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SUPEROXIDE DISMUTASE 3-MEDIATED CELL SURVIVAL AND PROLIFERATION

by

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ABSTRACT

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This dissertation studies the signaling events mediated by the extracellular superoxide dismutase (SOD3). SOD3 is an antioxidant enzyme which converts the harmful superoxide into hydrogen peroxide. Overproduction of these reactive oxygen species (ROS) in the cellular environment as a result of tissue injury or impaired antioxidant defense system has detrimental effects on tissue integrity and function. However, especially hydrogen peroxide is also an important signaling agent.

Ischemic injury in muscle causes acute oxidative stress and inflammation. We investigated the ability of SOD3 to attenuate ischemia induced inflammation and to promote recovery of skeletal muscle tissue. We found that SOD3 can downregulate the expression of several inflammatory cytokines and cell adhesion molecules thus preventing the accumulation of oxidant-producing inflammatory cells. Secondly, SOD3 was able to promote long-term activation of the mitogenic Erk pathway, but increased only briefly the activity of pro-survival Akt pathway at an early stage of ischemic inflammation, thus reducing apoptosis.

SOD3 is a prominent antioxidant in the thyroid gland where oxidative stress is constantly present. We investigated the role of SOD3 in normal thyroid follicular cells and the changes in its expression in various hyperproliferative disorders. We first showed that SOD3 is TSH-responsive which indicated its participation in thyroid function. Its principal function seems to be in follicular cell proliferation since knock-down cells were deficient in proliferation. Additionally, it was overexpressed in goiter tissue. However, SOD3 was consistently downregulated in thyroid cancer cell lines and tissues.

In conclusion, SOD3 is involved in tissue maintenance, cell proliferation and inflammatory cell migration. Its mechanisms of action are the activation of known proliferation/survival pathways, inhibition of apoptosis and regulation of adhesion molecule expression.

Key words: superoxide dismutase, oxidative stress, ischemia, Erk1/2, Akt, thyroid, proliferation, thyroid cancer

TIIVISTELMÄ

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Tämän tutkimuksen aiheena on solunulkoisen superoksididismutaasin (SOD3) välittämä solusignalointi. SOD3 on antioksidatiivinen entsyymi, joka muuntaa haitallisen superoksidin vetyperoksidiksi. Kudosvaurio tai heikentynyt antioksidanttipuolustus voi aiheuttaa näiden happiradikaalien ylituotantoa soluympäristössä ja siten haitata kudoksen normaalia toimintaa. Toisaalta jotkut happiradikaalit, erityisesti vetyperoksidi, toimivat tärkeinä viestimolekyyleinä.

Iskeeminen vaurio lihaksessa aiheuttaa äkillisen happistressin ja tulehdusreaktion. Tutkimme SOD3:n kykyä vähentää iskemian jälkeistä tulehdusta ja edistää luustolihaskudoksen korjautumista. Tuloksemme osoittivat, että SOD3 pystyy heikentämään useiden tulehdusvälittäjäaineiden ja solun tarttumismolekyylien ilmentymistä ja siten estämään happiradikaaleja tuottavien tulehdussolujen kulkemisen vaurioituneeseen kudokseen. Lisäksi osoitimme, että SOD3 aktivoi pitkäaikaisesti mitogeenistä Erk-signalointireittiä, mutta vain lyhytaikaisesti Akt-eloonjäämisreittiä tulehdusreaktion alkuvaiheessa vähentäen apoptoosia.

Kilpirauhaskudoksessa happistressi on jatkuvaa, minkä takia se tarvitsee vahvan antioksidanttipuolustuksen. SOD3 ilmentyykin vahvasti kilpirauhasessa. Tutkimme SOD3:n roolia normaalien follikulaaristen solujen toiminnassa sekä sen ilmentymisen muutoksia eri sairauksissa, joihin liittyy kudoksen liikakasvua. Tuloksemme osoittivat, että TSH-stimulaation jälkeen SOD3:n ilmentyminen kasvaa. Se ottaa myös osaa solujen lisääntymiseen, sillä SOD3-geenin ilmentymisen estämisen jälkeen solut lisääntyivät heikommin. Lisäksi sen ilmentyminen kasvoi struumakudoksessa. Toisaalta, SOD3:n ilmentyminen väheni voimakkaasti sekä kilpirauhassyöpäsolulinjoissa että -kudoksessa.

SOD3 ottaa osaa eri kudosten ylläpitoon ja paranemiseen vaikuttamalla solujen lisääntymiseen ja tulehdussolujen vaellukseen. Sen vaikutus-mekanismeina ovat kasvuun ja lisääntymiseen liittyvien signalointireittien aktivointi, apoptoosin estäminen ja tarttumismolekyylien ilmentymisen säätely.

Avainsanat: superoksididismutaasi, happiradikaalistressi, iskemia, Erk1/2, Akt, kilpirauhanen, soluproliferaatio, kilpirauhassyöpä

TABLE OF CONTENTS

ABBREVIATIONS

AA	autonomous adenoma
Akt/PKB	Akt/protein kinase B
AP-1	activating protein-1
ATC	anaplastic thyroid carcinoma
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma gene 2
Bim	BH3-interacting mediator of cell death
cAMP	cyclic adenosine monophosphate
CREB	cAMP responsive element binding protein
Duox	dual function oxidase
DuoxA	Duox activator
ECM	extracellular matrix
EGF	epidermal growth factor
Erk	extracellular signal-regulated kinase
Ets-1	E26 transformation specific sequence 1
FGF	fibroblast growth factor
FNAH	familial non-autoimmune hyperthyroidism
FoxO	Forkhead box class O
FTC	Follicular thyroid cancer
GPCR	G-protein coupled receptor
Gpx	glutathione peroxidase
H_2O_2	hydrogen peroxide
ICAM	intercellular cell adhesion molecule
IGF	insulin-like growth factor
IκB	inhibitor of κB
IL	interleukin
JNK	c-Jun NH2-terminal kinase
LacZ	β-galactosidase

MAPK	mitogen activated protein kinase
MCP	monocyte chemotactic protein
Mcl-1	myeloid cell leukemia sequence 1
MEK	MAP/Erk kinase
MIP	macrophage inflammatory protein
miR	microRNA
NFκB	nuclear factor kappa enhancer binding protein
NIS	sodium-iodide symporter
Nox	NADPH oxidase
O_2^-•	superoxide radical
OH•	hydroxyl radical
PI3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
Prx	peroxiredoxin
PTC	papillary thyroid carcinoma
PTEN	tensin homologue deleted on chromosome 10
PTP	protein tyrosine phosphatase
PTU	propylthiouracil
RET/PTC	rearranged during transfection/PTC
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SOD	superoxide dismutase
T3	tri-iodothyronine
T4	tetraiodothyronine, thyroxine
Tg	thyroglobulin
TGF-β	transforming growth factor β
TNF-α	tumor necrosis factor α

TNFR	TNF receptor
TPO	thyroid peroxidase
TSH	thyroid stimulating hormone, thyrotropin
TSH-R	TSH-receptor
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor

LIST OF ORIGINAL PUBLICATIONS

The dissertation is based on the following publications:

I Laurila JP, **Laatikainen LE**, Castellone MD, Laukkanen MO: SOD3 reduces inflammatory cell migration by regulating adhesion molecule and cytokine expression.
PLoS ONE 2009 Jun; 4(6):e5786.

II **Laatikainen LE**, Incoronato M, Castellone MD, Laurila JP, Santoro M, Laukkanen MO: SOD3 decreases ischemic injury derived apoptosis through phosphorylation of Erk1/2, Akt, and FoxO3a.
PLoS ONE 2011 Aug; 6(8):e24456.

III **Laatikainen LE**, Castellone MD, Hebrant A, Hoste C, Cantisani MC, Laurila JP, Salvatore G, Salerno P, Basolo F, Näsman J, Dumont JE, Santoro M, Laukkanen MO: Extracellular superoxide dismutase is a thyroid differentiation marker downregulated in cancer.
Endocrine Related Cancer 2010 Aug; 17(3): 785-796.

INTRODUCTION

The number of intracellular signaling pathways and factors involved in signaling in a single cell is overwhelming. Everything from the simplest exchange of ions through the plasmamembrane to the highly complex responses to the intra- and extracellular stimuli such as movement to a certain direction or destroying an invading pathogen are controlled by various simultaneous and overlapping signaling events. This is necessary, though, since without such a meticulous signal integration and coordination the cell would die. Appropriate signals tell the cell 'what the weather is like' out there – and inside – and when there are changes, the cell will alter its behaviour to remove them or adapt to them.

Survival signals are needed to keep the cell alive, and this applies also in tissue injuries where the cell has to decide between life and death. Initially, it is worthwhile to try and repair the damage inflicted on DNA, proteins and other macromolecules of the cell by e.g. oxygen radicals, with the prospect of restoring tissue function. This is not always enough. Occasionally, the cell confronts such harsh changes in the environment, brought on by e.g. ischemia and inflammation, that it needs to die to protect the tissue integrity. Even then the cell, in order to avoid harming the surrounding cells, will resort to mobilization of special signaling machinery which will lead to programmed cell death, apoptosis.

Sometimes, mutations in the DNA disrupt the well-regulated processes of a normal cell. If they promote hyperactive survival signaling and prevent apoptosis, the cell may transform into a cancer cell. Characteristically, such cells lose the distinct features and functions that define their original cell type, and thus become progressively undifferentiated. This phenomenon may help scientists to identify the malignant tissue before it can threaten the whole individual.

This study focuses on revealing the functions of extracellular superoxide dismutase (SOD3) in normal and in pathological tissues where the normal redox balance has been disrupted. It is an antioxidant enzyme with only one known enzymatic reaction: the conversion of superoxide into hydrogen peroxide. However, despite this restricted activity it has been proposed to have anti-inflammatory and protective effects in various types of tissue injury and cancer. We have studied ischemic skeletal muscle and oxidative stress prone thyroid gland as well as thyroid cancer to determine how this enzyme, formerly thought of as a simple antioxidant, participates in the survival and proliferation signaling in these tissues while attenuating the inflammatory reaction.

REVIEW OF LITERATURE

1 ROS, oxidative stress and antioxidants

1.1 Generation and removal of ROS

Reactive oxygen species (ROS) are derivatives of molecular oxygen which are characterized by an ability to react with other intra- and extracellular molecules. Some but not all of them are oxygen radicals and therefore may require a suitable catalyst in the cellular environment in order to carry out the chemical reaction. For example, iron acts as a catalyst in the Fenton reaction where hydrogen peroxide (H_2O_2) is reduced into two hydroxyl radicals (OH•) (Liochev and Fridovich, 1994). Besides H_2O_2 and OH•, superoxide (O_2^-•) is among the most common ROS in normal or pathological tissues, and is a precursor for many other oxidative species. Reactive nitrosative species (RNS), mainly nitric oxide (NO) and its derivative peroxynitrite ($ONOO^-$), often exist alongside ROS. Uncontrolled ROS generation can cause severe damage to cells and tissues since they can oxidize e.g. DNA, proteins and lipids (Konat, 2003; reviewed in Valko *et al.*, 2006; reviewed in Zadak *et al.*, 2009). On the other hand, some ROS or RNS, especially H_2O_2 and NO, are crucial signaling agents in normal tissues as well as in inflammation, infections and cancer (reviewed in Droge, 2002; reviewed in Stone and Yang, 2006).

Generation of ROS takes place in mitochondria, cytosol, on the plasma membrane as well as in the extracellular space through activity of a number of enzymes. NADPH oxidases (Nox) are a family of plasma membrane O_2^-• producing multisubunit enzymes found in a variety of cell types and tissues (reviewed in Lambeth, 2004). They participate in the innate immunity, cell proliferation, endothelial activation and receptor signaling reflecting the versatile role of O_2^-• (reviewed in Lambeth, 2004; reviewed in Alom-Ruiz *et al.*, 2008). Two enzymes closely related to Nox are the dual function oxidases (Duox; aka thyroid oxidase, Thox) which have an additional peroxidase domain for converting O_2^-• directly into H_2O_2 (De Deken *et al.*, 2000). They are found in a more limited set of tissues the most predominant being thyroid gland where they generate H_2O_2 for thyroid hormone synthesis (De Deken *et al.*, 2000; De Deken *et al.*, 2002; reviewed in Lambeth, 2004). Myeloperoxidase (MPO) is another important factor in pathogen eradication through its hypochlorous acid producing capacity (Lehrer *et al.*, 1969; Jiang *et al.*, 1997). The activity of O_2^-• and H_2O_2 producing xanthine oxidase (XO) increases under ischemic conditions and may thus contribute to skeletal muscle damage (Bishop *et al.*, 1985; Smith *et al.*, 1989a; Smith *et al.*, 1989b). The main O_2^-• scavengers are the three isoforms of superoxide dismutases (SOD) which are found specifically in mitochondria, cytosol and extracellular space where they convert O_2^-• into H_2O_2 thus reducing oxidative stress (reviewed in Zelko *et al.*,

2002). Nitric oxide synthases (NOS) in vasculature, muscle tissue and blood cells generate NO for a number of biological and pathological events (reviewed in Guzik *et al.*, 2003).

Due to their harmful effects, ROS generation must be kept under tight control and, therefore, there are a number of antioxidant enzymes that specifically reduce $O_2^{-}\bullet$, H_2O_2 and other oxidizing agents. Peroxiredoxins (Prx) are relatively abundant enzymes which reduce H_2O_2 and $ONOO^-$ by reversible oxidation of their active site cysteine residues but become inactivated under high ROS load (Kang *et al.*, 1998; Chae *et al.*, 1999; Woo *et al.*, 2003; Dubuisson *et al.*, 2004; reviewed in Rhee *et al.*, 2005a). Glutathione peroxidases (Gpx) resemble Prx enzymes in function except for having selenocysteine at their active site (Forstrom *et al.*, 1978). They also function more efficiently under high peroxide fluxes than Prx, and are therefore thought to form the primary antioxidant defense in tissues whereas Prx participate in e.g. regulation of local H_2O_2 based signal transduction (Wood *et al.*, 2003; reviewed in Fourquet *et al.*, 2008). One of the most efficient H_2O_2 decomposing enzymes is catalase which is mainly located in peroxisomes although some can be found in mitochondria, cytosol and nucleus (Yamamoto *et al.*, 1988; reviewed in Kirkman and Gaetani, 2007; Salvi *et al.*, 2007). Catalase seems to be dispensable to a certain extent under normal oxidative conditions since the other ROS scavengers can take up its function but it becomes an important protective antioxidant when severe oxidative stress occurs (reviewed in Ogata, 1991; Ho *et al.*, 2004). Additionally, there is a non-specific reductant pool consisting of e.g. free amino acids, peptides, glutathione, thioredoxin and vitamins which has an equally important impact on the cellular oxidant balance as the specific enzymatic ROS scavengers (reviewed in Droge, 2002).

