basic principles of quantitative PCR”. Mol. Biotechnol.; 15: 115 - 122.

141- Rappolee DA,Wang A, Mark D, Werb Z (1989). “Novel method for studying mRNA phenotype in single or small numbers of cells”. J, Cell. Biochem.; 39: 1 - 11.

142- Reddy JK, Rao MS (1986). “Peroxisome proliferators and cancer: mechanisms and implications”. Trends Pharmacol. Sci.; 7: 438 - 443.

143- Reinecke H, Poppa V, Murry C (2001). “Skeletal muscle stem cells do not transdifferentiate into cardiomyocytes after cardiac grafting”. J. Mol. Cell. Cardiol.; 34: 241 - 249.

144- Reinecke H, Zhang M, Bartosek T, Murry CE (1999). “Survival, integration, and differentiation of cardiomyocyte grafts: A study in normal and injured rat hearts”. Circulation; 100: 193 - 202.

145- Ribeiro RCJ, Kushner PJ, Baxter JD (1995). “The nuclear hormone receptor gene superfamily”. Annu. Rev. Med.; 46: 443 - 453.

146- Richter C, Gogvadze V, Laffranchi R, et al. (1995). “Oxidants in mitochondria: from physiology to diseases”. Biochim. Biophys. Acta.; 1271: 67 - 74.

147- Robertson E, Bradley A, Kuehn M (1986). “Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector”. Nature; 323: 445 - 448.

148- Robinson SW, Cho PW, Levitsky HI et al. (1996). “Arterial delivery of genetically labeled skeletal myoblasts to the murine heart: Long-term survival and phenotypic modification of implanted myoblasts”. Cell Transplant.; 5: 77 - 91.

149- Rochard P, Rodier A, Casas F, et al. (2000). “Mitochondrial activity is involved in the regulation of myoblast differentiation through myogenin expression and activity of myogenic factors”. J. Biol. Chem.; 275: 2733 - 2744.

150- Roche E, Ramirez M, Castillejo C.R, et al. (2007). “Stem cell potential for type 1 diabetes therapy”. Cent. Eur. J. Biol.; 2: 449 - 480.

151- Rosen ED, Sarraf P, Troy AE, et al. (1999). “PPARγ is required for the differentiation of adipose tissue in vivo and in vitro”. Mol. Cell.; 4: 611 - 617.

152- Rosen ED, Spiegelman BM (2001). “PPARγ: A nuclear regulator of metabolism, differentiation, and cell growth”. J. Bio. Chem.; 276: 37731 - 37734.

153- Rosenweig A (2006). “Cardiac cell therapy: mixed results from mixed cells”. N. Engl. J. Med.; 355: 1274 - 1277.

154- Rotman N, Michalik L, Desvergne B, Wahli W (2006). “PPARs in fetal and early postnatal development”. Adv. Develop. Biol.; 16: 33 - 64.

155- Rotrosen D, Leto TL (1990). “Phosphorylation of neutrophil 47-kDa cytosolic oxidase factor: Translocation to membrane is associated with distinct phosphorylation events”. J. Biol. Chem.; 265: 19910 - 19915.

156- Sack MN, Disch DL, Rockman HA, Kelly DP (1997). “A role for Sp and nuclear receptor transcription factors in a cardiac hypertrophic growth program”. Proc. Natl. Acad. Sci. USA; 94: 6438 - 6443.

157- Sack MN, Rader TA, Park S, et al. (1996). “Fatty acid oxidation enzyme gene expression is downregulated in the failing heart”. Circulation; 94: 2837 - 2842.

158- Sauer H, Neukirchen W, Rahimi G, et al. (2004). “Involvement of reactive oxygen species in cardiotrophin1-induced proliferation of cardiomyocytes differentiated from murine embryonic stem cells”. Exp. Cell Res.; 294: 313 - 324.

159- Sauer H, Rahimi G, Hescheler J, Wartenberg M (1999). “Effects of electrical fields on cardiomyocytes differentiation of embryonic stem cells”. J. Cell. Biochem.; 75: 710 - 723.

160- Sauer H, Rahimi G, Hescheler J, Wartenberg M (2000). “Role of reactive oxygen species and phosphatidylinositol 3-kinase in cardiomyocyte differentiation of embryonic stem cells”. FEBS Lett.; 476: 218 - 223.

