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REGULATION OF CARDIOTROPHIN-1 EXPRESSION DURING MOUSE EMBRYONIC STEM CELL DIFFERENTIATION BY HYPOXIA AND REACTIVE OXYGEN SPECIES

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Dedicated

To

My Beloved Family

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ABBREVIATIONS

ABCG 2	ATP-binding cassette superfamily G
AEBSF	4-(2-Aminoethyl)-benzensulfonyl fluoride
ANF	Atrial natriuretic factor
ANP	Atrial natriuretic peptide
ARNT	Aryl hydrocarbon receptor nuclear translocator
bHLH	Basic-helix-loop-helix
BIO	6-Bromoindirubin-3'-oxime
BSA	Bovine serum albumin
CBP	CREB-binding protein
cGMP	Cyclic guanosine-3', 5'-monophosphate
CHF	Congestive heart failure
CLSM	Confocal laser-scanning-microscope
CNTF	Ciliary neurotrophic factor
CREB	cAMP response element-binding
CT-1	Cardiotrophin -1
CTAD	C-terminal transactivation domain
Cy 2	Carbocyann
Cy 3	Indocarbocyanin
Cy 5	Indodicarbocyanin
DCF	2´-7´-Dichlorofluorescin
DMEM	Dulbecco´s modified Eagles medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxy-nucleoside triphosphate
DPI	Diphenylen-odonium
DTT	Dithiothreitol
EB	Embryoid bodies
EC cells	Embryonic carcinoma cells
ECL	Enhanced chemiluminescence
EDTA	Ethylendiamintetracetate
EG cells	Embryonic germ cells
ERK	Extracellular signal regulated kinase
ES cells	Embryonic stem cells
FAD	Flavin adenine dinucleotide
FCS	Foetal calf serum
FITC	Fluorescein-isothiocyanat
gp130R	Glycoprotein 130 receptor
GTP	Guanosintriphosphate
H_2DCF	2´-7´-Dichlorofluorescin

H_2DCF-DA	2',7'-Dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen Peroxide
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HIF	Hypoxia-Inducible Factor
HRE	Hypoxia-response element
Hsps	Heat shock proteins
ICM	Inner cell mass
IL-6	Interleukin 6
ILH	Mean helical hydrophobic moment
IMDM	Iscoves modified Dulbecco´s medium
JAK	Janus kinase
JNK	c-Jun-NH2 terminal kinase
KH_2PO_4	Potassium dihydrogen phosphate
LIF	Leukemia inhibitory factor
LIFR	Leukemia inhibitory factor receptor
L-NAME	NG-nitro-L-arginine methyl ester
LV	Left ventricular
M	Molar (mol/L)
MAPKs	Mitogen-activated protein kinases
MEF	Mouse embryonic fibroblast
MEM	Modified Eagles medium
MgCl2	Magnesium chloride
Min	Minute
mM	Milimolar
mRNA	messenger-Ribonucleicacid
Na_2HPO_4	di –Sodiumhydrogenphosphate dehydrate
NAC	N-acetyl L-cysteine
NaCl	Sodium chloride
NADH	reduced Nicotinamid-adenin-dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form
NEA	Non-essential amino acids
NFκB	Nuclear factor kappa B
NMPG	N-(2-mercaptopropionyl)glycine
NO	Nitric oxide
NOS	Nitric oxide synthase
Nox	NADPH oxidase
NTAD	N- terminal transactivation domain
NTR	Neurotrophin R
NYHA	New York heart association
ODD	Oxygen dependent degradation
ONOO	Peroxynitrite

OSM	Oncostatin M
p38	p38 mitogen-activated protein kinase
PAS	Per- Arnt- Sim
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Triton X-100
PCR	Polymerase chain reaction
p-gp130R	Phosphorylated Glycoprotein 130 receptor
PHD	Proline hydroxylases
PI-3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PMSF	Phenylmethylsulphonylflouride
POU	Pit- Oct- Unc
PSA -NCAM	Polysialic acid-neural cell adhesion molecule
qPCR	Realtime or quantitative colymerase-chain-reaction
RAS	Renin-angiotensin system
RLU	Relative light unit
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAPK	Stress-activated protein kinase
Sca-1	Stem cell antigen 1
sGC	Soluble guanylyl cyclase
SOD	Superoxide dismutase
SSEA	Stage specific embryonic antigen
STAT	Signal transducers and activators of transcription
TAD	Transactivation domain
TEA	Triethanolamine
TEMED	N,N,N',N'-Tetramethyl-1,3-propandiol
TNF-α	Tumour necrosis factor-α
Tris	2-Amino-2-hydroxymethyl-1,3-propanediol
U	unit
vol	Volume
VHL	Von Hippel-Lindau
VSMC	Vascular smooth muscle cell
W/O	Without
wt	Wild type

1 INTRODUCTION

Redox regulation, like phosphorylation, is a covalent regulatory system that controls many of the normal cellular functions of all living cells and organisms. In addition, it controls how cells respond to stress involving oxidants and free radicals. This area is undergoing a transition from general knowledge to specific description of the components and mechanisms involved. A progressive rise of oxidative stress due to altered reduction–oxidation (redox) homeostasis appears to be one of the hallmarks of the processes that regulate gene transcription in physiology and pathophysiology. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) serve as signalling messengers for the evolution and perpetuation of the inflammatory process that is often associated with the condition of oxidative stress, and involves genetic regulation [Halliwell, 1991; Foncea, 2000; Hensley *et al*, 2000, Droege, 2001; Hancock, Desikan and Neill, 2001]. Changes in the pattern of gene expression through ROS/RNS-sensitive regulatory transcription factors are crucial components of the machinery that determines cellular responses to oxidative/redox conditions. In order to study the effect of reactive oxygen species on the expression of cardiotrophin-1 (CT-1), we used stem cell derived embryoid bodies as *in vitro* model.

1.1 STEM CELLS

Stem cells are primal undifferentiated cells (self-renewing cells) that have the ability to proliferate and differentiate into cell types of different tissues *in vitro* and *in vivo*. Molecular cues provided by their cellular environment or niche and subsequently activated transcriptional factors appear to switch specific genetic programmes on or off in a very controlled manner. Execution of the right genetic programme and therefore differentiation into specific cell types depends crucially on the availability of right combination and sequences of cues [O'Shea, 2004]. Stem cells differ from other kinds of cells in the body. All stem cells regardless of their source have three general properties: (i) they are capable of dividing and renewing themselves for long periods; (i) they are specialised; (iii) they can give rise to specialised cell types. Based on the

developmental potential of stem cells, i.e., the number of different kinds of differentiated cell that they can become, they are classified as follows (see FIG. 1):

- **Totipotent cells;** these cells have the potential to become any cell type in the adult body and any cell of the extra embryonic membranes (e.g., placenta). The only totipotent cells are fertilised eggs.
- **Pluripotent stem cells;** these are cells that have the potential to differentiate into any cell in the body, but cannot contribute to making the extra embryonic membranes (which are derived from the trophoblast) [Wobus 2001]. Three types of pluripotent stem cells have been found
    - **Embryonic Stem (ES) Cells.** These cells are isolated from the **inner cell mass** (ICM) of the blastocyst — the stage of embryonic development when implantation occurs.
    - **Embryonic Germ (EG) Cells.** These cells are isolated from the precursor to the gonads in aborted fetuses.
    - **Embryonic Carcinoma (EC) Cells.** EC cells are isolated from teratocarcinomas, a tumor that occasionally occurs in a gonad of a fetus. Unlike the other two, they are usually aneuploid (having one or more extra (or fewer) chromosomes than the normal diploid (2n) set (e.g., 2n+1, 2n-1)).
- **Multipotent stem cells (adult stem cells);** these cells can only differentiate into a limited number of types. For example, the bone marrow contains multipotent stem cells that give rise to all the cells of the blood but not to other types of cells. Multipotent stem cells are found in adult animals; perhaps most organs in the body (e.g., brain, liver) contain them where they can replace dead or damaged cells.

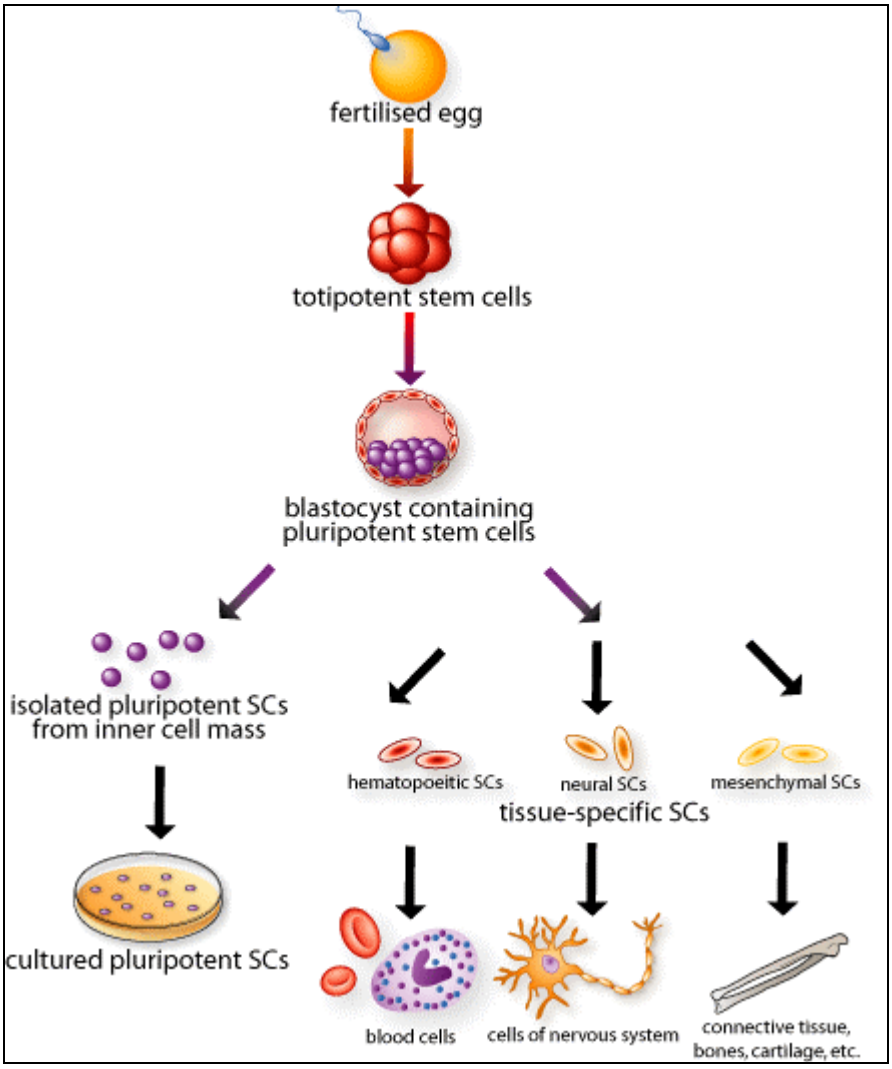

FIG. 1 Types of stem cells [www.bioteach.ubc.ca/ Bioengineering/StemCells/]

A focus of pluripotent stem cell research is the identification of signals that control stem cell differentiation and influence lineage specification. The replication of signalling events within the embryo, by providing defined signalling molecules in culture, may permit the directed differentiation of ES cells toward selected lineages. There exist both intrinsic and extrinsic molecular signals that drive stem cell renewal –a vital property of stem cells – and differentiation. Key signalling pathways that have been implicated in ES cell maintenance and differentiation include the Notch, TGFβ, Wnt pathways. A LIF-dependent JAK/STAT3 pathway [Raz *et al*, 1999; Bader *et al*, 2000; Bader *et al* 2001], and signalling by Nanog and Oct4 transcription factors have been shown to maintain ES cell self-renewal, whereas a MEK/ERK signalling mechanism prevents ES self-renewal [Burdon, Smith and Savatier, 2002; Saito, Liu and Yokoyama, 2004]. In contrast over-expression of Oct4 results in differentiation of ES cells into primitive endoderm; whereas over-expression of Nanog leads to a LIF-

independent ES cell self-renewal. Oct4 and Nanog signalling prevents differentiation into trophectoderm and primitive endoderm, respectively (see FIG. 2). The canonical Wnt pathway is activated upon binding of the Wnt protein to the Frizzled receptor [Rattis *et al*, 2004, Reya *et al* 2005]. Activation of the pathway leads to inhibition of glycogen synthase kinase-3 (GSK-3), subsequent nuclear accumulation of β-catenin and the expression of target genes. Sato and colleagues (2004) used the specific inhibitor of GSK-3, 6-bromoindirubin-3'-oxime (BIO), to demonstrate that the activation of the canonical Wnt pathway maintains the undifferentiated phenotype in ES cells and sustains expression of the pluripotent state-specific transcription factors Oct 4, Rex-1 and Nanog. They demonstrated that the process is fully reversible by removing BIO thereby leading to the subsequent onset of differentiation processes.

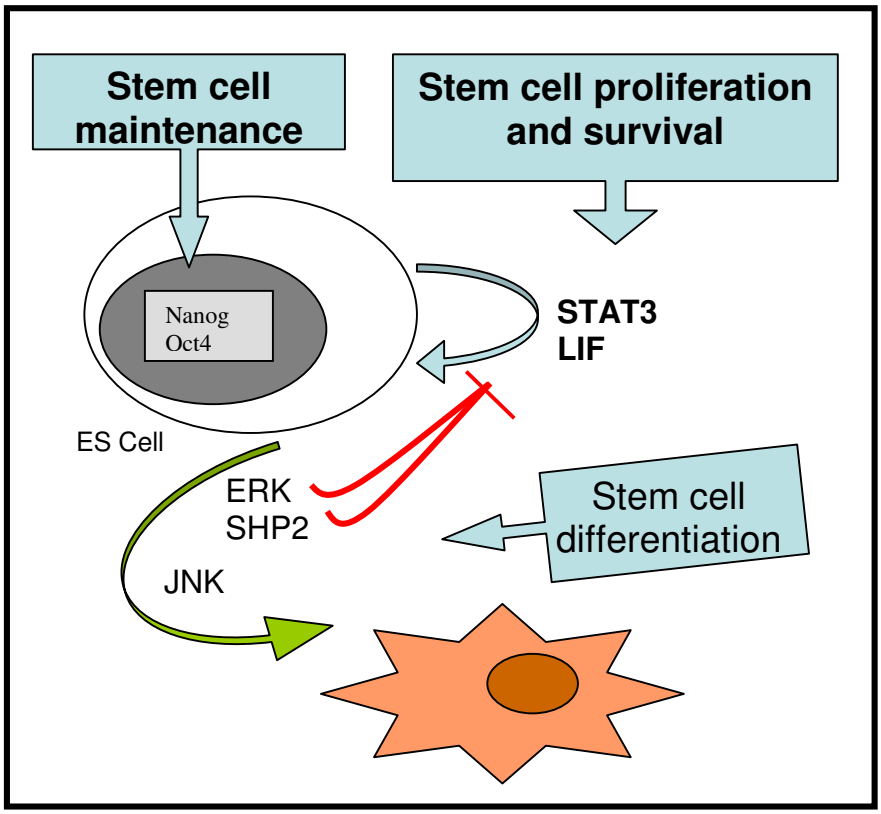

FIG. 2 Signalling pathways and transcription factors involved in the maintenance, proliferation, survival and differentiation of ES cells [adapted from Hearsley and Peterson, 2004]

1.1.1 EMBRYONIC STEM CELLS

ES cells are derived from embryos. Specifically, human ES cells are derived from embryos that developed from *in vitro* fertilised eggs and are then donated for research purposes with the consent of the donors. The embryos from which human embryonic stem cells are derived are typically 4 or 5 days old and are a hollow microscopic ball of cells called the blastocysts [Shamblott *et al*, 1998; Thomson *et al*, 1998; Thomson and Marshall, 1998]. Mouse ES cells are isolated from the inner cell mass of pre-implantation embryos or blastocyst at day 3.5 of mouse development. The blastocyst includes three structures: the trophoblast, which is the layer that surrounds the blastocyst; the blastocoel, which is the hollow cavity inside the blastocyst; and the inner cell mass (ICM), which is a group of cells at one end of the blastocoel and would normally give rise to the embryonic disk of the later embryo and, ultimately, the foetus. When the blastocysts are cultured, the outer layer of cells attaches to a feeder layer of mitotically inactivated embryonic fibroblasts and undifferentiated cells from the inner cell mass spontaneously form clumps [Evans and Kaufman, 1981; Martin, 1981]. When this occurs, they are removed gently and plated into several fresh cultured dishes. The process of re-plating the cells is repeated many times, and is called sub-culturing to yield ES cell lines. Each cycle of sub-culturing the cells is referred to as a passage. These cells are considered pluripotent as they can be maintained indefinitely in the undifferentiated state in culture, and when injected back into a blastocyst, have the ability to contribute to all tissues, including the germ cells. Maintenance of Es cells *in vitro* is achieved by co-culture on inactivated mouse fibroblast or on gelatinised plates with a differentiation inhibitory factor known as LIF [Martin, 1981; Williams *et al*, 1988]. Once ES cell lines have been established, batches of them can be frozen.

The *in vitro* differentiation of ES cells provides a basis both for detailed studies of developmental mechanisms and for the generation of specific cell types for tissue engineering and regenerative medicine applications. The *in vitro* differentiation of ES cells recapitulates the early processes of development into a variety of endodermal, mesodermal, and ectodermal lineages [Guan *et al*, 1999; Amit *et al*, 2000]. Both the pattern and the efficiency of differentiation are affected by parameters such as ES cell density, media components, growth factors and additives and the quality of fetal calf serum used. ES cell lines display different developmental properties *in vitro*. *In*

vitro differentiation requires the removal of differentiation inhibitory factors and the development of ES cells in aggregates, called embryoid bodies (EBs) [Doetschman *et al*, 1993; Wartenberg *et al*, 1998, Desbaillets *et al*, 2000; Doevendans *et al*, 2000; Dang *et al*, 2002]. Specialised cells from these germ layers constitute the complex assortment of tissues that make up an entire organism (TABLE 1).

EMBRYONIC GERM LAYER	CELL TYPE
Ectoderm	Neurons
	Oligodendrocytes
	Astrocytes
	Epithelial cells
Mesoderm	Adipocytes
	Cardiomyocytes
	Chondrocytes
	Hematopoietic (stem) cells
	Endothelial cells
	Osteoblasts
	Striated- and Smooth- muscle cells
Endoderm	Pancreatic-like islets
	Insulin-producing cells
	Lung cells
	Hepatocytes

TABLE 1 Differentiation of mouse embryonic stem cells *in vitro* [adapted from Faulkes S.Minireview. www.stemcell.com]

1.1.2 ADULT STEM CELLS

An adult stem cell is an undifferentiated cell found among differentiated cells in a tissue or organ, can renew itself, and can differentiate to yield the major specialized cell types of the tissue or organ. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found. Unlike embryonic stem cells, which are defined by their origin (the inner cell mass of the blastocyst), the origin of adult stem cells in mature tissues is unknown. Adult stem cells have been identified in many organs and tissues. They 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. As of now the following adult tissues

have been reported to contain resident stem cells: brain, bone marrow [Iscove, 1990; Mackay *et al*, 1998; Pittenger *et al*, 1999; Orlic *et al*, 2001], blood [Hall *et al*, 1989; Juttner *et al*, 1989; Kessinger et al 1991], skeletal muscle, skin, heart [Anversa *et al*, 2002], fat and liver.

Not so long ago, it was thought that only embryonic stem cells could generate all different cell types in mammalian body, and that adult stem cells are more restricted in their developmental potential. However, it has recently been shown that adult stem cells exhibit the ability to form specialized cell types of other tissues, which is known as transdifferentiation or plasticity (see FIG. 3) [Bjornson *et al*, 1999; Makino *et al* 1999; Goodell *et al*, 2001; Jackson *et al*, 2001; Orlic *et al*, 2001].

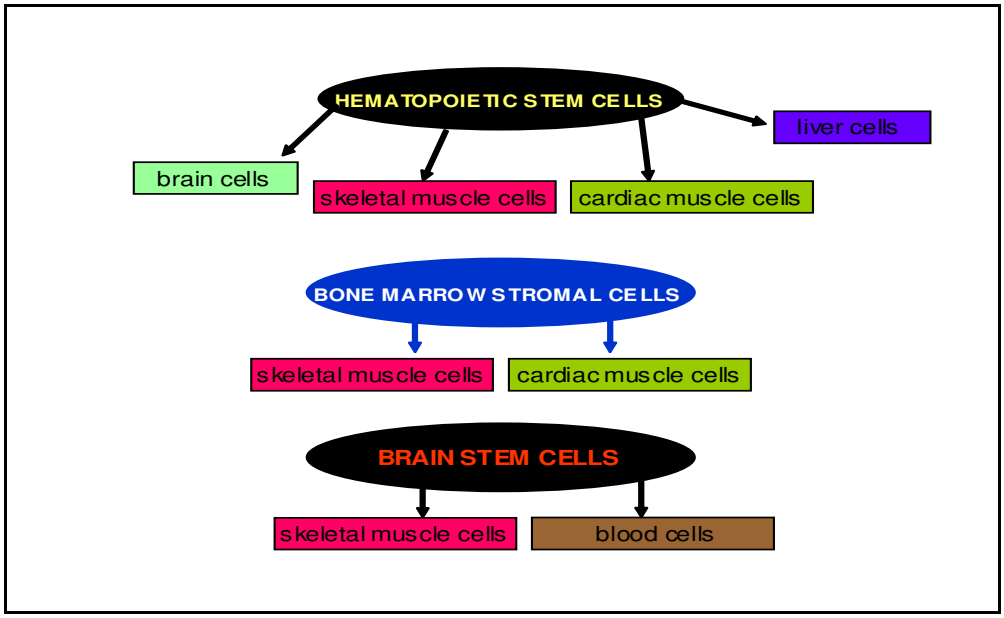

FIG. 3 Adult Stem Cell Plasticity And Transdifferentiation

In the past few years many scientists have been trying to find ways to grow adult stem cells in cell culture and manipulate them to generate specific cell types so they can be used to treat injury or disease. Some examples of potential treatments include replacing the dopamine-producing cells in the brains of Parkinson's patients, developing insulin-producing cells for type I diabetes and repairing damaged heart muscle following a heart attack with cardiac muscle cells. If successful, adult stem cell therapy will help to solve the ethical conflicts that exist with the ES cells. An advantage of adult stem cells is that, potential ethical issues and immunogenic rejection are averted, since they can be harvested from the patient.

1.1.3 STEM CELL MARKERS

While stem cells are best defined functionally, a number of molecular markers have been used to characterise various stem cell populations.

Embryonic stem cell markers

- Oct-4: Oct-4 (also termed Oct3 or Oct3/4), one of the POU transcription factors, was originally identified as a DNA-binding protein that activates gene transcription via a cis-element containing octamer motif. It is expressed in totipotent ES- and EG- cells. A critical level of Oct-4 is required to sustain stem cell self-renewal and pluripotency. Differentiation of ES cells result in down-regulation of Oct-4, an event essential for a proper and divergent development program [Scholer *et al*, 1989; Rosner *et al*, 1990; Niwa, Miyazaki and Smith, 200]. Oct-4 is not only master regulator of pluripotency that controls lineage commitment, but is also the first and most recognised marker used for the identification of totipotent ES cells.
- SSEAs (Stage Specific Embryonic Antigens): SSEAs were originally identified by the three monoclonal antibodies recognising defined carbohydrate epitopes associated with the lacto- and globo- series glycolipids, SSEA-1, -3 and -4. Undifferentiated murine pluripotent cells express SSEA-1 and do not exhibit any reactivity to the SSEA-3, SSEA-4 monoclonal antibodies. Following differentiation, murine EC and ES cells exhibit a decrease in the expression of SSEA-1 and an increase in the expression of SSEA-3 and SSEA-4 [Thomson *et al*, 1998; Thomson and Marshall, 1998]. In contrast, undifferentiated human EC, ES and EG cells express the antigens SSEA-3, SSEA-4. Differentiation of human EC and ES cells is characterized by an increase in SSEA-1 expression and a down regulation of SSEA-3 and SSEA-4. Unlike human EC and ES cells, only EG cells express SSEA-1.

ADULT STEM CELL MARKERS

In TABLE 2 below is a list of markers which are used to identify adult stem cells from hematopoietic [Sutherland *et al*, 1992; Spangrude and Brooks, 1993; Yu *et al*, 2002; Zhou *et al*, 2001], neural [Frederiksen *et al*, 1988; Mayer-Proschel *et al*, 1997; Morrison *et al*, 1999] and mesenchymal [Simmons and Rorok-Storb , 1991] origin.

STEM CELL TYPE	MARKERS
Hematopoietic stem cell	- CD34 - CD133 - stem cell antigen 1, Ly-6A/E (Sca-1) - ATP-binding cassette superfamily G member 2 (ABCG2)
Mesenchymal stem cell	STRO-1
Neural stem cell	- p75 Neurotrophin R (p75 NTR) - Polysialic acid-neural cell adhesion molecule (PSA-NCAM) - Nestin

TABLE 2 Adult Stem Cell Markers.

Since the initial derivation of mouse ES- cells in the early 1980s [Evans and Kaufman, 1981; Martin, 1981], the *in vitro* differentiation capacity of ES cells has provided a unique opportunity for experimental analysis of gene regulation and function during cell commitment and differentiation in early embryogenesis, and a potential source of cells for replacement following injury or disease. ES cells have also provided a platform to study pathways of differentiation and maintenance of pluripotency that are likely to have broad applications to the field of stem cell biology, cancer stem cell biology, and the understanding of how development may go awry.
In this project embryoid bodies (EBs) derived from mouse ES cell lines were used to study the effect of intracellular redox state in the expression of CT-1 during the differentiation of ES cells.

1.2 INTRA-CELLULAR REDOX STATE AND REDOX SIGNALLING

Free radicals and other ROS play a very important role in many cells/organisms. They are involved in many (patho-) physiological processes in the cell/organism. Every living organism possesses numerous cellular antioxidant systems to control the amount of free radicals and ROS available in the cellular system and maintain the redox balance of the cell (see FIG. 4). A shift in the equilibrium between free radicals/ROS and these control mechanism leads to oxidative stress. Free radicals are a cluster of atoms that contain an unpaired electron in their outermost orbit of electrons. This is an extremely unstable configuration, and radicals quickly react with other molecules or radicals to achieve the stable configuration. Most biological molecules are nonradicals containing only paired electrons.