A reduction-oxidation (or redox) homeostasis is effectively the balance between generation and clearance of various ROS and RNS, and it is actively maintained in order to keep the oxidants at subtoxic levels in cell interior and exterior. Individual cell types have their characteristic optimal concentration of ROS and RNS which, however, varies according to the metabolic activity of the cell. For instance, the intracellular concentration of H_2O_2, which is one of the most versatile ROS, ranges from 0.001-0.7 μM in mammalian cells (Oshino *et al.*, 1973; Chance *et al.*, 1979). Oxidative stress develops when the ROS are generated or otherwise increased in the cellular environment in excess to the capacity of their scavengers, or if the scavengers are diminished or defective. Increasing concentrations of ROS produce various responses in cell metabolism (Figure 1). Initially, cells alter their metabolism and gene expression pattern so that mild stress (e.g. 1-15 μM extracellular H_2O_2) induces e.g. Prx or Gpx inactivation by oxidation and survival gene expression (Wiese *et al.*, 1995; Guyton *et al.*, 1996; reviewed in Stone and Yang, 2006). Cells may even enhance their DNA synthesis and proliferation rate in

response to such low subtoxic H_2O_2 concentration (Wiese *et al.*, 1995; reviewed in Stone and Yang, 2006). Moderate oxidative stress (e.g. 20-150 μM extracellular H_2O_2) promotes more profound adaptation by e.g. altering cellular respiration towards anaerobic respiration and arresting cell growth temporarily in order to allow efficient DNA repair (Wiese *et al.*, 1995; reviewed in Davies, 2000; reviewed in Stone and Yang, 2006). High oxidative stress (e.g. 300-1000μM extracellular H_2O_2 and higher) induces permanent growth arrest and senescence, or can directly activate apoptosis or kill the cell by necrosis (Chen and Ames, 1994; Wiese *et al.*, 1995; reviewed in Stone and Yang, 2006).

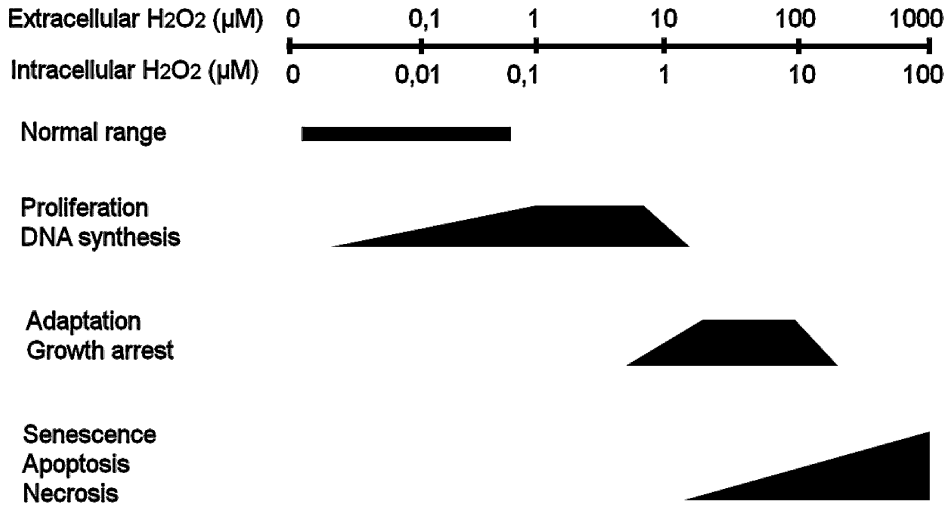

Figure 1. H_2O_2 induced responses in animal cells. The intracellular H_2O_2 concentration is approximately 10-fold lower than the extracellular concentration. Normal intracellular concentration varies between cell types. Mild oxidative stress (1-15μM extracellular) induces proliferation and DNA synthesis. Moderate stress (20-150μM extracellular) induces transient growth arrest and metabolic adaptation. Cell death rate increases starting from approximately 10μM, but acute high stress (300μM and above) may induce direct death. The specific thresholds for a given response vary among cell types. Adapted from the review by Stone and Yang, 2006.

1.2 ROS signaling

ROS mediated signal transduction is based on active production of oxygen radicals in a highly local manner in the cell interior. Globally it would cause too much harm to the cellular activities. A number of growth factors (EGF, PDGF, VEGF), cytokines (interleukins, IFN-γ, TNF-α), G-protein coupled receptor agonists and stress factors (e.g. shear stress, radiation, hypoxia) have been found to induce downstream H_2O_2 or $O_2^{-}\bullet$ generation in non-phagocytic cells (Masaki *et al.*, 1995;

reviewed in Thannickal and Fanburg, 2000; Sato *et al.*, 2005). These ROS in turn promote a variety of cell functions such as cytokine induction, transcription factor and kinase activation, cell growth, proliferation and differentiation (reviewed in Thannickal and Fanburg, 2000). However, the specific molecular mechanisms by which the ROS convey the signals are the least known details. Furthermore, the high reactivity, limited lifespan and relatively weak diffusion abilities complicate the physiological ROS mediated signaling as well as their research in the laboratory settings. Nox enzymes are the principal ROS producers upon receptor activation but their end-product, $O_2^{-}\bullet$, is often further converted into H_2O_2 (reviewed in Lambeth, 2004). H_2O_2 is possibly a better signaling agent due to its higher stability and absence of electrical charge which allow longer diffusion distances and lipid membrane crossing (reviewed in Cai, 2005).

A well documented signal transduction mechanism by ROS has been comprehensibly reviewed by Rhee *et al.* (2005b). At the core of this mechanism is the oxidative inhibition of protein tyrosine phosphatases (PTP), lipid phosphatase tensin homologue deleted on chromosome 10 (PTEN) and Prx enzymes which may otherwise hinder signal transduction. First, the subunits of a Nox enzyme are recruited into the vicinity of activated receptor either on the cell surface or on an internalized vesicle membrane where they then combine to form a functional enzyme (Figure 2A; reviewed in Lambeth, 2004). Since $O_2^{-}\bullet$ is a poor signaling agent, the H_2O_2 produced by e.g. SOD1 usually takes its place as the effector molecule, and is generated in relatively high concentration near the activated receptor (Figure 2B). The PTP, PTEN and Prx contain in their active site a cystein residue which is readily oxidized by H_2O_2 and other ROS (Denu and Tanner, 1998; Lee *et al.*, 2002; Woo *et al.*, 2003). This event inactivates the phosphatase enzymes or Prx until the cystein thiol group is reduced back to sulfhydryl by natural thiols, e.g. thioredoxin and glutathione. Normally, the H_2O_2 would be quickly neutralized by Prx but its relatively high concentration prevents the cystein reduction thereby allowing further local increase in H_2O_2 (Figure 2C). This initial step can then facilitate the oxidative inhibition of PTPs or PTEN and prolong the duration of receptor phosphorylation thus promoting signal transduction (Figure 2D). H_2O_2 is consumed in the process and the signal is likely to terminate when its concentration decreases under a certain threshold level after which PTPs or PTEN become reactivated (reviewed in Rhee *et al.*, 2005b).

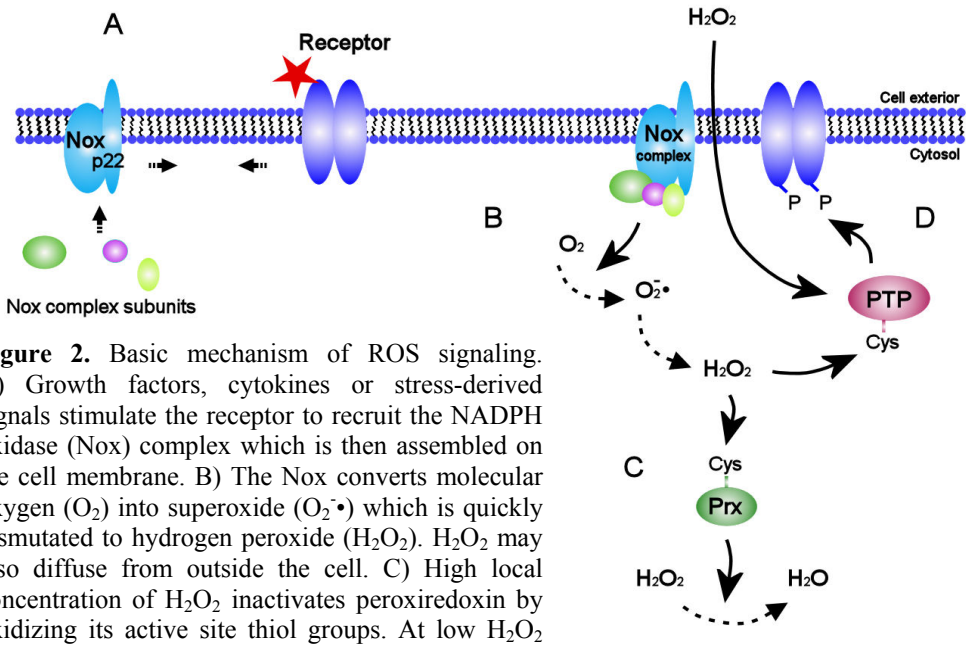

Figure 2. Basic mechanism of ROS signaling. A) Growth factors, cytokines or stress-derived signals stimulate the receptor to recruit the NADPH oxidase (Nox) complex which is then assembled on the cell membrane. B) The Nox converts molecular oxygen (O_2) into superoxide ($O_2^{-}\bullet$) which is quickly dismutated to hydrogen peroxide (H_2O_2). H_2O_2 may also diffuse from outside the cell. C) High local concentration of H_2O_2 inactivates peroxiredoxin by oxidizing its active site thiol groups. At low H_2O_2 concentrations, Prx reduces H_2O_2 into water. D) When Prx is inactive, H_2O_2 further oxidizes the thiol groups in protein tyrosine phosphatases (PTP) which become unable to dephosphorylate the activated receptor, and thus the signal duration is prolonged.

2 Extracellular superoxide dismutase, SOD3

2.1 Basic features

SOD3 is the extracellular member of the mammalian superoxide dismutase family the two other members being cytosolic SOD1 (Cu,Zn-SOD) and mitochondrial SOD2 (Mn-SOD). In general, they are considered to have little overlapping functions due to their different locations (Figure 3; Sentman *et al.*, 2006). Human SOD3 is found in most tissues and organs of the body but there are discrepancies between mRNA expression and protein activity. The main mRNA expression sites are heart, placenta, pancreas, lung and skeletal muscle (Folz and Crapo, 1994). On the other hand, SOD3 enzyme activity is highest in arterial wall, lung, kidney, thyroid, uterus and pancreas, and lower activity is seen in e.g. heart, liver, skeletal muscle and spleen (Marklund, 1984a; Marklund, 1984b; Stralin *et al.*, 1995). The proportion of SOD3 from total SOD in tissues is relatively small compared to SOD1 or 2, but it is the major isoform in various extracellular fluids such as plasma, lymph and cerebrospinal fluid (Figure 3; Marklund, 1982; Marklund *et al.*, 1982; Marklund, 1984b). Unlike with the other

isoforms, there are also marked differences in tissue SOD3 content which do not seem to correlate with either the content of the other isoforms or tissue properties such as metabolic activity (Marklund, 1984a; Marklund, 1984b). Additionally, there are major differences in the SOD3 expression between mammalian species: human, mouse, rabbit, pig, cow and sheep had, in general, higher SOD3 levels than rat, dog or cat (Marklund, 1984a).

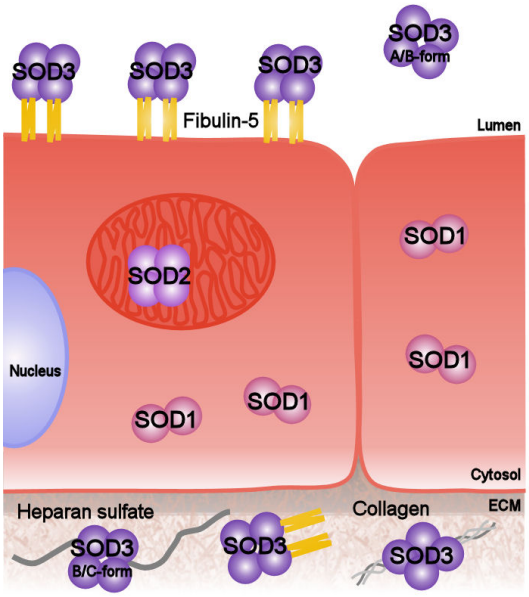

Figure 3. Superoxide dismutases in the vascular wall. SOD1 is the principal cytosolic SOD enzyme. SOD2 is almost exclusively found in mitochondria. SOD3 is extracellular. The A-form has no affinity for heparin, B-form has low affinity and C-form has high affinity. Small amount of SOD3 may circulate freely in blood, while most stays bound to cell surfaces and extracellular matrix (ECM) through Fibulin-5, collagen type I or heparan sulfate proteoglycan.

SOD3 is a glycoprotein as indicated by its binding to lectin, and contains a somewhat similar Cu,Zn active site as SOD1, but in other domains their amino acid composition and therefore also antigenic properties are very different (Marklund, 1982; Marklund, 1984c; Antonyuk *et al.*, 2009). SOD3 is synthesized in intact form and before secretion a portion of SOD3 pool is cleaved at C-terminal to lower its affinity to extracellular matrix (ECM) and cell surface ligands in a tissue specific manner (Enghild *et al.*, 1999). Some SOD3 therefore stays in the tissues while some diffuses into the extracellular fluids or blood stream. In general, SOD3 forms tetramers from two disulphide-linked dimers (Oury *et al.*, 1996). In the extracellular space SOD3 can bind heparan sulfate proteoglycan, type I collagen and fibulin-5 in the extracellular matrix and cell surfaces (Figure 3; Marklund, 1982; Nguyen *et al.*, 2004; Petersen *et al.*, 2004). SOD3 enzymes can be classified by two methods: according to the degree of heparin affinity of the tetramer, and to the subunit composition of the tetramer (Marklund, 1982; Karlsson and Marklund, 1987; Adachi *et al.*, 1995). The classifications are summarized in Table 1.

Table 1. Classification of SOD3.			
Heparin affinity of tetrameric enzyme		Monomer composition of enzyme	
A	very weak affinity	I	A-A-A-A
B	weak affinity	II	A-A-A-C
C	strong affinity	III	A-A-C-C
		IV	A-C-C-C
		V	C-C-C-C

The main function of SODs in tissues is the removal of O_2^-• radical and prevention of e.g. $ONOO^-$ and OH• radical formation which all could otherwise cause serious damage to DNA and other macromolecules, cell membranes, enzymes and signaling pathways (Liochev and Fridovich, 1994). SOD3, as well as the other isoforms, catalyze a very rapid dismutation reaction with copper as catalyst in the active site (Klug *et al.*, 1972; Marklund, 1982):

$$2O_2^-\bullet + 2H^+ \rightarrow H_2O_2 + O_2$$

Studies with SOD3 knock-out mice have shown that such an animal can develop normally to adulthood and be fertile; however, elevated plasma stress markers, some ventricular hypertrophy and fibrosis of the heart was detected (Carlsson *et al.*, 1995; Sentman *et al.*, 2006; van Deel *et al.*, 2008). Compensatory upregulation of other antioxidant enzymes was not detected (Carlsson *et al.*, 1995). However, when these animals were exposed to lung hyperoxia, skeletal muscle ischemia-reperfusion or myocardial infarction they suffered from more severe tissue inflammation, edema and increased cardiac dysfunction than the wild type control mice due to the inability to dampen the oxidative stress involved in these conditions (Carlsson *et al.*, 1995; Park *et al.*, 2005; van Deel *et al.*, 2008). In striking contrast to these studies, Gongora et al. (2008) showed that conditional deletion of SOD3 with the Cre-lox system in adult animals and following exposure to normal ambient air results in severe inflammation and damage in the lungs with mortality rate of 85%. Furthermore, they confirmed that SOD3 knock-out mice do have altogether 37 differentially regulated genes compared to wild type animals thus offering an explanation why they remain viable (Gongora *et al.*, 2008).