161- Sauer H, Wartenberg M (2005). “Reactive oxygen species as signaling molecules in cardiovascular differentiation of embryonic stem cells and tumor-induced angiogenesis”. Antioxid. Redox Signal.; 7: 1423 - 1434.

162- Sauer H, Wartenberg M, Hescheler J (2001). “Reactive oxygen species as intracellular messengers during cell growth and differentiation”. Cell Physiol. Biochem.; 11: 173 - 186.

163- Schmelter M, Ateghang B, Helmig S, et al. (2006). “Embryonic stem cells utilize reactive oxygen species as transducers of mechanical strain-induced cardiovascular differentiation”. FASEB J.; 20: 1182 - 1184.

164- Segal AW, Abo A (1993). “The biochemical basis of the NADPH oxidase of phagocytes”. Trends Biochem. Sci.; 18: 43 - 47.

165- Shalev A, Siegrist-Kaiser CA, Yen PM, et al (1996). “The peroxisome proliferator-activated receptor alpha is a phosphoprotein: regulation by insulin”. Endocrinology; 137: 4499 - 4502.

166- Shamblott MJ, Axelman J, Wang S, et al. (1998). “Derivation of pluripotent stem cells from cultured human primordial germ cells”. Proc. Natl. Acad. Sci. USA; 95: 13726 - 12731.

167- Shatwell KP, Segal AW (1996). “NADPH oxidase”. Int. J. Biochem. Cell Biol.; 28: 1191 - 1195.

168- Singla DK, Schneider DJ, LeWinter MM, Sobel BE (2006). “wnt3a but not wnt11 supports self-renewal of embryonic stem cells”. Biochem. Biophys. Res. Comm.; 345: 789 - 795.

169- Skerjanc IS, Petropoulos H, Ridgeway AG, Wilton S (1998). “Myocyte enhancer factor 2C and Nkx2.5 up-regulate each other’s expression and initiate cardiomyogenesis in P19 cells”. J. Biol. Chem.; 273: 34904 - 34910.

170- Smith DF (1993). “Dynamics of heat shock protein 90-progesterone receptor binding and the disactivation loop model for steroid receptor complexes”. Mol. Endocr.; 7: 1418 - 1429.

171- Snir M, Kehat I, Gepstein A, et al. (2003). “Assessment of the ultrastructural and proliferative properties of human embryonic stem cell-derived cardiomyocytes. Am. J. Physiol. Heart Circ. Physiol.; 285: H2355 - 2363.

172- Sorescu D, Griendling KK (2002). “Reactive oxygen species, mitochondria, and NADPH oxidases in the development and progression of heart failure”. Conges. Heart Fail.; 8: 132 - 140.

173- Spiegelman B.M (1997). “Peroxisome proliferator-activated receptor g: A key regulator of adipogenesis and systemic insulin sensitivity”. Eur. J. Med. Res.; 2: 457 - 464.

174- Spitkovsky D, Sasse P, Kolossov E, et al. (2004). “Activity of complex III of the mitochondrial electron transport chain is essential for early heart muscle cell differentiation”. FASEB J.; 18: 1300 - 1302.

175- Srivastava D, Ivey KN (2006). “Potential of stem cell-based therapies for heart disease”. Nature; 441: 1097 - 1099.

176- St. Clair D, Zhao Y, Chaiswing L, Oberley T (2005). “Modulation of skin tumorigenesis by SOD”. Biomed. Pharmacother.; 59: 209 - 214.

177- Staels B, Koenig W, Habib A, et al. (1998). “Activation of human aortic smooth-muscle cells is inhibited by PPARα but not by PPARα activators”. Nature; 393: 790 - 793.

178- Steele A, Jones O.Y, Gok F, et al. (2005). “Stem-like cells traffic from heart ex vivo, expand in vitro, and can be transplanted in vivo”. J. Heart Lung Transplant.; 24: 1930 - 1939.

179- Steinmetz M, Quentin T, Poppe A, et al. (2005). “Changes in expression levels of genes involved in fatty acid metabolism: Up-regulation of all three members of the PPAR family and the newly described adiponectin receptor-2, but not adiponectin receptor-1 during neonatal cardiac development of the rat”. Basic Res. Cardiol.; 100: 263e - 269e.