1.2.1 SOURCES OF ROS

The main source of ROS is the mitochondrion which converts about 2% of consumed molecular oxygen into superoxide anion ($O_2^{\cdot -}$) during mitochondrial respiration. Mitochondrial respiration converts carbohydrates into high-energy metabolites such as adenosine triphosphate (ATP). This requires the sequential oxidation and reduction of the substrates using respiratory complexes. After oxidation by mitochondrial and plasma membrane oxidases, oxygen is reduced and the superoxide radical is formed. Other sources for $O_2^{\cdot -}$ generation include NADPH oxidase, lipooxygenase, hypoxanthine/xanthine oxidase, cyclooxygenase [Droge, 2002]. $O_2^{\cdot -}$ is a relatively short-lived ROS and is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) (equation 1-3), and H_2O_2 is degraded to water by several cellular enzymes such as catalase and glutathione (GSH) peroxidase. Reaction of $O_2^{\cdot -}$ with $NO^{\cdot}$ generates peroxynitrite, a potentially deleterious ROS (equation 4).

$$O_2 + e^- \rightarrow O_2^{\cdot -} \quad (1)$$

$$2O_2 + NADPH \rightarrow 2O_2^{\cdot -} + NADP^+ + H^+ \quad (2)$$

$$2O_2^{\cdot -} + H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

$$NO^{\cdot} + O_2^{\cdot -} \rightarrow OONO^- \quad (4)$$

H_2O_2 is also produced in the thyroid gland as a substrate for thyroperoxidase, which catalyzes the attachment of iodine to thryoglobulin, an important protein for the synthesis of thyroid hormone. H_2O_2 is generated in peroxisomes to aid in the degradation of fatty acids and other molecules, and H_2O_2 is used for detoxification reactions involving the liver cytochrome P-450 system. H_2O_2 can also react with reduced transition metals (Me) or semiquinones through the Haber-Weiss- or Fenton reactions [Stadtman and Berlett, 1998] to be converted to the highly reactive hydroxyl radical ($^{\cdot}OH$) as shown in equation 5, or it can be metabolized by myeloperoxidase (MPO) to form hypochlorous acid (HOCl).

$$H_2O_2 + Me^{(n-1)} \rightarrow {}^{\cdot}OH + OH^- + Me(n) \qquad (5)$$

e.g. $H_2O_2 + Fe^{2+}$ or $Cu^{+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+}$ or Cu^{2+}

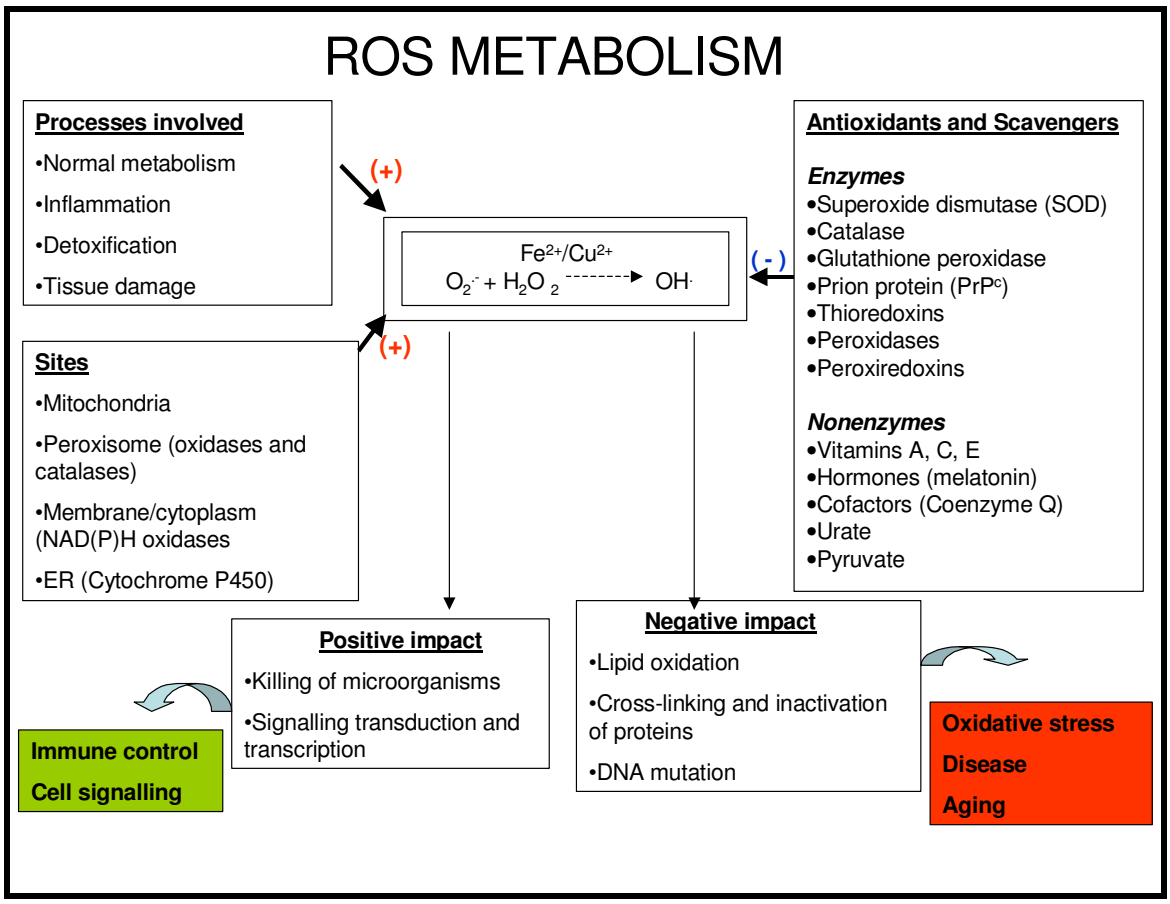

FIG. 4 Schematic presentation of sources of ROS and antioxidant defence system. The levels of intracellular ROS are balanced by the intracellular antioxidant defence system which consists of enzymatic and nonenzymatic components.

Some cells, such as phagocytic leukocytes, have evolved the use of H_2O_2 as a bactericidal defense chemical, a phenomenon known as oxidative burst. In these inflammatory cells, NADPH oxidase associated with the plasma membrane reduces molecular oxygen to generate $O_2^{\cdot -}$. $O_2^{\cdot -}$ is spontaneously or enzymatically converted to H_2O_2 which can then freely pass through the membrane. While these oxidants are important in protecting us from infection, they can cause oxidative damage during chronic inflammatory activity [Halliwell, 1991; Bruene *et al*, 2003; Kreeger, 2003]. Oxidative stress is caused by an excessive accumulation of ROS as a result of a defective antioxidant defense system of the cell (see FIG. 5).Oxidative stress can generally be imposed on cells as a result of one of three factors:

1) an increase in oxidant generation,
2) a decrease in antioxidant protection,
3) a failure to repair oxidative damage.

The importance of oxidative stress is that it increases the susceptibility of cellular constituents to oxidative molecular damage such DNA fragmentation, lipid peroxidation, activation of oncogenes or repression of tumor suppressor genes and release of Ca^{2+} within the cells, leading to the activation of Ca^{2+}-dependent proteases and nucleases. Inflammatory processes often overshoot in their reaction leading to excessive production of ROS, destruction of healthy body tissue, and development of auto-destructive disease. The relationship of oxidative stress and inflammation is undisputed. Mounting evidence points to chronic inflammation as not only being the problem of well recognized inflammatory diseases such as tuberculosis, rheumatoid arthritis or inflammatory bowel disease [Hensley *et al*, 2000], but also as a contributor to a growing number of mechanistically unconnected illnesses such as atherosclerosis, Alzheimer disease, aging and some cancers [Beckman & Ames, 1998; McNally *et al,* 2003; Forrester, 2004].

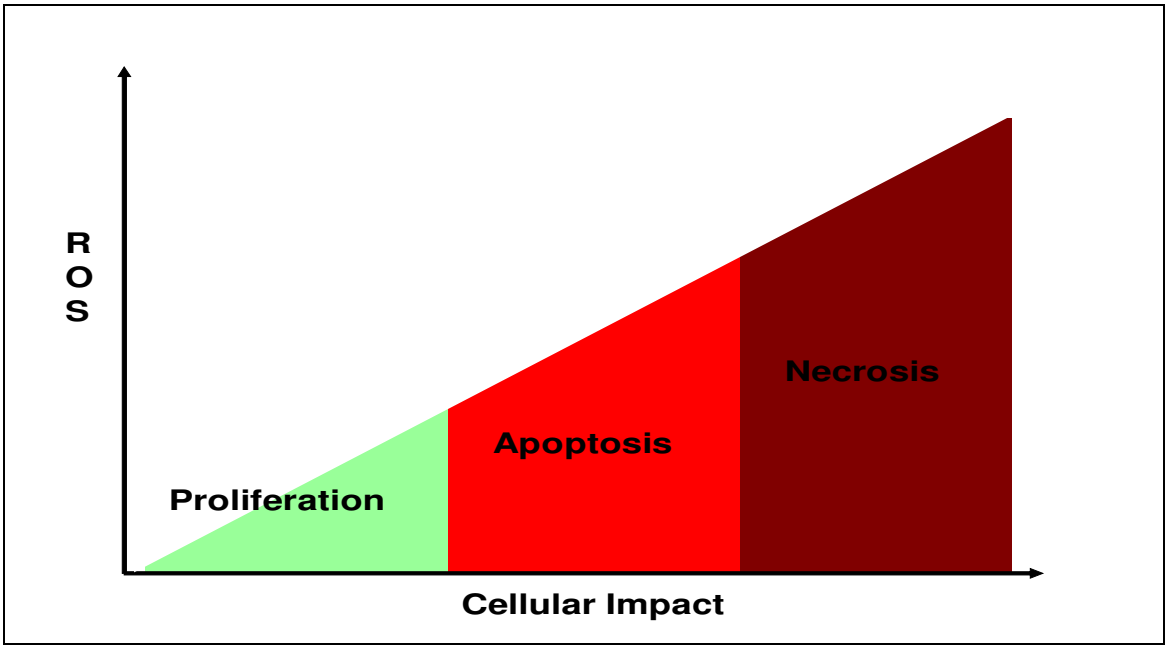

FIG. 5 Schematic illustration of the cellular impact of ROS. At low concentrations, ROS appear to exert a growth-stimulatory effect on a wide variety of cells and organisms. However, when ROS levels increase, other signaling pathways may be activated that lead to apoptosis. When ROS levels rise even higher, a cell will probably die a sudden necrotic death. Only the latter 2 modes of ROS action are currently being considered to be due to "oxidative stress." [Adapted from Buetler *et al*, 2004]

1.2.2 REDOX SIGNALLING

Signal transduction is an event of conversion of signals from extracellular stimuli carried by first messengers such as hormones, growth factors, cytokines, and neurotransmitters across plasma membranes to intracellular responses that lead to changes in gene expressions and cellular phenotypic modulations.

Evidence is rapidly accumulating to suggest that intracellular oxidation-reduction (redox) reactions play a critical role in the regulation of several (patho) physiological processes including cell proliferation, senescence, differention, and apotosis. ROS including $O_2^{\cdot -}$, H_2O_2, $^{\cdot}OH$, and organic hydroperoxides are continuously generated by-products of O_2 metabolism and have traditionally been thought of as unwanted and toxic by-products of living in aerobic environment. The long held view that ROS is detrimental to the biological tissue was challenged after recent discovery that ROS can function as signalling molecules. An important feature of the signal transduction is that the first messenger molecules need not enter the cell and their biological effects are mediated inside the cell by second messenger molecules such as cAMP, cGMP, inositol 1,4,5-trisphosphate (Ins 1,4,5-P3), nitric oxide, and phosphatidylinositol 1,3,4,5-tetrakisphosphate (PtdIns 1,3,4,5-P4) (pioneered by the

work on cAMP by Earl W. Jr. Sutherland Recipient of the 1970 Nobel Prize in Physiology and Medicine). The second messenger generation processes involve various cellular components, like specific receptors, transducers, adaptor proteins, protein kinases, and protein phosphatases, and eventually lead to the induction of physiological responses. ROS fulfil the definition of secondary messenger, which are either up-regulated or down-regulated after physiologic stimuli. ROS have been shown to be generated in a wide variety of cell types stimulated by hypoxia [Chandel *et al*, 1998; Duranteau *et al*, 1998; Chandel *et al*, 2000; Kulisz *et al* 2002; Schäfer *et al*, 2003], cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1,-6 (IL-1, IL-6), and angiotensin II (Ang II) [Ohba *et al*., 1994; Thannickal and Fanburg, 1995; Meier *et al*.,1989; Lo, Wong and Cruz, 1996; Lassegue *et al*, 2001], growth factors [Krieger-Brauer and Kather, 1995; Sundaresan *et al*., 1995; Lo, Wong and Cruz, 1995; Bae *et al*., 1997; Irani *et al*, 1997; Chandel et al, 2001; Park *et al*, 2004], and other agonist acting through tyrosine kinase and G protein-coupled receptors [Krieger-Brauer and Kather, 1995; Sundaresan *et al*., 1995; Bae *et al*., 1997; Lo, Wong and Cruz, 1996].

The mitogenic signals mediated through the generation of ROS activate transcription factors including NF-kB [Schreck *et al*., 1991] and AP-1 [Pahl and Baeuerle, 1994; Lo and Cruz, 1995; Diamond *et al*, 1999], HIF-1α [Chandel *et al*, 1998; Chandel *et al*, 2000; Sauer, Wartenberg and Hescheler, 2001; Soberman, 2003], mitogen-activated protein [MAP] kinases [Chen *et al*., 1995; Sundaresan *et al*., 1995; Guyton *et al*, 1996; Hashimoto *et al*, 2001; Haddad and land, 2002; Blanc *et al*, 2003; Gorin *et al*, 2004], phospholipase A2 [Zor *et al*., 1993], protein kinase C [Konishi *et al*., 1997], phosphatidylinositol 3-kinase [Sauer *et al*, 2000; Park *et al*, 2004] and phospholipase D [Natarajan *et al*., 1993; Min *et al*., 1998]. Furthermore, ROS increase cytosolic calcium [Suzuki *et al*., 1997], trigger apoptosis [Jacobson, 1996], inhibit protein tyrosine phosphatases [PTPase] [Hecht and Zick, 1992; Sullivan *et al*., 1994; Lee and Esselman, 2002], and alter ion transport mechanisms [Kourie, 1998]. A further feature of ROS is that they regulate the expression of antioxidant enzymes like thioredoxin system [Arner and Holmgren, 2000; Mustacich and Powis, 2000; Zhao and Holmgren, 2002], superoxide dismutase [Decraene *et al*, 2004] catalase, glutathione peroxidase and small anti-oxidant molecules like α-tocopherol, lipoic acid, ascorbic acid, and uric acid and the antiapoptotic protein BCL2.

1.2.3 REDOX SIGNALLING IN THE CARDIOVASCULAR SYSTEM

ROS are important signalling molecules in the vasculature, acting as intermediates in biochemical pathways involved in processes ranging from acute vasodilation to vascular growth and remodelling. Although it is well established that NADPH oxidase is the major source of ROS in the artery wall, it still remains unclear which isoform (s) of this enzyme contributes to ROS production during normal physiology and importantly, to the oxidative stress associated with many vascular diseases [Byrne, 2003]. In vascular cells ROS are known to mediate the K^+ channel-opening and vasodilation effects of bradykinin and other endothelium-dependent relaxing agonists. ROS also regulate long-term vasculature processes, such as cell growth and division, by acting as second messengers for the effects of growth factors such as angiotensin II and platelet-derived growth factors [Sundaresan *et al*, 1995], thrombin and cytokines such as TNF-α and IL-1 [Meier *et al*, 1989], protein kinases and transcription factors and on gene expression. It has been shown, using the stem cell-derived EBs model [Wartenberg *et al*, 1998] that ROS stimulate and promote cardiomyogenesis. This effect is attenuated with antioxidants and NADPH oxidase inhibitors [Sauer *et al*, 2000; Sauer *et al*, 2004].

Under patho-physiological conditions, ROS have the potential to cause cellular damage and dysfunction. Whether the effect is beneficial or harmful will depend upon site, source and amount of ROS produced, and the overall redox status of the cell. All cardiovascular cell types are capable of producing ROS, and the major enzymatic sources in heart failure are mitochondria, xanthine oxidases, and non-phagocytic NADPH oxidases (Noxs) [Griendling, 1997; Lassegue *et al*, 2001; Kulisz *et al*, 2002; Suzuki and Griendling, 2003]. In addition to their effects on cellular enzymatic and protein function, ROS have been implicated in the development of agonist-induced cardiac hypertrophy, cardiomyocyte apoptosis and remodelling of the failing myocardium. ROS production in the artery wall has been shown to increase so that the anti-oxidant systems are overwhelmed in vascular diseases such as hypertension, diabetes and atherosclerosis [Sorescu *et al*, 2002]. This shift in equilibrium between ROS production and elimination, leads to oxidative stress, a hall mark of virtually all vascular patho-physiological states. ROS have been implicated as a major cause for the pathogenesis of myocardial ischemia [Sudgen and Clerk,

1998] and reperfusion injury. These alterations in phenotype are driven by redox-sensitive gene expression, and in this way ROS act as potent intracellular second messenger. Despite advances in treatment, chronic congestive heart failure carries poor prognosis and remains a leading cause of cardiovascular death. Accumulating evidence suggests that ROS play an important role in the development and progression of heart failure, regardless of the ethiology. Keith and colleagues (1998) found in a clinical study with 58 patients a progressive increase in lipid peroxidation, and a reduction in antioxidant reserve with the progression of congestive heart failure (CHF). Mallat and colleagues (1998) also reported an increase in levels of 9-iso-prostaglandin $F_{2\alpha}$, a marker of oxidative stress, in the pericardial fluid of CHF patients undergoing surgery, which are closely related with NYHA functional class and left ventricular (LV) chamber dimensions. Cardiac hypertrophy is a compensatory response that allows the heart to cope with the pathogenic stimuli found in many cardiovascular diseases. Cardiac hypertrophy occurs in response to diverse stimuli, including mechanical stress [Komuro *et al*, 1990; Sadoshima and Izumo, 1993; An *et al*, 1999; Aikawa *et al*, 2001; Sugden, 2001; Sugden, 2003], and neurohormonal stimuli such as angiotensin II [Ushio-Fukai *et al*, 1996; Griendling *et al,* 2000; Lassegue *et al,* 2001; Sadoshima *et al*, 1993], endothelin-1 [Yamazaki *et al,* 1996] and norepinephrine [Zimmer *et al,* 1995] The functional importance of ROS in cardiovascular cells has been most widely studied in VSMCs where $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH^-$ have been shown to be prohypertrophic [Ushio-Fukai *et al*, 1998; Finkel, 1999; Griendling *et al,* 2000; Aikawa *et al*, 2001]. The treatment of neonatal rat ventricular myocytes with vitamin E, N-acetylcysteine, N-2-mercaptopropionyl-glycine and catalase completely inhibited angiotensin II and TNF-α induced myocyte hypertrophy [Nakamura *et al*, 1998; Aikawa *et al*, 2001; Xiao *et al,* 2002]. Laursen and colleagues (1997) showed that angiotensin II-induced hypertension was associated with increased vascular $O_2^{\cdot-}$ production and that treatment with SOD reduced blood pressure by 50 mmHg in angiotensin II-infused rat. In addition, Hamaguchi and colleagues (1998) reported that the increase in MAP kinase activity was sustained in the angiotensin II-infused rat. These findings suggest that hypertension caused by chronically elevated angiotensin II and MAP kinase activity is mediated in part by $O_2^{\cdot-}$. Increases in ROS may affect the pathogenesis of atherosclerosis. Hypercholesterolemic animals and patients exhibit impaired endothelial dependent relaxation that can be restored with antioxidants. It was clinically observed that

vascular superoxide production by NADH/NADPH oxidase is associated with endothelial dysfunction in patients with hypercholesterolemia [Guzik *et al*, 2000; Djordjevic *et al*, 2005]. Vessel remodeling is also a ROS-sensitive process. It was reported that p22phox, a component of NADH/NADPH oxidase, expressed highly in atherosclerotic remodeled specimens of the coronary artery. It should be noted that NADH/NADPH oxidase is a potent mediator of MAP kinase activation. Recent findings revealed that remodeling occurs in patients after the PTCA procedure and remodeling is a key process for restenosis. *In vivo* experiment have shown that antioxidants inhibit restenosis implying the role of ROS in vascular remodelling [Azumi *et al*, 1999; Mintz *et al*, 1996, Numes *et al*, 1995; Tardif *et al,* 1997].

1.2.4 VASCULAR NADPH-OXIDASES

The major sources of ROS in the vasculature, the vascular NAD(P)H oxidases, are similar in structure to the phagocytic NADPH oxidase, which consist of 4 major subunits: a cytochrome b558, comprising of a large catalytic β-subunit, gp91phox that contains binding sites for NADPH and molecular oxygen, as well as flavin and 2 heme groups to allow electron transport between the two substrates and a smaller α-subunit, p22phox. The cytoplasmic protein complex is composed of p47phox, p67phox and p40phox, and a regulatory low-molecular weight G protein rac. The function of p40 subunit which is not essential for oxidase activity is still to be clarified. In order to be activated, the oxidase requires the translocation of the cytoslic components to the membrane (see FIG. 6). There exist at least three isoforms of NADPH oxidase expressed in the vascular wall, each differing with respect to the flavin-containing catalytic subunit it uses to transfer electrons from NADPH to molecular oxygen. Thus, although endothelial cells and adventitial fibroblasts express a gp91phox-containing NADPH oxidase (Nox2) similar to that originally identified in phagocytes, vascular smooth muscle cell and cardiomyoctes may rely on homologes of gp91phox, namely Nox1 [Suh *et al*, 1999; Bànfi *et al*, 2000; Sorescu *et al*, 2004] and Nox4 [Geiszt *et al*, 1997; Shiose *et al,* 2001] to produce superoxide. Other Noxs have been found in non-vascular cells, these include Nox3 [Cheng *et al,* 2001; Lambeth, 2004] found in embryonic kidney, Nox5 which is distinguished from the other family members by its longer N-terminus and is found in testis, B- and T-lymphocyte-rich area of spleen and lymph nodes, Duox1 and Duox2 with an additional peoxidase domain [Krause *et al*, 2004; Harper *et al*, 2005]. Duox1 is found in thyroid and lung, and Duox2 in thyroid and colon.

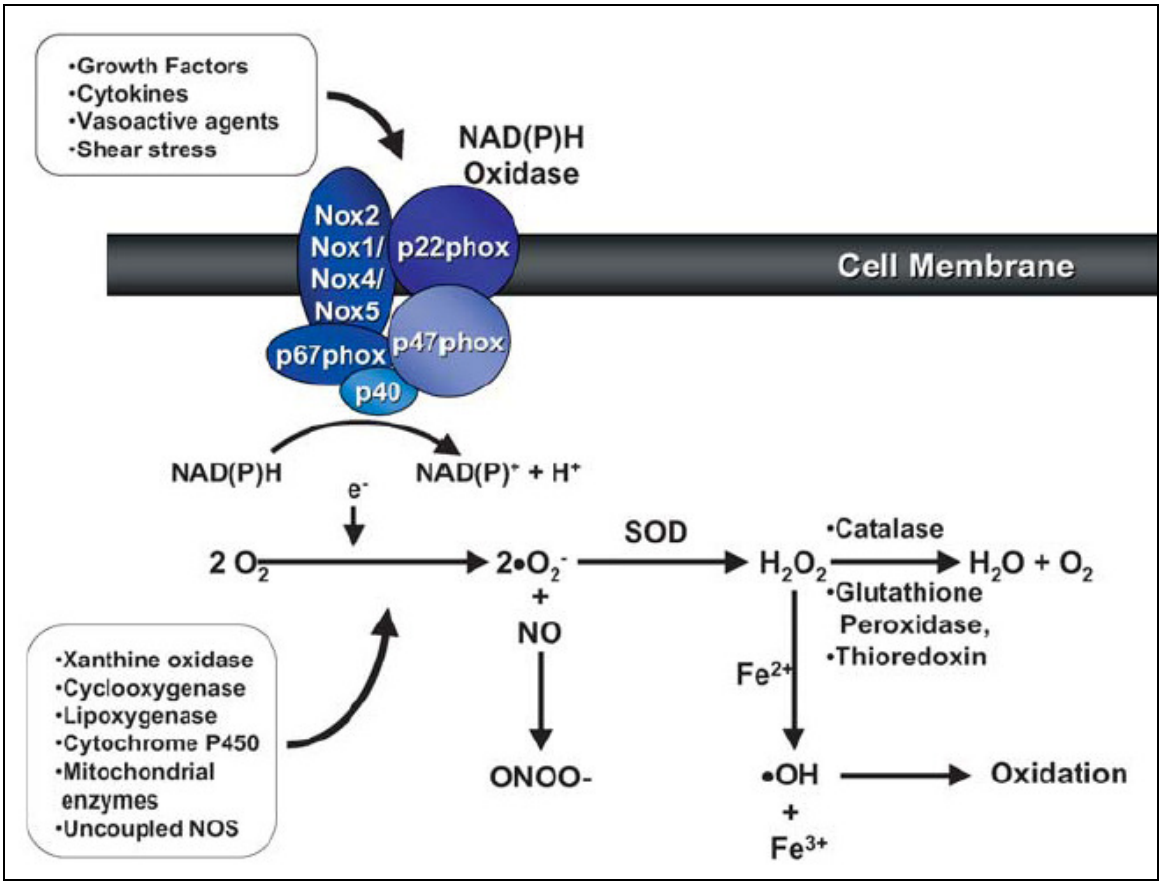

FIG. 6 Generation of $O_2^{\cdot-}$ and H_2O_2 from O_2 in vascular cells. Many enzyme systems, including NAD(P)H oxidase, xanthine oxidase, and uncoupled nitric oxide synthase (NOS) among others, have the potential to generate ROS. Superoxide acts either as an oxidizing agent, where it is reduced to H_2O_2 by SOD, or as a reducing agent, where it donates its extra electron (e^-) to form $ONOO^-$ with NO. Hydrogen peroxide is scavenged by catalase, glutathione and thioredoxin systems, and can also be reduced to generate ·OH in the presence of Fe^{2+} [Touyz and Schiffrin, 2004].

1.2.5 HYPOXIA, HIF-1α REDOX SIGNALLING

In order to achieve oxygen homeostasis, a process that is essential for survival, pO_2 delivery to the mitochondrial electron transport chain must be tightly maintained within a narrow physiological range. This system may fail with subsequent induction of hypoxia, resulting either in a failure to generate sufficient ATP to sustain metabolic activities or in a hyperoxic condition that contributes to the generation of ROS, which in excess could be cytotoxic. Variation in ΔpO_2 in particular, differentially regulates the compartmentalisation and functioning of transcription factors such as hypoxia-inducible factor-1α (HIF-1α) and nuclear factor-*kappa* B (NF-κB). Oxygen-evoked regulation of HIF-1α and NF-κB is closely coupled with the intracellular redox state, such that modulating redox equilibrium affects their responsiveness at the molecular level (expression/transactivation). Hypoxia initiates transcription of a number of gene products that help to sustain the supply of O_2 to tissues and to enhance cell survival

during severe O_2 deprivation. The induction of these genes is mediated by hypoxia-inducible factor 1 (HIF-1), a heterodimeric transcription factor consisting of HIF-1α, and the aryl hydrocarbon nuclear translocator (ARNT or HIF-1β) subunits, both belonging to the basic helix-loop-helix Per-aryl hydrocarbon receptor nuclear translocator Sim (PAS) family of transcription factors (see FIG 7) [Chun, Kim and Park, 2002; Wang et al, 1995; Semenza, 2001; Semenza, 2002].

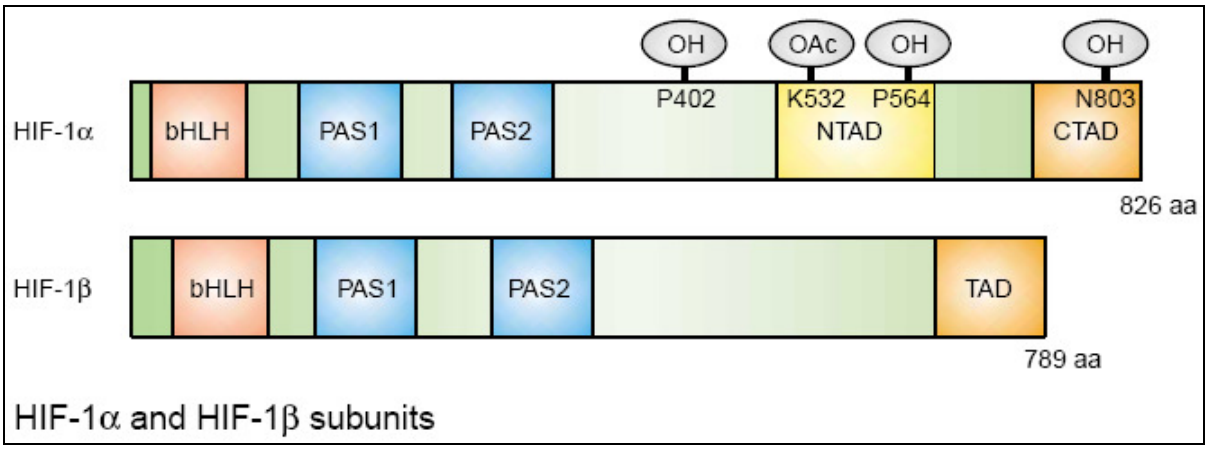

FIG 7 HIF-1α and HIF-1β subunits. HIF-1α and HIF-1β contain one basic–helix–loop–helix (bHLH) domain and two PER–ARNT–SIM (PAS1 and PAS2) domains in their N-terminal regions. The positions of post-translational hydroxylation (OH) and acetylation (OAc) sites of HIF-1α are indicated. Hydroxylation of two proline residues (at P402 and P564) and acetylation of lysine (at K532) within the oxygen dependent degradation (ODD) domain (residues 401–603) and close to the N-terminal transactivation domain (NTAD) confers recognition by pVHL (the product of the von Hippel–Lindau tumour suppressor gene), leading to degradation of the α-subunit. Hydroxylation at N803 in the C-terminal transactivation domain (CTAD) of HIF-1α inhibits recruitment of coactivators required for HIF1α transcriptional activity. HIF-1α contains one transactivation domain (TAD) in its C-terminus. [www-ermm.cbcu.cam.ac.uk/ 05009130h.htm]

HIF-1 binds to the core DNA sequence 5'-[AG]CGTG-3' within the hypoxia response element (HRE) of target gene promotors. Activation requires recruitment of transcriptional coactivators such as CREB-binding protein (CBP) and p300.