SOD3 expression and activity are regulated at transcriptional, post-transcriptional and post-translational levels to keep the tissue ROS content under strict control. A computer analysis has revealed e.g. metal regulatory element (MRE), cyclic AMP-responsive element (CREB), AP-1/TPA responsive element (TRE), skeletal muscle-specific trans-activating factor responsive element (M-CAT), glucocorticoid responsive element (GRE), xenobiotic responsive element (XRE) and antioxidant

responsive element (ARE) in the promoter sequence of SOD3 (Folz and Crapo, 1994) which regulate SOD3 expression in response to e.g. oxidative stress, inflammation and mitogenic stimuli. There is a marked difference in the amount of SOD3 mRNA and translated protein in tissues indicating that post-transcriptional regulation is an important point of determination on how much functional protein is available (Laukkanen *et al.*, 2000). The mechanisms are, however, incompletely understood.

Post-translational regulation of SOD3 involves proteolytic furin- and carboxypeptidase-mediated cleavage of the C-terminal heparin binding domain leading to lower affinity to ECM and cell surfaces (Enghild *et al.*, 1999; Bowler *et al.*, 2002; Olsen *et al.*, 2004; Petersen *et al.*, 2005). The tissue where SOD3 is expressed may control which type of SOD3 is preferentially produced. For instance, systemic SOD3 gene transfer by an intravenous injection resulting in high liver and spleen expression mainly produced the B-form (Laukkanen *et al.*, 2001a). Both intact and cleaved forms are produced in defined proportions from a single source and the ratio is tissue-specific suggesting this mechanism to be an important determinant of SOD3 tissue distribution and half-life under normal conditions (Enghild *et al.*, 1999; Bowler *et al.*, 2002). This allows a highly localized SOD3 expression in the vicinity of O_2^-• generating or sensitive cells as well as a more diffuse SOD3 activity in a tissue environment.

2.2 SOD3 in tissue injuries and cancer

Hypoxic/hyperoxic conditions, ischemia-reperfusion injuries and inflammation increase ROS production leading to cellular damage and tissue dysfunction if their generation is not controlled. The effect of SOD3 overexpression or absence has been studied under such conditions mainly in heart, vascular system and lungs (Dewald *et al.*, 2003; Iida *et al.*, 2005; Ozumi *et al.*, 2005; Fattman *et al.*, 2006; Nozik-Grayck *et al.*, 2008). These studies have established the role of SOD3 as an important and beneficial enzyme in various types of tissue injuries but changes in its endogenous expression have been less studied.

In myocardial ischemia, endogenous SOD3 protein expression was initially increased during the acute phase i.e. 1-3 days after injury in human patients (Horiuchi *et al.*, 2004) whereas in human patients with chronic coronary artery disease, osteoarthritis or corneal pathologies the SOD3 levels were consistently lower than in the control groups (Landmesser *et al.*, 2000; Behndig *et al.*, 2001; Regan *et al.*, 2005; Tasaki *et al.*, 2006). *In vitro* studies have shown upregulation of SOD3 in various human vascular smooth muscle cell lines after exposure to many cytokines including the pro-inflammatory IFN-γ and interleukin (IL)-1 which are secreted early in the tissue response to injury (Stralin and Marklund, 2000; Stralin and Marklund, 2001). However, an important pro-inflammatory cytokine tumor necrosis factor (TNF-) α suppressed SOD3 expression (Stralin and Marklund, 2000; Ueda *et al.*, 2008). Similar

results were obtained from an *in vitro* study of human fibroblast cell lines (Marklund, 1992). On the other hand, transforming growth factor (TGF)-β and several other growth factors such as fibroblast, platelet-derived and epidermal growth factor (FGF, PDGF and EGF, respectively) which usually emerge later in the inflammatory response attenuated SOD3 expression in the same cell lines (Marklund, 1992; Stralin and Marklund, 2000; Stralin and Marklund, 2001). SOD3 thus seems to be briefly induced in the acute phase after an injury, but may be reduced to a deficient level in chronic conditions due to e.g. continuous damage to tissues by oxidative stress.

The strict regulation of SOD3 expression, synthesis and activity can, however, be problematic in determination of its actual impact on the injury healing. Firstly, the varying tissue expression level in different species is likely to impose a significant effect on the function of SOD3 in a given experimental setting (Marklund, 1984a). Furthermore, in several animal models with induced inflammation or ischemia-reperfusion the SOD3 protein or activity levels were decreased during the first few hours or days after injury (Laukkanen *et al.*, 2002; Park *et al.*, 2005; Ueda *et al.*, 2008) which is contradictive to the presumed positive regulation by the inflammatory cytokines. However, SOD3 protein synthesis seems to react to the cytokine stimulus relatively slowly. In cultured human fibroblasts and vascular smooth muscle cells the response to cytokine or growth factor incubation took several days (Marklund, 1992; Stralin and Marklund, 2000; Stralin and Marklund, 2001). Accordingly, *in vivo* SOD3 protein content can be initially reduced. Later, towards the beginning of healing phase after approximately one week, the amount of protein was increased in e.g. balloon denuded aortas, although the activity was not fully restored (Laukkanen *et al.*, 2002; Leite *et al.*, 2003). Lastly, the type of injury may affect the SOD3 expression and synthesis: in ischemia-reperfusion injury of mouse skeletal muscle mRNA expression was quickly upregulated and the protein downregulated while in lungs after induction of systemic inflammation they both were downregulated (Park *et al.*, 2005; Ueda *et al.*, 2008).

Animal studies with SOD3 overexpression or knock-out and gene therapy have elucidated some of the mechanisms by which SOD3 works to attenuate inflammation. Binding of SOD3 to ECM components promotes tissue retention of SOD3 which e.g. hinders the generation of very reactive OH• from O_2^-• and enhances NO bioavailability in the vascular wall (Jung *et al.*, 2003; reviewed in Qin *et al.*, 2008). Furthermore, tissue bound SOD3 protects the ECM from ROS mediated degradation and fragmentation thus preventing the leukocyte chemoattractant function of heparan sulfate, collagen or hyaluronan debris (Petersen *et al.*, 2004; Gao *et al.*, 2008; Kliment *et al.*, 2008). SOD3 also contributes to overall lowered levels of O_2^-•, reduced inflammatory leukocyte infiltration as well as downregulation of inflammatory cytokines such as IFN-γ, IL-1 and TNF-α (Folz *et al.*, 1999; Ross *et al.*, 2004; Park *et al.*, 2005; reviewed in Qin *et al.*, 2008). Consequently, area of apoptotic and necrotic

tissue after ischemia/reperfusion in heart, brain or other organ under study is consistently smaller and the tissue function is restored better in animals that have received SOD3 as a transgene (Laukkanen *et al.*, 2001b; Li *et al.*, 2001; Laukkanen *et al.*, 2002; Rabbani *et al.*, 2005; Ha *et al.*, 2006; reviewed in Qin *et al.*, 2008; Kliment *et al.*, 2009). The heparin binding domain seems to be essential for the protective function of SOD3 in the target tissue possibly because it enables higher local concentration of SOD3 (Chu *et al.*, 2005; Iida *et al.*, 2005). However, to date, few clinical studies with SOD enzymes have been conducted and none of them has involved SOD3 gene therapy in humans.

The role of $O_2^{-}\bullet$ and other ROS as signaling agents has been established in normal tissue, inflammation as well as in benign and malignant neoplasms. Characteristically, the reduction-oxidation balance is disturbed in pathological tissues due to mutated or defective antioxidants and increased ROS generation from mitochondria and various NADPH oxidases. SOD1 and SOD2 have been associated with many cancer types thus establishing their importance in regulation of redox balance in malignant tissues (De Palma *et al.*; Zhao *et al.*, 2001; Weydert *et al.*, 2006; Connor *et al.*, 2007). However, the number of studies investigating the role of SOD3 in malignant tissues is smaller and the SOD enzyme may not specified. Therefore, more detailed studies are needede to estimate the importance of SOD3 since the relative expressions of SODs do not always follow the same pattern in different pathologies.

SOD enzymes show different expression patterns in cancerous tissues depending on the tissue itself and the degree of malignancy. In lungs, one of the major SOD3 expression sites, SOD1 and SOD2 levels were increased whereas that of SOD3 was decreased as compared to normal tissue (Svensk *et al.*, 2004). In thyroid, total SOD expression was slightly decreased in benign follicular adenoma but increased in more malignant follicular and papillary carcinomas (Sadani and Nadkarni, 1996). On the other hand, in breast cancer tissue and patient blood samples total SOD was decreased, and plasma SOD was inversely correlated with the tumor grade (Sinha *et al.*, 2009). However, in earlier breast cancer studies serum or plasma SOD content was found to be higher (Ray *et al.*, 2000; Khanzode *et al.*, 2004). Considering that the predominant SOD isoform in blood is SOD3 (Marklund *et al.*, 1982; Marklund, 1984a) and that cancer often involves inflammation (reviewed in Grivennikov and Karin, 2011) these results are in line with those showing decreased SOD3 levels in chronic inflammatory conditions in which the tissues, e.g. vascular wall or joint cartilage, can no longer sustain adequate antioxidant defenses (Landmesser *et al.*, 2000; Regan *et al.*, 2005; Tasaki *et al.*, 2006). This suggests that as the cancer advances, mutations and continuous oxidative stress gradually destroy the antioxidant system, thus offering some advantage for the tumor cells. For instance, $O_2^{-}\bullet$ has been shown to be crucial for cell invasion in prostate cancer cells and tumor angiogenesis in

general (Kumar *et al.*, 2008; reviewed in Ushio-Fukai and Nakamura, 2008). However, the removal of O_2^{-}• by overexpression of SOD3 or the other isoforms prevented proliferation and migration of cancer cells (Zhao *et al.*, 2001; Kim *et al.*, 2005a; Weydert *et al.*, 2006; Teoh *et al.*, 2007; Chaiswing *et al.*, 2008).

3 Muscle ischemia

3.1 Characteristics of ischemia and inflammation

Ischemia is defined as a condition where blood circulation is inadequate or completely blocked in a certain tissue area leading to acute lack of nutrient and oxygen delivery to the site. Studies with skeletal muscle and myocardial ischemia have shown the development of acute hypoxia, mitochondrial dysregulation, vascular endothelial cell activation, an inflammatory reaction and cell death (reviewed in Ferrari, 1995; reviewed in Gute *et al.*, 1998; reviewed in Blaisdell, 2002). In the absence of reperfusion the ischemic condition becomes chronic but free radicals are still formed from residual oxygen left in the tissue (reviewed in Clanton, 2007). On the other hand, reperfusion itself has also been considered to contribute to oxidative damage due to sudden availability of oxygen to the reduced mitochondrial electron transport chain and xanthine oxidase system, as well as to the ROS generating leukocytes attracted to the inflammation site (reviewed in Ferrari *et al.*, 1993; reviewed in Gute *et al.*, 1998).

Cardiovascular diseases and various other disease conditions, surgical procedures, tissue traumas and thromboembolism may cause acute ischemia in the myocardium or skeletal muscles with ensuing tissue damage due to inadequate oxygenation. Oxidative damage to tissues is a result of oxidative stress and generation of free oxygen radicals which oxidize DNA, lipids, proteins and other cellular structures thus impairing their functions (reviewed in Valko *et al.*, 2006). The damage mediating ROS are generated mainly in the mitochondria of inflicted tissue and by infiltrating phagocytes which initiate the oxidative burst, an automatic defense mechanism against possible invading pathogens (Cadenas *et al.*, 1977; Nathan, 1987a; reviewed in Clanton, 2007). Damage caused by ischemia with or without reperfusion shows as tissue dysfunction due to disrupted metabolism and tissue integrity with symptoms such as reduced contractile capacity in muscle, interstitial edema and systemic inflammation which may lead to further kidney, heart and/or lung complications (Carvalho *et al.*, 1997; reviewed in Eliason and Wakefield, 2009).

3.1.1 Time course of ischemic injury

Starting from the site of the occlusion, gradually increasing hypoxia determined by the availability of collateral blood flow turns into absolute ischemia. After the circulation has stopped in the muscle the first events occurring are oxygen and glucose depletion,

and intracellular acidification which signal the muscle tissue the need to reduce oxygen and energy consumption. Subsequently, conversion from aerobic to anaerobic respiration and lactate generation aim to preserve cell viability and low-level energy supply (reviewed in Ferrari, 1995; reviewed in Eliason and Wakefield, 2009). Animal studies have shown that skeletal muscle tissue tolerates ischemia up to 3-4 hours (Makitie and Teravainen, 1977; Belkin *et al.*, 1988; Hickey *et al.*, 1992). More than 4 hours will induce irreversible damage and after 6 hours the muscle will be mostly necrotic (Makitie and Teravainen, 1977; Hickey *et al.*, 1992). Due to leakage of cell breakdown products and other inflammatory factors from the damaged muscle into the circulation, long-term ischemia involving a large mass of muscle tissue has been shown to increase the risk of post-reperfusion pulmonary and cardiac complications, the main causes for mortality in limb ischemia (reviewed in Blaisdell, 2002; reviewed in Eliason and Wakefield, 2009). At the cellular level, the mature myocytes seem to be more susceptible to oxidative damage than undifferentiated tissue (Franco *et al.*, 1999). The major antioxidant enzymes, including SOD1, SOD2, Gpx and catalase were downregulated in cultured myoblasts after exposure to differentiation conditions (Franco *et al.*, 1999), and thereby rendered the cells more vulnerable after maturation.

Histological findings in the ischemic skeletal muscle of mouse include cellular swelling, foci of necrosis, interstitial edema and infiltration of inflammatory cells (Paoni *et al.*, 2002). During the first few days after acute ischemia induced by permanent femoral artery ligation the muscle fibers appeared shrunken, but the myocyte precursors, satellite cells, were already proliferating actively (Paoni *et al.*, 2002). The inflammatory cell accumulation as well as the number of differentially expressed genes was found to peak three days after induction of ischemia (Paoni *et al.*, 2002). According to Paoni *et al.* (2002) the most prominent groups of upregulated genes involved several chemokines and cytokines, their receptors, inflammatory cell markers and general proliferation markers, while many genes associated with energy metabolism and muscle contractile function were downregulated. They suggested that this early response to ischemia, lasting approximately the first week post-injury, is to suppress final myocyte differentiation to allow functional recovery through improved contractility and proliferation of satellite cells.

The same study showed that the initial inflammatory phase is largely over by the day 7, after which the satellite cells stop proliferating and begin to differentiate into mature myocytes (Paoni *et al.*, 2002). Several growth factors, including FGF, PDGF, TGF-β and HGF, derived from various infiltrated and resident cells are bound by satellite cells and promote their proliferation whereas insulin-like growth factor (IGF)-I and -II from circulation or secreted in para- or autocrine fashion finally switch the program towards differentiation (Ewton *et al.*, 1994; reviewed in Husmann *et al.*, 1996; Adi *et al.*, 2002; Halevy and Cantley, 2004). Additionally, around this time the number of accumulated macrophages and contractile function had returned to normal

levels, while the gene expression profile was at the original state later, after 28 days (Paoni *et al.*, 2002). Receptor for IL-10, a key anti-inflammatory cytokine, was found upregulated around the days 3-10 after induction of ischemia indicating a gradual decline of the inflammatory phase (Paoni *et al.*, 2002). IL-10 induces the suppression of e.g. the pro-inflammatory cytokines IL-1α, IL-1β, TNF-α, IL-6, IL-8 and IL-12, and regulates the expression of matrix metalloproteinases and their inhibitors as well as promotes angiogenesis (reviewed in Frangogiannis *et al.*, 2002). Accordingly, receptors for several of these were found downregulated around the days 10 and 14 after injury (Paoni *et al.*, 2002).