180- Stewart CL, Gadi I, Bhatt H (1994). “Stem cells from primordial germ cells can reenter the germ line”. Dev. Biol.; 161: 626 - 628.

181- Sumanasekera WK, Tien E, Davis JW, et al. (2003a). “Heat shock protein 90 (Hsp90) acts as a repressor of peroxisome proliferator activated receptor-α and PPARβ activity”. Biochem.; 42: 10726 - 10735.

182- Sumanasekera WK, Tien ES, Turpey R, et al. (2003b). “Evidence that Peroxisome proliferator activated receptor-α is complexed with the 90 kDa heat shock protein and the hepatitis virus B X-associated protein 2”. J. Biol. Chem.; 278: 4467 - 4473.

183- Takagi Y, Takahashi J, Saiki H, et al. (2005). “Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model”. J. Clin. Invest.; 115: 102 - 109.

184- Takeshige K, Minakami S (1979). “NADH- and NADPH-dependent formation of superoxide anions by bovine heart submitochondrial particles and NADH-ubiquinone reductase preparation”. Biochem. J.; 180: 129 - 135.

185- Tanaka K, Honda M, Takabatake T (2001). “Redox regulation of MAPK pathways and cardiac hypertrophy in adult rat cardiac myocyte”. J. Am. Coll. Cardiol.; 37: 676 - 685.

186- Taylor DA, Atkins BZ, Hungspreugs P, et al. (1998). “Regenerating functional myocardium: Improved performance after skeletal myoblast transplantation”. Nat. Med.; 4: 929 - 933.

187- Teissier E, Nohara A, Chinetti G, et al. (2004). “Peroxisome proliferator activated receptor-α induces NADPH oxidase activity in macrophages, leading to the generation of LDL with PPARα activation properties”. Circ. Res.; 95: 1174 - 1182.

188- Tenbaum S, Bahiahmad A (1997). “Nuclear receptors: Structure, function and involvement in disease”. Int. J. Biochem. Cell Biol.; 29: 1325 - 1341.

189- Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. (1998). “Embryonic stem cell lines derived from human blastocystes”. Science; 282: 1145 - 1147.

190- Tontonoz P, Nagy L, Alvarez J.G.A, et al. (1998). “PPARγ promotes monocyte/macrophage differentiation and uptake of oxidize LDL”. Cell; 93: 241 - 252.

191- Tsunawaki S, Mizunari H, Nagata M, et al. (1994). “A novel cytosolic component, p40phox, of respiratory burst oxidase association with p67phox and is absent in patients with chronic granulomatous disease who lack p67phox”. Biochem. Biophys. Res. Commun.; 199: 1378 - 1387.

192- Van Laake L.W, Haaink R, Doevendans P.A, Mummery C (2006). “Heart repair and stem cells”. J. Physiol.; 577: 467 - 478.

193- Van Laake L.W, Passier R, Doevendans P.A, Mummery C.L (2008). “Human embryonic stem cell derived cardiomyocytes and cardiac repair in rodents”. Circ. Res.; 102: 1008 - 1010.

194- Ventura C, Maioli M (2000). “Opioid peptide gene expression primes cardiogenesis in embryonal pluripotent stem cells”. Circ. Res.; 87: 189 - 194.

195- Wang T, Brown MJ (1999). “mRNA quantification by real time TaqMan polymerase chain reaction: Validation and comparison with RNase protection”. Anal. Biochem. 269: 198 - 201.

196- Wang Y, Haider H.K, Ahmad N, et al. (2006). “Combining pharmacological mobilization with intramyocardial delivery of bone marrow cells over-expressing VEGF is more effective for cardiac repair”. J. Mol. Cell. Cardiol.; 40: 736 - 745.

197- Wartenberg M, Günther J, Hescheler J, Sauer H (1998). “The embryoid body as a novel in vitro assay system for antiangiogenic agent”. Lab. Invest.; 78: 1301 - 1314.

198- Watt A.J, Battle M.A, Duncan S.A (2004). “GATA4 is essential for formation of the proepicardium and regulates cardiogenesis”. Proc. Natl. Acad. Sci. USA; 101: 12573 - 12578.

199- Wei H, Juhasz O, Li J, et al. (2005). “Embryonic stem cells and cardiomyocyte differentiation: Phenotype and molecular analysis”. J. Cell. Mol. Med.; 9: 804 - 817.