Since its original description, the study of the transcription factor HIF-1 has demonstrated its central role in regulating the body's response to changing oxygen levels. HIF-1 is a key component of a widely operative transcriptional response activated by hypoxia, cobaltous ions, and iron chelation. HIF-1α, first identified *in vitro* through its DNA-binding activity expressed under hypoxic conditions, has its concentration and activity increased exponentially when oxygen tensions are decreased over physiological relevant ranges (hypoxia). The ubiquitous activity of HIF-1α is thus consistent with the significant role that it plays in coordinating adaptive responses to hypoxia [Wang and Semenza, 1995; Jewell, 2001; Semenza, 2001; Lutz and Prentice, 2002; Page, 2002]. It is the paradigm for how molecular oxygen

can regulate transcription. The functions of HIF-1 target genes have been divided into categories that include cell proliferation and viability, erythropoiesis and iron metabolism, as well as vascular development and remodeling. The expression of these genes is induced when oxygen tension decreases over physiological relevant ranges. To prevent the continuous overexpression of these genes, cells utilize prolyl hydroxylases [Hofer *et al*, 2001; Elkins *et al*, 2003; Huang and Bunn, 2003] to hydroxylate HIF-1α. Hydroxylation targets HIF-1α for binding to the von Hippel-Lindau protein (pVHL), which is the recognition component of an E3 ubiquitin protein ligase, and ubiquitination of HIF-1α. These results in the targeting of HIF-1α for destruction by the proteasome. Cells lacking functional pVHL cannot degrade HIF and thus overproduce mRNAs encoded by HIF target genes. Oxygenation of asparagine also blocks the recruitment of coactivating proteins [Lando *et al*, 2002].

Many researchers have shown that there is an increase in intracellular ROS levels during hypoxia [Chandel *et al*, 1998; Duranteau *et al*, 1998; Kulisz *et al*, 2002; Schaefer *et al*, 2003], indicating that redox signaling pathways may be involved in the regulation of hypoxia responsive genes. This hypothesis was supported by the fact that pro-oxidants, that induce an increase in intracellular ROS, stabilized and activated HIF-1α leading to the increased expression of various genes [Duyndam *et al*, 2001; Haddad and Land, 2001; Gao *et al*, 2002].

1.3 CARDIOTROPHIN-1 AND INTERLEUKIN-6 SUPERFAMILY

1.3.1 INTERLEUKIN-6 SUPERFAMILY AND RECEPTOR-COMPLEXES

CT-1 is member of the cytokine family known as the IL-6 superfamily that also includes interleukin-6 (IL-6), interleukin-11 (IL-11, leukaemia-inbibitory factor (LIF), Oncostatin M (OSM), cardiotrophin-like cytokine (CLC) and ciliary neurotrophic factor (CNTF). CT-1 has overlapping functions with other IL-6 family members in a variety of cell types [Pennica *et al*, 1995]. Since the molecular cloning of IL-6 in 1986, members of the IL-6 family of cytokines, LIF, CNTF, OSM, IL-11 and CT-1 have been molecularly cloned. During the last decade, many findings have been made concerning the structure and function of IL-6 family cytokines and their receptors. These findings, together with a large number of studies on many cytokines, have greatly contributed to the establishment of a variety of concepts about these cytokines in general: the establishment of pleiotropy and redundancy as properties of cytokine function, the cytokine receptor superfamily, the sharing of a signal-transducing receptor subunit among several cytokine receptors (see FIG. 8), and the agonistic activity of certain soluble cytokine receptors. In fact, the IL-6 cytokine family plays pivotal roles in the immune, hematopoietic, nervous, cardiovascular, and endocrine systems, as well as in bone metabolism, inflammation, and acute phase response. Furthermore, they often exert overlapping biological activities; the molecular mechanism of this functional redundancy is explained at least in part by the sharing of gp130, a signal-transducing receptor subunit among the receptors for the IL-6 cytokine family. Important questions yet to be resolved are: how a single cytokine can exert functional pleiotropy and how it can induce only a specific biological activity in a given target cell. Another important receptor that plays a role in the signalling by members of the IL-6 superfamily of cytokines is the LIFR. These cytokines relay their signals into the cells either by homodimerisation of gp130R (like in the case of IL-6, IL-11) or heterodimerisation of the LIFR and gp130R (LIF, CT-1, CNTF) [Taga *et al*, 1997; Heinrich *et al*, 1998; Hermans *et al*, 1999]. In the case of OSM, a third receptor has been cloned i.e. the OSMR [Mosley *et al*, 1996]; the receptor combination depends on the type of tissue (either LIFR and gp130R or OSMR and gp130R). Robledo and colleagues in 1997 reported the existence of a third receptor for CT-1. This receptor is highly glycosylated and has a molecular

weight of about 80 kDa (gp80). It is postulated that the gp80R regulates the cell specifity and sensitivity of CT-1, since it is not expressed in all cell types that express the LIFR.

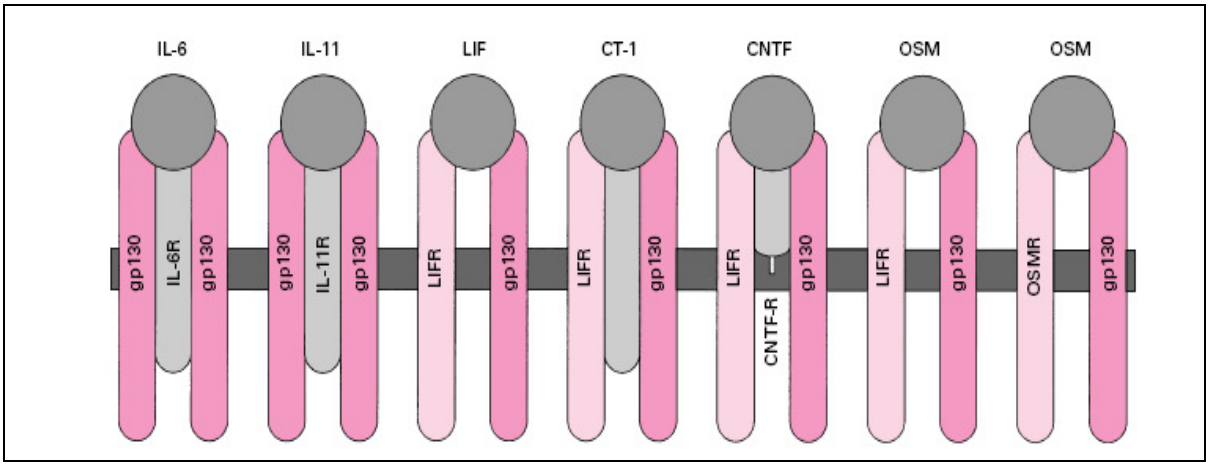

FIG. 8 Receptor complexes of IL-6-type cytokines. IL-6-type cytokine receptor complexes signal through different combinations of the signalling receptor subunits gp130, LIFR and OSMR, with gp130 being used by all the family members [Heinrich *et al*, 1998].

1.3.2 CARDIOTROPHIN-1

CT-1 was discovered by Pennica and colleagues (1995) via expression cloning of mouse embryoid bodies, using a cardiac myocyte hypertrophy screen to identify positive clones. The mouse CT-1 protein contains 203 amino acids and shares 80% amino acid homology with the human 201-amino acid CT-1 sequence however, unlike the mouse CT-1, the human CT-1 protein has 2 rather than 1 cys and has no N-gylcosylation site. They reported that the mouse CT-1 gene constitutes 5.4 kilobases (kb) in length and consists of three exons and two introns. When nucleotide sequences of the coding regions of exons were compared with those of human exon 1, 2 and 3, they were shown to share 96%, 84% and 81% homology, respectively. The amino acid sequence of CT-1 has some 24% identity and 19% identity with that of LIF and CNTF, respectively (see FIG. 9). While members of the IL-6 superfamily are only distantly related in primary sequence (15-20% amino acid identity), they are predicted to have similar tertiary structures containing four amphipathic helices. Analysis of the helices predicted for CT-1, based on the sequence alignment (see FIG. 9), indicates that they are amphipathic, as would be expected for a member of this family. CNTF, like CT-1, lacks a hydrophobic N-terminal secretion signal sequence. Funamoto and colleagues (2000) isolated and characterized the mouse

CT-1 gene. They showed that the 2.2 kb of 5 flanking region of the mouse CT-1 gene contains a variety of transcription factor binding motif (e.g. CREB, HIF-1, MyoD, NF-IL6, Nkx2.5, and GATA). Fluorescent *in situ* hybridization (FISH) analysis demonstrated that the mouse CT-1 gene was located on chromosome 7F3.

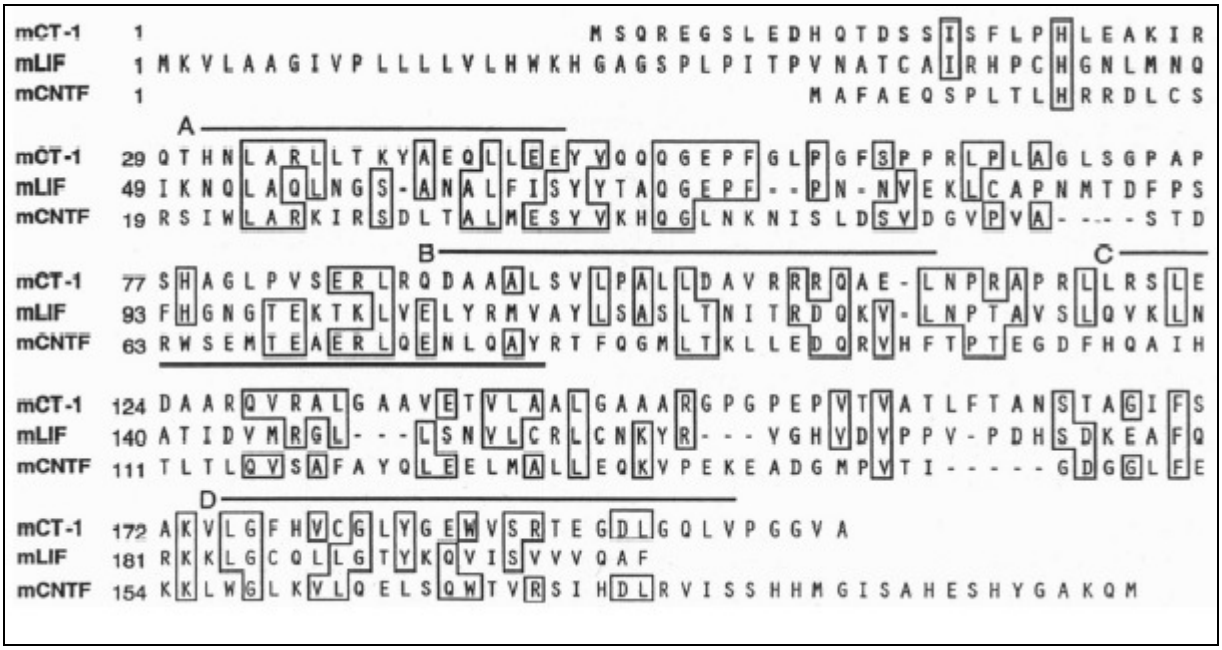

FIG. 9 Encoded amino acid sequence of mouse CT-1 (mCT-1) aligned with that of mouse LIF (mLIF) and mouse CNTF (mCNTF). Overlining indicates the location of four amphipathic helices based on their proposed locations in CNTF. As a quantitative measure of their amphipathic character, the mean helical hydrophobic moments ((ILH)) for the four CT-1 segments (maximum of 18 residues) are 0.59, 0.34, 0.59, and 0.34 for helicesA-D, respectively. [Pennica *et al*. 1995]

CT-1 is a pleiotropic cytokine which is highly expressed in heart, skeletal muscle, prostate and ovary and to lower levels in lung, kidney, pancreas, thymus, testis and small intestine [Asai *et al*, 2000]. CT-1 signals through the LIF receptor and the gp130 receptor subunit. The LIF receptor binds CT-1, and then gp130 associates with the ligand-receptor complex and transduces the proximal signal. CT-1 intracellular signaling pathways include extracellular signal regulated kinases (ERK), mitogen activated protein (MAP) kinases, the janus kinase (JAK)/signal transducers and activators of transcription (STAT) system, and PI3-kinase/Akt. Downstream mediators of CT-1's cellular effects include multiple ERK-coupled transcription factors, STAT-3, nuclear factor-κB, and heat shock proteins 56, 70, and 90 [Freed *et al*, 2005; Funamoto *et al*, 2000; Kunisada *et al*, 1998; Kuwahara *et al*, 2000; Robledo *et al*, 1997; Pennica *et al*, 1996; Yasukawa *et al*, 2001]. CT-1 has the ability to induce cardiac myocyte hypertrophy, and enhances the survival of cardiomyocyte (see FIG. 10 for mechanisms involved). CT-1 promotes cardiac myocyte hypertrophy by

directing sarcomere assembly in series through gp130 signalling [Wollert et al. 1996]. At the ventricular structural level, in-series sarcomeric assembly leads to “eccentric” hypertrophy and chamber dilation. CT-1 has been shown to be elevated in the serum of patients with ischemic heart disease, including unstable angina pectoris [Talwar *et al*, 2000], myocardial infarction [Takimoto *et al*, 2002], and heart failure [Jougasaki *et al*, 2000; Talwar *et al*, 2000; Zolk *et al*, 2002]. CT-1 has also a neurotrophic function [Li *et al*, 2003; Sakamoto *et al*, 2003]. Oppenheim and colleagues (2001), reported that CT-1 deficiency causes increased motoneuron cell death in spinal cord and brainstem nuclei of mice during a period between embryonic day 14 and the first postnatal week. Bordet and colleagues (2001) reported that intramuscular injection of an adenoviral vector encoding CT-1 in SOD1G93A newborn mice resulted in systemic delivery of CT-1, supplying motor neurons with a continuous source of trophic factor. Furthermore, they showed that CT-1 delayed the onset of motor impairment. CT-1 is a hepatocyte survival factor that efficiently reduces hepatocellular damage in animal models of acute liver injury [Robledo *et al*, 2003]. Richards CD and colleagues (1996), showed that murine CT-1 is a strong acute-phase mediator for rat hepatocytes in vitro and that its activity is similar to LIF on rat hepatocytes, H35 cells, and HepG2 cells. CT-1 expression has been shown to be augmented after hypoxic stimulation, and it can protect cardiac cells when added either prior to simulated ischemia or at the time of reoxygenation following simulated ischemia (Liao *et al*, 2002; Bristow and Long, 2002). CT-1 also can induce expression of protective heat shock proteins (Hsps) in cardiac cells. CT-1 increased ventricular expression of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and angiotensinogen mRNA [Ishikawa *et al*, 1999]. So far, the molecular regulation of CT-1 expression is unknown. The property of CT- 1 as a cardioprotective cytokine in stress conditions predicts upregulation during cardiac diseases which are characterized by an environment of hypoxia, inflammation and oxidative stress. It could be hypothesized that stress stimuli occurring during cardiac diseases regulate the expression of CT-1 which subsequently exerts its cardioprotective effect. A comparable microenvironment of hypoxia and elevated ROS generation may prevail in the heart of the early post-implantation embryo and may regulate CT-1 expression in the embryonic heart. Hypoxia and robust endogenous ROS production have been previously shown to occur in differentiating

ES cells and may represent one key stimulus for regulation of CT-1 expression as well as induction of the cardiomyogenic cell lineage [Sauer *et al*, 2000].

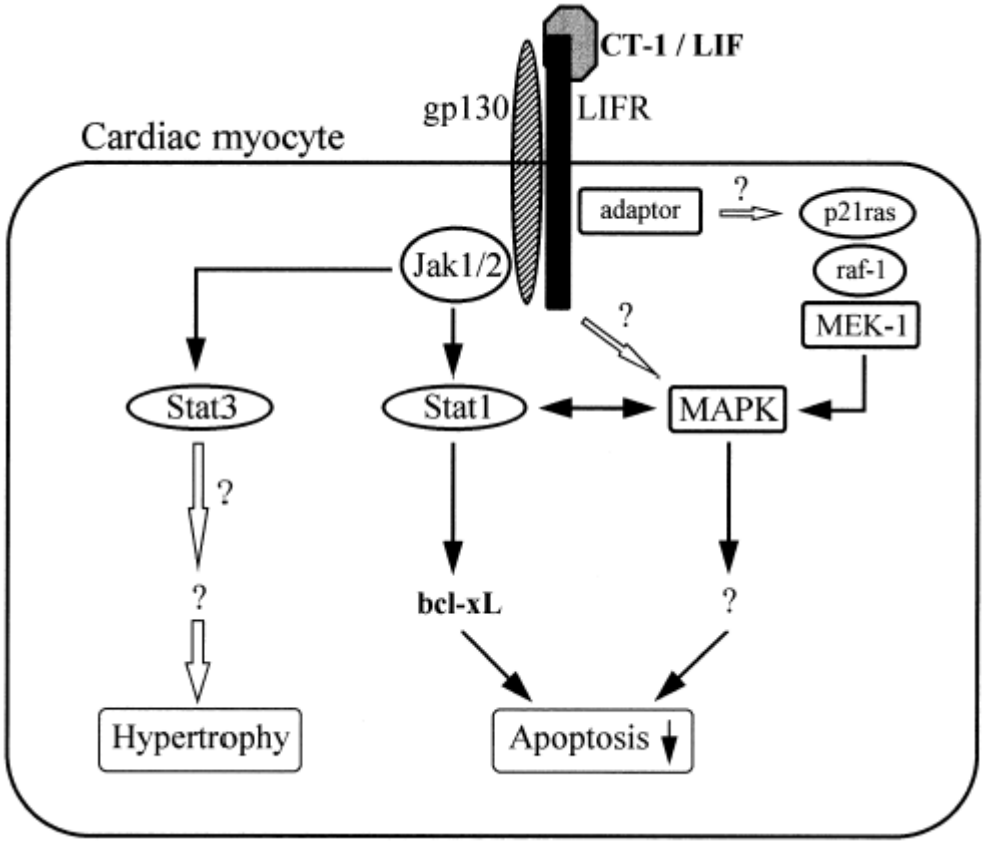

FIG. 10 Schematic representation of signal pathways involved in CT-1 signalling. There exists evidence for the involvement of JAK, Stat1 / 3, MAPK and there is possibility of cross talk [Wollert, 1997]

1.4 AIM AND OBJECTIVES

The current study was performed to evaluate the impact of the stress factors hypoxia and ROS on the signalling cascades resulting in CT-1 expression. Since differentiating ES cells into embryoid bodies mimic cardiomyogenic differentiation [Desbaillets *et al*, 2000; Itskovitz-Eldor *et al*, 2000; Kehat *et al*, 2003], and knowing that vascularised embryoid bodies are well oxygenated [Gassmann *et al*, 1996], we made use of this cell culture model.
ES cell-derived embryoid bodies endogenously generate ROS – and express CT-1 as well as the oxygen sensing element HIF-1α. The aim of this project was to find out whether:

(i) CT-1 expression is regulated by ROS
(ii) HIF-1α is involved in ROS dependent CT-1 expression

In order to do this the following objectives were drawn;

- Evaluation of the effect of exogenous ROS and hypoxia induced ROS generation on the expression of CT-1 and HIF-1α

- Analysis of the site of ROS generation by analysing the expression of NADPH-oxidase

- Analysis of the involvement of MAPKs in ROS dependent expression of CT-1 and HIF-1α

- Analysis of the involvement of the JAK/STAT pathway in ROS induced CT-1 and HIF-1α up-regulation

- Analysis of cellular localisation of CT-1 after stimulation of cells with exogenous ROS and chemical hypoxia.

2 MATERIALS AND METHODS

2.1.1 MEDIUM AND CHEMICALS

MEDIUM AND CHEMICALS		
Name	**Catalogue-Number**	**Supplier**
β-Mercaptoethanol	M7522	Sigma
17β-Estradiol	E4389	Sigma
2´7´-dichlorodihydro-fluorescein diacetate (H2DCFDA)	D-399	Molecular Probes
2-Amino-methyl-pyridine	164575	Calbiochem
2-Methoxyestradiol	S-540	Biomol
AEBSF Hydrochloride	101500	Calbiochem
AG 18	658395	Calbiochem
AG 490	658401	Calbiochem
Apocynin	178385	Calbiochem
IMDM	F0465	Biochrom
Collagenase B	1088807	Roche
Detergent 7X	34205.01	Serva
Diphenyleniodonium chloride	D2926	Sigma
DMEM high glucose	D5671	Sigma
DMSO	317275	Calbiochem
DNAse I	18068-015	Invitrogen
dNTP's	18427-013	Invitrogen
Dulbecco´s PBS (1x) w/o Ca&Mg	H15-002	PAA
Ebselen	E3520	Sigma
ESGRO (LIF)	ESG1106	Chemicon
FCS	F7524	Sigma
Glycerol	49781	Sigma
JNK Inhibitor	420119	Calbiochem
JumpStart ReadyMix	P-0982	Sigma
KH_2PO_4	3094.2	Roth
L-Glutamin 200mM (100x)	M11-004	PAA
Ly 294002	440202	Calbiochem
MEK Inhibitor UO 126	V112A	Promega
MEM (50x) aminoacids	K0363	Biochrom
Menadion 2-methyl-1,4-naphtochinon 98%	M5740-5	Sigma
Mitomycin C	M4287	Sigma

MMLV RT		Biorad
Monothioglycerol	M6145	Sigma
N-(2-mercaptopropionyl)glycine	M6635	Sigma
N-acetyl-L-cysteine cell culture tested	A9165	Sigma
NaCl	3957.1	Roth
$NaH_2PO_4x2H_2O$	4984.2	Roth
NEA (100x)	K0293	Biochrom
Penicillin/Streptomycin	P11-010	PAA
Primer		Invitrogen
Random hexamer primer	48190-011	Invitrogen
RNAlater-ICE 25ml 2833AE	#7030	Ambion
SB 202190	559388	Calbiochem
SB 203580	Alx-270-179-M001	Alexis
Sigmacote	SL-2	Sigma
SKF 86002	567305	Calbiochem
Sodiumpyruvat (100mM)	L0473	Biochrom
Sterillium	pharmacy	
Superscript II RTase	18064-014	Invitrogen
Sytox Green Nucleic Acid Stain 5mM solution in DMSO	S7020	Invitrogen
Thymochinon	27.4666-6	Sigma
Tosyl-l-phenylalanin-chloromethyl ketone	T-4376	Sigma
Triton X-100	8787	Sigma
Trizol reagent	15596-018	Invitrogen
Trolox	238813	Aldrich
Trypsin /EDTA	25300-062	Invitrogen
Water for molecularbiology, DEPC-treated, steril	A2864.0500	AppliChem
Wortmannin	681675	Calbiochem

TABLE 3 List of Reagents and Media Used

2.1.2 ANTIBODIES

ANTIBODIES		
Name	**Cataloge-Number**	**Supplier**
cardiotrophin 1 (recombinant mouse)	438-010CF	R&D Systems
CT1 anti-mouse monoclonal (IgG)	MAB438	R&D Systems
Cy 2 goat anti-mouse (IgG + IgM (H +L))	115-225-044	Dianova
Cy 2 mouse anti-rat (IgG (H +L))	212-225-082	Dianova
Cy 3 donkey anti-goat (IgG (H +L))	705-166-147	Dianova
Cy 3 rabbit anti-rat (IgG, F(ab')2 fragment)	312-165-047	Dianova
Cy 5 goat anti-rabbit (IgG (H +L))	111-175-144	Dianova
Cy 5 mouse anti-goat (IgG (H +L))	205-175-108	Dianova
Cy 5 rabbit anti-rat (IgG, Fcg-fragment)	AP164S	Chemicon
Cy 5 sheep anti mouse (IgG, F(ab')2 fragment)	515-175-072	Dianova
FITC anti-guinea-pig (IgG, Fab-specific)	F7762	Sigma
FITC goat anti-mouse (IgG)	F8771	Sigma
FITC sheep anti-rabbit (IgG)	F7512	Sigma
gp130 (IgG) --goat	AF468	R&D Systems
gp91-phox (C-15) (IgG) --goat	sc-5827	santa cruz
HIF 1-α --mouse	ABR-MA1-516-R100	Alexis
JAK2 (C-20) (IgG) --rabbit	sc-294	Santa Cruz
MOX 1 (M-15) (IgG) --goat	sc-5819	Santa Cruz
NOX 4 (M-15) (IgG) --goat	sc-21860	Santa Cruz
p-gp130 (Ser 782) (IgG) --goat	sc-12978	santa cruz
p-JAK2 (IgG) --goat	sc-21870	santa cruz
p22-phox(C-17) (IgG) --goat	sc-11712	santa cruz
p67-phox (H-300) (IgG) --goat	sc-15342	santa cruz
PI3-Kinase P85 --rabbit	4292	Cell Signaling
phospho p38 MAP Kinase --rabbit	9211S	Cell Signaling
phosphop44/42 MAPKinase (Thr202/Tyr204) --rabbit	9101S	Cell Signaling
phospho SAPK/JNK (Thr183/Tyr185) --rabbit	9251S	Cell Signaling
Phospho STAT3 (Ser727) (6E4)	9136 L	Cell Signaling

TABLE 4 List of Primary and Secondary Antibodies

2.1.3 MEDIA, BUFFERS AND SOLUTIONS

10x PBS	4 g KCl (26,82 mM) 4 g KH_2PO_4 (14,70 mM) 160 g NaCl (1,37M) 23 g $Na_2HPO_4 * H_2O$ (64,61 mM) dissolved in 2L H_2O, set pH to 7,4 with HCl
1x PBS	10 x PBS 100 ml + Water 900 ml
0,01%PBST	1 x PBS 999 ml + Triton 100 µl
0,1%PBST	1 x PBS 999 ml + Triton X-100 1 ml
Blocking solution (10% FCS in 0,01% PBST)	5 ml FCS + 45 ml 0,01% PBST
Medium for feeder layer cultivation --IMDM with 10 % FCS	- 500 ml IMDM-MEDIUM - 1,5 ml Penicillin / Streptomycin (100x) - 3,5 ml β-Mercaptoethanol (10 µM) - 6,25 ml L-Glutamine (100x) - 6,25 ml NEA (100x) - 6,25 ml MEM-Aminoacids (100x) - 6,25 ml Na- Pyruvat (100x) - 59 ml Heat-inactivated FCS
Medium for embryoid body (EB) cultivation, **Differentiation medium** -- IMDM with 20 % FCS	- 500 ml IMDM-MEDIUM - 2,5 ml Penicillin / Streptomycin (100x) - 3,5 ml β-Mercaptoethanol (10 µM) - 6,25 ml L-Glutamine (100x) - 6,25 ml NEA (100x) - 6,25 ml MEM-Aminoacids (100x) - 6,25 ml Na- Pyruvat (100x) - 132,5 ml Heat-inactivated FCS
ES maintenance medium	49 ml IMDM with 10 % FCS + 1 ml LIF solution (1000U/ ml)

TABLE 5 Composition of Cell Culture Media and Buffers

2.2 EMBRYONIC STEM CELL CULTURE

Mouse ES-cells have been established as permanent lines of undifferentiated pluripotent cells as early as 1981 [Evans and Kaufman, 1981; Martin, 1981]. These cells are generally isolated from the inner cell mass of the preimplantation blastocyst. They can be maintained in a pluripotent state for indefinite periods of time in the presence of the leukemia inhibitory factor (LIF) or in coculture with mouse embryonic fibroblasts (MEFs). When cultured in the absence of LIF, ES cells differentiate spontaneously, forming three-dimensional (3D) aggregates called EBs. By cultivation *in vitro* as EBs, ES cells differentiate into derivatives of all 3 primary germ layers (ectoderm, mesoderm, and endoderm). The EB system is an extremely valuable tool for the investigation of embryonic development in vitro.

Three mouse cell lines were used during this project: HIF-1α $^{-/-}$, HIF-1α $^{+/+}$, and CCE mouse ES cell lines. The HIF-1α $^{-/-}$ and HIF-1α $^{+/+}$ cell lines were kindly donated by Max Gassmann Department of Veterinary Physiology, Zurich Switzerland. The CCE S103 line is a mouse ES cell line derived from 129/Sv mouse strain. It was made by the separate efforts of both Dr. Robertson E and Dr. Keller G [Robertson *et al*, 1986; Keller *et al*, 1993]

The cells were cultured on inactivated feeder layers. Feeder layer cells are LIF producing cells from mouse fibroblasts, LIF hinders the stem cells from differentiating spontaneously. The ES cell maintenance medium (Iscoves medium) was supplemented with LIF in order to strengthen this effect. Medium change was performed every day.

All cell-handling procedures, unless otherwise noted, were performed in a sterile laminar flow hood to prevent contamination.