3.1.2 Chemokines and cytokines in ischemic injury

The severely injured and necrotic muscles release locally several factors that stimulate the blood clotting system and inflammation, such as purines, amino acids, various enzymes, myoglobin, interleukins, TNF-α, ROS, ECM components and platelet activating factor (PAF) which may be further distributed systemically after the circulation is re-established in reperfusion (reviewed in Blaisdell, 2002). The pro-inflammatory cytokines launch the cytokine cascade, a coordinated sequential activation of secondary cytokines and adhesion molecules, which is necessary for the inflammatory cell recruitment (reviewed in Eliason and Wakefield, 2009). The transcription factor nuclear factor κB (NFκB) is in a central position to initiate the inflammatory response: its activation and nuclear translocation leads to upregulation of an array of cytokines, chemokines, cell adhesion proteins, complement factors and anti-apoptotic factors (reviewed in Pahl, 1999; reviewed in Eliason and Wakefield, 2009). Subsequently, the infiltrated leukocytes of the innate immunity, mainly phagocytic neutrophils and monocyte/macrophages, produce a respiratory burst characterized by massive generation of ROS, and later direct the clearance of the site and the healing process itself (Nathan, 1987a; reviewed in Tidball, 2005). Cardiomyocyte and macrophage-derived IL-1α induces the expression of other pro-inflammatory cytokines in these cells thus amplifying the reaction (Kamari *et al.*, 2011; Turner *et al.*, 2009).

In ischemic skeletal muscle, TNF-α, IL-1β, IL-6, IL-8 and macrophage chemotactic protein (MCP)-1 are the predominant factors, and are released from the resident mast cells and infiltrating neutrophils in response to the various inflammatory stimuli (Frangogiannis *et al.*, 1998; reviewed in Kasama *et al.*, 2005; reviewed in Eliason and Wakefield, 2009). Some generally occurring chemokines and cytokines in skeletal muscle and myocardial tissue are listed in Table 2. Besides promoting the expression of other cytokines, TNF-α and IL-1β induce e.g. intercellular cell adhesion molecule (ICAM) expression and endothelial permeability thus upregulating the transmigration of leukocytes, and contribute to remote organ specific dysfunction (Welbourn *et al.*, 1991; Yi and Ulich, 1992; Hashimoto *et al.*, 1994; Yassin *et al.*,

2002). ROS, IL-1β and TNF-α stimulate the expression of IL-6, a multifunctional pro- and anti-apoptotic cytokine, in an NFκB dependent manner in skeletal muscle cells and myotubes (Frangogiannis *et al.*, 1998; Nagaraju *et al.*, 1998; Kosmidou *et al.*, 2002; reviewed in Kabe *et al.*, 2005). IL-6 has been shown to modulate energy metabolism and immune responses so that it induces release of glucose for fuel and opposes many TNF-α-mediated events (reviewed in Febbraio and Pedersen, 2002). It can also initiate angiogenic processes in ischemic tissues (Hernandez-Rodriguez *et al.*, 2003). On the other hand, IL-6 promotes transmigration of leukocytes by upregulating e.g. ICAM expression in the myocytes, and thus contributes to neutrophil mediated tissue damage (Youker *et al.*, 1992; Sawa *et al.*, 1998). IL-8 acts mainly as a chemoattractant for neutrophils whereby it is contributing to tissue damage, but more recently it has also been associated with neovascularization in ischemic myocardium (Ivey *et al.*, 1995; Kukielka *et al.*, 1995; Boyle *et al.*, 1998; Kocher *et al.*, 2006).

Table 2. Chemokines and cytokines in ischemic skeletal and cardiac muscle tissues.

Name	Main function	Name	Main function
TNF-α	cytokine cascade, transmigration	MIP2	neutrophil accumulation
IL-1α	pro-inflammatory response	IP-10	T lymphocyte migration
IL-1β	cytokine cascade, transmigration	MIG	T lymphocyte migration
IL-6	transmigration, metabolism	IL-12	T lymphocyte stimulation
IL-8	neutrophil accumulation	IL-10	suppression of inflammation
MCP-1	macrophage accumulation	TGF-β	survival, proliferation, fibrosis

3.1.3 Towards healing

Accumulation of neutrophils and macrophages is known to markedly increase the oxidative stress in ischemic or otherwise injured tissues, caused by the oxidative burst initiated by these cells. $O_2^-\bullet$, H_2O_2 and their derivatives are potent inducers of tissue damage through activation of e.g. stress-responsive c-Jun NH2-terminal kinase (JNK) and p38 MAPK pathways and, therefore, their removal and restoration of the redox balance is essential in the recovery process (reviewed in Gabbita *et al.*, 2000). However, ROS have also been implicated in tissue healing but their concentration needs to be markedly lower than those observed during the inflammatory phase. Reduced oxidative stress itself can markedly promote the healing response due to attenuated inflammation signaling caused by active leukocytes, lyzed cells and oxidized macromolecules. Elimination of $O_2^-\bullet$ promotes availability of NO which has multiple anti-inflammatory functions including inhibition of leukocyte trafficking by downregulation of adhesion molecules and increasing the blood supply to the tissues

by vasodilatation (reviewed in Tidball, 2005). The activation of survival pathways, such as Ras-mitogen activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)-Akt, is also enhanced by subtoxic ROS concentrations, especially H_2O_2 (reviewed in Gabbita *et al.*, 2000; reviewed in Rhee *et al.*, 2005b).

The process of tissue healing can be divided into three slightly overlapping phases. In the *inflammatory phase* infiltrated leukocytes clear the site from dead cells and granulation tissue forms upon macrophage-derived stimuli. The most prominent factors present are various pro-inflammatory cytokines and chemokines. Too early scar formation is prevented by e.g. interferon γ inducible protein 10 (IP-10) and monokine induced by interferon γ (MIG) which possess anti-angiogenic and anti-fibrotic properties (reviewed in Frangogiannis, 2007). Towards the end of the phase the neutrophil numbers and pro-inflammatory cytokine expression are reduced whereas endothelial cells and fibroblasts start proliferating following the cues from growth factor secreting leukocytes, mainly macrophages and to some extent T lymphocytes, mast cells, and tissue cells including myocytes (reviewed in Frangogiannis *et al.*, 2002). During the *proliferative phase* fibroblasts generate new ECM and the capillary system develops to meet their needs in response to hypoxia-induced and macrophage-derived vascular endothelial growth factor (VEGF)(reviewed in Frangogiannis *et al.*, 2002). Additionally, the myocyte precursors, satellite cells, proliferate to regenerate the lost muscle tissue (reviewed in Ten Broek *et al.*, 2010). The myocyte proliferation signals are mediated among others by two prominent transcription factors, NFκB and activating protein (AP-) 1, which become suppressed later during myocyte maturation (Lehtinen *et al.*, 1996). In the last phase, *maturation*, the scar is formed as the excess fibroblasts and vascular cells die by apoptosis leaving only the collagenous ECM behind (Desmouliere *et al.*, 1995; Dobaczewski *et al.*, 2006).

3.2 Leukocytes and transmigration

3.2.1 Major leukocyte populations in ischemic tissues

Tissue resident mast cells and macrophages provide the initial cytokine cues of tissue damage which in turn induce endothelial activation. The infiltration begins within minutes from injury and may continue for several days at varying intensities and with different leukocyte subpopulations emerging at different times. Neutrophils and monocyte/macrophages are the first leukocyte populations which arrive at the site of muscle injury where they mount the rapid innate immune response (reviewed in Butterfield *et al.*, 2006). Lymphocytes are responsible for adaptive immune response, and until recently were not thought to play a role in ischemic injuries or tissue healing. However, T lymphocytes and to some extent also B lymphocytes are now recognized to contribute to both processes (reviewed in Linfert *et al.*, 2009).

Neutrophils are considered mainly a harmful cell population due to their ability to generate large amounts of cytolytic and cytotoxic molecules, such as Nox2-derived O_2^-• and myeloperoxidase (MPO)-derived hypochlorous acid (HOCl), in the oxidative burst in response to pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α (Nathan, 1987a; Suzuki *et al.*, 1996; reviewed in Hampton *et al.*, 1998; Best *et al.*, 1999; Brickson *et al.*, 2001). Accordingly, several studies have shown how neutrophil depletion attenuates ischemia-reperfusion damage in the myocardium and muscle tissue (Walden *et al.*, 1990; Crinnion *et al.*, 1994; Kin *et al.*, 2006). *In vivo*, O_2^-• itself is not the likely direct mediator of tissue damage. It can be rapidly converted by SODs into H_2O_2 which is a more stable and diffusible molecule than O_2^-• thus being able to travel away from the site of generation (reviewed in Hampton *et al.*, 1998). Moreover, H_2O_2 is a substrate for MPO producing HOCl as well as in the OH• generating Fenton reaction thus acting as a source for these highly oxidative ROS, and it could therefore be considered more harmful than O_2^-• (reviewed in Hampton *et al.*, 1998). Additionally, neutrophils themselves secrete and promote activation of several proteases, such as elastase and matrix metalloproteinases, which contribute to increased EC permeability and neutrophil accumulation at the injury site as well as to tissue damage away from the original ischemic site e.g. in lung (Goldman *et al.*, 1990; Carden and Korthuis, 1996; Muhs *et al.*, 2003; Plitas *et al.*, 2003). However, basement membrane and ECM degradation are necessary for revascularization to occur. Neutrophils may thus be beneficial as judged by their participation to angiogenesis, and also in that they modulate monocyte chemotaxis (reviewed in Soehnlein *et al.*, 2009).

Monocyte/macrophages are the second distinct population to invade injured sites and temporally they arrive within 24 hours after the neutrophils lured by endothelium, mast cell or fibroblast derived chemokines/cytokines as well as hypoxia and ROS (McLennan, 1996; Trautmann *et al.*, 2000; Hirani *et al.*, 2001; Lakshminarayanan *et al.*, 2001). It was recognized two decades ago that there are different macrophage subpopulations which predominate in the different stages of muscle recovery. In a muscle reloading induced inflammation model, the infiltrating CD68+/ED1+ macrophage subpopulation, which demonstrates highest activity 1-3 days after the ischemic insult, was initially engaged in phagocytosis in order to clear the site from cell debris, whereas tissue resident CD163+/ED2+ macrophages were found later in areas of muscle regeneration (Honda *et al.*, 1990; McLennan, 1993; St Pierre and Tidball, 1994; reviewed in Butterfield *et al.*, 2006). It seems that they are trapped at the hypoxic sites due to downregulation of MMPs (reviewed in Butterfield *et al.*, 2006). Macrophages are a rich source of cytokines and other factors which orchestrate the inflammatory reaction and the healing response (reviewed in Nathan, 1987b). In general, CD68+/ED1+ macrophages are pro-inflammatory: they secrete many mediators including IL-1, TNF-α and prostaglandin E2, as well as attract more neutrophils thereby creating a positive feedback loop to amplify the inflammatory reaction (reviewed in Butterfield *et al.*, 2006). On the other hand, CD163+/ED2+

macrophages are believed to contribute to the tissue repair by secreting such growth and angiogenesis promoting factors as HB-EGF, FGF, IGF-I, VEGF and TGF-β1 (reviewed in Butterfield *et al.*, 2006; reviewed in Shireman, 2007). Additionally, macrophage derived MMPs release more ECM-bound TGF-β1 which together with growth factors induces fibroblast activation and granulation tissue formation (Imai *et al.*, 1997).

The leukocyte and tissue cell populations have their own tasks and extensive collaboration in the healing muscle, but the monocyte/macrophages are essential also in orchestrating the recovery process. Infiltrating activated monocytes and lymphocytes are the main sources of IL-10 which acts to switch the environment from pro-inflammatory to anti-inflammatory and towards recovery (reviewed in Frangogiannis *et al.*, 2002). CD163+/ED2+ macrophages and mast cells in the inflicted but non-necrotic areas provide the cues for fibroblast proliferation and ECM deposition as well as participate in ECM production and remodeling (Ruoss *et al.*, 1991; DiPietro and Polverini, 1993; reviewed in Kovacs and DiPietro, 1994; reviewed in Sunderkotter *et al.*, 1994). Furthermore, they promote endothelial cell proliferation and migration by secreting the essential factors and recruiting other cell types to aid in the process, and also potentiate the vessel formation through secretion of various proteinases (reviewed in Sunderkotter *et al.*, 1994; reviewed in Norrby, 2002). Additionally, macrophages have been shown to increase satellite cell proliferation and delay their differentiation, and satellite cells in turn secrete factors that attract macrophages until they start to differentiate (Cantini *et al.*, 1994; Merly *et al.*, 1999; Chazaud *et al.*, 2003).

The T lymphocytes have been associated with the antigen-mediated adaptive immune responses to various pathogens or autoantigens, but more recently they have been shown to participate in pathogen-free ischemic injuries, as well, in e.g. brain, kidney, liver, myocardium, lung and intestines (reviewed in Linfert *et al.*, 2009). They are recruited to the injured site by e.g. the macrophage derived factors IP-10 and MIG (Gillitzer, 2001). The first populations seem to migrate relatively early, within a few hours, and activate soon after (reviewed in Linfert *et al.*, 2009). They are thought to activate in response to toll-like receptor (TLR) stimulation, complement system, ROS or the various pro-inflammatory cytokines present independently of antigens (reviewed in Huang *et al.*, 2007). The CD4+ helper T cell subset has been shown to drive the tissue injury, as demonstrated by smaller damage area in CD4+ depleted mice, while CD8+ cytotoxic T cells seem to function in recruitment of the CD4+ cells through IL-16 secretion (Stabile *et al.*, 2006; Yang *et al.*, 2006). The mechanism by which T cells induce the damage remains elusive, but interaction with endothelium and platelets and augmentation of neutrophil extravasation was suggested by a study with hepatic ischemia (Khandoga *et al.*, 2006). However, they have been shown to enable tissue healing, as well. Previous

studies have shown markedly impaired tissue recovery and arteriogenesis after T lymphocyte depletion (Stabile *et al.*, 2003). These findings indicate that, as with the monocyte/macrophages, also different T lymphocyte subpopulations have specific tasks in tissue recovery from ischemic insult.

3.2.2 Leukocyte transmigration through the endothelium

Ischemia-reperfusion, hypoxia and the release of various inflammatory agents from injured and necrotic tissue promote endothelial activation in the viable blood vessels (reviewed in Alom-Ruiz *et al.*, 2008). This phenomenon is crucial for the inflammatory reaction since it orchestrates the transmigration of leukocytes into the tissues. Soon after the initial stimuli by tissue-resident mast cells and macrophages, intracellular ROS generation for signaling purposes is induced in endothelial cell mitochondria and by e.g. Nox enzymes, xanthine oxidase, cytochrome P450 and endothelial nitric oxide synthase (eNOS) (reviewed in Alom-Ruiz *et al.*, 2008). The ROS and especially H_2O_2, in turn, activate directly or indirectly a number of redox sensitive signaling pathways such as those involving the transcription factors NF-κB, AP-1, hypoxia inducible factor (HIF)-1α, or the MAP kinases and Akt/protein kinase B (Schreck and Baeuerle, 1991; Wang and Semenza, 1993; Chen *et al.*, 1995; Lo and Cruz, 1995; Knight and Buxton, 1996; Aikawa *et al.*, 1997; Wang *et al.*, 2000b). These signaling pathways then execute major changes in endothelial cell gene expression, cytoskeleton organization, morphology and permeability of the vascular wall.