200- Weisiger RA, Fridovich I (1973). “Mitochondrial superoxide dismutase”. J. Biol. Chem.; 248: 4793 - 4796.

201- Wientjes FB, Hsuan JJ, Totty NF, Segal AW (1993). “p40phox, a third cytosolic component of the activation of the NADPH oxidase to contain src homology 3 domain”. Biochem. J.; 296: 557 - 561.

202- Wiles MV, Keller G (1991). “Multiple hematopoietic lineages develop from embryonic stem cells in culture”. Development; 111: 259 - 267.

203- Wobus AM, Kaomei G, Shan J, et al. (1997). “Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes”. J. Mol. Cell. Cardiol.; 29: 1525 - 1539.

204- Wolstenholme DR (1992). “Animal mitochondrial DNA: structure and evolution”. Int. Rev. Cytol.; 141: 173 - 216.

205- Xu HE, Stanley TB, Montana VG, et al. (2002). “Structural basis for antagonist-mediated recruitment of nuclear co-repressors by PPARα”. Nature; 415: 813 - 817.

206- Zhang B, Berger J, Zhou G, et al. (1996). “Insulin- and mitogen-activated protein kinase-mediated phosphorylation and activation of Peroxisome proliferator-activated receptor gamma”. J. Biol. Chem.; 271: 31771 - 31774.

207- Zhang M, Methot D, Poppa V, et al. (2001). “Cardiomyocyte grafting for cardiac repair: Graft cell death and anti-death strategies”. J. Mol. Cell. Cardiol.; 33: 907 - 921.

208- Zheng B, Wen JK, Han M (2003). “Regulatory factors involved in cardiogenesis”. Biochemistry; 68: 650 - 657.

209- Zoete V, Grosdidier A, Michielin O (2007). “Peroxisome proliferator-activated receptor structures: ligand specificity, molecular switch and interactions with regulators”. Biochim. Biophys. Acta; 1771: 915 - 925.

210- Zuo L, Clanton TL (2002). “Detection of reactive oxygen and nitrogen species in tissue using redox-sensitive fluorescent probes”. Methods Enzymol.; 352: 307 - 325.

7- MISCELLANEOUS

7-1- PUBLICATION

- *Sharifpanah F.*, Wartenberg M., Hannig M., Piper H.M., Sauer H. (2008). "Peroxisome Proliferator-Activated Receptor α agonists enhance cardiomyogenesis of mouse ES cells by utilization of a reactive oxygen species-dependent mechanism". Stem cells; 26 (1): 64 – 71.

7-2- ORAL AND POSTER PRESENTATIONS

1- *Sharifpanah F.*, Wartenberg M., Sauer H. (2006). "PPARα induces cardiomyogenesis during differentiation of mouse ES cells by utilization of a Reactive Oxygen Species dependent mechanism". *85th Annual meeting of The German Society of Physiology (DPG) and The Federation of European Physiological Societies (FEPS), Munich, Germany*.

- Published in "Acta Physiologica" 2006, vol. 186, suppl. 1.

2- *Sharifpanah F.*, Wartenberg M., Sauer H. (2006). "PPARα induces cardiomyogenesis during differentiation of mouse ES cells by utilization of a Reactive Oxygen Species dependent mechanism". *38th European Human Genetics Conference (ESHG), Amsterdam, Netherland.*

- Published in "European Journal of Human Genetics" 2006, vol. 14, suppl. 1.

3- *Sharifpanah F.*, Wartenberg M., Sauer H. (2007). "The redox control of peroxisome proliferator activated receptor α-mediated cardiomyogenesis of embryonic stem cell". *86th Annual meeting of The German Society of Physiology (DPG) and The Federation of European Physiological Societies (FEPS), Hannover, Germany*.

- Published in "Acta Physiologica" 2007, vol. 189, suppl. 1.

4- *Sharifpanah F.*, Wartenberg M., Sauer H. (2007). "The redox control of peroxisome proliferator activated receptor α-mediated cardiomyogenesis of embryonic stem cell". *Stem Cell Manchester Meeting: The Northwest Initiative, Manchester, England.*

5- *Sharifpanah F.*, Wartenberg M., Arnold B., Sauer H. (2007). "PPARα stimulation induces cardiomyogenesis in mouse embryonic stem cell". *2nd Annual congress of The German Society for Stem Cell Research, Wuerzburg, Germany*.