2.2.1 CULTURE AND MAINTENANCE OF MOUSE FIBROBLASTS (FEEDER LAYER CELLS)

The feeder layer cells seeded at a density of 9 x 10^4 were grown in 10% FCS IMDM to about 80% confluence (normally attained after 2-days) at 37°C and 5% CO_2. The proliferation of the feeder layer cells was stopped by treating with mitomycin C. Mitomycin C is an antibiotic produced from *Streptomyces caespitosus*. It is an alkylating agent with anti-neoplastic properties. While mitomycin C is not cell cycle specific, it is most active in the late G1 and early S phase of the cell cycle. It acts by suppressing the synthesis of nucleic acids. In cell culture, mitomycin C mitotically arrests cells.

- **Inactivation of feeder layer cells**
  - The cells were washed once with 1x PBS
  - 3 ml of mitomycin C dissolved in IMDM to an end concentration of 10 µg/ ml was then given onto the cells and incubated for 3h at 37°C, 5% CO_2
  - The cells were washed 3x with 10% FCS IMDM
  - Medium change was performed every 2 days. Inactivated Feeder cells should be stored not more than a week

The ES cells can then be seeded on to the feeder layer cells using the ES maintenance medium.

2.2.2 ES CELL CULTURE AND SPINNER-CULTURE TECHNIQUE FOR CULTIVATION OF EMBRYOID BODIES

The ES cell lines were thawed at 37°C within a waterbath after storage in liquid nitrogen (-196°C) and centrifuged with the Heraeus Labofuge 300 at 65 g for 5 min in 5 ml Iscove's medium. The cell pellet was then re-suspended in 2 ml ES-cell medium from which 200 µl was transferred into a 60 x 20 mm cell culture plate containing mitotically inactivated feeder layers preincubated with 5 ml ES maintenance medium (see TABLE 5). The cells were cultured at 37°C, 5% CO_2 with 95% relative humidity, and passaged every 2-3 days.

At day 0 of differentiation adherent cells were dissociated using 0.2% trypsin and 0.05% EDTA in PBS and seeded at a density of $1x10^7$ cells / ml in 250 ml siliconised spinner flasks (in order to prevent the cells from adhering to the spinner flask walls) [Integra] containing 125 ml differentiation medium (see TABLE 5). After 24 h 125 ml medium was added to give a final volume of 250 ml. The cells in the spinner flask are held in steady movement at 37°C on a magnetic stirrer [Integra] with 20 rpm and change of direction after four rotations. About 150 ml cell culture medium was exchanged every day from the spinner flask. In the spinner flasks the cells aggregate to form EBs and start differentiating spontaneously due to the absence of LIF (see FIG. 11). The incubator condition was the same as mentioned above.

For endogenous gene expression analysis during the course of differentiation, aliquotes of EBs were removed from day 2 to day 10 and analysed using immunohistochemistry for protein- or using real-time PCR for mRNA expression. For other experiments 4-day-old EBs were treated for 24h in medium supplemented with various test substances under the same condition as mentioned above.

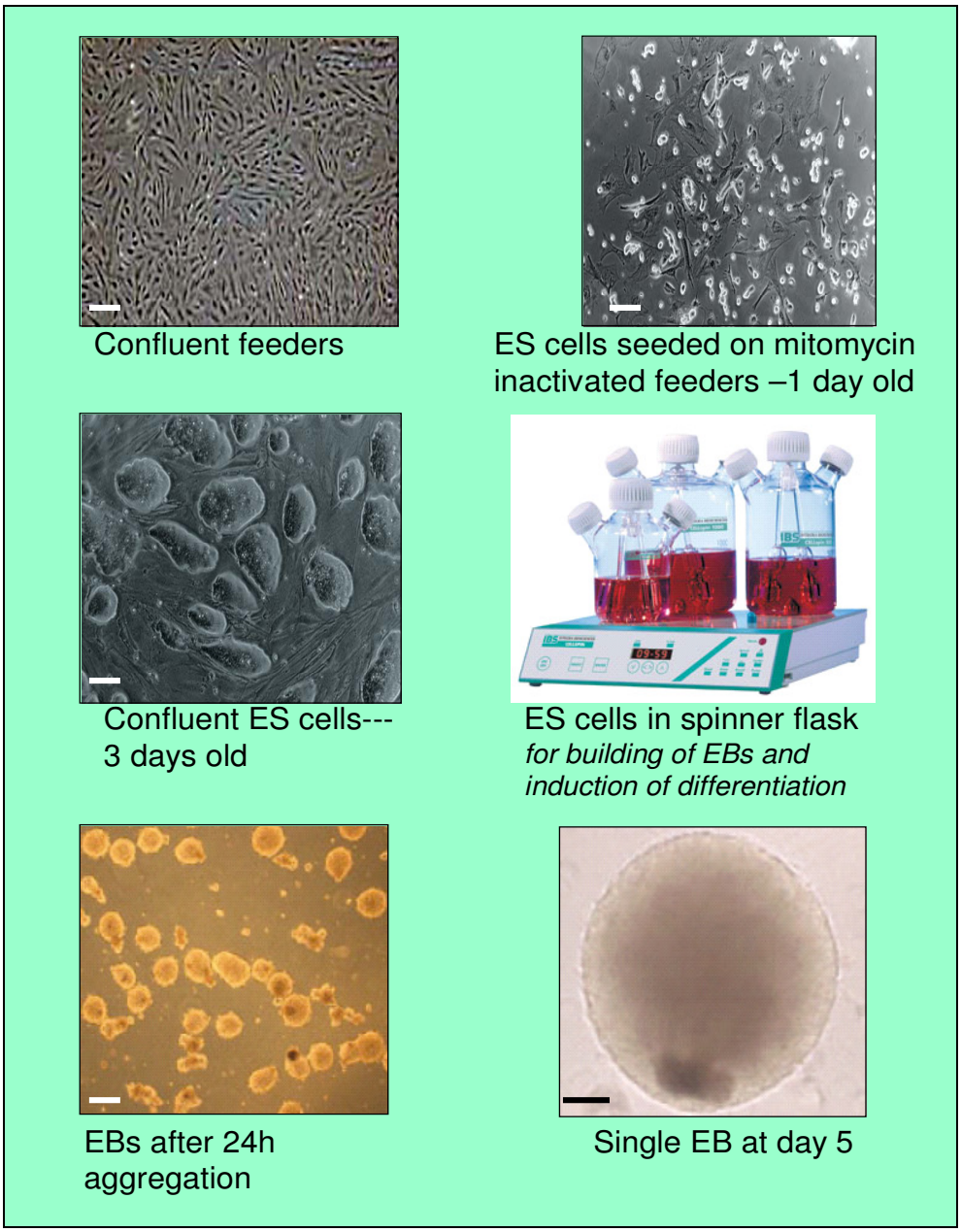

FIG. 11 Culture and Differention of Feeder Cells Dependent ES Cells

2.3 TREATMENT OF EBs WITH VARIOUS SUBSTANCES

STIMULANTS:

In order to study the effect of changes in redox milieu on gene expression during the differentiation of ES cells the substances listed in TABLE 6 were used. 4-day-old EBs were treated for 24h or for times as indicated and analysed either by immunohistochemistry or real time PCR.

SUBSTANCE	CONCENTRATION
Cobalt chloride	50 µM
Hydrogen peroxide	10 µM
Menadione	20 µM
Physiological hypoxia (N_2)	100 mbar 95% N_2, 1% O_2

TABLE 6 List and Concentration of Substances Used as Pro-Oxidants

ANTIOXIDANTS:

Listed in TABLE 7 are the substances and their concentrations that were used to study the effect of ROS scavenging on the expression of CT-1 and HIF-1α.

SUBSTANCE	CONCENTRATION
Trolox	20 µM
N-(2-mercaptopropionyl)glycine (NMPG)	20 µM

TABLE 7 Concentration of Anti-Oxidants Used

NADPH OXIDASE INHIBITORS: In order to find out whether NADPH-oxidase is involved in the redox-dependent regulation of CT-1 and HIF-1α expression, NADPH oxidase inhibitors were used as shown in TABLE 8 below.

SUBSTANCE	CONCENTRATION
Apocynin	10 µM
Diphenyleniodonium chloride (DPI)	10 µM

TABLE 8 Concentration of NADPH-Oxidase Inhibitors Used

INHIBITORS:

In order to analyse the possible signal transduction pathways that are involved in redox-dependent regulation of CT-1 and HIF-1α, inhibitors were used as indicated below (see TABLE 9).

SUBSTANCE	SUBSTRATE	CONCENTRATION
2-Methoxyestradiol	HIF-1α	3 µM
AG 490	JAK2	50 µM
LY294002	PI-3K	20 µM
SKF 86002	p38 MAPK	10 µM
SP 600125	JNK	10 µM
U0126	ERK 1/2	10 µM

TABLE 9 Concentration and Substrate of Inhibitors Used

2.4 FIXATION AND IMMUNOFLUORESCENCE STUDIES

Depending on the target protein, the EBs were fixed either by incubating for 20 min at -20°C in 100% methanol or by incubating for 1h on ice in ice-cold 4% paraformaldehyde. The EBs were then washed 2x with 1x PBS to remove the excess fixative. In order to reduce unspecific binding of the antibodies, the EBs were incubated in blocking buffer (either 10% FCS in 0.01% PBST or 10% skimmed milk in 0.01% PBST) for 1h at RT. The primary antibodies were diluted in blocking solution as shown in TABLE 10 below, and were incubated either for 1h at RT or overnight at 4°C. The EBs were then washed 5x with 0,1x PBST to remove the unbound antibodies. The secondary antibodies were also diluted in blocking solution as shown in TABLE 11 below; the seconary antibody incubation lasted usually for 1h at RT. After that the EBs were washed 5x with 0,1x PBST and stored in 0.01% PBST. The protein fluorescence analysis was performed using the Zeiss LSM 510 confocal laser scanning microscope (CLSM).

2.4.1 PRIMARY ANTIBODY STAINING

	PRODUCED IN	FIXATION	Dil.
HIF 1-α	mouse	Methanol 20min -20℃	1:100
CT-1	rat	„	1:100
gp130	goat	„	1:100
p-gp130 (Ser 782)	goat	„	1:100
MOX 1 (M-15)	goat	Methanol 20min -20℃	1:50
NOX 4 (M-15)	goat	„	1:50
gp91-phox (C-15)	goat	„	1:50
P47-phox (H-300)	goat	„	1:50
p22-phox(C-17)	goat	„	1:50
p67-phox (H-300)	rabbit	„	1:50
p-STAT3	rabbit	„	1:50
PI3-Kinase P85	rabbit	4% PFA 1h on ice	1:50
phospho p38 MAP Kinase	rabbit	„	1:50
phosphop44/42 MAPKinase (Thr202/Tyr204)	rabbit	„	1:50
phospho SAPK/JNK (Thr183/Tyr185)	rabbit	„	1:50

TABLE 10 Fixation Method and Dilution of Primary Antibodies Used

SECONDARY ANTIBODIES

SECONDARY ANTIBODIES	DILUTION
Cy 2 goat anti-mouse	1:200
Cy 2 mouse anti-rat	1:200
Cy 3 donkey anti-goat	1:200
Cy 3 rabbit anti-rat	1:200
Cy 5 goat anti-rabbit	1:200
Cy 5 mouse anti-goat	1:200
Cy 5 rabbit anti-rat	1:200
Cy 5 sheep anti mouse IgG	1:200
FITC anti-guinea-pig IgG	1:300
FITC goat anti-mouse	1:300
FITC sheep anti-rabbit IgG	1:300

TABLE 11 Dilution of Secondary Antibodies Used

2.4.2 VISUALISATION AND DATA ANALYSIS: CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

CLSM is a conventional microscope which is equipped with a laser light source, the laser scanning head, and an automatic focusing stage, connected to a monitor and PC. Operation is possible in two modes: in reflected light or in fluorescence mode. The basic principle of a confocal microscope is that light from a laser is focused onto a pinhole. The pinhole is imaged onto the object by an objective lens. Reflected light and/or fluorescence from the object are imaged by the same objective lens onto a detection pinhole via a beamsplitter. A photomultiplier-detector records the light transmitted through the pinhole. In this way, light rays generated from outside the focused region are effectively suppressed by the detection pinhole (optical sectioning-effect). An image of the object is obtained by scanning the light beam in x/y-directions, in raster-like manner. Finally, a 3D-image is obtained by scanning the object in z-direction (along the optical axis) and stacking the images obtained for each z-value. Using these arrangement images of objects with a resolution up to 1.4 x higher than in a non-confocal system can be obtained. Confocal microscopes use specific wavelengths of light to excite fluorescent molecules within a sample. Our Zeiss 510 uses the following lasers: 458/488nm Argon, 543nm HeNe (Helium/Neon), and a 633nm HeNe. Following excitation of fluorescent molecules with certain wavelengths of light, those molecules then relax. Upon this relaxation, the molecules emit light at specific wavelengths. This emission is the signal we detect when doing confocal microscopy. In TABLE 12 below are the spectral data of the fluorochromes in this work.

Fluorochrome	**Spectral data [nm]**	
	Absorption	**Emission**
Cy2	489	506
Cy3	514	566
Cy5	649	666
Dichlorodihydrofluorescein Diacetate (H_2DCFDA)	505	535
FITC	490, 494	520, 525

TABLE 12 Spectral Data of Fluorochromes used.

2.5 POLYMERASE CHAIN REACTION (PCR)

The coupling of reverse transcription (RT) of mRNA with PCR amplification of the resulting cDNA (RT-PCR) was initially described by Powell and colleagues in 1987. Subsequently, RT-PCR has been widely used in research laboratories as a rapid and sensitive technique for the detection, quantification of specific mRNAs. RT-PCR (reverse transcription-polymerase chain reaction) is the most sensitive technique for mRNA detection and quantitation currently available. Compared to the two other commonly used techniques for quantifying mRNA levels, Northern blot analysis and RNase protection assay, RT-PCR can be used to quantify mRNA levels from much smaller samples.

Real-time is a method of simultaneous DNA quantification and amplification. DNA is specifically amplified by polymerase chain reaction (PCR). After each round of amplification, the DNA is quantified. Common methods of quantification include the use of fluorescent dyes that intercalate with double-strand DNA and modified DNA oligonucleotides (called probes) that fluoresence when hybridized with a complementary DNA. Frequently, real-time PCR is combined with reverse transcription-polymerase chain reaction (RT-PCR) to quantify low abundance messenger RNA, enabling a researcher to quantify relative gene expression at a particular time, or in a particular cell or tissue type. The combined technique is often called quantitative RT-PCR.

2.5.1 RNA ISOLATION AND DNase DIGESTION

RNA EXTRACTION USING TRIZOL PROTOCOL

EBs were transferred into 1.5 ml Eppendorf tubes, washed once with 1x PBS and centrifuged (Heraeus Biofuge 15R) for 1min at 11000 g at RT. 300 µl TRIzol reagent (Invitrogen) was then added. Homogenisation of EBs was achieved by pipetting for at least 10 times and vortexing. After 5min incubation at RT the homogenate was centrifuged at 11000 g for 20 min at 4°C in pre-cooled centrifuge. The supernatant was removed to a fresh Eppendorf tube and 300 µl of chloroform was added and mixed by vortexing before incubation at RT for 5 min. After 10 min centrifugation at 11000 g, at 4°C, the aqueous phase (~500 µl) was transferred to a fresh Eppendorf tube. RNA was precipitated with 0.5 ml of isopropanol for 20 min at – 20°C. The precipitate was pelleted by centrifugation at 11000 g for 20 min at 4°C. The

supernatant was removed and the pellet was washed twice with 1 ml 70% EtOH by centrifugation at 4500 g for 5 min at 4℃, air dried, and re-suspended in ca 33 µl RNase-free water. In order to eliminate DNA contamination, DNase digestion was performed with Dnase I RNase-free. DNase I is a nonspecific endonuclease that degrades double-and single-stranded DNA and chromatin. It functions by hydrolyzing phosphodiester linkages, producing mono and oligonucleotides with a 5'-phosphate and a 3'-hydroxyl group.

DNase DIGESTION

To remove genomic DNA contamination, the RNA solution was treated with 10 U of RNase-free DNase I in the presence of 50 mM Tris pH 7.5, 1 mM MgCl2, 5 mM DTT, and 1 U/µl RNasin at 37 ℃ for 1h. Water was added up to 250 µl, and the RNA was purified by extraction with phenol-chloroform (3:1) and chloroform. The RNA was precipitated from the aqueous phase by adding sodium acetate up to 0.3 M and 2.5 vol of 100 % ethanol. The RNA pellet was redissolved in 20 µl of RNase-free water and the RNA concentration was measured with a spectrophotometer.
RNA concentration was estimated by measuring A260 in a spectrophotometer (Eppendorf) and applying the formula: [RNA] = A260 x D x 40 µg/µL, where D is the RNA dilution factor. RNA quality was judged from A260/280 (ratio 1.8-2.0, indicates low protein contamination) and A260/230 (ratio ≥ 2.0, indicating low polysaccharide contamination).

2.5.2 cDNA SYNTHESIS

Each RNA sample was reverse transcribed using oligo-dT primer. For the reactions, 2 µg of DNase-treated RNA was filled with water to volume of 10 µl. The RNA solution was incubated at 60℃ for 15 min to denature the RNA and quick chilled on ice for 1 min, microcentrifuged at 11000 g for 10 sec. A mix containing 4 µl of 5x Reverse Transcription buffer, 1 µl of 0.1 M DTT, 2 µl of 100 µM 4dNTP mix, and 2 µl of 10 µM oligo-dT primer and 1 µl of 200 U/µl MMLV reverse transcriptase was added into each tube, giving a final reaction volume of 20 µl. The reactions were incubated at 37℃ for 60min. To inactivate the reverse transcriptase, the samples were incubated at 95℃ for 5 min, then microcentrifuged at 10000 g for 10sec, and placed on ice or stored at –20℃ for later PCR amplification.

2.5.3 QUANTITATIVE PCR

The real-time PCR system is based on the detection and quantification of a fluorescent reporter (Lee, 1993; Livak, 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.

Real-time PCR requires an instrumentation platform that consists of a thermal cycler, a computer, optics for fluorescence excitation and emission collection, and data acquisition and analysis software.

Currently four different chemistries, TaqMan® (Applied Biosystems, Foster City, CA, USA), Molecular Beacons, Scorpions® and SYBR® Green (Molecular Probes), are available for real-time PCR. All of these chemistries allow detection of PCR products via the generation of a fluorescent signal. TaqMan probes, Molecular Beacons and Scorpions depend on Förster Resonance Energy Transfer (FRET) to generate the fluorescence signal via the coupling of a fluorogenic dye molecule and a quencher moeity to the same or different oligonucleotide substrates. SYBR Green was used during this work. SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding to double-stranded DNA.

2.5.3.1 DATA ANALYSIS

Two strategies are commonly employed to quantify the results obtained by real-time RT-PCR; the standard curve method and the comparative threshold method. The comparative threshold method was used in this project. This involves comparing the C_t (cycle number at which a PCR reaction enters its exponential phase) values of the samples of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. The C_t values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping gene. The comparative C_t method is also known as the $2^{-[delta]\,[delta]Ct}$ method, where

$$[delta][delta]C_t=[delta]C_{t,sample}-[delta]C_{t,reference}$$

Here, $[delta]C_{T,sample}$ is the C_t value for any sample normalized to the endogenous housekeeping gene and $[delta]C_{t,\ reference}$ is the C_t value for the calibrator also normalized to the endogenous housekeeping gene. For the $[delta][delta]C_t$ calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal. This can be established by looking at how $[delta]C_t$ varies with template dilution. If the plot of cDNA dilution versus delta C_t is close to zero, it implies that the efficiences of the target and housekeeping genes are very similar.

2.5.3.2 REACTION CONDITIONS

PCR amplification was carried out in 20 µl reaction mixtures per tube containing 10 µl of SYBR Green PCR Master Mix, 10 pM of each primer, and 3 µl of cDNA using the ICycler Optical Module (Biorad). PCR conditions were set as follows: 15 min at 95°C to activate the polymerase followed by 45 cycles consisting of denaturation at 95°C for 30sec and annealing 60-64°C for 30 sec (see primer sequences and annealing temperatures in TABLE 13 below) and elongation at 72°C for 30 sec. The data were analysed with the Bio-Rad ICycler iQ software. Signals were regarded positive if the fluorescence intensity (increase of fluorescence ΔRn) exceeded 10 times the standard deviation of the baseline fluorescence (threshold cycle [CT]). CT values of 45 were regarded as negative. The threshold cycle or CT value is the cycle at which a statistically significant increase in ΔRn is first detected.

		SEQUENCES	Temperature
BACT	forward	GATGACCCAGATCATGTTTGAG	60℃
BACT	reverse	CCATCACAATGCCTGTGGTA	60℃
GAPDH	forward	TCGTCCGGTAGACAAAATGG	64℃
GAPDH	reverse	GAGGTCAATGGGGTCGT	64℃
CT-1	forward	GCGTCGCCTGAGAGTGAATAC	64℃
CT-1	reverse	GAACACACAGGACACAGATGGAG	64℃
HIF1a	forward	TCACCAGACAGAGCAGGAAA	60℃
HIF1a	reverse	CTTGAAAAAGGGAGCCATCA	60℃
NOX 1	forward	AATGCCCAGGATCGAGGT	60℃
NOX 1	reverse	GATGGAAGCAAAGGGAGTGA	60℃
NOX 4	forward	GATCACAGAAGGTCCCTAGCAG	64℃
NOX 4	reverse	GTTGAGGGCATTCACCAAGT	64℃

TABLE 13 Lists of primers and their annealing temperatures

HIF-1α and NOX 1 data were normalised against the β –actin (BACT) data and CT-1 and NOX 4 data were normalised against the GAPDH data.

2.6 ENZYMATIC DISSOCIATION OF EMBRYOID BODIES INTO SINGLE CELLS USING COLLAGENASE

To obtain single cells and cardiomyocytes, EBs were removed from the spinner flasks on day 4 of differentiation and plated on petriperm plates. Three days after plating (i.e. day 7 of differentiation) a number of EBs displayed spontaneous contractions, indicating cardiomyocyte differentiation. On day 8 of differentiation, EBs with beating areas were cut out with a sterile microscalpel and collected in IC-buffer (see TABLE 14). The tissue was then incubated in enzyme medium (collagenase B (1 mg/ ml); 30 µM $CaCl_2$) for 20 min at 37℃ with gentle rotation on a shaker and then centrifuged (Eppendorf 5417C) at 665 g for 5 min. The supernatant was discarded and the pellet resuspended in Iscove's medium. Isolated cells were plated on sterile coverslips and kept in the incubator for 96 h. The isolated cells were treated for 24 h and the cellular localisation of CT-1 in cardiomyocytes as well as non-cardiomyocyte was analysed.

	Molecular weight (g/Mol)	**mmol/l**	**g/l**
NaCl	58,44	120	7
KCl	74,56	5,4	0,4
$MgSO_4$	246,48	5	1,23
Na-Pyruvat	0,1	5	50 ml
Glucose	198,19	20	3,96
Taurin	125,15	20	2,5
HEPES	238,31	10	2,38
→ adjusted to pH 6,9 with NaOH			

TABLE 14 Cardiomyocyte Isolation Buffer (IC-Buffer)

2.7 MEASUREMENT OF REACTIVE OXYGEN SPECIES

ROS generation in EBs was assessed using the probe 2,7-dichlorofluorescin (DCF). The membrane-permeable diacetate form of the dye (H_2DCF-DA) was added to the EBs that were incubated in serum-free medium at a final concentration of 20 µM. H_2DCF-DA is a low molecular-weight non-fluorescent and non-polar compound that enters cells freely. Within the cells, esterases cleave the acetate groups on the H_2DCF-DA, resulting into a polar and non-fluorescent DCFH thus trapping the reduced probe (DCFH) intracellularly. ROS in the cells oxidize DCFH, yielding the fluorescent product DCF. After 20 min of incubation, DCF fluorescence was measured using the 488-nm band of the argon ion laser of the confocal setup. The intensity of green fluorescence of DCF is an indicator of the levels ROS production.

2.8 STATISTICS

Results are expressed as means ± SD, were *n* represents the number of experimental replicates ($n \geq 3$). Statistical significance was assessed by Student's t-test and ANOVA. $P < 0.05$ was considered significant.

3 RESULTS

3.1 CT-1 AND GP130 EXPRESSION IN DIFFERENTIATING CCE ES CELLS

Sauer *et al.* 2004 showed that treatment of ES cell-derived embryoid bodies with CT-1 results in stimulation of cardiomyogenesis, and that CT-1 is endogenously expressed in embryoid bodies. To correlate CT-1 with the time course of embryoid body differentiation and expression of the transducing receptor (gp130), protein levels of CT-1 and gp130 were monitored. Expression of CT-1 (n = 6) and gp130 (n = 4) followed a similar expression pattern until day 8 in culture and subsequent downregulation on day 10 and day 11 (FIG. 12*A, B*). Expression of CT-1 and gp130R culminated at 175 $\pm$ 25 % and 188 + 6 %, respectively, on day 8 as compared to day 2 (set to 100 %). It has been previously shown by Hescheler *et al*. 2002 that cardiomyogenic differentiation of mouse ES cells occurs between day 6 and 8, i.e. during the time of maximal CT-1 and gp130 expression.

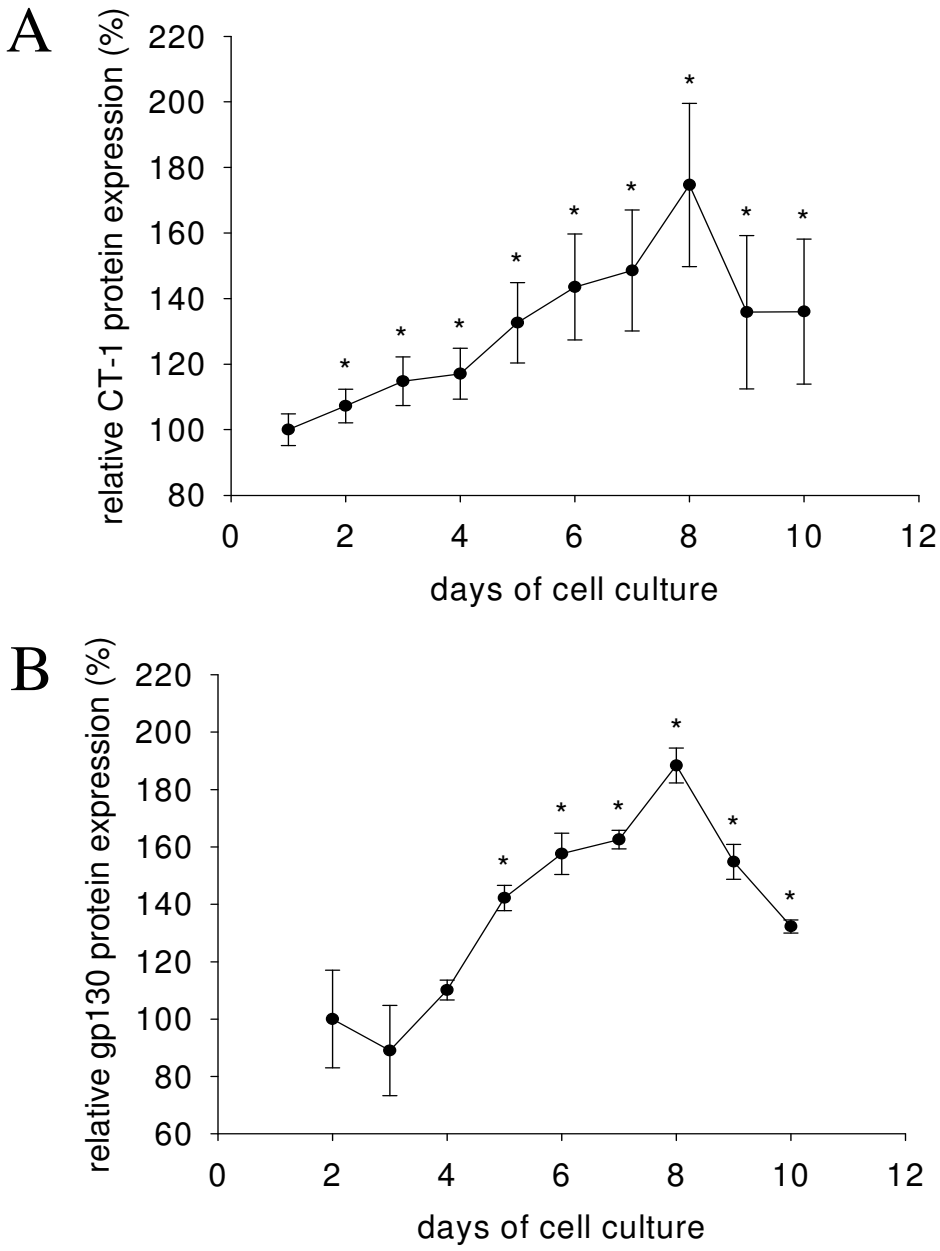

FIG. 12 Protein expression of CT-1 (*A*) and gp130 (*B*) during ES cell differentiation. Determination was performed within the three-dimensional tissue of embryoid bodies by semiquantitative immunohistochemistry. Note that cardiac cell differentiation occurs during day 6 and 8 of embryoid body cell culture. $*P < 0.05$, significantly different to day 1 and day 2 of cell culture in (*A*) and (*B*), respectively.