The activated endothelial cells produce a number of chemokines to lure circulating leukocytes to the site of injury. One of the best studied is MCP-1 which is a potent attractant for monocytes, T lymphocytes and natural killer cells (reviewed in Frangogiannis, 2007). *In vivo* myocardial ischemia studies have shown that it is expressed in endothelial cells soon after the injury peaking at day 3 (Kumar *et al.*, 1997; Lakshminarayanan *et al.*, 2001; Shireman *et al.*, 2006). It is upregulated in response to TNF-α and IL-1 via the ROS-dependent NFκB and AP-1 pathways (Sica *et al.*, 1990; Martin *et al.*, 1997; Lakshminarayanan *et al.*, 2001; Chen *et al.*, 2004). Removal of the effective MCP-1 by antibody administration or genetic knock-out revealed significantly lower macrophage infiltration and downregulation of adhesion molecules as well as several other important cytokines such as TNF-α, IL-1 and TGF-β (Ono *et al.*, 1999; Dewald *et al.*, 2005). Also a role in angiogenesis has been suggested (Ito *et al.*, 1997). Other relevant endothelial cell-derived chemokines in leukocyte trafficking are the macrophage inflammatory proteins (MIPs). MIP-1α and β function similarly to MCP-1, but MIP-2 is specific for neutrophils (Uguccioni *et al.*, 1995; Zhang *et al.*, 2001a; Zhang *et al.*, 2001b). They are expressed in muscle and endothelial cells at the sites of active transmigration, and respond to ROS through NFκB and AP-1 (Nossuli *et al.*, 2001; Hua *et al.*, 2005). However, TNF-α seems to be

optional for MIP-2 activation (Nossuli *et al.*, 2001; Zhang *et al.*, 2001b). MIP-2 induces transmigration through e.g. P-selectin, an important adhesion molecule on the endothelial surface (Zhang *et al.*, 2001a).

Transmigration of neutrophils, monocytes and other leukocytes is a complicated and dynamic event which takes place as soon as the endothelium is activated, and aims to quickly establish an immune response in the tissues. The current model has been reviewed lately by Ley *et al.* (2007). It comprises the initial rolling, activation and firm adhesion of leukocytes, and subsequent intraluminal crawling and extravasation by the para- or transcellular routes. Briefly, initial attachment and rolling of leukocytes on the endothelial surface is mediated mainly by endothelial P- and E-selectins which interact with various glycosylated ligands on leukocytes such as P-selectin glycoprotein ligand (PSGL)-1 and CD44. Selectin-ligand binding and intense chemokine signaling induce integrin activation leading to stronger adhesion of leukocytes to the vascular wall. Also G-protein coupled receptors have been implicated in the integrin activation but the intracellular signaling pathway has not been determined. Leukocyte integrins, such as very late antigen (VLA)-4 and lymphocyte function-associated antigen (LFA)-1, bind intercellular and vascular cell-adhesion molecule (ICAM and VCAM, respectively) on the endothelial cell surface. Attached leukocytes then crawl to an appropriate site and initiate the transmigration cascade in which both the leukocytes and the endothelial cells actively participate. The process is characterized by extensive changes in cell shape and expression of adhesion and junctional molecules such as platelet/endothelial adhesion molecule (PECAM)-1, junctional adhesion molecules (JAMs) and CD99. Lastly, the leukocyte has to cross a basement membrane and a pericyte layer beneath the endothelium before reaching the underlying tissues.

In general, the prevention of leukocyte trafficking, especially of neutrophils, has been considered an effective method to reduce the tissue damage involved in ischemic injuries. However, the numerous signaling events through either direct contact or via chemokines, cytokines and other factors between the different leukocyte subtypes and the tissue cells themselves indicate dependence upon each other, which culminates in the activation of the beneficial CD163+/ED2+ macrophages at the injury site (reviewed in Tidball, 2005; reviewed in Butterfield *et al.*, 2006). Thus, if their mutual signaling or the relative numbers of the cells are altered by e.g. administration of glucocorticoid drugs or inhibitors to the signaling molecules, or depletion of one of the leukocyte subpopulations, the outcome may be slower and improper recovery (Mishra *et al.*, 1995; Lescaudron *et al.*, 1999; Warren *et al.*, 2002; Warren *et al.*, 2004; Shireman *et al.*, 2007). Accordingly, the attempted treatments require thorough understanding of the biology of inflammation and should take into account the importance of the inflammatory reaction to the healing process.

4 Apoptosis vs. survival in tissue injuries and cancer

4.1 Apoptotic signaling pathways

H_2O_2, other ROS, growth factors and cytokines bombard the cells in injured tissue with a multitude of apoptotic and survival signals. Apoptosis or programmed cell death is the final option when the cell cannot adapt to changes in the environment, is incapable of repairing its damaged parts or is potentially dangerous to the organism. Such occasions may arise in ischemic injuries, aging and transformation into cancerous cells, due to lack of oxygen and nutrients, ROS formation, scar formation, telomere shortening and escape from normal growth regulation. Apoptosis is also a very important facet in the development and normal tissue turnover, but these subjects are beyond the scope of this text. Apoptosis protects the surrounding cells and the whole organism by allowing highly controlled removal of deficient cells. As the name implies, it is a highly regulated process in which the cell in a stepwise manner shuts down all its functions and disaggregates itself leaving behind only small vesicles, apoptotic bodies, to be eaten by phagocytes (reviewed in Fiers *et al.*, 1999). In a microscope, an apoptotic cell can be recognized by typical loss of attachement to surface, membrane blebbing, shrinkage, and DNA condensation and fragmentation. By committing a suicide the cell avoids the leakage of its contents into the extracellular space that may cause an inflammatory reaction, and is therefore aptly named a "silent" process. However, when the injury is severe, the cell may die by necrosis characterized by swelling and eventual rupture which evokes the nearby inflammatory cells. Lately, necrosis has also been determined to be a regulated event to some extent (reviewed in Fiers *et al.*, 1999).

According to the severity of the ischemic injury and the site of arterial damage or occlusion, zones with variable level of ischemia develop (reviewed in Blaisdell, 2002). The core area is likely to die by necrosis in the complete absence of blood supply, but depending on the availability of collateral blood flow the surrounding tissues, or the peri-infarct zone in e.g. myocardium, is dominated by apoptosis (reviewed in Abbate *et al.*, 2006). As compared to necrosis, apoptosis has been shown to be the major form of myocardial damage already a few hours after the occlusion, and it continues for several days afterwards (Kajstura *et al.*, 1996). Skeletal muscles are more resistant to apoptosis than other types of muscle tissue, but given sufficient ischemic time will eventually succumb to necrosis and apoptosis (reviewed in Blaisdell, 2002). Solid tumors often display hypoxia and necrosis in their core regions due to high growth rate and malformed vasculature incapable of providing adequate circulation; for the same reason apoptosis is also very common, especially in the initial phase (reviewed in Wouters *et al.*, 2003). Tumor cells are constantly under oxidative stress due to the mitochondrial ROS overproduction and reduced antioxidant enzyme activity (Szatrowski and Nathan, 1991; Laurent *et al.*, 2005). However, they

proliferate fast and are tolerant to hypoxia, malnutrition and apoptosis which often means poor prognosis since hypoxia and ROS can induce angiogenesis and a more invasive and metastatic phenotypes in cancer cells (reviewed in Wouters *et al.*, 2003; reviewed in Kim *et al.*, 2004; Laurent *et al.*, 2005).

Apoptosis signaling can initiatate from two sources: the death domain-containing plasmamembrane receptors (extrinsic) or mitochondria (intrinsic). Additionally, various extracellular stresses can activate e.g. NFκB or p53 routes which co-operate with the basic apoptotic cascades. The activation of these major signaling pathways has been verified in various ischemic conditions involving e.g. heart, liver, kidney and brain (reviewed in Lopez-Neblina *et al.*, 2005). In cancer tissue, however, the NFκB and p53 mediated death signals are frequently suppressed (reviewed in Green and Evan, 2002). The apoptotic mechanisms can be further divided to caspase-dependent and independent, the former being studied most. Caspases, or *c*ysteine-dependent *asp*artate specific prote*ase*s, are expressed at relatively high levels as pro-caspases which can be rapidly activated upon stimulus and start to degrade the cellular proteins. Altogether 13 mammalian caspases have been cloned and classified traditionally as apoptotic or inflammatory (reviewed in Chowdhury *et al.*, 2008).

Extrinsic pathway. TNF receptors (TNFR) 1 and 2, Fas/CD95/APO-1 and TNF-related apoptosis inducing ligand receptors (TRAIL-R) 1 and 2 belong to the TNF superfamily and are situated on the plasmamembrane of a wide variety of cell types (reviewed in Schultz and Harrington, 2003; reviewed in Lopez-Neblina *et al.*, 2005). They bind a number of signaling factors from the extracellular space including e.g. TNF-α, Fas ligand (FasL), TRAIL and agonistic antibodies (reviewed in Schultz and Harrington, 2003; reviewed in Lopez-Neblina *et al.*, 2005). Upon ligand binding they recruit various adapter proteins, such as TNFR- or Fas-associated death domain (TRADD and FADD, respectively) or p53-induced protein with death domain (PIDD), which may possess affinity for more than one of the death receptors (reviewed in Lopez-Neblina *et al.*, 2005). Subsequently, the dimerization-dependent proteolytic activation of initiator caspases 2, 8, and 10 is potentiated by formation of an activation complex gathered around the ligand-activated death receptor (reviewed in Pop and Salvesen, 2009). The different initiator caspases are associated with different activation platforms such as death inducing signaling complex (DISC) or PIDDosome (reviewed in Pop and Salvesen, 2009).

Intrinsic pathway. A number of stress factors, such as hypoxia, ROS, radiation or genotoxic drugs, are detected by the B-cell lymphoma (Bcl-) 2 family of proteins which is a central mediator of the mitochondrial events in apoptosis (reviewed in Adams and Cory, 2007). It is divided into anti- and pro-apoptotic groups: the anti-apoptotic group includes Bcl-2, Bcl-XL, Bcl-w, A1 and Mcl-1, while the pro-apoptotic members are divided to Bcl-2 homology domain 3 (BH3) only proteins Bid, Bad, Bim, Blk, BiK and Hrk, and multidomain proteins Bax, Bok and Bak which

contain several other BH domains (reviewed in Schultz and Harrington, 2003). Under normal conditions the Bcl-2 homologous antagonist/killer (Bak) and Bcl-2-associated X protein (Bax) are bound by the anti-apoptotic Bcl-2 proteins which keep them inactive (reviewed in Adams and Cory, 2007). The BH3 only proteins reside in the cytosol and function as sensors for the cellular stresses (reviewed in Adams and Cory, 2007; reviewed in Ewings *et al.*, 2007b). They seem to have different preferences for the activating stimuli; for instance, BH3-interacting mediator of cell death (Bim) is freed upon cytokine deprivation independently of caspase activation, Bid is directly activated by caspase-8 and Bad is activated after reduction in Akt survival signaling (Zha *et al.*, 1996; Li *et al.*, 1998; Puthalakath *et al.*, 1999). The activated BH3-only proteins then bind to the anti-apoptotic Bcl-2 proteins which results in the recruitment and oligomerization of Bax and Bak on the mitochondrial outer membrane (reviewed in Ewings *et al.*, 2007b). Consequently, several pro-apoptotic factors such as cytochrome *c*, apoptosis inducing factor (AIF) and second mitochondria-derived activator of caspases (Smac)/DIABLO leak into the cytosol (reviewed in Schultz and Harrington, 2003; reviewed in Lopez-Neblina *et al.*, 2005). Cytochrome *c* promotes the assembly of apoptosis protease-activating factor (Apaf-) 1 and procaspase-9 to form the apoptosome activation platform which cleaves caspase-9 and the downstream effector caspases (reviewed in Schultz and Harrington, 2003). AIF mediates caspase-independent apoptosis by translocating to nucleus where it induces DNA condensation and subsequent p53 activation (reviewed in Schultz and Harrington, 2003). Smac/DIABLO, in turn, prevents the functions of the inhibitors of apoptosis (IAPs), a family of active caspase inhibitors (Chai *et al.*, 2000).

Both the extrinsic and intrinsic pathways ultimately activate the dimeric effector procaspases 3, 6 and 7 by targeted proteolysis and subsequent autoproteolysis (reviewed in Pop and Salvesen, 2009). Since proteolysis is irreversible, the appearance of cleaved effector caspases can be considered a commitment to apoptosis pathway. They have slightly different preferences for substrates, the caspase-3 having a wider repertoire than the others whereby it is considered the major apoptosis-inducing caspase (Faleiro *et al.*, 1997; Walsh *et al.*, 2008). Furthermore, the major changes seen in cell and chromatin morphology of apoptotic cells seem to be largely dependent on the presence of caspase-3 (Woo *et al.*, 1998; reviewed in Porter and Janicke, 1999). The number of cellular caspase substrates is suggested to rise to hundreds, but all of them are not likely to be direct targets (reviewed in Timmer and Salvesen, 2007). However, a few true targets have been established. Firstly, the effector caspases are obviously targeted by the initiators, but there may be co-operation between pathways, as well: extrinsicly and intrinsicly activated caspases may cleave each other and thus amplify the apoptotic signal (reviewed in Timmer and Salvesen, 2007). Other targets include e.g. Bid, retinoblastoma-associated protein (RB), IAPs, inhibitor of caspase-activated DNase (ICAD) and poly(ADP-ribose) polymerase (PARP) which participate

in the apoptosis signaling, DNA fragmentation and DNA repair (reviewed in Timmer and Salvesen, 2007).

4.1.1 Transcription factors as the key determination points

Paradoxically, the apoptotic pathways may also induce pro-survival signaling (Liu *et al.*, 2005). This could be a mechanism to extend the lifespan enough to enable repair system activation and possible recovery, or to accomplish successful apoptosis instead of uncontrolled necrosis. Typical pro-survival factors upregulated by apoptosis-induced survival signaling are e.g. mitochondrial respiratory chain molecules to maintain energy production, anti-apoptotic Bcl-2 family proteins, antioxidants to protect from ROS and induction of cell cycle arrest through cyclin-dependent kinase inhibitors (Joshi *et al.*, 1999; Chandra *et al.*, 2002; Javelaud and Besancon, 2002; Liu *et al.*, 2005). The decision between life and death requires integration of a vast amount of signals from the cell exterior and interior. However, the existence of master regulators has been proposed that function as points of signal convergence. In general, the multiple signaling pathways activated by some stimulus interact through their downstream effectors either amplifying or suppressing each other and the signals may need to pass checkpoints until they reach the key switch with the power to direct the fate of the cell. For instance, transcription factors p53, NFκB, E2F1 and FoxO3a have been suggested to possess such a role.