- Published in "Journal of Stem Cells & Regenerative Medicine" 2007, vol. 2, issue 1.

6- *Sharifpanah F.*, Wartenberg M., Piper H.M, Sauer H. (2008). "PPARα activators enhance cardiomyogenesis of mouse ES cells by utilization of a ROS-dependent mechanism". *The Excellent Cluster of Cardio-Pulmonary System Area C Meeting (ECCPS), Frankfurt, Germany*.

7- *Sharifpanah F.*, Wartenberg M., Piper H.M, Sauer H. (2008). "Signal transduction pathway leading to cardiomyogenesis of embryonic stem cells after treatment with peroxisome proliferator activated receptor alpha agonists". *87th Annual meeting of The German Society of Physiology (DPG) and The Federation of European Physiological Societies (FEPS), Cologne, Germany*.

- Published in "Acta Physiologica" 2008, vol. 192, suppl. 663.

8- *Sharifpanah F.*, Wartenberg M., Piper H.M, Sauer H. (2008). "Signal transduction pathway leading to cardiomyogenesis of embryonic stem cells after treatment with peroxisome proliferator activated receptor alpha agonists". *Mini-symposium of the Excellence Cluster of Cardio-Pulmonary System (ECCPS), Bad Neuheim, Germany*.

7-3- AFFIDAVIT

I hereby declare in lieu of an oath that the PhD thesis "*Role of Peroxisome Proliferator Activated Receptor α (PPARα) in cardiomyogenesis of mouse embryonic stem cells*" which was carried out under the supervision of **Prof. Dr. Heinrich Sauer** at the Physiology Institute of Justus Liebig University Giessen, is the product of my original research, without unauthorised outside help, and that I did not use any sources or aids but those that were quoted, and that I did clearly identify the quotes taken from the sources either literally or with regard to content.

In addition, I declare that this thesis is not submitted to any another evaluation, neither in this form nor in another.

I have not acquired or tried to acquire any other academic degree than that documented in the application.

Giessen, July 2008 **Fatemeh Sharifpanah**

8- CURRICULUM VITAE

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ACADEMIC QUALIFICATIONS:

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- *B.Sc.* in Biology, Biology Department, Faculty of Science, Azad University, Damghan, Iran (09.1993 – 02.1997)
- *High School* (Diploma in Life Science), Tehran, Iran (09.1988 – 06.1992)

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PUBLICATIONS:

- *Sharifpanah F.*, Wartenberg M., Hannig M., Piper H.M., Sauer H. (2008). "Peroxisome Proliferator-Activated Receptor α agonists enhance cardiomyogenesis of mouse ES cells by utilization of a reactive oxygen species-dependent mechanism". *Stem cells, 26 (1): 64 – 71.*
- Houshmand M., *Sharifpanah F.*, Tabasi A., Sanati M.H, Vakilian M., Lavasani Sh., Joughedoust S. (2004). "Leber's Hereditary Optic Neuropathy: The spectrum of mitochondrial DNA mutations in Iranian patients". *Annals of New York Academy of Science, 1011: 345-349.*
- Houshmand M., Sanati M.H, Rashedi I., *Sharifpanah F.*, Asgharzadeh A., Lotfi M. (2004). "Lack of association between Multiple Sclerosis and LHON primary point mutations in Iranian families". *European Neurology, 51(2): 59-67.*
- Houshmand M., *Sharifpanah F.* (2003). "Review article: Mitochondrion and its inheritance". *Genetics in 3rd Millennium, 1(1): 13-23.*

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- The best poster award at "The 8th Iranian Genetics Congress", 2003, Tehran, Iran.
- Young scientist travel fellowship and invited speaker for "The 7th Pakistan Society for Biochemistry and Molecular Biology Conference (PSBMB)", 2003, Lahore, Pakistan.
- Young scientist travel fellowship for "The First Scientific Meeting of Asian Society for Mitochondrial Research and Medicine (ASMRM),", 2003, Seoul, Korea.

- Young scientist travel fellowship for "The 9th Federation of Asian and Oceanian Biochemists and Molecular Biologists Congress (FAOBMB)", 2001, Lahore, Pakistan.
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