3.2 REGULATION OF CT-1 AND HIF-1α EXPRESSION BY VITAMIN E

It was previously shown that differentiating embryoid bodies express HIF-1α and robustly generate ROS that may be utilized as signalling molecules regulating CT-1 expression [Bichet *et al*, 1999; Sauer *et al,* 2000]. To support this assumption embryoid bodies were treated with the free radical scavenger vitamin E (20 µM) from day 2 to day 8 (FIG. 13). Subsequently, CT-1 expression was assessed in untreated and vitamin E-treated embryoid bodies. Since CT-1 has been previously shown to be upregulated by hypoxic stress in cardiac myocytes [Hishinuma *et al*, 1999) the expression of HIF-1α was assessed in parallel to investigate possible correlation with CT-1 expression. It was apparent that vitamin E treatment downregulated CT-1 as well as HIF-1α expression in 8-day-old embryoid bodies (untreated control set to 100 %), indicating regulation by ROS endogenously generated in differentiating ES cells (n = 4).

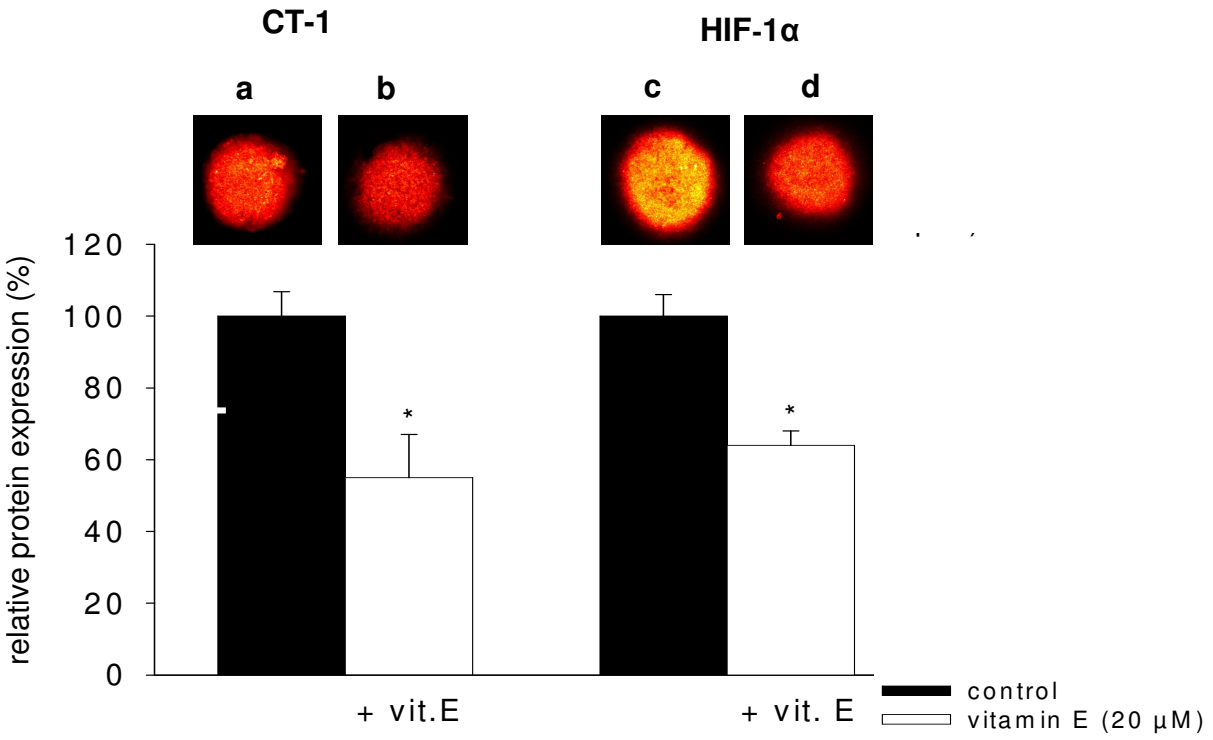

FIG. 13 Regulation of CT-1 and HIF-1α expression in embryoid bodies by endogenous ROS. Embryoid bodies were incubated from day 2 to day 8 of cell culture with 20 µM vitamin E. On day 8 protein expression of CT-1 and HIF-1α was assessed by semiquantitative immunohistochemistry in whole mount embryoid bodies. The images show representative embryoid bodies labelled with antibodies either against CT-1 or HIF-1α which remained untreated (*a*,*c*) or were treated with vitamin E (*b*,*d*). The bar represents 100 µm. **P* < 0.05, significantly different to the untreated control.

3.3 REGULATION OF CT-1 AND HIF-1α EXPRESSION BY EXOGENOUS PRO-OXIDANTS

The data above suggest regulation of CT-1 and HIF-1α by endogenous generation of ROS during differentiation. To validate these findings, 4-day-old embryoid bodies were treated with either the prooxidants menadione or H_2O_2 (FIG. 14*A, B*). Treatment of embryoid bodies with H_2O_2 in concentrations ranging from 1 nM to 100 µM resulted in dose-dependent increase of CT-1 protein expression (data not shown). Maximum effects were achieved at 10 µM H_2O_2 which increased CT-1 protein (FIG. 14*A*) and mRNA expression (FIG. 14*B*) to 145 $\pm$ 8% and 140 $\pm$ 9%, respectively (n = 3) (untreated control set to 100 %). Likewise, treatment with 10 µM (data not shown) and 20 µM menadione resulted in increased CT-1 expression with maximum values of 157 $\pm$ 8 % and 175 $\pm$ 7 % for CT-1 protein (n = 3) (FIG. 14*A*) and mRNA (n = 3) (FIG. 14*B*) expression, respectively, at 20 µM menadione concentration. Under the same experimental conditions increased protein as well as mRNA expression of HIF-1α was observed. HIF-1α protein expression amounted to 140 $\pm$ 8% and 184 $\pm$ 9% (see FIG. 14*A*), and mRNA expression to 220 $\pm$ 24% and 264 $\pm$ 7% for H_2O_2 and menadione, respectively.

3.4 UP-REGULATION OF CT-1 AND HIF-1α EXPRESSION BY HYPOXIA

Regulation of CT-1 on the protein as well as mRNA level paralleled the regulation of HIF-1α. Consequently, it was hypothesized that hypoxia vice versa results in up-regulation of CT-1. To address this assumption, 4-day-old embryoid bodies were exposed to either 24 h of physiological (1% O_2) or chemical (50 µM $CoCl_2$) hypoxia (FIG. 15*A, B*). Physiological (n = 3) as well as chemical hypoxia (n = 4) upregulated CT-1 protein expression to 191 $\pm$ 18% and 157 $\pm$ 13%, respectively, whereas increased levels of HIF-1α protein amounting to 192 $\pm$ 20% (n = 3) and 183 $\pm$ 16 % (n = 3) were observed upon physiological and chemical hypoxia, respectively (see FIG. 15*A*). A significant increase in *CT-1* as well as *HIF-1α* mRNA expression amounting to 192 $\pm$ 8 % (n = 3) and 279 $\pm$ 24 % (n = 4), respectively, was only achieved with chemical hypoxia (FIG. 15*B*).

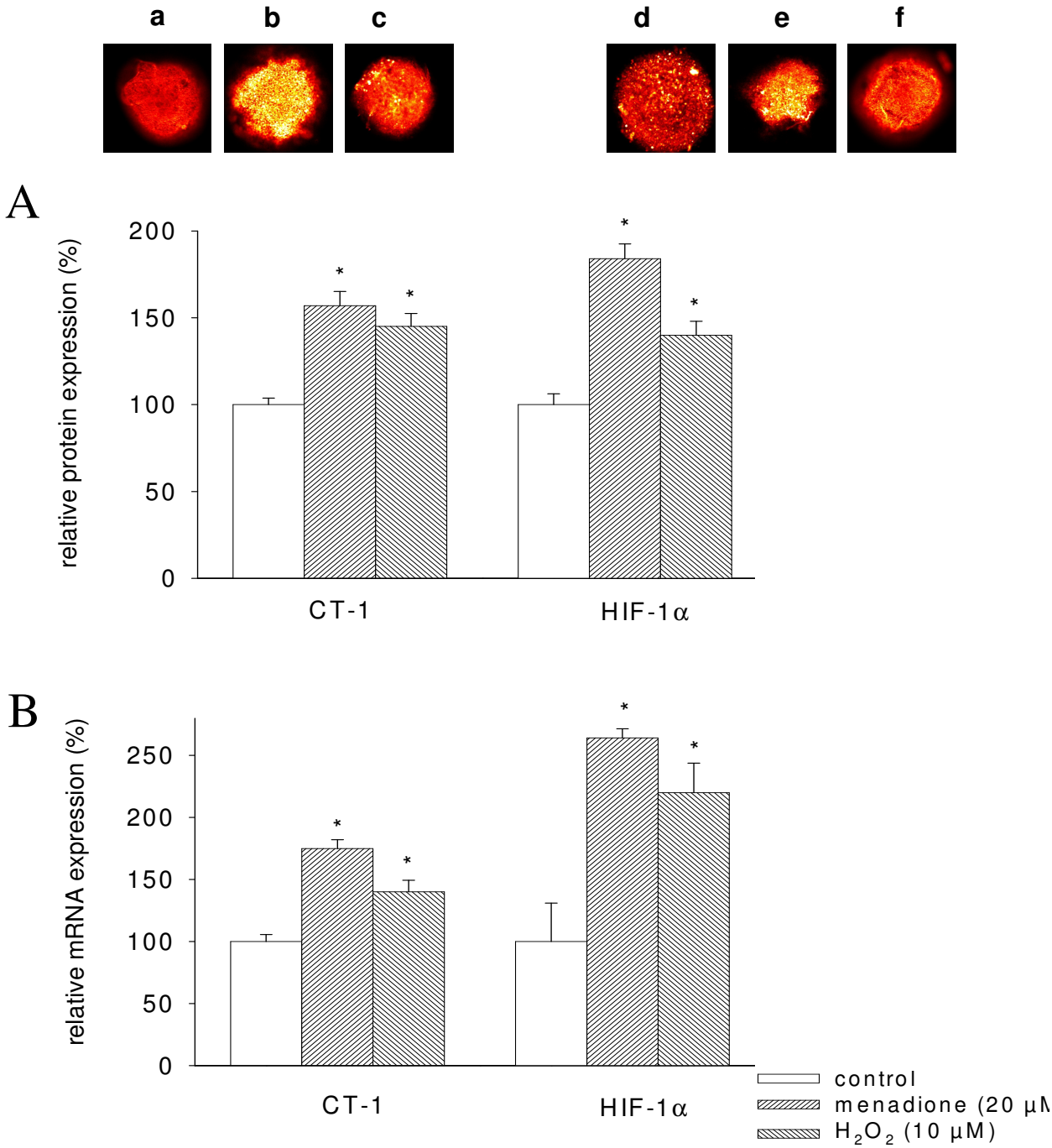

FIG. 14 Upregulation of CT-1 and HIF-1α protein (*A*) and mRNA (*B*) upon treatment with the prooxidants menadione (20 µM) and H_2O_2 (10 µM). Embryoid bodies were treated on day 4 with the respective substances and were examined 24 h thereafter. The images show representative embryoid bodies labelled with antibodies against CT-1 (left panel) and HIF-1α (right panel) which remained either untreated (*a*,*d*) or were treated with menadione (*b*,*e*) or H_2O_2 (*c*,*f*). The bar represents 200 µm. *$P < 0.05$, significantly different to the untreated control.

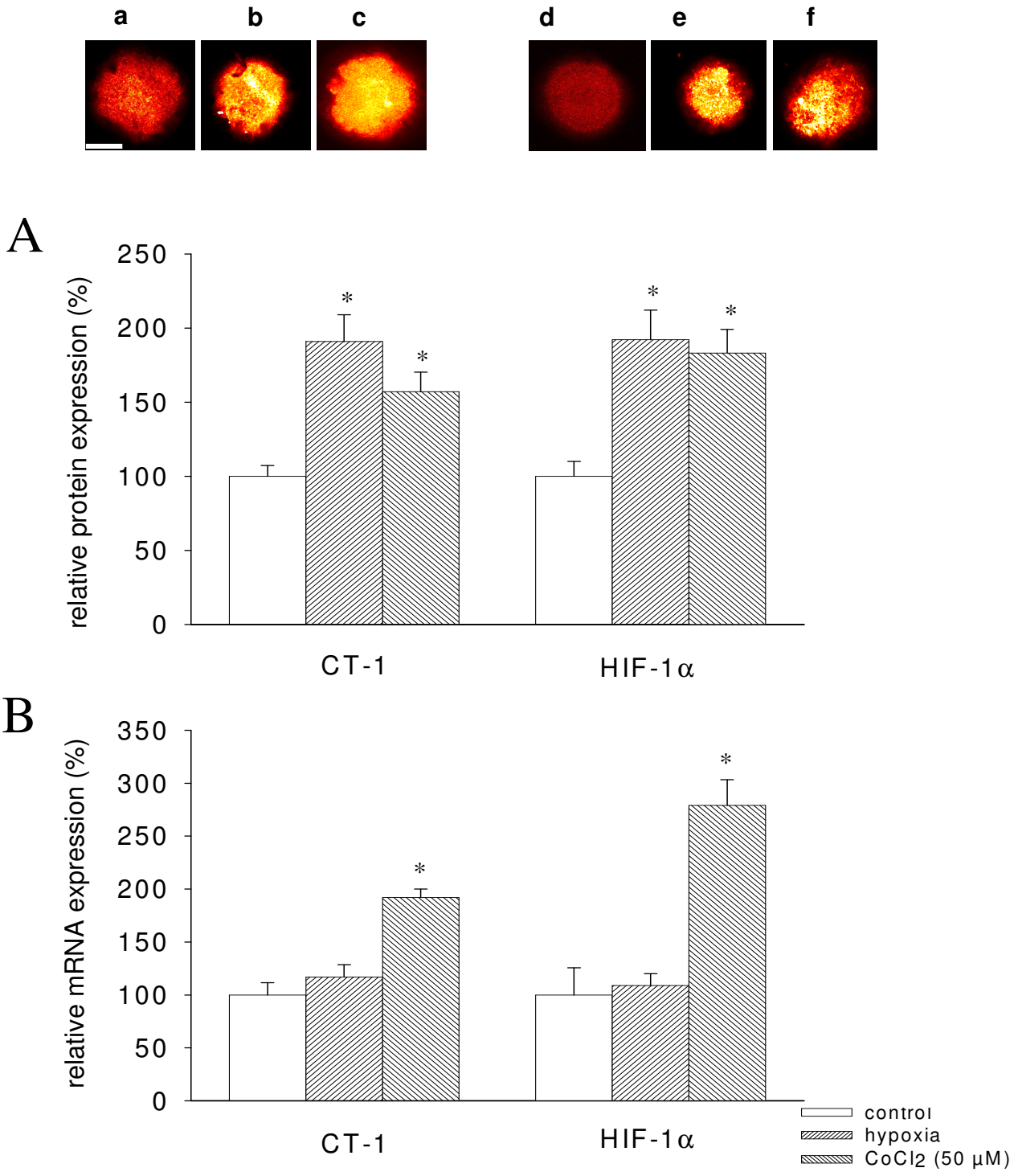

FIG. 15 Upregulation of CT-1 and HIF-1α protein (*A*) and mRNA (*B*) upon exposure to hypoxia. Embryoid bodies were treated on day 4 of cell culture with either physiological (1% O_2) or chemical ($CoCl_2$) hypoxia and were examined 24 h thereafter. The images show representative embryoid bodies labelled with antibodies against CT-1 (left panel) and HIF-1α (right panel) which remained either untreated (*a*,*d*) or were treated with 1% O_2 (*b*,*e*) or $CoCl_2$ (50 µM) (*c*,*f*). The bar represents 200 µm. *$P < 0.05$, significantly different to the untreated control.

3.5 DIFFERENTIAL UP-REGULATION OF CT-1 AND HIF-1α EXPRESSION BY MENADIONE AND CHEMICAL HYPOXIA

The above data shows that both ROS and hypoxia lead to the up-regulation of HIF-1α as well as CT-1. In order to correlate these expression data and our hypothesis that HIF-1α might be involved in the redox dependent expression of CT-1, it was necessary to perform time course experiments to find out whether and/or these proteins are differentially expressed. Assessment of the time course of HIF-1α and CT-1 (FIG. 16*B*) expression upon treatment with either menadione (FIG. 16*A*) (n = 3) or $CoCl_2$ (FIG. 16*B*) (n = 4) revealed that up-regulation occurred already after 2 h of incubation. Statistical significance was achieved at earlier time points for HIF-1α expression as compared to CT-1 expression, i.e. after 2 and 4 h in menadione and $CoCl_2$ treated samples, respectively, suggesting that HIF-1α precedes CT-1 expression.

3.6 GENERATION OF ROS BY MENADIONE AND CHEMICAL HYPOXIA IN EMBRYOID BODIES

Pro-oxidants as well as chemical hypoxia may increase intracellular ROS generation. When embryoid bodies were treated for 24 h with 20 µM menadione, a significant increase in ROS generation was observed which remained at an elevated level even 24 h after placement into normal medium, but was not significantly different to the control after 4 h (FIG. 17*A*) (n = 4). An increase in ROS generation was likewise achieved when embryoid bodies were treated for 24 h with $CoCl_2$ (FIG. 17*B*) (n = 3), suggesting that indeed ROS may act as signalling molecules regulating the expression of CT-1 and HIF-1α following treatment with menadione and chemical hypoxia.

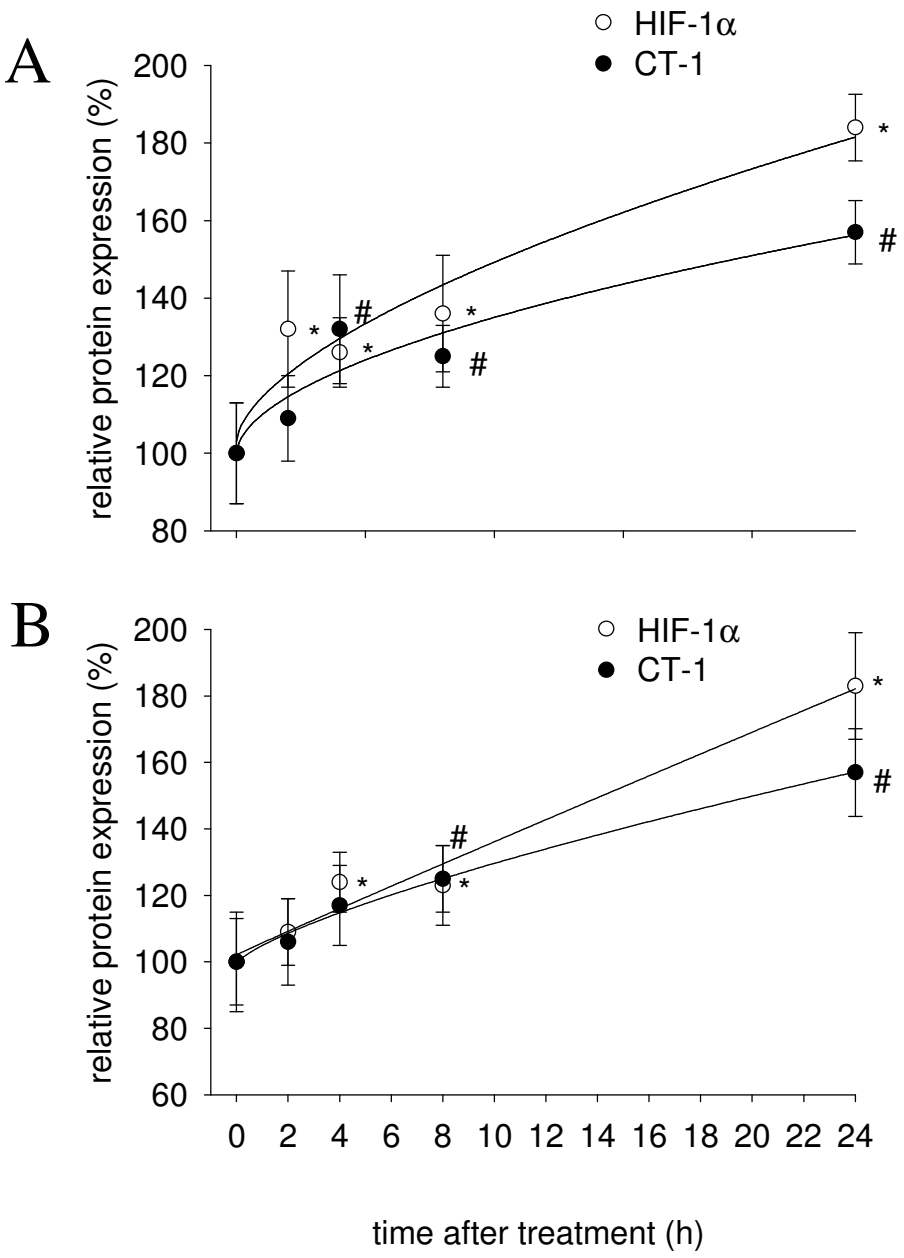

FIG. 16 Time course of HIF-1α and CT-1 up-regulation upon treatment with either menadione (*A*) or $CoCl_2$ (*B*). Protein expression was assessed 2, 4, 8, and 24 h after treatment. $^{*,\#}P < 0.05$, significantly different as compared to untreated samples.

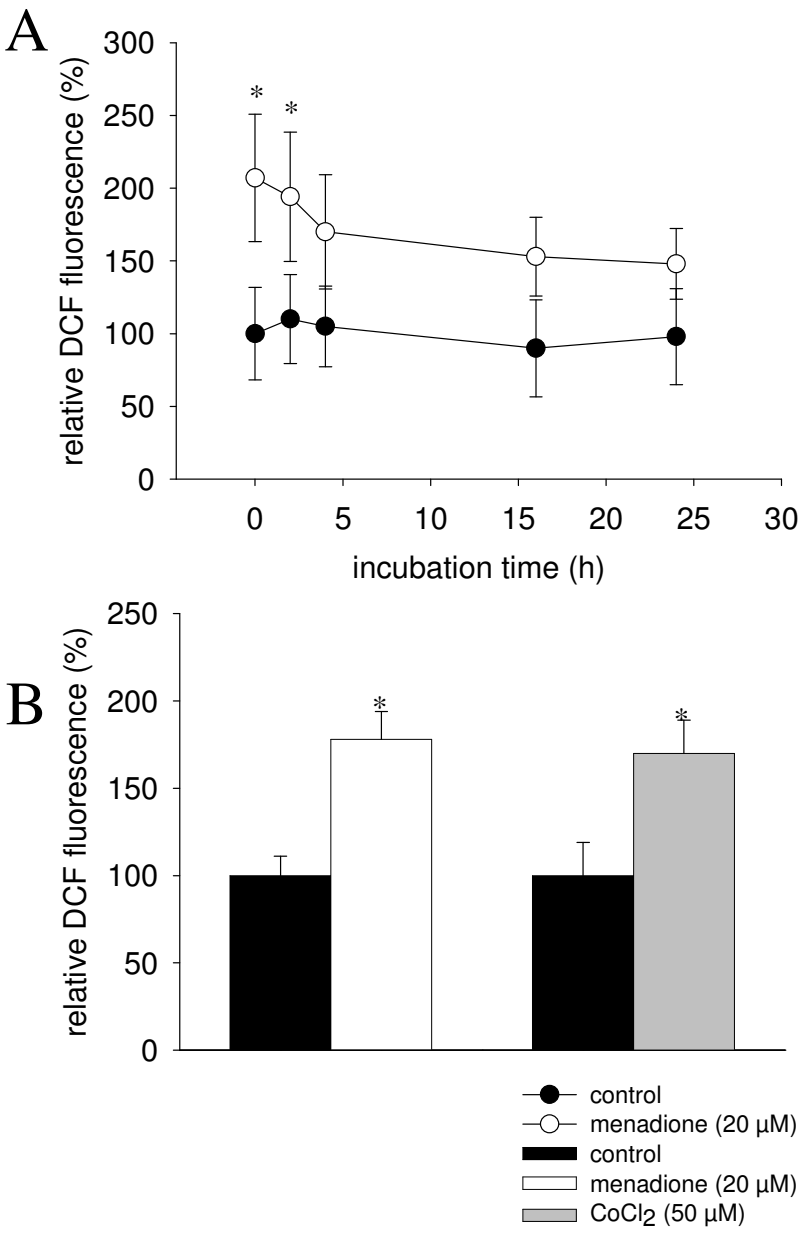

FIG. 17 Generation of ROS following exposure to menadione (A) or chemical hypoxia ($CoCl_2$ treatment) (*B*). In *A*, embryoid bodies were treated for 24 h with 20 µM menadione. Subsequently, the cell culture medium was exchanged with medium devoid of menadione, and ROS were monitored at different times after removal of menadione. *B*, ROS generation in the presence of either menadione (20 µM) or $CoCl_2$ (50 µM). Determination was performed immediately after exposure. $^*P < 0.05$, significantly different to the untreated control.

3.7 ATTENUATION OF MENADIONE- AND CHEMICAL HYPOXIA-INDUCED CT-1 UP-REGULATION BY FREE RADICAL SCAVENGERS

The hypothesis of involvement of ROS in the menadione and $CoCl_2$ induced CT-1 up-regulation mentioned above was further strengthened by experiments where CT-1 expression following treatment with menadione (FIG. 18*A*) (n = 3) and $CoCl_2$ (FIG. 18*B*) (n = 4) was assessed in the presence of the free radical scavengers vitamin E (20 µM) and NMPG (20 µM) which resulted in significant down-regulation of CT-1 expression, thus pointing towards regulation of CT-1 expression by intracellular ROS.

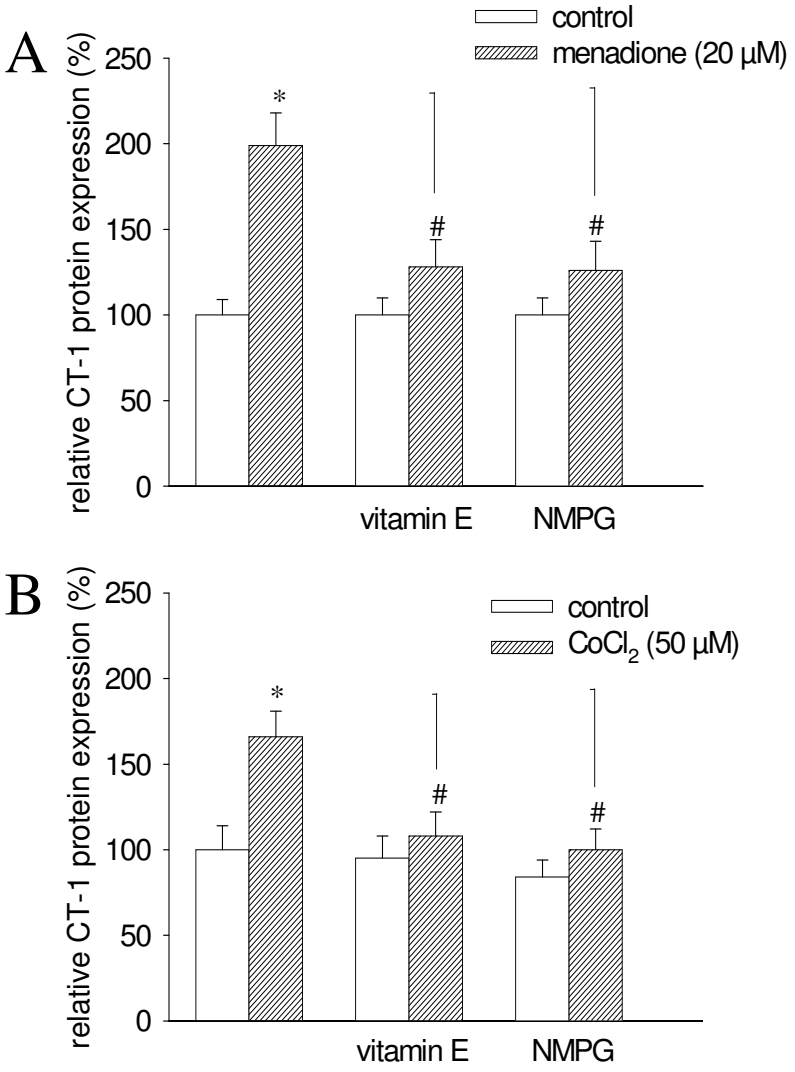

FIG. 18 Inhibition of menadione- (*A*) and $CoCl_2$-mediated (*B*) upregulation of CT-1 upon preincubation with either the free radical scavenger vitamin E (20 µM) or NMPG (20 µM). Embryoid bodies were treated at day 4 of cell culture with either menadione (20 µM) or $CoCl_2$ (50 µM) in the presence of free radical scavengers. Following 24 h CT-1 protein expression was determined by semiquantitative immunohistochemistry. $^{*,\#}P < 0.05$, significantly different as indicated.