Recently, the transcription factor Forkhead box class O3a (FoxO3a) was found to act as a switch between apoptotic and survival signaling (Liu *et al.*, 2005; reviewed in van der Horst and Burgering, 2007). It belongs to a FoxO subfamily of transcription factors comprising mammalian FoxO1, FoxO3a, FoxO4 and FoxO6 that are involved in metabolism, differentiation, transformation, oxidant detoxification and cell cycle regulation among other things (reviewed in Accili and Arden, 2004; reviewed in van der Horst and Burgering, 2007). Due to this intricate involvement in numerous cell functions, their activity is strictly controlled. FoxO3a activity is mainly regulated post-translationally by Akt mediated phosphorylation and CREB-binding protein (CBP)/p300 mediated acetylation which both decrease its DNA binding and transcriptional activity resulting in a cytosolic translocation (Figure 4, left panel; Brunet *et al.*, 1999; reviewed in Calnan and Brunet, 2008). However, several other kinases including extracellular signal regulated kinase (Erk) 1/2, phosphatases, acetylases and deacetylases can modify and fine-tune its functions according to the additional upstream signals thus supporting its role as a key signaling factor (reviewed in Calnan and Brunet, 2008; Yang *et al.*, 2008). In general, survival and proliferation signaling promote FoxO3a inactivation while stress signals such as ROS activate it, and the specific post-translational modification pattern determines its transcriptional target genes promoting either apoptosis or cell cycle arrest (reviewed in Calnan and Brunet, 2008).

The FoxO3a targets include both pro- and anti-apoptotic genes (Figure 4, right panel). Many factors of the extrinsic and intrinsic apoptotic cascades are transcribed by FoxO3a such as Fas ligand, Bim, TRADD and TRAIL (Dijkers *et al.*, 2000; Liu *et al.*, 2005; Lee *et al.*, 2008). Thus, it is able to amplify the TNFR mediated signals and induce the caspase activation. Furthermore, it can indirectly suppress NFκB activity and promote JNK activity after TNFR stimulation directing the cell towards apoptosis (Lee *et al.*, 2008). Some of the negative FoxO3a-responsive cell cycle regulatory factors are $p27^{KIP1}$, P130, cyclin G2 and GADD45a which become upregulated in the absence of growth factors or under cellular stress, and induce transition to G_0 or arrest the cycle at G_2 (reviewed in Furukawa-Hibi *et al.*, 2005). Simultaneously with these, FoxO3a can downregulate e.g. microRNA-21, cyclins D1 and D2 to enhance the inhibitory effect on cell cycle progression (Wang and Li, 2010; reviewed in Furukawa-Hibi *et al.*, 2005). The additional expression of many antioxidant enzymes such as the SOD isoforms, catalase, Prx enzymes, thioredoxin and thioredoxin reductase in response to FoxO3a activation implies that it is an important factor in the protection of cells against ROS generated during various stress conditions (Kops *et al.*, 2002; Liu *et al.*, 2005; Tothova *et al.*, 2007; Olmos *et al.*, 2009).

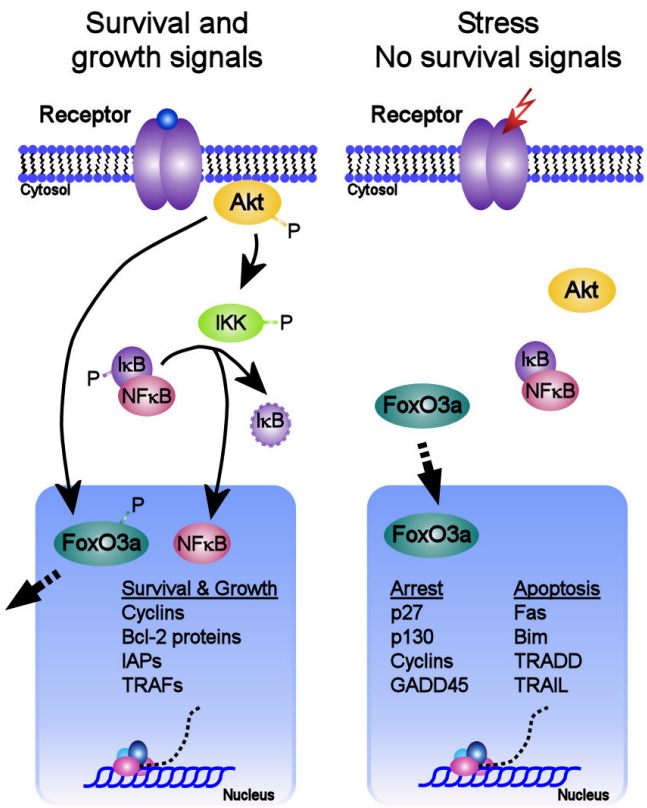

Figure 4. The antagonistic effects of NFκB and FoxO3a. Left panel: Upon receptor activation by e.g. growth factors, Akt is activated and able to phosphorylate IκB kinase complex (IKK). IKK marks inhibitor of κB (IκB) for degradation which allows nuclear factor κB (NFκB) to translocate to the nucleus, and start gene expression. Nuclear Akt induces export of Forkhead box class O3a (FoxO3a) transcription factor. Right panel: Under stress or in the absence of survival signals Akt and NFκB remain inactive while FoxO3a is translocated to nucleus. There FoxO3a induces cell cycle arrest or apoptosis-related genes depending on the status of the cell.

The NFκB acts in general to oppose many FoxO3a-mediated events in cell stress and apoptosis, and is thus more distinctly a survival factor (reviewed in Finnberg and El-Deiry, 2004). It is a ubiquitous transcription factor with multiple functions in the immune system, cell growth, stress responses and cell survival. It is activated, for instance, by growth factors, inflammatory cytokines, oxidative stress and microbial antigens (reviewed in Pahl, 1999). Ultimately the various signaling pathways result in activation of the IκB kinase complex (IKK) which phosphorylates the NFκB-bound inhibitor of κB (IκB) thus marking it for degradation, after which the NFκB is free to translocate to the nucleus (Figure 4, left panel; reviewed in Perkins, 2000). The important pro-survival kinase Akt regulates NFκB positively by phosphorylating the IKK (reviewed in Datta *et al.*, 1999). Moreover, TNF-α stimulated TNFR controls the activity of several factors responsible for the eventual NFκB release: the IκB and the IKK, NFκB-inducing kinase (NIK) upstream of IKK as well as the MAPKs JNK and p38 (reviewed in Kabe *et al.*, 2005). The nuclear NFκB promotes the transcription of several survival-related genes such as cyclins, IAPs, TNFR-associated factors (TRAFs) and anti-apoptotic Bcl-2 family proteins (Figure 4, left panel; reviewed in Barkett and Gilmore, 1999; reviewed in Pahl, 1999). To complicate the matters, NFκB may also promote apoptosis under special conditions by inducing Fas ligand expression or co-operating with the p53 tumor suppressor; the final role of NFκB in apoptosis seems to depend on the cellular context (reviewed in Barkett and Gilmore, 1999).

4.2 Cellular survival and proliferation pathways

Cells need a constant flow of survival signals from their environment in order to live; when these signals are absent, they initiate apoptosis (reviewed in Raff, 1992). The signals can be e.g. growth factors, mitogens, attachment to ECM or other surface, hormones, cytokines or ROS, which bind their respective receptors on the plasmamembrane such as receptor tyrosine kinases (RTK) and G-protein coupled receptors (GPCR), or cytosolic steroid hormone receptors. In malignant transformation the cell becomes independent of external mitogenic and survival signals due to mutations in a number of growth-regulatory genes (reviewed in Hanahan and Weinberg, 2000). Simultaneous inactivation of the apoptotic pathways is required, though, since pro-survival stimuli and active oncogenes often launch them, as well (reviewed in Harrington *et al.*, 1994).

Tissue healing after an injury requires well-coordinated survival/proliferation and apoptosis signaling to regenerate the original properties and functionality of the tissue. Initially, the apoptosis-promoting environment and infiltrating phagocytes remove the non-viable cells and tissue debris, thus creating a more suitable ground for proliferation (reviewed in Tidball, 2005). Macrophages and mast cells provide the growth signals in the later phases of inflammation which induce the appropriate

responses in fibroblasts, satellite cells and endothelial cells (Ruoss *et al.*, 1991; Cantini *et al.*, 1994; reviewed in Crivellato *et al.*, 2004; reviewed in Tidball, 2005). The activated survival and proliferation signaling routes are relatively universal with regard to cell type, and different receptors can activate the same route or amplify it in response to another growth stimulating factor. Two survival/proliferation pathways found in nearly all cells have been under intense research, namely the Ras-Erk1/2 and PI3K-Akt. They activate in response to many types of receptors and stimuli, and are frequently found mutated in cancer tissue (reviewed in Bos, 1989; reviewed in Yuan and Cantley, 2008). However, they are not merely for survival purposes, but also have a role in e.g. myocyte differentiation (Rommel *et al.*, 1999; Halevy and Cantley, 2004; De Alvaro *et al.*, 2008).

4.2.1 The Ras small GTPase

A common upstream activator for both of these pathways is the small GTPase Ras. However, Ras is not restricted to these two routes, but is a versatile regulator of a variety of pathways involved in cell survival, growth and proliferation. There are four Ras proteins, N-, Ha- and two Ki- isoforms, which have different capabilities to activate Raf-MEK-Erk1/2 and PI3K-Akt routes (Yan *et al.*, 1998). Ras is mainly activated through RTK signaling, but GPCRs can also modulate its activity in a receptor and cell type specific manner through e.g. protein kinase A (PKA), phospholipase C (PLC) or PI3K (reviewed in McKay and Morrison, 2007; reviewed in Ramjaun and Downward, 2007). In the classical model, upon RTK stimulation Ras is recruited to the plasmamembrane into the Grb2/SOS complex which induces the Ras GDP-GTP exchange thus enabling the downstream effector activation (Figure 5A). Recent research has discovered specific Ras activation in intracellular compartments such as Golgi, endoplasmic reticulum and endosomes which may have influence on the outcome of the signaling event (reviewed in McKay and Morrison, 2007). Constitutively active Ras mutations are found in approximately 30% of all cancers which renders it one the most frequent oncogenes (reviewed in McCubrey *et al.*, 2007).

ROS-mediated activation of the Ras and its downstream pathways can occur by different mechanisms. However, direct ROS-dependent activation of Erk1/2 or Akt is not supported by current data, but instead seems to affect the members upstream of the signaling pathways (Lander *et al.*, 1995; Aikawa *et al.*, 1997; Lee *et al.*, 2002). A well-documented mechanism is the inhibition of cytosolic lipid or protein tyrosine phosphatases, especially by H_2O_2, which then allows enhanced phosphorylation of the receptors and kinases leading to Ras-Erk1/2 or PI3K-Akt cascade activation (reviewed in Rhee *et al.*, 2005b). Specificity for a certain downstream MAPK pathway may arise from the differential inactivation of a set of RTK associated phosphatases (Lee and Esselman, 2002). Ras may also be directly affected by ROS through a mechanism in

which the GDP-GTP exchange is enhanced by oxidation of the GTPase (Lander *et al.*, 1995; Heo and Campbell, 2005). Furthermore, Ras may be dispensable when the signaling takes place through a GPCR. In this case, the signal to Erk1/2 is conveyed in a protein kinase C (PKC)- and Raf-dependent manner thus bypassing the tyrosine kinases (Zou *et al.*, 1996).

4.2.2 The Raf-MEK-Erk1/2 pathway

The MAPK Erk1/2 signaling cascade is a very important mediator of RTK signals. In addition to Erk1/2, there are JNK, p38 and the more recently discovered Erk5 MAP serine/threonine kinases in the family (reviewed in Raman *et al.*, 2007). While Erk1/2 is mainly activated by growth stimuli, the other MAPKs are more inclined to stress-mediated activation, and regulate many stress responses as well as apoptosis (Xia *et al.*, 1995; reviewed in Kyriakis and Avruch, 2001). MAPKs and their upstream kinases (MAP2K and MAP3K) form a cascade characterized by sequential phosphorylation of each kinase finally leading to activation or inactivation of the target factors (Figure 5B). The three or more tiered structure of these pathways is probably for signal regulation purposes, as it creates many interaction points with other modulatory signaling events. For instance, the Raf-MEK-Erk1/2 and the corresponding JNK and p38 cascades have their own specific scaffold proteins which are involved in the assembly and activation of the MAP3K-MAP2K-MAPK complex and direct its localization within the cell to achieve a specified signal in a certain target site (reviewed in Kolch, 2005; Casar *et al.*, 2009). The scaffolds may bind additional factors such as phosphatases or kinases which aid in the regulation of the MAPKs (reviewed in Kolch, 2005; reviewed in Raman *et al.*, 2007). Furthermore, positive and negative modulators can interact with Erk1/2 or it can induce feedback signaling to alter the duration and strength of the signal which is essential in determination of its outcome (reviewed in Ramos, 2008). Erk1/2 binding specificity is also controlled by several docking motifs on the kinase itself and its interacting regulators and substrates (reviewed in Raman *et al.*, 2007).

The preferential MAP3K for Erk1/2 is Raf which exists in three isoforms, A-Raf, B-Raf and C-Raf/Raf-1. They have specific as well as partly overlapping roles in regulation of signal transduction, but display variable activation potencies towards the downstream MAP2K, the MAP/Erk kinase (MEK) (Alessi *et al.*, 1994; reviewed in O'Neill and Kolch, 2004). Raf kinases are activated by Ras in a complex, multistep manner, retain several phosphorylation sites for various other kinases and are modulated by e.g. heat shock protein 90 and chaperonins (reviewed in McKay and Morrison, 2007), indicating that they are a major point of signal regulation and integration in the Ras-Erk1/2 cascade. However, Braf seems to be the most powerful activator of the downstream cascade due to its simpler activation mechanism (Mercer and Pritchard, 2003). Raf proteins have been shown to induce additional MEK-Erk1/2

independent functions in the apoptosis and cell cycle regulation, as well (reviewed in Hindley and Kolch, 2002).

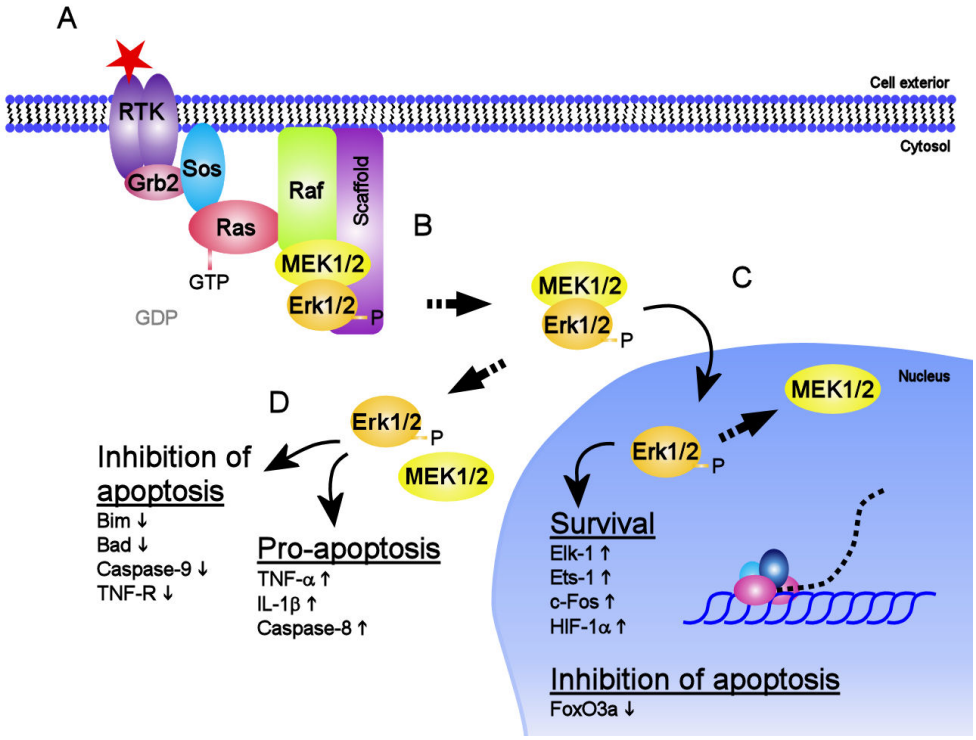

Figure 5. The Ras-Erk1/2 pathway. A) Activated receptor tyrosine kinase (RTK) recruits the Grb2 and Sos adaptor proteins which bind and activate Ras by inducing GDP-GTP exchange. B) Raf activation by Ras induces a sequential phosphorylation of downstream MEK1/2 and Erk1/2. The three kinases are usually bound to one of several scaffold proteins which enable more efficient signal transduction and determine the location of the complex. C) MEK1/2-Erk1/2 remain bound to each other while they are translocated to the nucleus where they quickly detach. Erk1/2 can then directly activate or inactivate several transcription factors which regulate the appropriate gene expression programs. D) Earlier MEK1/2-Erk1/2 detachement results in phosphorylation of cytosolic targets. Erk1/2 can inhibit several apoptotic factors, and thus promote survival. However, Erk1/2 can also promote apoptosis by inducing the activation of some pro-apoptotic factors.