3.8 UP-REGULATION OF NADPH-OXIDASE IN EMBRYOID BODIES BY MENADIONE AND HYPOXIA

Pro-oxidants may regulate the expression of ROS generating NADPH-oxidase thereby providing a feed-forward loop of increased ROS generation even in the absence of external pro-oxidants. To evaluate this notion, we determined expression of NADPH-oxidase subunits. Pre-incubation for 24 h with either menadione (20 µM) or $CoCl_2$ (50 µM) significantly increased protein expression of p22-phox, p47-phox, p67-phox, Nox-1 and Nox-4 (FIG. 19*A*) (n = 4) as well as mRNA expression of *Nox-1* and *Nox-4* (FIG. 19*B*) (n = 5), suggesting regulation of CT-1 expression by NADPH-oxidase-derived ROS. Furthermore, treatment with physiological hypoxia (1% O_2) significantly increased protein expression of p22-phox, p47-phox and Nox-4 (FIG. 19*C*).

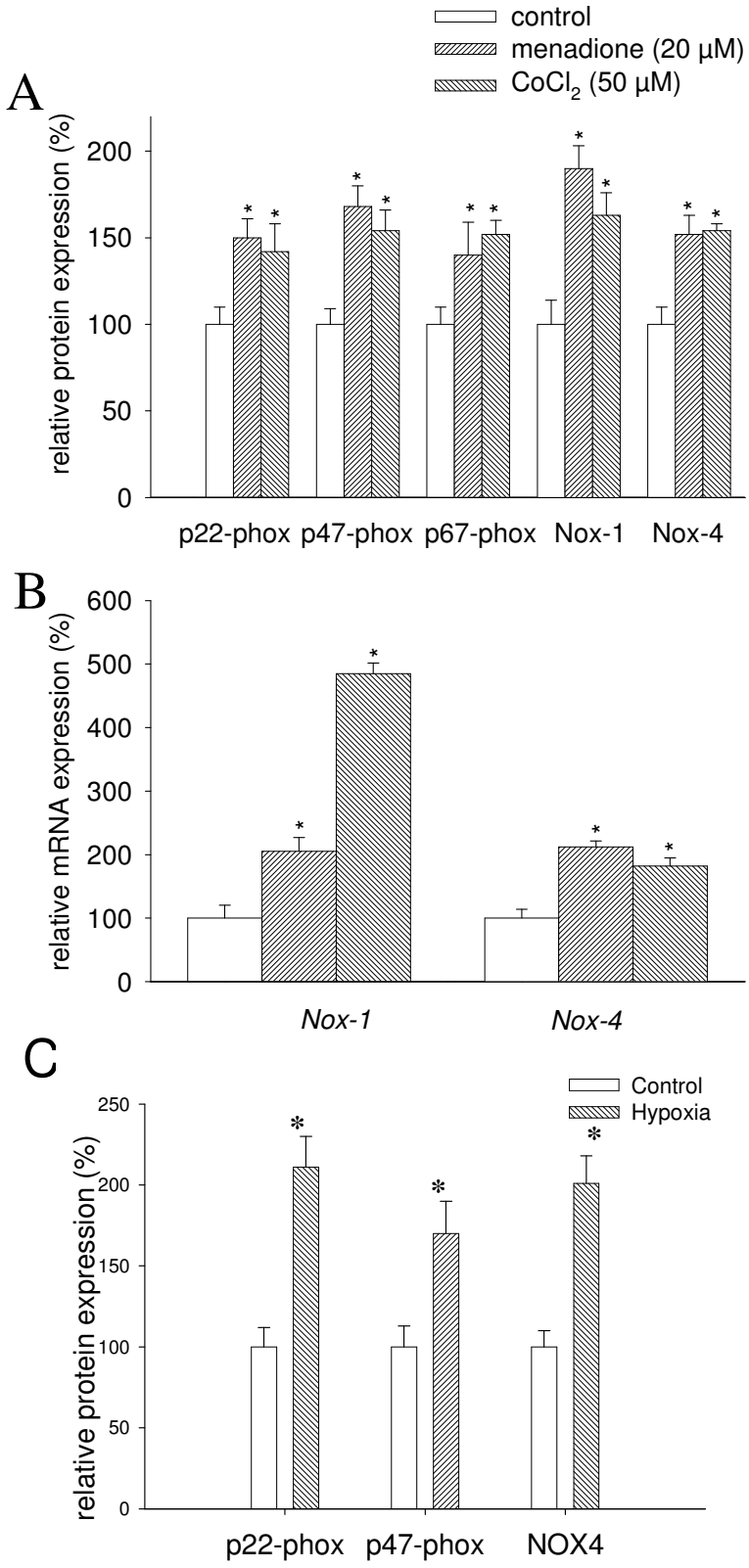

FIG. 19 Upregulation of NADPH-oxidase subunits upon incubation of embryoid bodies with menadione and $CoCl_2$. *A*, increase in protein expression of the NADPH-oxidase subunits p22-phox, p47-phox, p67-phox, Nox-1 and Nox-4 which were determined 24 h after treatment of 4-day-old embryoid bodies by semiquantitative immunohistochemistry. *B*, mRNA expression of *Nox-1* and *Nox-4* upon either menadione (20 µM) or $CoCl_2$ (50 µM) treatment. *C*, increase in protein expression of the NADPH-oxidase subunits p22-phox, p47-phox, Nox-4 upon treatment with physiological hypoxia (1% O_2). $^*P < 0.05$, significantly different to the untreated control.

3.9 ATTENUATION OF MENADIONE- AND CHEMICAL HYPOXIA-INDUCED CT-1 UP-REGULATION BY NADPH-OXIDASE INHIBITORS

The involvement of NADPH-oxidase in the regulation of CT-1 expression was further substantiated by incubating embryoid bodies with either menadione (20 µM) or $CoCl_2$ (50 µM) in the presence of the NADPH-oxidase inhibitors DPI (10 µM) (n = 3) or apocynin (10 µM) (n = 3) (FIG. 20). This treatment significantly inhibited the increase in CT-1 expression following treatment with either menadione or $CoCl_2$, thus indicating that NADPH-oxidase-derived ROS are involved in up-regulation of CT-1 by pro-oxidants and hypoxia.

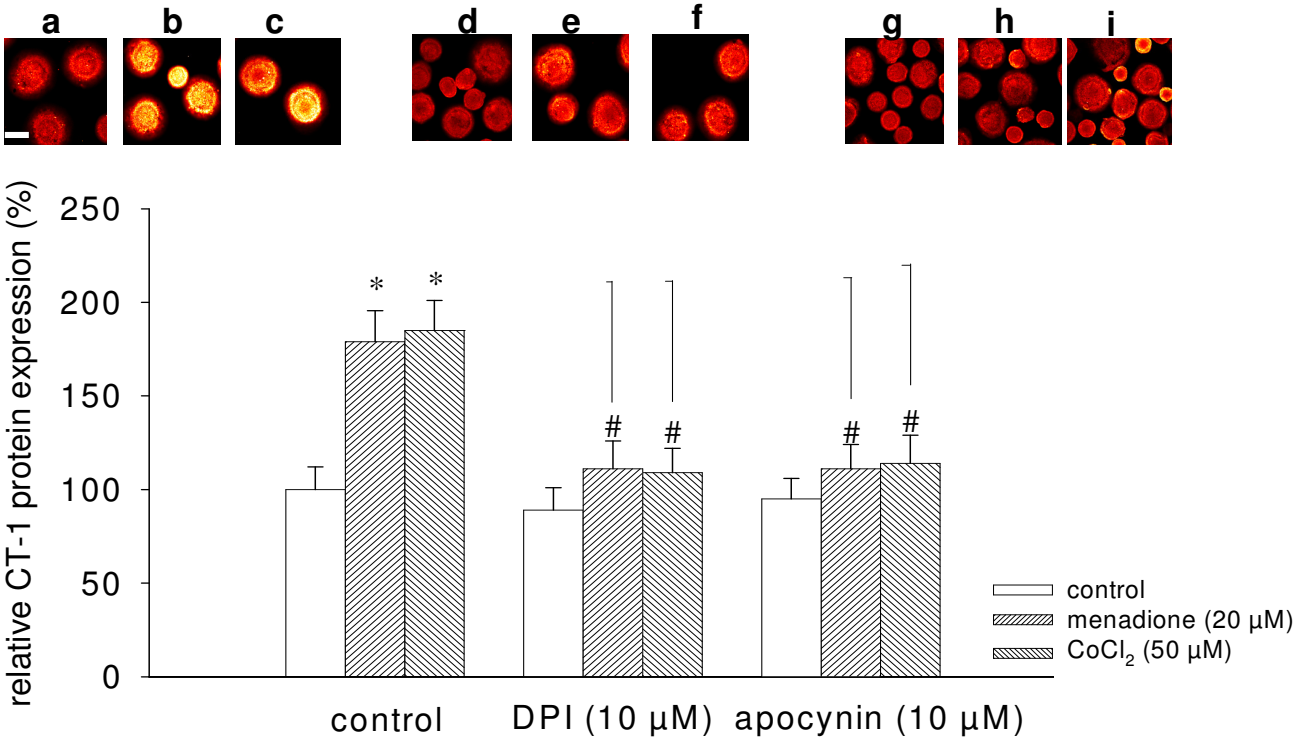

FIG. 20 Inhibition of menadione- and $CoCl_2$-mediated upregulation of CT-1 upon preincubation with the NADPH-oxidase inhibitors DPI (10 µM) or apocynin (10 µM). Embryoid bodies were treated at day 4 of cell culture with either menadione (20 µM) or $CoCl_2$ (50 µM) in the presence of either DPI or apocynin. Following 24 h CT-1 protein expression was determined by semiquantitative immunohistochemistry. The images show representative embryoid bodies: *a*, untreated; *b*, menadione-treated; *c*, $CoCl_2$-treated; *d*, DPI-treated; *e*, DPI- + menadione-treated; *f*, DPI- + $CoCl_2$-treated; *g*, apocynin-treated; *h*, apocynin- + menadione-treated; *i*, apocynin- + $CoCl_2$-treated. The bar represents 100 µm. $^{*,\#}P < 0.05$, significantly different as indicated.

3.10 SIGNALLING CASCADES INVOLVED IN THE UP-REGULATION OF CT-1 IN EMBRYOID BODIES BY MENADIONE AND CHEMICAL HYPOXIA

Upon binding of CT-1 to a LIFR-*ß*: gp130 heterodimer a complex signalling cascade is activated which involves the MAPK members ERK1,2, JNK and p38 as well as PI3-kinase and the JAK/STAT pathway [Sauer *et al*, 2003].

The data in section 3.2 to section 3.7 above shows clearly that pro-oxidants as well as hypoxia influenced the regulation of both CT-1 and HIF-1α gene expressions using a mechanism that involves ROS. Furthermore, the results in section 3.9 implicated the involvement of NADPH-oxidase in the ROS dependent regulation of CT-1 expression.

In this section the involvement of the MAPKs, PI-3 kinase as well as gp130, JAK2 and STAT3 was analysed.

3.10.1 EXPRESSION AND PHOSPHORYLATION OF GP130 IN EMBRYOID BODIES BY PRO-OXIDANTS AND CHEMICAL HYPOXIA

Regulation of CT-1 expression may be mediated through phosphorylation of the gp130 signal transduction receptor. To assess changes in gp130 expression as well as gp130 phosphorylation in the presence of prooxidants and chemical hypoxia, semiquantitative immunohistochemistry was performed using antibodies against the unphosphorylated as well as the phosphorylated form of gp130. It was found that prooxidants as well as chemical hypoxia significantly increased gp130 expression as well as phosphorylation, suggesting activation of gp130-mediated signal transduction cascades (n = 3) (FIG. 21).

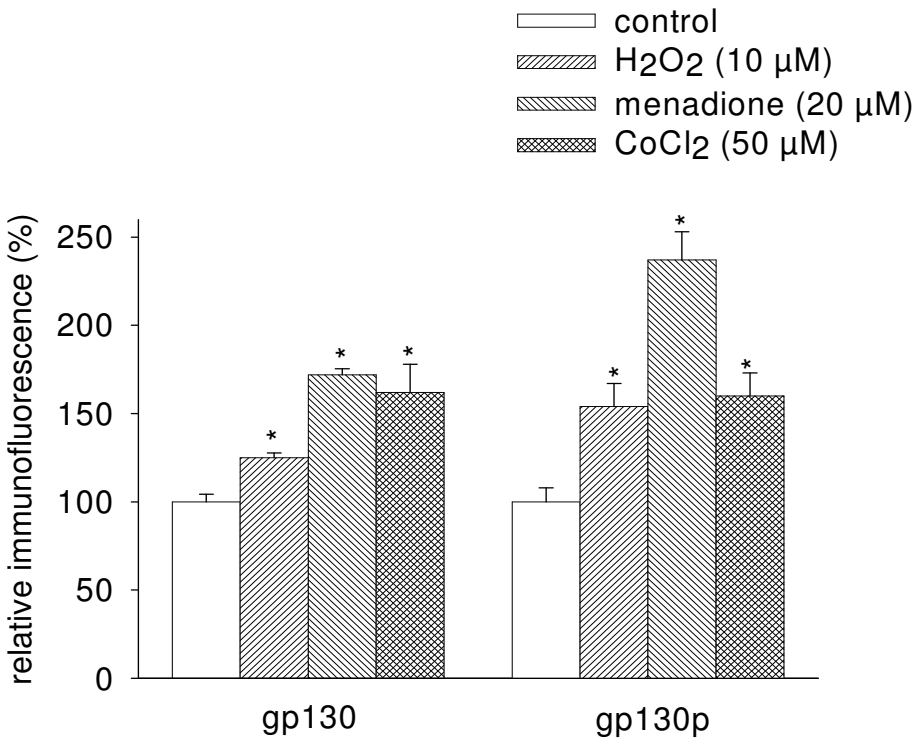

FIG. 21 Upregulation of gp130 protein expression and phosphorylation upon treatment of embryoid bodies with prooxidants or chemical hypoxia. Embryoid bodies were treated at day 4 of cell culture with either H_2O_2 (10 µM), menadione (20 µM) or $CoCl_2$ (50 µM). After 24 h gp130 protein expression and phosphorylation was assessed by semiquantitative immunohistochemistry using an antibody directed against unphosphorylated gp130 or the phosphorylated form of gp130. *$P < 0.05$, significantly different to the untreated control.

3.10.2 EXPRESSION AND PHOSPHORYLATION OF JAK2 AND STAT3 IN EMBRYOID BODIES BY PRO-OXIDANTS AND CHEMICAL HYPOXIA

By using phospho-specific antibodies it was found that treatment of EBs with menadione (n = 3) and $CoCl_2$ (n = 3) led to the phosphorylation of both JAK2 and STAT3 (FIG. 22 *A* and *B*). To correlate the activation of JAK2 to the expression of CT-1, embryoid bodies were treated with the JAK-2 antagonist AG490 which significantly down-regulated the menadione- and $CoCl_2$-induced up-regulation of CT-1 (n = 3) (FIG. 23).

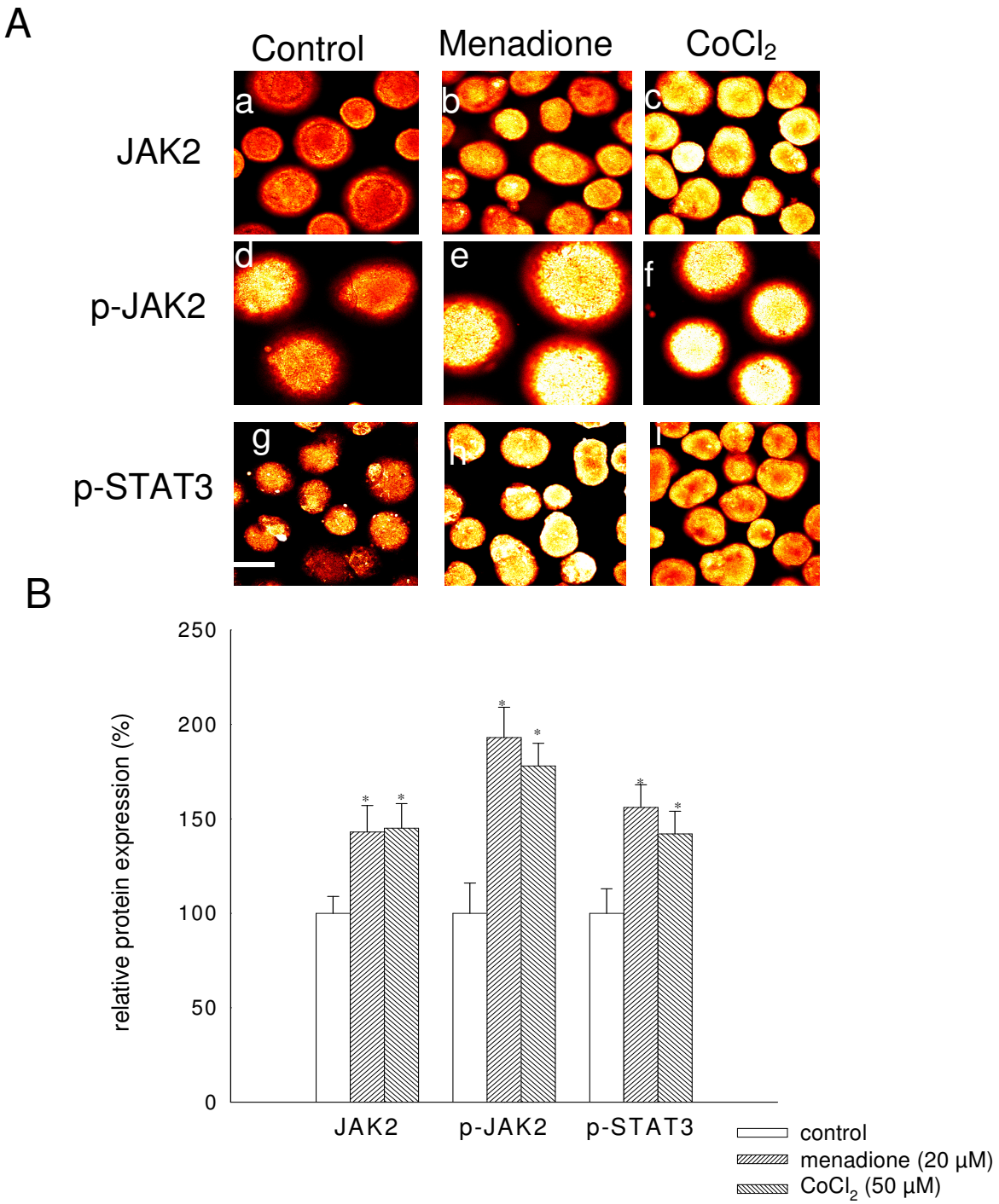

FIG. 22 Expression and activation of JAK2 and STAT3 upon incubation of embryoid bodies for 24h with either menadione (20 µM) or $CoCl_2$ (50 µM). Activation was assessed by semiquantitative immunohistochemistry using phospho-specific antibodies. There was a significant up-regulation of JAK2 expression and activation of JAK2 and p-STAT3. The image show representative embryoid bodies: *a, d* and *g*, untreated; *b, e* and *h*, menadione-treated; *c*, *f* and *i*, $CoCl_2$-treated. The bar represents 400 µM. $^*P < 0.05$, significantly different to the untreated control.

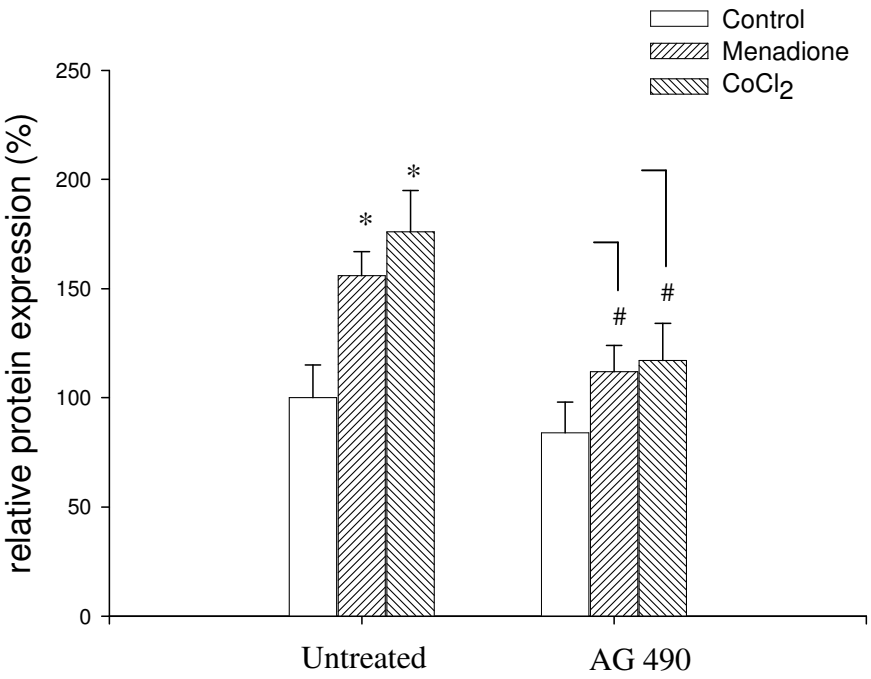

FIG. 23 Inhibition of menadione and $CoCl_2$ induced CT-1 expression by the JAK2 inhibitor AG 490. There was a significant attenuation of the effect of menadione (20 µM) and $CoCl_2$ (50 µM) when EBs were pre-incubated for 2 h with 10 µM AG 490. $*^{\#}P < 0.05$, significantly different as indicated.

3.10.3 ACTIVATION OF MAPKs AND PI-3K IN EMBRYOID BODIES BY PRO-OXIDANTS AND CHEMICAL HYPOXIA

By using phospho-specific antibodies it was found that phosphorylation of ERK1,2 (menadione, n = 3; $CoCl_2$, n = 5), JNK (menadione, n = 3; $CoCl_2$, n = 5), p38 (menadione, n = 4; $CoCl_2$, n = 4), and PI3-kinase (n = 4) occurred (FIG. 24).

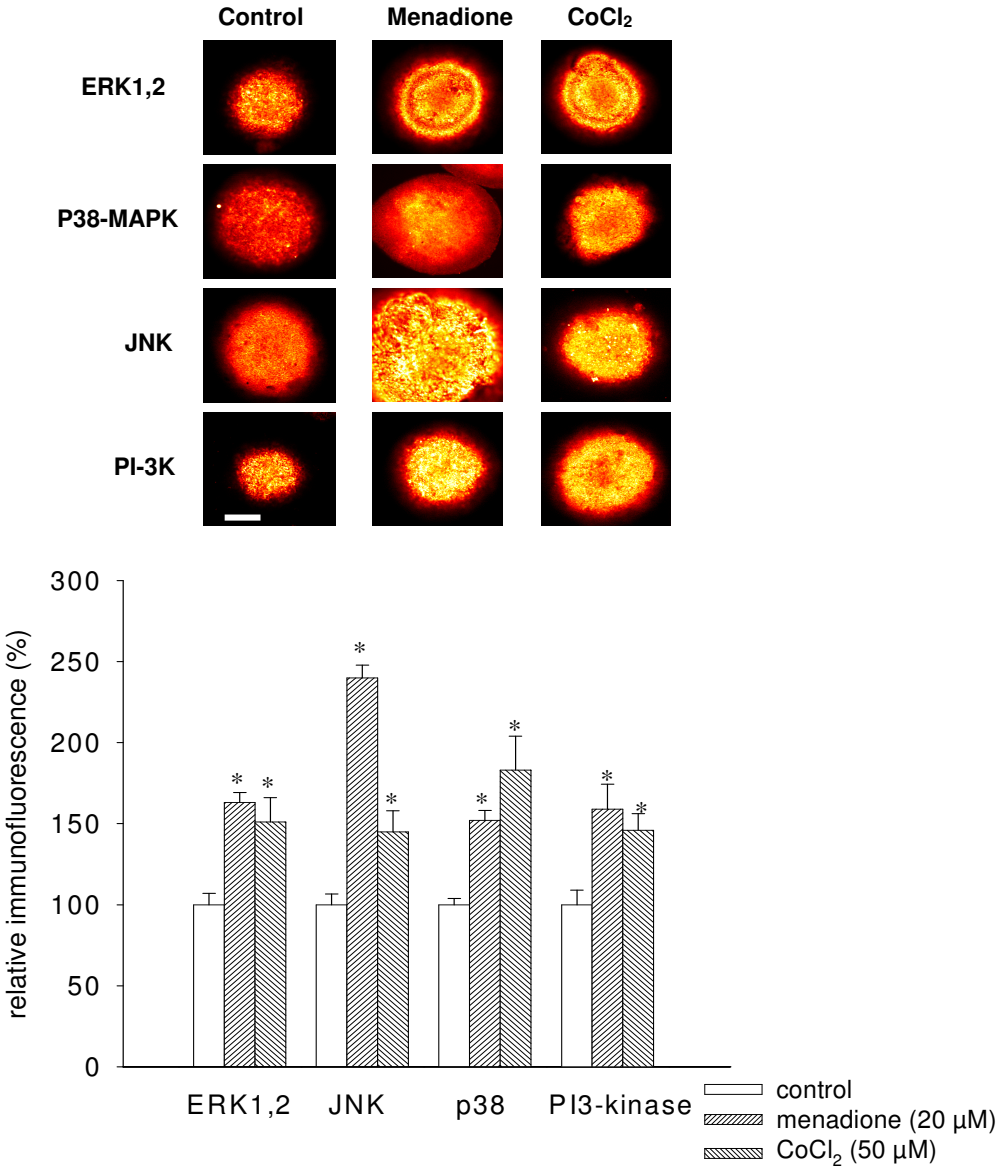

FIG. 24 Activation of ERK1,2, JNK p38 and PI3-kinase upon incubation of embryoid bodies with either menadione (20 µM) or $CoCl_2$ (50 µM). Activation was assessed by semiquantitative immunohistochemistry using phospho-specific antibodies. Maximum activation of ERK1,2 was achieved after 15 min; maximum activation of JNK was at 15 min for menadione treatment and 30 min for $CoCl_2$ treatment; maximum activation of p38 was at 30 min for menadione treatment and 60 min for $CoCl_2$ treatment; maximum activation of PI3-kinase was at 60 min. The bar represents 400 µm. *$P < 0.05$, significantly different to the untreated control.

3.10.4 EFFECT OF ERK1/2- (U0126), JNK-(SP600125), P38 (SKF 86002) AND PI3-KINASE (LY294002) INHIBITORS ON PRO-OXIDANT- AND CHEMICAL HYPOXIA- INDUCED CT-1 UP-REGULATION

To correlate the activation of MAPK pathways and PI3-kinase to the expression of CT-1, embryoid bodies were treated with the ERK1,2 inhibitor UO126 (10 µM), the JNK inhibitor SP600125 (10 µM) the p38 inhibitor SKF86002 (10 µM) as well as the PI3-kinase inhibitor LY294002 (20 µM) which significantly down-regulated the menadione- and $CoCl_2$-induced up-regulation of CT-1 (n = 3) (FIG. 25).

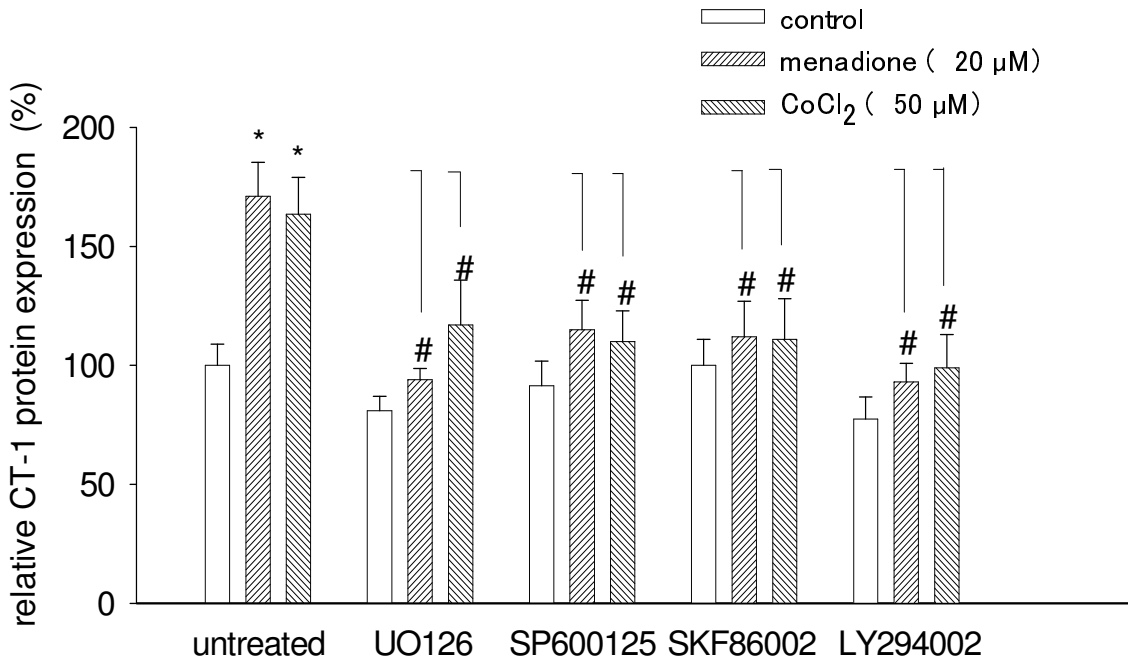

FIG. 25 Inhibition of menadione- and $CoCl_2$-mediated upregulation of CT-1 by the ERK1,2 inhibitor UO126 (10 µM), the JNK inhibitor SP600125 (10 µM), the p38 inhibitor SKF86002 (10 µM), and the PI3-kinase inhibitor LY294002 (20 µM). Embryoid bodies were treated at day 4 of cell culture with either menadione (20 µM) or $CoCl_2$ in the presence of the respective inhibitor. CT-1 protein expression was assessed following 24 h by semiquantitative immunohistochemistry. $*^{,\#}P < 0.05$, significantly different as indicated.

3.10.5 EFFECT OF ERK1/2- (U0126), JNK-(SP600125), P38 (SKF 86002) INHIBITORS ON PRO-OXIDANT- AND CHEMICAL HYPOXIA-INDUCED HIF-1α UP-REGULATION

It has previously been shown that the MAPKs are activated under conditions of increased ROS production. Thus, it was necessary to find out whether inhibiting these kinases would affect the menadione and $CoCl_2$ induced regulation of HIF-1α expression.

The EBs were preincubated for 2 h with U0126 (10 µM), SP600125 (10 µM) and SKF 86002 (10 µM) prior to adding of either menadione or $CoCl_2$. All three inhibitors significantly attenuated the effect of both menadione (20 µM) and $CoCl_2$ (50 µM) as shown in FIG. 26.

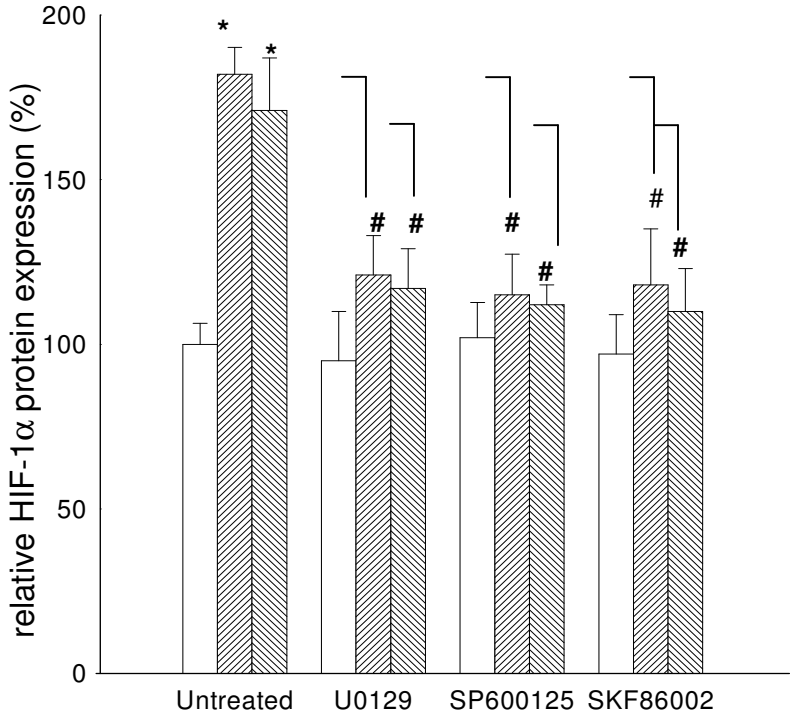

FIG. 26 Inhibition of menadione- and $CoCl_2$-mediated upregulation of HIF-1α by the ERK1,2 inhibitor UO126 (10 µM), the JNK inhibitor SP600125 (10 µM), the p38 inhibitor SKF86002 (10 µM). Embryoid bodies were treated at day 4 of cell culture with either menadione (20 µM) or $CoCl_2$ (50 µM) in the presence of the respective inhibitor. HIF-1α protein expression was assessed following 24 h by semiquantitative immunohistochemistry. $^{*,\#}P < 0.05$, significantly different as indicated.

3.11 EFFECT OF THE INHIBITION OF HIF-1 α ON CT-1 EXPRESSION

3.11.1 EFFECT OF HIF-1α INHIBITOR (2-METHOXYESTRADIOL) ON PRO-OXIDANTS AND CHEMICAL HYPOXIA INDUCED CT-1 AND HIF-1α UP-REGULATION

Our working hypothesis suggested that the ROS mediated CT-1 expression was HIF-1α dependent. This implies that inhibition of HIF-1α would lead to inhibition of CT-1 up-regulation by pro-oxidants and hypoxia thereby decreasing cardiomyogenesis.

To test our theory, embryoid bodies grown from the parental CCE cell line were treated with the HIF-1α inhibitor 2-methoxyestradiol (2-ME) (3 μM). In corroboration of our working hypothesis it was apparent that in the presence of 2-ME menadione- and chemical hypoxia-induced upregulation of CT-1 was significantly attenuated (FIG. 27*A*) (n = 5). Furthermore, treatment with 2-ME significantly inhibited the increase HIF-1α expression observed after menadione (20 μM) and $CoCl_2$ (50 μM) exposure (FIG. 27*B*).

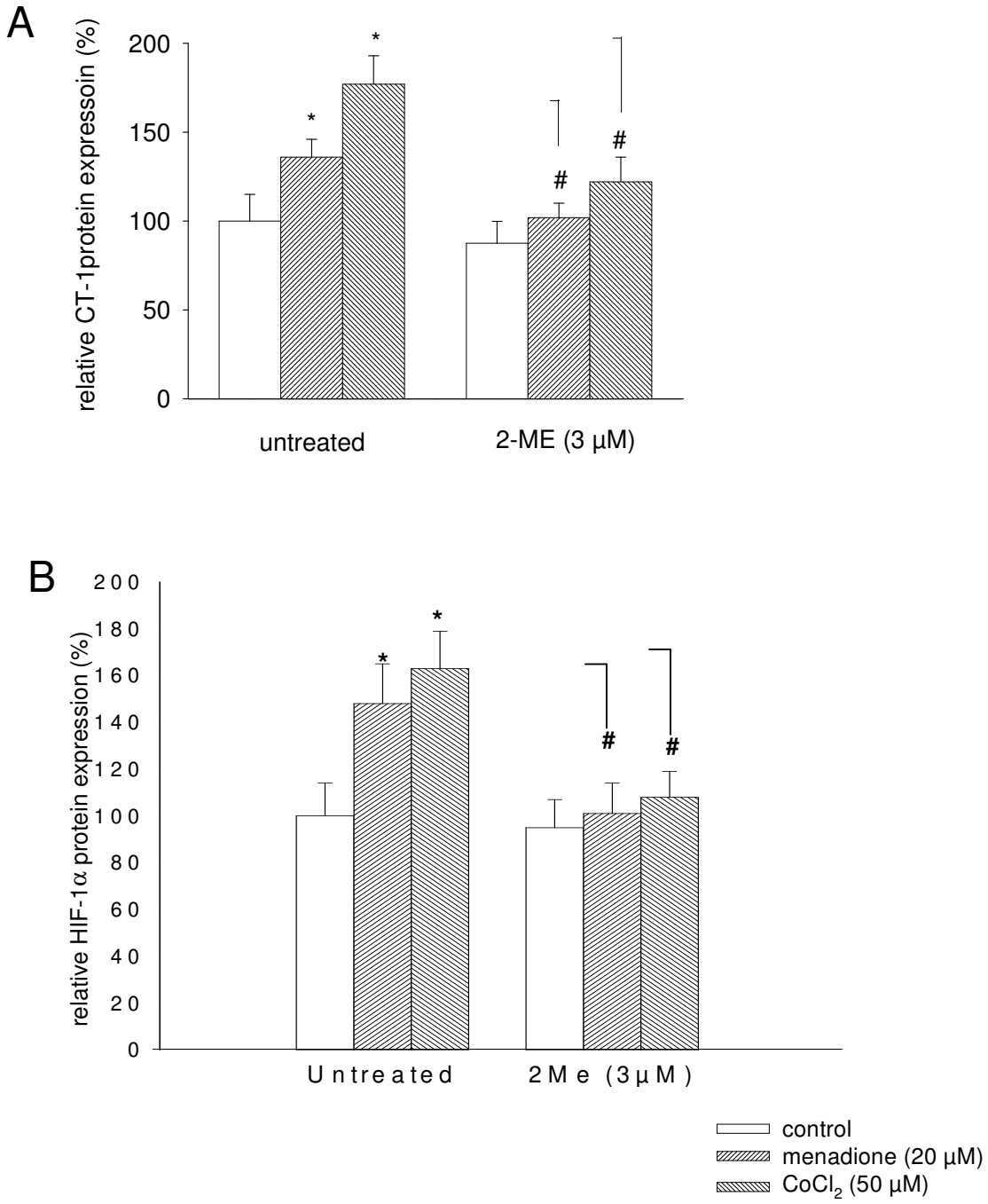

FIG. 27 Inhibition of menadione- and $CoCl_2$-mediated upregulation of CT-1 by the HIF-1α inhibitor 2-ME (3 µM). Embryoid bodies were treated at day 4 of cell culture with either menadione (20 µM) or $CoCl_2$ (50 µM) in the presence of 2-ME. CT-1 (*A*) and HIF-1α (*B*) protein expression was assessed following 24h by immunohistochemistry. $*^{,\#}P < 0.05$, significantly different as indicated.

3.11.2 ABSENCE OF CARDIOMYOGENESIS AND STIMULATION OF CT-1 EXPRESSION IN HIF-1$\alpha^{-/-}$ ES CELLS

To proof the involvement of HIF-1α in the regulation of CT-1 expression, we made use of the homozygous HIF-1α-deficient HM ES cell line (Hopfl *et al*, 2000). In differentiating HIF-1$\alpha^{-/-}$ ES cells cardiomyogenesis was completely absent as assessed by determination of the number of beating embryoid bodies (FIG. 28). The lack of cardiomyogenesis in the EBs derived from the HIF-1$\alpha^{-/-}$ ES cells suggest the involvement of HIF-1α in the cardiomyogenic activities of CT-1 since it has previously been shown by Sauer and colleagues (2004) that CT-1 promotes cardiomyogenesis using a ROS dependent mechanism.

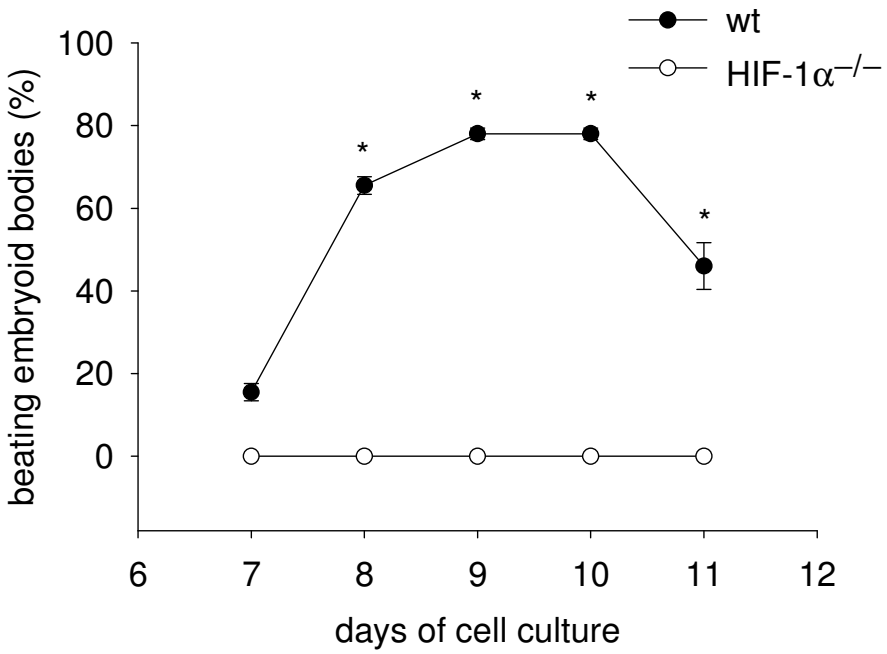

FIG. 28 Cardiac cell differentiation in wt and HIF-1$\alpha^{-/-}$ ES cells assessed by counting the number of beating EBs between day 6 and day 12. There was no cardiomyogenesis observed in the HIF-1$\alpha^{-/-}$ derived EBs. There was a significant progressive increase in the number of EBs with beating areas in the wt cells. $*P < 0.05$, significantly different to wt embryoid bodies.

Moreover, as compared to wt HM-1 ES cells (FIG. 29) and ES cells of the CCE cell line (FIG. 12*A*) no upregulation of CT-1 during the time course of differentiation was observed (n = 5) although there was basal CT-1 protein expression.

Furthermore, the upregulation of CT-1 mRNA (FIG. 30*A*) (n = 6) and protein (FIG. 30*B*) (n = 4) following treatment of embryoid bodies with menadione and $CoCl_2$ was completely abolished in HIF-1$\alpha^{-/-}$ ES cells. Taken together, our data strongly support the hypothesis that HIF-1α regulates CT-1 expression.

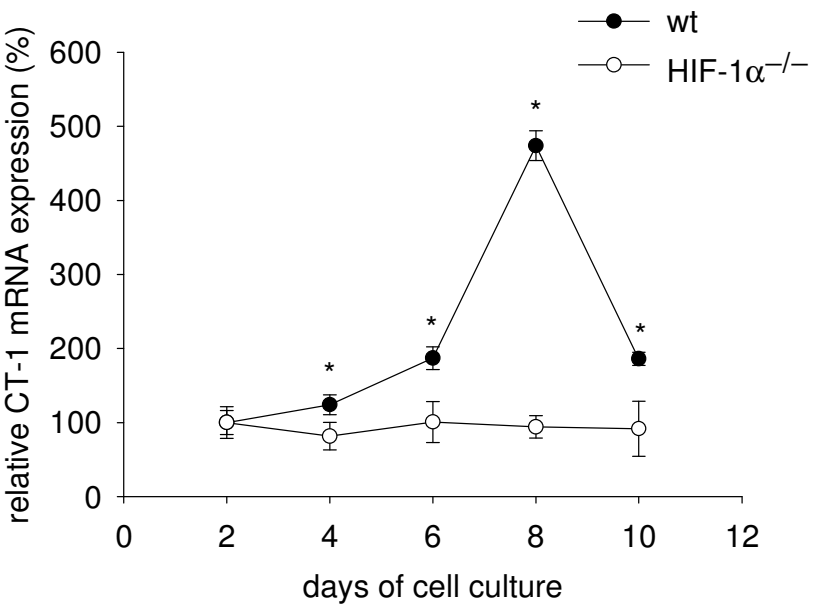

FIG. 29 Analysis of CT-1 expression during the time of differentiation. No upregulation of CT-1 mRNA was observed in HIF-1$\alpha^{-/-}$ as compared to the wt which showed a similar trend as observed in CCE ES cell derived EBs as assessed by semiquantitative immunohistochemistry. *$P < 0.05$, significantly different to wt embryoid bodies.

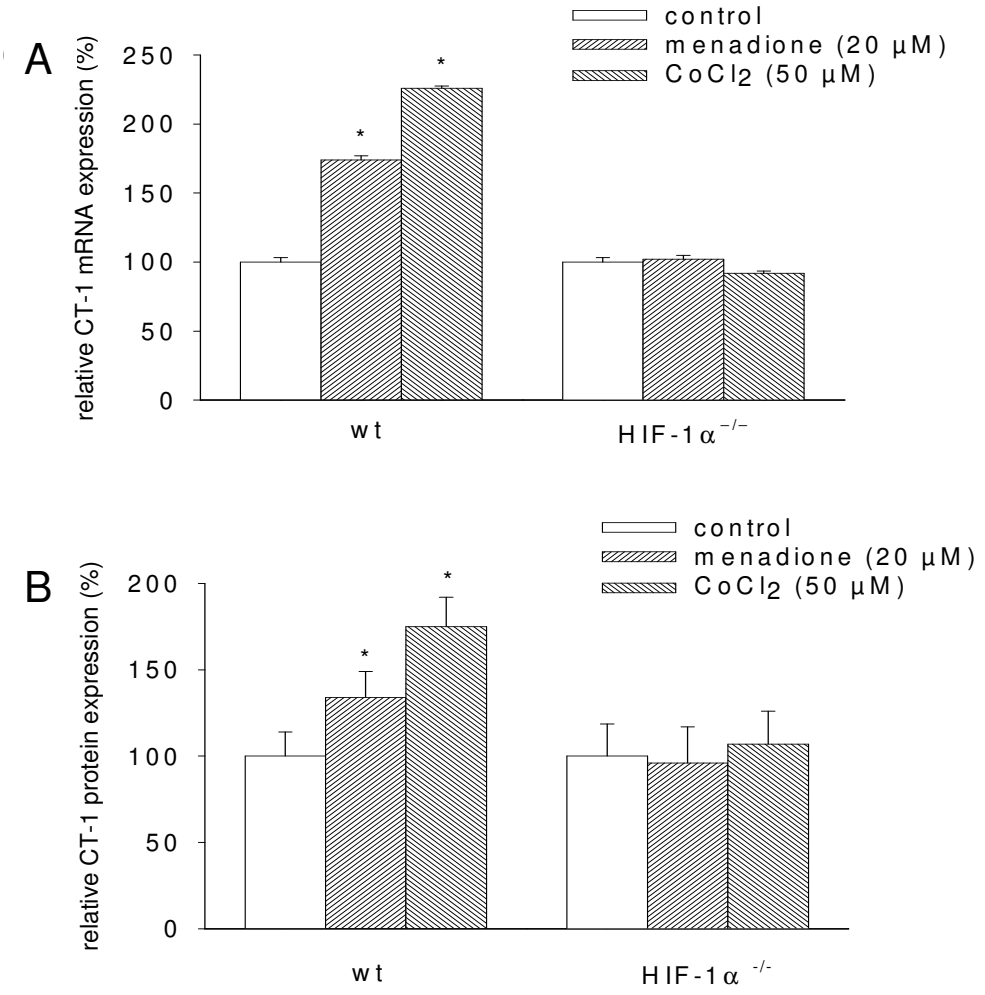

FIG. 30 Expression of CT-1 during the time of differentiation in wt and HIF-1$\alpha^{-/-}$ ES cells upon incubation with menadione (20 µM) and $CoCl_2$ (50 µM) (*A, B*). In HIF-1$\alpha^{-/-}$ ES cells no upregulation of CT-1 protein (*A*) and mRNA (*B*) was observed as compared to the wt. This was asssed by semiquantitative immunohistochemistry and real-time PCR respectively. **P* < 0.05, significantly different to control in wt embryoid bodies.

3.12 INCREASED CARDIOMYOGENESIS IN EBS TREATED WITH PRO-OXIDANTS AND CHEMICAL HYPOXIA

Since CT-1 promotes cardiomyogenesis, ROS- and hypoxia-mediated up-regulation of CT-1 in EBs should lead to the stimulation of cardiomyogenesis. To test this notion, EBs were treated with menadione (20 µM) and $CoCl_2$ (50 µM) (n=3) on day 4 of differentiation and cultivated on petriperm plates till day 8. ROS and hypoxia significantly increased cardiomyogenesis in comparison to control as assessed by determination of the number of beating embryoid bodies (FIG. 31).

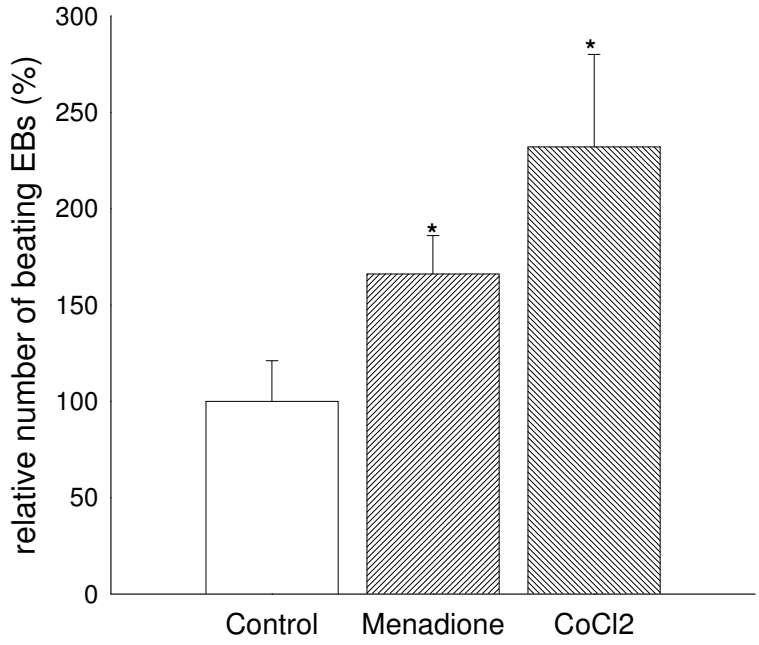

FIG. 31 Cardiomyogenesis in CCE ES cells. Incubation with menadione (20 µM) and $CoCl_2$ (50 µM) (n=3) lead to a significant increase in the number of beating EBs as compared to control. $^{*}P < 0.05$, significantly different as indicated.

In order to find out whether ROS was involved in the chemical hypoxia induced cardiomyogenesis, plated EBs were preincubated with either the free radical scavengers vitamin E (20 µM) and NMPG (20 µM) or the specific NADPH oxidase inhibitor apocynin (10 µM) for 2 h prior to adding $CoCl_2$. The effect of $CoCl_2$ was significantly attenuated by free radical scavengers vitamin E (20 µM) (n=3), NMPG (20 µM) (n = 4) and NADPH oxidase inhibitor apocynin (10 µM) (n = 4) (FIG. 32), indicating that ROS was involved.

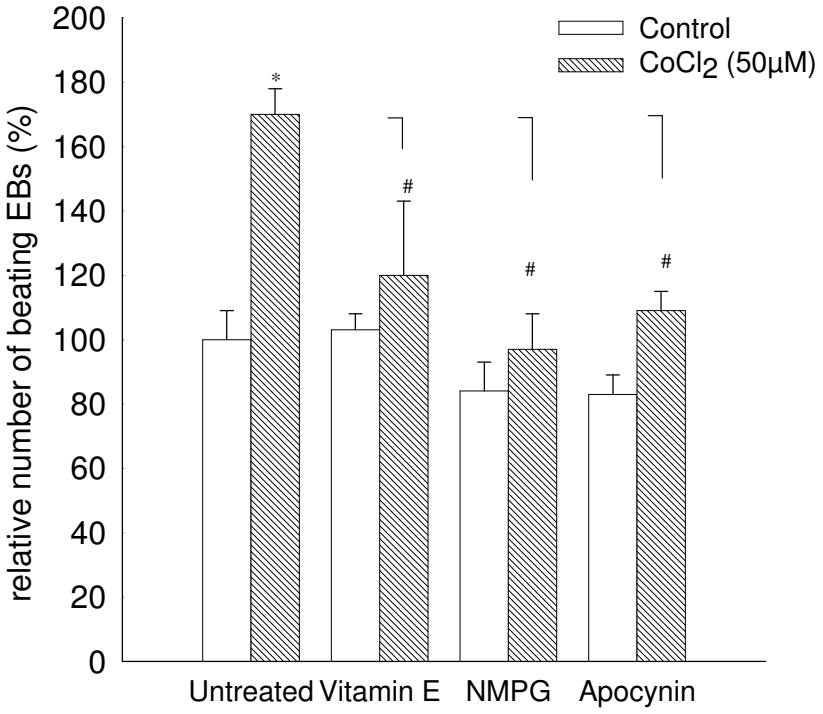

FIG 32 Inhibition of cardiomyogenesis in CCE ES cells. The effect induced by $CoCl_2$ was significantly abolished when EBs were preincubated with vitamin E, NMPG and apocynin. This was evaluated by counting the number of spontaneously contracting embryoid bodies. $^{*,\#}P < 0.05$, significantly different as indicated.

3.13 CELLULAR LOCALISATION OF CT-1 PRE- AND POST-STIMULATION WITH PRO-OXIDANT AND CHEMICAL HYPOXIA

In order to find out where CT-1 is localised in the cells pre- and post stimulation, eight days old EBs with beating foci (cardiomyocytes) were dissociated into single cells with 1mg/ ml collagenase and plated on coverslips in 24 wells cell culture plates. Four days after dissociation, cells were treated for 4h with either menadione (20 µM) or $CoCl_2$ (50 µM) and stained for CT-1 and against α-actinin to identify cardiomyocytes. There was up-regulation and translocation of CT-1 from the cytoplasm to the nucleus in cell treated with menadione and $CoCl_2$ in comparison to control (FIG. 33).

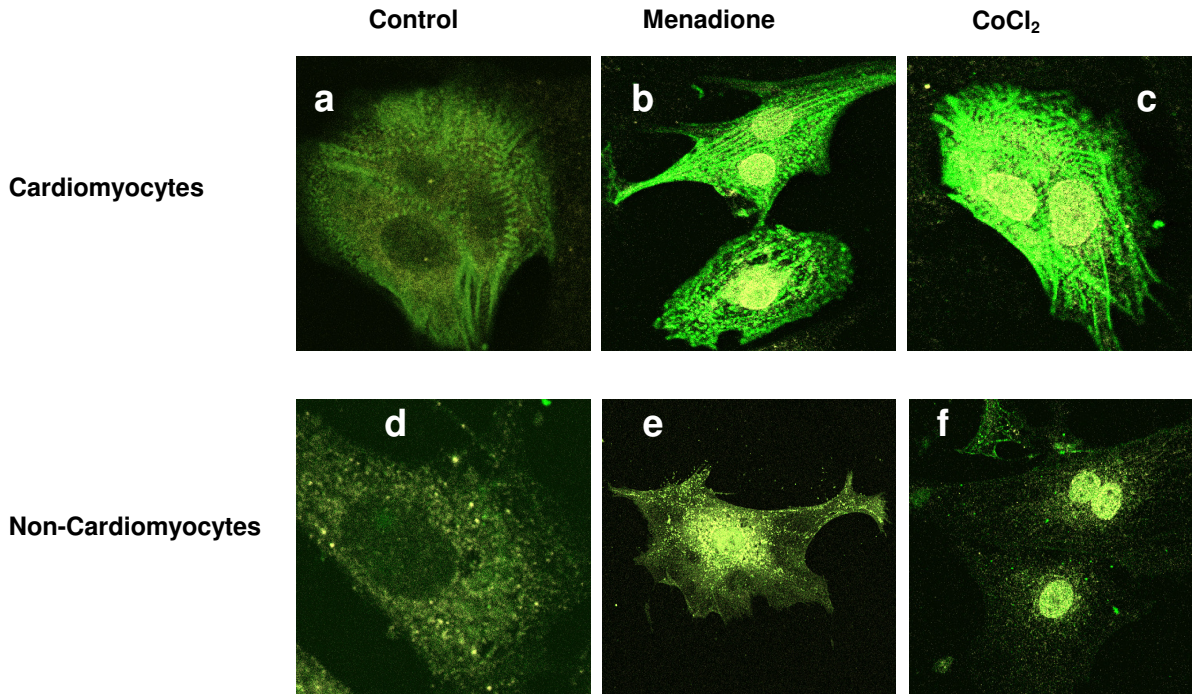

FIG. 33 Induction of nuclear translocation of CT-1 in cardiomyocytes (b, c) as well as non-cardiomyoctes (e, f) after treatment with 20 µM Menadione (b, e) and 50 µM $CoCl_2$ (c, f).