In contrast to Raf activation by Ras, MEK activation by Raf is a simple phosphorylation event at defined residues (Alessi *et al.*, 1994; Zheng and Guan, 1994). There are at least seven MEKs with different target MAPKs, and MEK1 and 2 are specific for phosphorylating Erk1/2 and responsible for its transport to the nucleus (Figure 5B; Payne *et al.*, 1991; Zheng and Guan, 1993b; Zheng and Guan, 1993a; Fukuda *et al.*, 1997; Jaaro *et al.*, 1997). Rafs, MEKs and Erks usually dimerize upon activation so that Rafs will form either homo- or heterodimers, MEKs prefer to form heterodimers and Erks homodimers (Khokhlatchev *et al.*, 1998; reviewed in Cobb and Goldsmith, 2000; Weber *et al.*, 2001; Rushworth *et al.*, 2006; Catalanotti *et al.*, 2009).

Originally, MEKs and Erks were thought to possess redundant functions in the cells, hence the nomenclature, but more recent findings suggest they can have differential impacts on each other as well as on substrate specificities (Sarbassov *et al.*, 1997; reviewed in Pelech, 2006; Catalanotti *et al.*, 2009).

Unlike MEK1/2, Erk1/2 has over 150 direct substrates in both cytosol and nucleus (Figure 5C, D). Firstly, it can extensively modify its own activity by phosphorylating e.g. the upstream kinases and several phosphatases in nucleus or cytosol (reviewed in Ramos, 2008). When other than the autoregulatory substrates are considered, the variety of Erk1/2 substrates reflects its participation in most cellular functions. A comprehensive list of Erk1/2 targets has been compiled by Yoon and Seger (2006), and it includes e.g. factors involved in metabolism, cell cycle regulation and cell migration; immediate early gene products in response to e.g. growth factors or stresses; apoptotic factors; transcription factors; cytoskeleton-interacting proteins; cell junction-interacting proteins and even viral proteins. Due to the large number of these factors, the remainder of this section will concentrate on the substrates relevant for the topic.

One of the best studied transcription factor substrates of Erk1/2 is the Elk-1 (Figure 5C; Marais *et al.*, 1993; Gille *et al.*, 1995). It belongs to the Ets family and, more precisely, the ternary complex factor (TCF) subfamily of transcription factors which is responsive to the Ras-MAPK pathway (reviewed in Wasylyk *et al.*, 1998). Other members of the TCF family are Sap1a, Sap1b, Fli1 and Net (reviewed in Wasylyk *et al.*, 1998). On growth factor stimulation they become phosphorylated by Erk1/2, and mainly act by binding to the Ras and serum response elements (RRE and SRE, respectively) on the target gene promoter region and subsequently recruiting other transcriptional factors which confer cell type specific gene expression (Whitmarsh *et al.*, 1995; reviewed in Yordy and Muise-Helmericks, 2000). Many Elk-1 target genes encode immediate early factors, such as c-Fos, JunB and Egr-1 which further enhance the transcription of relevant genes in response to growth factor stimulation (Gille *et al.*, 1995; Hodge *et al.*, 1998), and thus give it a central role in the Ras-Erk1/2 mediated cellular responses. However, the TCFs can also be activated by the JNK and p38 MAPKs and the PI3K pathway, and thus respond to stress stimuli, as well (reviewed in Yordy and Muise-Helmericks, 2000). Of the Elk-1 target genes, c-Fos is an important factor since it promotes the activity of c-Jun, whose activity can be further enhanced by Erk1/2, by forming with it a dimeric complex, the activating protein (AP-) 1 (Halazonetis *et al.*, 1988; Kouzarides and Ziff, 1988; Schutte *et al.*, 1989; Morton *et al.*, 2003). AP-1 in turn acts as a multifunctional transcription factor in proliferation, survival and apoptosis (reviewed in Shaulian and Karin, 2001). Stabilization of c-Fos requires phosphorylation by Erk1/2, and in unphosphorylated state it is quickly degraded, which indicates that it can also act as a sensor for Erk1/2 signal duration (Murphy *et al.*, 2002). Another Ets family transcription factor, from a

subfamily with the same name, activated by Erk1/2 phosphorylation is the E26 transformation specific sequence (Ets-) 1 which regulates a set of pro-proliferative and migration-related genes such as VEGF, VEGF receptor and matrix metalloproteinases as well as its own expression together with the AP-1 (reviewed in Dittmer, 2003).

In addition to changing the gene expression profile of a stressed cell towards survival and proliferation, Erk1/2 can directly inhibit the intrinsic apoptotic pathway by phosphorylating several factors involved in the process (Figure 5D). For instance, Bim phosphorylation by Erk1/2 induces its dissociation from the anti-apoptotic myeloid cell leukemia sequence (Mcl)-1 and promotes its subsequent proteasomal degradation while Mcl-1 is released from inhibition and can associate with the Bax (Luciano *et al.*, 2003; Ley *et al.*, 2004; Ley *et al.*, 2005; Ewings *et al.*, 2007a; Hubner *et al.*, 2008). Erk1/2 may also hinder Bim synthesis by inhibiting FoxO3a activity through phosphorylation and cytosolic retention (Yang *et al.*, 2008). Similarly, phosphorylation of Bad by Erk1/2, other Erk1/2 downstream kinases, Akt or PKA promotes its dissociation from the Bcl-xL on the mitochondrial membrane and subsequent degradation (reviewed in Balmanno and Cook, 2009). The anti-apoptotic Bcl-2 proteins are synthetized upon MEK-Erk1/2 activation (Boucher *et al.*, 2000). However, their overexpression can expose the cell to transformation, and Erk1/2 driven Mcl-1 stabilization has been suggested as one mechanism to confer such sustained survival (reviewed in Balmanno and Cook, 2009). Erk1/2, in concert with Akt, can also reduce the activity of caspase-9 towards the effector caspase-3, and induce a relocalization of the TNFR thereby blocking the apoptotic cascades (Cardone *et al.*, 1998; Cottin *et al.*, 1999; Allan *et al.*, 2003).

As stated above, the proliferation signaling also promotes apoptotic cues in order to control the net growth of the tissue. The Raf-MEK-Erk1/2 pathway is not an exception as the first implications of its involvement in apoptosis were obtained over a decade ago (Figure 5D; Bhat and Zhang, 1999). Since then, similar findings have been made in various renal, neuronal, hepatic and bone marrow cells as well as in animal models of cerebral and renal ischemia (reviewed in Zhuang and Schnellmann, 2006). The stimuli are equally variable including H_2O_2 and ROS, nutrient or growth factor depletion and chemicals (reviewed in Zhuang and Schnellmann, 2006). In most studies the upstream signal has been transduced through the Ras-Raf-MEK pathway, but it does not seem to be compulsory (Lee *et al.*, 2003; reviewed in Zhuang and Schnellmann, 2006). In the intrinsic pathway, Erk1/2 may promote Bax and the tumor suppressor p53 expression, or activate the p53 by phosphorylation which can then induce the cytochrome *c* release by sequestering Bcl-xL or upregulating Bak activity (reviewed in Zhuang and Schnellmann, 2006). In the extrinsic pathway Erk1/2 can likewise promote the expression of pro-apoptotic factors including e.g. TNFα and IL-1β, or activate the central initiator caspase-8 (reviewed in Zhuang and Schnellmann, 2006). Erk1/2-mediated cytochrome *c* release and caspase-3 activation have been

proposed, as well (Wang *et al.*, 2000a; Nowak, 2002; Kim *et al.*, 2005b). Another mechanism is the suppression of the PI3K-Akt pathway by the MAP kinase pathway, but the details of their interaction remain unclear. However, two modes have been proposed: a multienzyme complex regulating Akt activity and a Raf-MEK-Erk1/2-mediated feedback inhibition of Ras (Sinha *et al.*, 2004; Menges and McCance, 2008).

4.2.3 The PI3K-Akt pathway

The RTKs and GPCRs that activate the Raf-Erk1/2 pathway can also induce the PI3K-Akt pathway. These two pathways are often activated simultaneously, since Ras can be found upstream of both of them. PI3K has several substrates, but Akt, also known as protein kinase B (PKB), is thought to be its principal effector. There are three isoforms of Akt, named Akt1, Akt2 and Akt3, which are very similar in structure, size and substrate specificity, but differing slightly in tissue expression, for instance, Akt2 is the prevalent isoform in skeletal muscle (reviewed in Kandel and Hay, 1999). PI3Ks also exist in several isoforms according to their structure, specificity and mode of activation, and the classes IA and IB are the most studied (reviewed in Oudit *et al.*, 2004). The class IA PI3Ks respond mainly to RTKs, and growth factor and cytokine receptors (reviewed in Oudit *et al.*, 2004). This type of activation is initiated by binding of a PI3K regulatory subunit directly or through adaptor proteins to the cytosolic docking site of the receptor after which a PI3K catalytic subunit is recruited and the functional complex is formed (reviewed in Oudit *et al.*, 2004; reviewed in Rommel *et al.*, 2007). The class IB PI3Ks, on the other hand, respond mainly to GPCRs (reviewed in Oudit *et al.*, 2004). In this mode of activation the stimulated receptor binds the regulatory and catalytic subunits whose activity is further enhanced by G-protein βγ subunit and Ras (reviewed in Oudit *et al.*, 2004; reviewed in Rommel *et al.*, 2007).

The Akt activity is induced by a vast number of factors including cytokines, growth factors, cAMP/PKA agonists, phosphatase inhibitors, hypoxia and other cellular stresses (reviewed in Datta *et al.*, 1999). Initially, the active PI3K phosphorylates the cellular phosphatidylinositol-4,5-phosphate (PIP2) to form phosphatidylinositol-3,4,5-phosphate (PIP3) which promotes the binding of phosphoinositol-dependent kinase (PDK) 1 and Akt to the receptor complex (Figure 6A; reviewed in Scheid and Woodgett, 2003; reviewed in Oudit *et al.*, 2004). PDK1 is a direct upstream kinase of Akt required for its activation in a reciprocal manner: a phosphorylated motif on Akt is needed for initial PDK1 activation (Figure 6B; reviewed in Scheid and Woodgett, 2003). Additionally, a second phosphorylation event is needed for full Akt activity, and this is mediated by PDK2 which was recently identified as the mammalian target of rapamycin complex 2 (Figure 6B; Gao *et al.*, 2005; Sarbassov *et al.*, 2005). Active Akt then dissociates from the plasmamembrane

complex and translocates to the cytosol or nucleus where it can phosphorylate its substrates (Figure 6C, D).

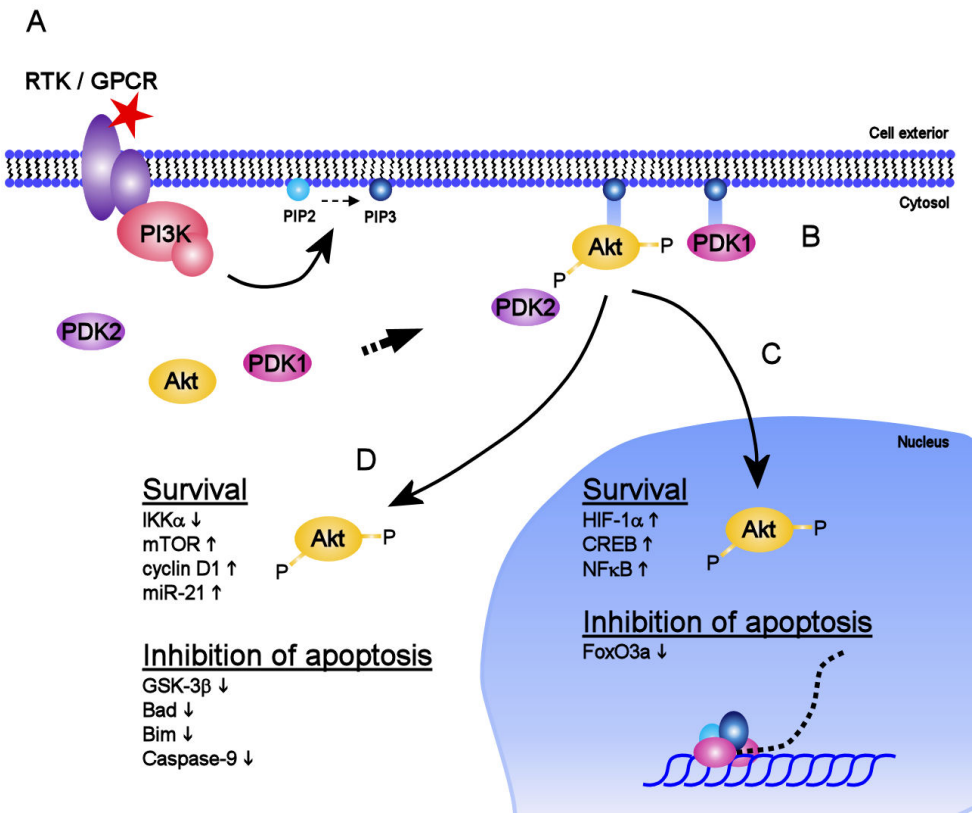

Figure 6. The PI3K-Akt pathway. A) Activated receptor tyrosine kinase (RTK) or G-protein coupled receptor (GPCR) recruits phosphatidyl-inositol-3-kinase (PI3K). PI3K induces the production of phosphatidylinositol-3,4,5-phosphate (PIP3) from PI-4,5-phosphate (PIP2). B) PIP3 serves as a plasmamembrane anchor to the phosphoinositol-dependent kinase (PDK-) 1 and Akt which are recruited from the cytosol. PDK1 and PDK2 activate Akt by phosphorylation. C) In the nucleus, Akt can promote cell survival by regulating directly or indirectly several transcription factors. D) Akt has also many cytosolic targets under direct or indirect regulatory phosphorylation that contribute to cell survival and inhibition of apoptosis.