4 DISCUSSION

Exposure of cells to ROS such as $O_2^{\cdot -}$ and H_2O_2 has been associated to a variety of physiological and pathological events [Cross *et al*, 1987; Miller and Britigan, 1995]. The cellular consequences of oxidant exposure, as well as the relative importance of different cellular antioxidant systems in protecting cells from such exposures, varies with both the nature of the oxidant species generated and the intracellular and/or extracellular location of generation [Fujimoto *et al*, 1990; Miller and Britigan, 1995].

The aim of the present study was to analyse the role of redox signalling for the expression of CT-1 in differentiating mouse ES cells in response to pro-oxidants and hypoxia and to find out whether HIF-1α is involved in the ROS dependent regulation of CT-1. To induce changes in the intracellular redox state, we incubated the mouse ES cell-derived EBs with either H_2O_2, menadione (pro-oxidant), $CoCl_2$ (chemical hypoxia) or nitrogen gas (physiological hypoxia – 1% O_2). Menadione is a quinone which has been widely used to induce cellular oxidative stress [Chen and Cederbaum, 1997; Han *et al*, 2002; Kawasaki *et al*, 2004; Mauzeroll and Bard, 2004]. Quinones form an important group of substrates for flavoenzymes and can undergo either one-electron reduction, producing semiquinone radicals, or two-electron reduction, resulting in hydroquinones. In the presence of O_2 the semiquinones and hydroquinones are transformed back to the mother quinone (redox-cycle), thereby generating $O_2^{\cdot -}$ which is transformed to the more stable H_2O_2. $CoCl_2$ has been widely used to mimic hypoxia and has been previously shown to induce an increase in intracellular ROS concentration and up-regulation of HIF-1α [Liu *et al*, 1999; Chachami *et al*, 2004; BelAida *et al*, 2004].

We found that the up-regulation of CT-1 in differentiating mouse ES cells by pro-oxidants and hypoxia depends on ROS, gp130/JAK2/STAT3 as well as MAPKs, PI-3K and HIF-1α. Furthermore, NADPH-oxidase was found to be the source of ROS generation (see FIG. 35). .

Very often, reduction or oxidation of protein redox active groups is a key event in enzymatic reactions. The function of most proteins crucially depends on the redox state of the solution [Ullmann and Knapp 1999]. During recent years, considerable

evidence has been accumulated to show that the intracellular redox state can regulate cell proliferation, differentiation, cellular growth and development [Hutter *et al*, 1997; Smith *et al*, 2000; Sauer *et al*, 2001; Droege 2002]. ROS are produced after activation of cell surface receptors, such as cytokine- and G protein-coupled receptors. ROS generated in response to cytokine receptor activation are implicated in the control of apoptotic pathways. Conversely, ROS generated in response to activation of G protein-coupled receptors are involved in triggering cell proliferation and hypertrophy.

Sauer and colleagues (1999, 2000) showed that differentiation of stem cells into cardiomyocytes is critically regulated by exogenous as well as endogenous ROS. Changes in the intracellular redox state initiate various signalling pathways and regulate the transcriptional and post transcriptional events that control gene expression [Haddad 2002]. Napoli and colleagues (2001), showed by using cDNA microarray, that about 100 genes are induced in response to oxidant stress. Kokura and colleagues (1999) showed that changes in the endothelial cell redox state cause transcriptional-independent and transcriptional-dependent surface expression of different endothelial adhesion surface molecules, which leads to neutrophil-endothelial adhesion. ROS are involved in the signalling cascade for cardio-protection induced by brief exposure to volatile anaesthetic, a procedure known as anaesthetic preconditioning. Up-regulation of transcription factors such as NF-kB and AP-1 occur after treatment with H_2O_2 [Schreck, Rieber and Baeuerle, 1991].

Hypoxia is a (patho-) physiological situation occurring during conditions, where increased expression of CT-1 has been reported: (*a*) during cardiac diseases like angina pectoris, cardiac infarction and heart failure [Freed *et al*, 2003]; (*b*) in the embryonic heart where the heart mass increases through cardiac cell hyperplasia [Wikenheiser *et al*, 2005]; (*c*) during the growth of ES cells within the 3-dimensional tissue of embryoid bodies [Wartenberg *et al*, 2001]. Recently, it has been demonstrated that hypoxia is associated with increased ROS generation produced either through the mitochondrial respiratory chain or NADPH-oxidase activity [Wolin *et al*, 2005]. Elevated ROS are known to occur in cardiac infarction and are suspected to cause ischemia-reperfusion injury [Berg *et al*, 2005].

Although hypoxia has been previously demonstrated to induce CT-1 expression [Hishinuma *et al*, 1999], the underlying signaling cascades have not been clarified

nor has any connection between hypoxia, ROS generation and CT-1 expression been established. In differentiating ES cells within EBs hypoxia occurs [Wartenberg *et al*, 2001] and endogenous generation of ROS via NADPH-oxidase [Sauer *et al*, 1999; Sauer *et al,* 2000] has been previously corroborated, which led to the working hypothesis of the present study that hypoxia and ROS may regulate CT-1 expression. Treatment of EBs with vitamin E which is a free radical scavenger led to the down-regulation of CT-1 and HIF-1α expression. Vice versa pro-oxidants as well as physiological and chemical hypoxia up-regulated CT-1 as well as HIF-1α protein and mRNA expression. Furthermore, either menadione treatment or chemical hypoxia resulted in increased ROS generation with subsequent up-regulation of ROS-generating NADPH-oxidase, supporting the notion that under conditions of pro-oxidant incubation and hypoxia the same ROS-mediated signaling pathways are triggered. The up-regulation of HIF-1α by exogenously applied ROS and hypoxia is in line with its role in the control of cellular oxygen homeostasis. Many studies reported the up-reguation of HIF-1α by exogenously applied ROS and hypoxia [Iyer *et al*, 1997; Chandel *et al*, 1998; Duranteau *et al*, 1998; Chandel *et al*, 2000; Goda *et al*, 2003], consequently leading to the regulation of many other redox sensitive genes.

Elevated ROS levels during hypoxia have been previously shown to occur in pulmonary myocytes [Marschall *et al*, 1996], cardiac myocytes, Hep3B cells, HeLa cells [Chandel *et al*, 1998] as well as adipocytes [Carriere *et al,* 2003], the sources being either the mitochondrial respiratory chain or NADPH-oxidase. These ROS may regulate the so far not quite defined non-hypoxic pathway of HIF- 1α stabilization, translocation and activation [Haddad, 2002; Haddad and Harb, 2005]. On the basis of the properties of NOX oxidases it has been recently proposed that hypoxia could cause an acute increase in ROS production by augmenting rates of electron transport to cytochrome b558, and this could occur through hypoxia-promoting oxidase activation and/or increasing the availability of its NADH or NADPH substrates [Wolin, Ahmad and Gupte, 2005]. The data of the present study clearly point towards a distinct role of NADPH-oxidase-derived ROS in the regulation of CT-1 expression in ES cells (see FIG. 35) since inhibition of NADPH-oxidase by apocynin as well as DPI abolished pro-oxidant and chemical hypoxia-mediated induction of CT-1. The involvement of ROS in the regulation of CT-1 expression was further validated by experiments demonstrating that the effects observed with the pro-oxidant menadione

and chemical hypoxia ($CoCl_2$) were significantly attenuated when EBs were pre-incubated with the free radical scavengers vitamin E and NMPG.

The regulation of cytokine expression by HIF-1α and ROS has been recently discussed [Haddad and Harb, 2005]. Expression of cytokines is generally associated to states of hypoxia, ROS generation and inflammation, i.e. patho-physiologic situations that require an immunological response as well as protection from tissue injury. Previously, stimulation of mRNA expression as well as secretion of IL-6 in response to exposure with pro-oxidants have been shown [Kosmidou *et al*, 2002; Haddad *et al*, 2002; Kida *et al*, 2005] and may act in paracrine as well as autocrine manner, thereby activating a loop mechanism disposed to stimulate the cell´s IL-6 receptors. CT-1 is a naturally occurring protein member of the interleukin (IL)-6 cytokine family and signals through the gp130/leukemia inhibitory factor receptor (LIFR) heterodimer. The formation of gp130/LIFR complex triggers the auto/trans-phosphorylation of associated Janus kinases, leading to the activation of Janus kinase/STAT and MAPK (ERK1 and -2) signalling pathways [Igaz, Tóth and Falus, 2001; Zvonic *et al*, 2004].

In this respect, our data demonstrated stimulation of gp130 protein expression as well as phosphorylation by pro-oxidants and chemical hypoxia, and downstream activation of several members of the previously described CT-1 activated signal transduction cascade, i.e. the MAPKs, ERK1,2, JNK, p38 as well as PI3-kinase which indeed suggests activation of the CT-1-mediated signalling cascade by ROS (see FIG. 35). Mitogen-activated protein kinase (MAPK) signaling pathways consist of a sequence of successively acting kinases that ultimately result in the dual phosphorylation and activation of terminal kinases such as p38, JNKs, and ERKs [Widman *et al*, 1999]. The MAPK signaling cascade is initiated in cardiac myocytes by G protein–coupled receptors (angiotensin II, endothelin-1, and adrenergic receptors), receptor tyrosine kinases (insulin-like growth factor, transforming growth factor-ß, and fibroblast growth factor receptors), CT-1 (gp130 receptor), and by stress stimuli [Sugden *et al*, 1998]. The requirement of ROS for the functioning of the MAPK pathways has been demonstrated in a variety of studies. Our data are in line with reports that hypoxia and exogenously applied ROS lead to the activation of the MAPKs (p38, JNK, ERK1,2) and PI-3K [Baas and Berk, 1995; Kulisz *et al*, 2001; Schäfer *et al*, 2003]. Menadione [Czaja, Liu and Wang, 2003; Dabrowski *et al*, 2000] as well as $CoCl_2$ [Liu *et al*, 1999; Yan *et al*, 2005] have been reported to activate the

MAPKS. Furthermore, there was significant up-regulation and activation of gp130R as well as JAK2 and STAT3. Consequently, the data of the present study demonstrated that inhibition of all investigated MAPK pathways, PI3-kinase as well as the JAK/STAT pathway, abolished the pro-oxidant- and hypoxia-mediated increase in CT-1 and HIF-1α expression. The present study clearly shows redox sensitivity of ERK1,2, JNK as well as p38, which link the observed increase in intracellular ROS generation to the signalling cascades involved in initiation of CT-1 gene regulation, promoting for example cardiomyocytes survival [Sheng *et al,* 1996; Sheng *et al*, 1997; Latchman, 1999; Craig *et al*, 2001; NG *et al*, 2002; Freed *et al*, 2003] and proliferation [Pennica *et al*, 1995; Sauer *et al*, 2004].

The mouse *CT-1* gene has been recently isolated. It constitutes 5.4 kilobases (kb) in length and consists of three exons and two introns. When nucleotide sequences of the coding regions of exons were compared with those of human, it was observed that exon 1, 2 and 3 share 96%, 84% and 81% homology, respectively. Interestingly, potential binding sites for several ubiquitous transcription factors including Nkx2,5, NF-IL6, CREB, GATA, AP-1, and most important, HIF-1α were present in the 5´-flanking region extending 2174-bp upstream from the transcription initiation site [Funamoto *et al*, 2000]. The presence of the HIF-1α binding site on the CT-1 gene accentuates the notion of the present study of CT-1 regulation by signal transduction pathways that involve HIF-1α. If CT-1 expression is indeed regulated by HIF-1, absence of hypoxia- and pro-oxidant-mediated up-regulation of CT-1 should be anticipated under conditions of either pharmacological or genetic inactivation of the α subunit of HIF-1. This was investigated by either a pharmacological approach using 2-methoxyestradiol (2-ME), which has been recently shown to downregulate HIF-1α at the posttranscriptional level and inhibits HIF-1-induced transcriptional activation of VEGF expression [Mabjeesh *et al*, 2003], or by ES cells homozygous deficient for HIF-1α [Hopfl *et al*, 2002] (see FIG. 34). 2-ME significantly inhibited the effect of menadione and chemical hypoxia ($CoCl_2$) on the expression of CT-1 and HIF-1α in differentiating EBs. Although basal mRNA and protein expression of CT-1 was found in HIF-1$\alpha^{-/-}$ cells, up-regulation during the time course of ES cell differentiation as occurring in wt cells was not observed, and cardiomyogenesis was completely absent. The absence of cardiomyogenesis in the HIF-1$\alpha^{-/-}$ ES cells indicates the importance of redox signalling pathway involving CT-1 and HIF-1α in the growth and

development of cardiomyocytes during embryogenesis, since treatment of CCE S103 ES cells, which have intact HIF-1α gene, with menadione and $CoCl_2$ lead to significant increase in cardiomyogenesis in comparison to control as observed by increase in number of beating (contracting) EBs. This effect was inhibited when the EBs were pre-incubated with free radicals scavengers and NADPH-oxidase inhibitor, indicating the involvement of ROS. Furthermore, pro-oxidants as well as chemical hypoxia failed to up-regulate CT-1 in HIF-1$\alpha^{-/-}$ ES cells both at the protein and mRNA levels which clearly demonstrate regulation of CT-1 expression by HIF-1α.

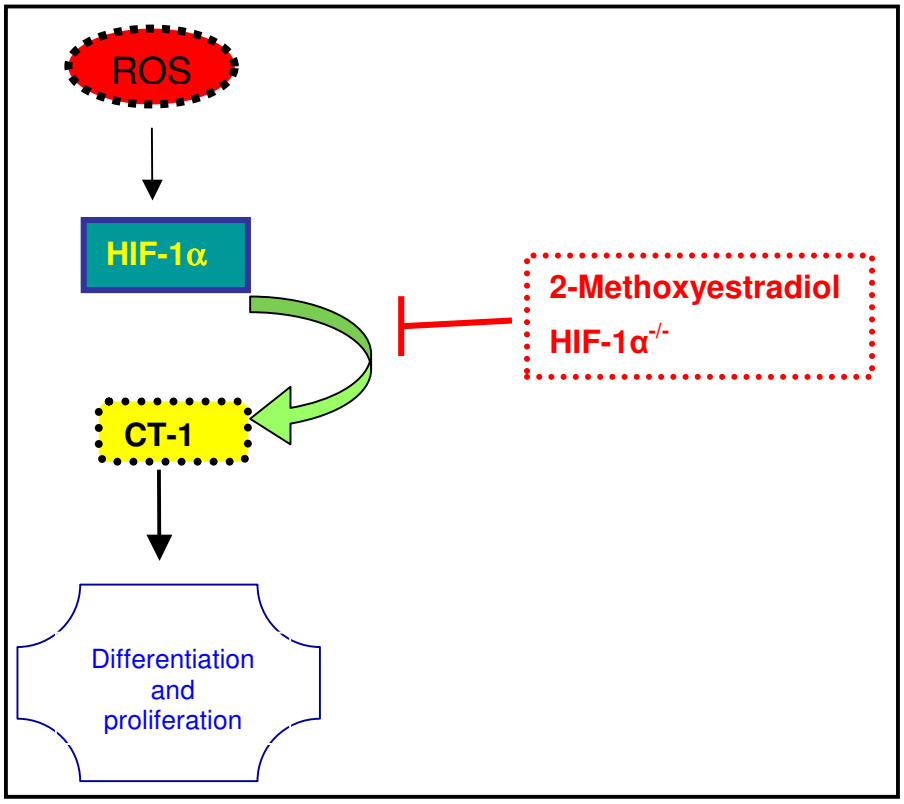

FIG. 34 Inhibition of ROS-dependent CT-1 up-regulation by 2-Methoxyestradiol (HIF-1a inhibitor) and through knockout of HIF-1α gene.

Although the classical view of HIF-1 regulation proposed stabilization of HIF-1α under hypoxic conditions and down-regulation at normoxia by process of pVHL-mediated ubiquitin-proteasome pathway, mechanisms of HIF-1α stabilization under normoxic conditions have been recently proposed [Lee *et al*, 2004]. These non-hypoxic pathways are utilized by many growth factors and cytokines, including insulin-like growth factors [Feldser *et al*, 1999], transforming growth factor and platelet-derived growth factor [Gorlach *et al*, 2001] and IL-1*ß* [Hellwig-Burgel *et al*, 1999], which are all known to utilize ROS as signalling molecules within their signal

transduction cascade. In this regard it was demonstrated that inhibition of ROS generation abolished hormone and growth factor-mediated increase in HIF-1α expression [Richard *et al*, 2000]. The data of the present study demonstrate ROS generation under conditions of either chemical or physiological hypoxia which was recently also reported to occur in intrapulmonary arteries of mice [Liu *et al*, 2005], in skeletal muscle [Zuo and Clanton, 2005] and in human hepatoma cells [Chandel *et al*, 2000]. This was underscored by the observation that oxygen sensing during hypoxia is dependent on mitochondrial-generated ROS [Brunelle *et al*, 2005; Emerling *et al*, 2005].

Another observation which has as of now not yet been reported, was the translocation of CT-1 from the cytoplasm to the nucleus in cells treated with pro-oxidant or chemical hypoxia. This nuclear translocation process was complete between two to four hours after stimulation in cardiomyocytes as well as non-cardiomyocytes. Whether nuclear translocation is required for CT-1-mediated gene activation remains to be determined.

Regulation of CT-1 expression by HIF-1α and ROS sounds reasonable in light of its biological function in inhibiting cardiac cell apoptosis, promoting cardiac cell hypertrophy and stimulating embryonic cardiac cell differentiation and proliferation, which are phenomena occurring under conditions of hypoxia and/or ROS-mediated inflammation. The investigation of the physiological microenvironment that regulates CT-1 expression will not only assist to unravel the role of CT-1 in cardiac hypertrophy and cardiac repair in the infarcted heart, but also give clues to the understanding of the mechanisms of cardiac cell hyperplasia in the embryonic heart and cardiomyogenic differentiation of ES cells.

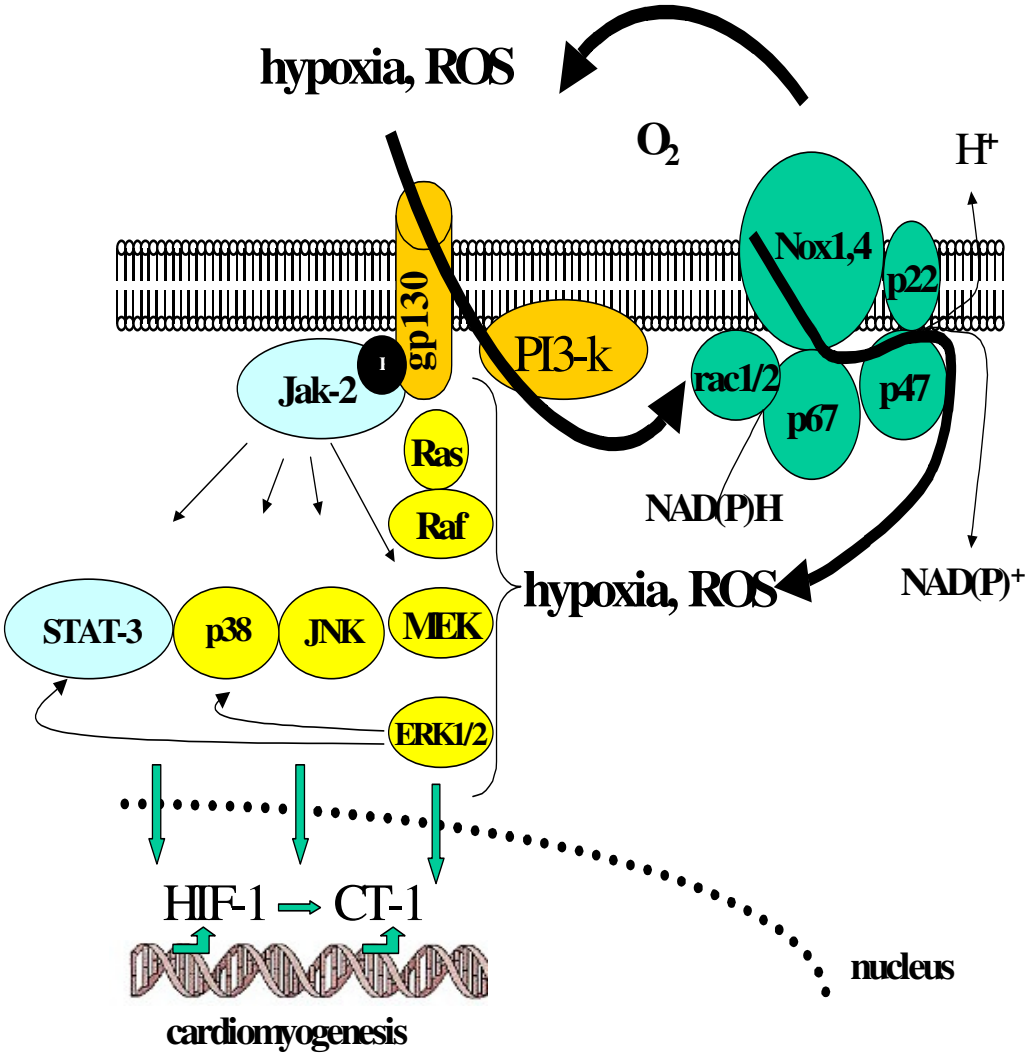

FIG. 35 Scheme of the proposed signal transduction cascade activated by ROS and hypoxia. Either hypoxia (initiating elevated ROS generation) or exogenous addition of ROS induces phosphorylation of gp130 and initiates a feed-forward cycle of NADPH-oxidase activity and expression. ROS generated by NADPH-oxidase act as signalling molecules within the CT-1/gp130 signalling cascade which finally results in HIF-1 upregulation and stimulation of CT-1 expression.

5 SUMMARY

Cardiomyogenesis in differentiating mouse embryonic stem (ES) cells is promoted by cardiotrophin-1 (CT-1), a member of the IL-6 interleukin superfamily and acts through the gp130 cytokine receptor. It was shown that prooxidants (menadione, hydrogen peroxide) as well as chemical ($CoCl_2$) and physiological (1% O_2) hypoxia increased CT-1 as well as HIF-1α protein and mRNA expression in embryoid bodies, indicating that CT-1 expression is regulated by reactive oxygen species (ROS) and hypoxia. Treatment with either prooxidants or chemical hypoxia increased gp130 phosphorylation and protein expression of NADPH-oxidase subunits p22-phox, p47-phox, p67-phox, as well as *Nox-1* and *Nox-4* mRNA. Consequently, inhibition of NADPH-oxidase activity by diphenylen iodonium chloride (DPI) and apocynin abolished prooxidant- and chemical hypoxia-induced up-regulation of CT-1 and HIF-1α. Prooxidants and chemical hypoxia activated ERK1,2, JNK and p38 as well as PI3-kinase, JAK2 and STAT3. The pro-oxidant and $CoCl_2$-mediated up-regulation of CT-1 and HIF-1α was significantly inhibited in the presence of the ERK1,2 antagonist UO126, the JNK antagonist SP600125, the p38 antagonist SKF86002, the PI3-kinase antagonist LY294002, the JAK-2 antagonist AG490 as well as in the presence of free radical scavengers. Moreover, developing embryoid bodies derived from HIF-1$\alpha^{-/-}$-ES cells lack cardiomyogenesis, and prooxidants as well as chemical hypoxia failed to upregulate CT-1 expression. Treatment of cells obtained by collagenase dissociation of beating EBs with pro-oxidants and chemical hypoxia resulted in the translocation of CT-1 into the nucleus within 2-4 h. In summary our data demonstrate that CT-1 expression in ES cells is regulated by ROS and HIF-1α and imply a crucial role of CT-1 in survival and proliferation of ES cell-derived cardiac cells. The importance of CT-1 nuclear transport to CT-1 mediated gene expression is still to be determined.

5.1 ZUSAMMENFASSUNG

Die Kardiomyogenese in differenzierenden embryonalen Stammzellen der Maus wird durch Cardiotrophin-1 (CT-1) gefördert, einem Mitglied der IL-6 Interleukin Superfamilie, das seine Signale durch den gp130 Rezeptor vermittelt. Es wurde gezeigt, dass sowohl Prooxidantien (Menadione, Wasserstoffperoxid) als auch chemische ($CoCl_2$) und physiologische Hypoxie (1% O_2) die CT-1 sowie HIF-1α Protein- und mRNA-Expression in den Embryonalkörperchen erhöhen, was zeigt, dass die CT-1 Expression durch reaktive Sauerstoff- Spezies (ROS) und Hypoxie geregelt wird. Die Behandlung mit Prooxidantien oder mit chemischer Hypoxie erhöhte die gp130 Phosphorylierung und Proteinexpression der NADPH-Oxidase-Untereinheiten p22-phox, p47-phox, p67-phox, sowie die mRNA-Expression von Nox-1 und Nox-4. Dementsprechend führte die Hemmung der NADPH-Oxidase Aktivität durch Diphenylen Iodonium Chloride (DPI) und Apocynin zur Aufhebung der durch Prooxidantien und chemische Hypoxie abhängigen Steigerung der CT-1 und HIF-1α Expression. Prooxidantien und chemische Hypoxie aktivierten ERK1,2, JNK und p38 sowie PI3-kinase, JAK2 und STAT3. Die Menadione- und $CoCl_2$-vermittelte Steigerung von CT-1 and HIF-1α wurde in Anwesenheit des ERK1,2 Antagonisten UO126, des JNK Antagonisten SP600125, des p38 Antagonisten SKF86002, des PI3-Kinase Antagonisten LY294002, des JAK-2 Antagonisten AG490 sowie in Anwesenheit von freien Radikalfängern signifikant gehemmt. Außerdem zeigten die aus HIF-1$\alpha^{-/-}$ ES Zellen entwickelten Embryonalkörperchen keine Kardiomyogenese, und die Behandlung mit Prooxidantien sowie chemischer Hypoxie führte nicht zu einer Steigerung der CT-1 Expression. Nach Kollagenaseverdau der schlagenden Areale aus EBs wurden die vereinzelten Zellen mit Menadione und $CoCl_2$ behandelt. Dies führte innerhalb von 2-4 Stunden zum Transport von CT-1 in den Kern.

Zusammenfassend zeigen unsere Daten, dass die CT-1 Expression in den ES Zellen von ROS und HIF-1α reguliert wird und deuten eine entscheidende Rolle von CT-1 im Überleben und der Proliferation der ES-abgeleiteten Herzzellen an. Die Bedeutung des CT-1 Kerntransportes für die CT-1- vermittelte Genexpression muss noch festgestellt werden.

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7 MISCELLANOUS

7.1 PUBLICATIONS

B. Ateghang, M. Wartenberg, M. Gassmann H. Sauer**.** Regulation of cardiotrophin-1 (CT-1) expression in mouse embryonic stem cells by HIF-1 and intracellular reactive oxygen species.
In Press. Journal of Cell Science

M. Schmelter, **B. Ateghang**, S. Helmig, M. Wartenberg, H. Sauer. Embryonic stem cells utilize reactive oxygen species as transducers of mechanical strain-induced cardiovascular differentiation.
In Press. FASEB Journal

ABSTRACTS IN CONFERENCES

B. Ateghang, M. Wartenberg, M. Gassmann H. Sauer**.**
Hypoxia Induces Cardiotrophin-1 Up-Regulation and Nuclear Translocation in Differentiating Embryonic Stem Cells by a ROS Dependent Mechanism.
DPG-Munich March 29-29, 2006. Abstract number 12734

B. Ateghang, M. Wartenberg, M. Gassmann H. Sauer.
Reactive Oxygen Species Dependent Cardiotrophin-1 Up-Regulation and Nuclear Translocation in Differentiating Embryonic Stem Cells.
GBM-Mosbach April 6-8, 2006

B. Ateghang, M. Wartenberg, H. Sauer
Cobalt Chloride induces cardiotrophin-1 (CT-1) expression in mouse embryonic stem cells by a ROS, PI3K and MAPK-dependent mechanism.
[doi:10.1240/sav_gbm_2005_h_001132]

B. Ateghang M. Wartenberg, H. Sauer. Regulation of cardiotrophin-1 (CT-1) expression during mouse embryonic stem cell differentiation by the intracellular redox state.
European J. Phy. 2005; 449:S139

7.2 AFFIDAVIT

I hereby declare in lieu of an oath, that the thesis «REGULATION OF CARDIOTROPHIN-1 EXPRESSION DURING MOUSE EMBRYONIC STEM CELL DIFFERENTIATION BY INTRACELLULAR REDOX STATE» 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 other 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, 16-02-2006 Bernadette Ateghang