The PI3K-Akt cascade is under strict regulation since it is a powerful inducer of proliferation; activating mutations in the pathway or its regulators are frequently encountered in cancers, as well (reviewed in McCubrey *et al.*, 2006). The best known inhibitors are the lipid phosphatases such as PTEN, myotubularin (MTM) and SH2-domain containing inositol 5-phosphatase 2 (SHIP2) which counteract the PI3K activity (reviewed in Oudit *et al.*, 2004). PTEN is able to dephosphorylate several types of PIPs and is considered an important tumor suppressor, while MTM and SHIP2 have a more restricted repertoire of substrates, but nevertheless play essential

roles in physiology of their respective expression sites (reviewed in Oudit *et al.*, 2004). PTEN and MTM display dual function phosphatase activity with an ability to dephosphorylate proteins (reviewed in Oudit *et al.*, 2004). Moreover, SHIP2 may act as either positive or negative regulator of Akt (reviewed in Oudit *et al.*, 2004). They function by removing the PIPs required for the activation of Akt and other substrates. However, upon receptor stimulation the PIP3 can induce transient local increase in Nox activity and subsequent H_2O_2 generation which will then inactivate the nearby PTPs and PTEN thus allowing unobstructed Akt signaling (reviewed in Rhee *et al.*, 2005b; reviewed in Rommel *et al.*, 2007).

Early on, Akt was recognized as a link between growth factor signaling and metabolic functions such as gene transcription, carbohydrate metabolism and protein synthesis which drive the cell growth, but soon it was also associated more directly with cell survival. It has also established a role as a protective factor in ischemic injuries (reviewed in Mullonkal and Toledo-Pereyra, 2007). It seems to be sufficient and necessary for growth factor-driven survival as suggested by studies where dominant negative Akt impaired the ability of IGF-I or serum to induce survival signaling, and vice versa, i.e. constitutively active Akt rendered the cells independent of growth factor stimuli (Dudek *et al.*, 1997; Kauffmann-Zeh *et al.*, 1997; Kennedy *et al.*, 1997; Kulik *et al.*, 1997). Accordingly, Akt induces a number of signaling pathways involved in the aforementioned functions, and the glycogen synthase kinase (GSK) 3 is a central effector in many of them due to the large number of its substrates. Glucose metabolism is enhanced e.g. by upregulation of glucose transporters and glycolysis, and inactivation of GSK3 which enables glycogen synthase activation (reviewed in Kandel and Hay, 1999). The activation of mTOR, cyclin D1 and again inhibition of GSK3 by Akt promote protein synthesis and cell growth by contributing to translation initiation (Figure 6D; reviewed in Kandel and Hay, 1999; reviewed in Huang and Manning, 2009). Akt also mediates survival gene transcription through upregulation of transcription factors, such as NFκB, HIF-1α and cAMP responsive element binding protein (CREB), which are mainly positive regulators of cell survival (Figure 6C; Mazure *et al.*, 1997; Du and Montminy, 1998; Kane *et al.*, 1999; Wang *et al.*, 1999). Recent studies have even suggested NFκB along with FoxO3a as the major effectors for PI3K-Akt signaling in cell proliferation (Tullai *et al.*, 2004; Terragni *et al.*, 2008). However, there are indications that chronic Akt activation could induce apoptosis by increasing ROS generation and subsequent FoxO3a activation (van Gorp *et al.*, 2006).

The pro-apoptotic factors Bad and caspase-9 of the intrinsic pathway are targeted by Akt which inactivates them through phosphorylation (Datta *et al.*, 1997; del Peso *et al.*, 1997; Cardone *et al.*, 1998). Moreover, CREB can mediate the anti-apoptotic Mcl-1 expression while NFκB can antagonize the apoptotic p53 tumor suppressor by promoting its degradation through murine double minute (Mdm) 2

upregulation and subsequent ubiquitination (Wang *et al.*, 1999; reviewed in Finnberg and El-Deiry, 2004). This results in maintenance of mitochondrial integrity, blockade of cytochrome *c* release and caspase activation, and thus inhibition of the apoptosis pathway (reviewed in Kandel and Hay, 1999). Additionally, Akt concensus phosphorylation sites have been found on e.g. several other caspases and IAPs (reviewed in Datta *et al.*, 1999). The inactivation of FoxO3a by Akt blocks the expression of pro-apoptotic and cell cycle arrest-related genes, and thereby allows the expression of many growth promoting genes. For instance, the expression of pro-apototic Bcl-2-protein Bim is markedly downregulated after Akt activation (Stahl *et al.*, 2002; Urbich *et al.*, 2005; Zhu *et al.*, 2008). Moreover, a recent study has shown direct suppression by active FoxO3a of the microRNA (miR-) 21 gene transcription leading to enhanced apoptosis (Wang and Li, 2010).

MicroRNAs (miRNAs) silence protein synthesis post-transcriptionally by binding to their target mRNAs in the cytosol, and then either preventing their translation or promoting their degradation (reviewed in Du and Zamore, 2005). An increasing number of miRNAs are associated with normal cellular processes, e.g. proliferation, survival, differentiation and apoptosis, in most tissues of the body (reviewed in Sayed and Abdellatif, 2011). The relatively widely expressed miR-21 has been implicated in e.g. survival of endothelial cells and myocytes through downregulation of PTEN and FasL, protection of myocardium from oxidative stress and in hyperproliferative cardiovascular diseases (reviewed in Cheng and Zhang, 2010; Ji *et al.*, 2007; Cheng *et al.*, 2009; Sayed *et al.*, 2010). Cancer-related miRNAs, oncomirs, have recently gained attention as important inducers or suppressors of malignant cell functions (reviewed in Sotiropoulou *et al.*, 2009). They target central genes involved in proliferation, apoptosis and angiogenesis such as Ras, p53, Bcl-2 and VEGF (Johnson *et al.*, 2005; Hua *et al.*, 2006; Bommer *et al.*, 2007; Tazawa *et al.*, 2007). MiR-21 is considered an oncomir, as well, due to its ability to promote invasion and metastatic growth of tumor cells by suppressing e.g. tumor suppressors programmed cell death (PDCD) 4 and PTEN (Meng *et al.*, 2007; Asangani *et al.*, 2008).

4.2.4 Ras-Erk1/2 and PI3K-Akt interactions

The two pathways discussed above have partly overlapping roles in cell survival, proliferation and metabolism, and they also share a number of downstream effectors. However, they can also promote opposing programs such as apoptosis-survival and proliferation-differentiation. This requires cross-talk between these routes in order not to waste the resources. Indeed, several studies have shown modulatory effects of these pathways directed to each other.

The first indications for Raf-Erk1/2 and PI3K-Akt interaction came from the myocyte differentiation studies by Rommel *et al.* (1999) and Zimmermann and

Moelling (1999). Their results suggested that Akt can inactivate Raf, presumably by phosphorylation, in differentiated myocytes, and that Akt activity induced muscle cell differentiation and hypertrophy while Raf-MEK-Erk1/2 did not. Previous studies had dealt with only one signaling pathway at the time, although indicating similar functions (Bennett and Tonks, 1997; Coolican *et al.*, 1997; Jiang *et al.*, 1998; Kaliman *et al.*, 1998). Further research revealed an intensive collaboration between the Raf-MEK-Erk1/2 and PI3K-Akt in myogenesis. Upon mitogenic stimuli in myoblasts the Erk1/2 pathway becomes activated and further induces e.g. Elk-1, thus resulting in a proliferation program and suppression of differentiation as determined by reduced muscle-specific marker, myogenin, expression (Rommel *et al.*, 1999; De Alvaro *et al.*, 2008). The IGF-I is a key factor in myogenic proliferation/differentiation, and has a biphasic effect on myocytes: before differentiation it induces Erk1/2 phosphorylation and promotes proliferation, but later switches to suppress Erk1/2 leading to enhanced differentiation (Adi *et al.*, 2002). The mechanism behind this remained obscure, however, although the researchers suggested the involvement of phosphatases acting negatively on Erk1/2. Upon differentiation stimuli, the PI3K-Akt is activated along with p38 and other kinases, but this is not sufficient since simultaneous downregulation of Raf-MEK-Erk1/2 pathway seems to be obligatory (Tiffin *et al.*, 2004; De Alvaro *et al.*, 2008). The appropriate activation/inactivation of the downstream transcription factors FoxO3a and NFκB was also found important for successful differentiation (De Alvaro *et al.*, 2008). The relative amounts of the Erk1 and Erk2 seem to in part determine the fate of the cell. In two studies Erk2 was found to be associated with mature myocyte formation and production of contractile proteins while Erk1 was dispensable (Sarbassov *et al.*, 1997; Li and Johnson, 2006).

5 Thyroid gland

5.1 Basic features and functions

The thyroid gland is the largest endocrine gland in human, weighing approximately 10-20 grams in adults. It situates in front of the neck, the two lobes covering the anterior part of trachea. The thyroid, and sometimes the nearby parathyroid glands as well, are enveloped in a connective tissue capsule. Thyroid tissue is follicular in structure and each follicle is surrounded by a basement membrane. The follicles are lined with a single layer of follicular epithelial cells, or thyrocytes (Strum and Karnovsky, 1970). They are the main functional cell type in thyroid gland being responsible for iodine uptake, thyroid hormone synthesis and secretion upon appropriate stimuli (Rousset *et al.*, 1989a; Taurog *et al.*, 1994; Dai *et al.*, 1996). Microvilli protrude from the thyrocyte surface to provide more area for hormone synthesis which takes place in the lumen immediately adjacent to the cell membrane (Strum and Karnovsky, 1970; Ofverholm and Ericson, 1984). The follicle lumen is

filled with colloid, a proteinous substance consisting mainly of thyroglobulin (Tg) which participates in the hormone synthesis and storage (Berndorfer *et al.*, 1996). Connective tissue between follicles contains an extensive vascular network to allow efficient exchange of nutrients, iodine and thyroid hormones. Additionally, embedded in the connective tissue are parafollicular, or C, cells which regulate blood calcium homeostasis together with the parathyroid glands.

The thyroid gland regulates development, growth and the rate of metabolism of cells, organs and whole individuals through the function of thyroid hormones triiodothyronine (T3) and thyroxine (T4). During the embryonic stage T3 and T4 participate in e.g. development of brains and muscles (Butler-Browne *et al.*, 1990; reviewed in Morreale de Escobar *et al.*, 2004). In adults they maintain the metabolic homeostasis by controlling energy and heat production as well as regulate e.g. bone formation and degradation, architecture of the nervous tissue and cardiovascular function (Monzani *et al.*, 1997; reviewed in Gomes *et al.*, 2001; Lebon *et al.*, 2001; reviewed in Bassett and Williams, 2003; reviewed in Klein and Danzi, 2007). Accordingly, excess or diminished amounts of either T3 or T4 cause a variety of symptoms of the nervous and cardiovascular systems, muscle function, gastrointestinal tract and energy metabolism.

Majority of thyroid hormones are produced in the T4 form which is processed into bioactive T3 or inactive reverse-T3 and T2 forms by thyroidal or tissue deiodinases, a family of enzymes specialized in cleaving the iodide residues from the hormone molecule (Maia *et al.*, 2005; reviewed in Gereben *et al.*, 2008). In circulation over 99% of both T3 and T4 are bound to carrier proteins, and the rest forms a pool of free hormone in blood plasma (reviewed in Sterling and Lazarus, 1977). The carrier proteins facilitate the transport and equal distribution of the hydrophobic hormones but release it readily to maintain the proportion of plasma free hormone at the correct level (Mendel *et al.*, 1987; reviewed in Schussler, 2000). After uptake into a cell, T3 exerts its function by forming a complex with one of the thyroid hormone receptors (TR) which then act as transcription factors. In most tissues, at basal level approximately 40-50% of all TRs are occupied (reviewed in Oppenheimer, 1979), and changes in plasma and cellular T3 content are quickly reflected in the amount of T3-TR complexes. This determines directly the impact of the thyroid hormone on the target tissue by increasing or decreasing expression of the appropriate genes.

The thyroid hormonogenesis and, therefore, the function of the gland are dependent on iodine supply from food and drink since iodide is integrated into the hormone molecule. Thus, the iodine level in the blood determines in part the activity of the thyroid gland so that abundant iodine suppresses thyroid growth and a deficiency stimulates it (reviewed in Dumont *et al.*, 1992). However, the main regulator of thyroid functions is the hypothalamus-pituitary-axis. Hypothalamus secretes thyrotropin releasing hormone (TRH) which induces the secretion of

thyrotropin (thyroid stimulating hormone, TSH) from the pituitary gland (reviewed in Chiamolera and Wondisford, 2009). TSH travels to the thyroid gland via blood circulation and binds specific TSH receptors (TSH-R) on the plasmamembrane of thyrocytes (reviewed in Chiamolera and Wondisford, 2009). TSH stimulates directly thyroid growth, differentiation, hormone synthesis and secretion depending on its concentration (Allgeier *et al.*, 1994). T3, in turn, exerts a negative feedback on the hypothalamus thereby downregulating its own synthesis and secretion (reviewed in Chiamolera and Wondisford, 2009). Other factors participating in positive thyroid regulation are insulin and IGF, and to lesser extent, FGF and EGF (reviewed in Dumont *et al.*, 1992).

5.1.1 TSH-R pathway

The TSH receptor is the most important thyroid growth and function regulating receptor. It is a G-protein coupled receptor with intra- and extracellular domains, and is located on the basolateral surface of follicular cells (Nagayama *et al.*, 1989a; Parmentier *et al.*, 1989; Loosfelt *et al.*, 1992). Post-translational modifications such as glycosylation and cleavage into two subunits ensure that the functional receptor can only be found in mature follicles (Misrahi *et al.*, 1994; Vu *et al.*, 2009). TSH is the main ligand for the TSH-R but it can also bind stimulating or blocking autoantibodies leading to hyperthyroid Grave's disease or hypothyroidism by activating or inhibiting the TSH-R function, respectively. In addition, other growth factors such as FGF, EGF, insulin and IGF-I have been associated with stimulation of thyroid function (Coulonval *et al.*, 2000; Medina and Santisteban, 2000; reviewed in Kimura *et al.*, 2001). Incubation with TSH also enhanced the effect of insulin/IGF in *in vitro* models (Takahashi *et al.*, 1991; Burikhanov *et al.*, 1996). However, there are species specific differences in the response of thyroid follicular cells to various stimulatory factors and the downstream pathways that become activated after TSH binding to its receptor (reviewed in Kimura *et al.*, 2001). Upon ligand binding the receptor-TSH complex may be internalized but the receptor is recycled back to the plasmamembrane while the TSH molecule is degraded in the lysosomes (Baratti-Elbaz *et al.*, 1999). Internalization is not obligatory, though, and receptor activation can be detected for relatively long even in the presence of TSH-R inhibitors (Neumann *et al.*, 2010).

Being a GPCR, the TSH-R interacts with intracellular G-proteins and can initiate two separate downstream signaling cascades (Figure 7A; Van Sande *et al.*, 1990). $G_s\alpha$ subunit promotes the upregulation of second messenger cyclic AMP leading to follicular cell proliferation, iodide organification and thyroid hormone secretion, whereas $G_q\alpha$ subunit promotes the PLC/Ca^{2+} route resulting in thyroid hormone synthesis (Allgeier *et al.*, 1994; Corvilain *et al.*, 1994). Furthermore, the pathways seem to exert active inhibition on each other thus avoiding simultaneous activation (Corvilain *et al.*, 1994). The role of the $G\beta\gamma$ subunit still remains largely

undiscovered but it has been associated with thyroid specific gene regulation (Zaballos *et al.*, 2008). The major factor determining which pathway is activated seems to be the concentration of TSH. Low TSH (in the range 1-5 mU/ml) has been shown to induce cAMP while higher concentration (in the range 3-30 mU/ml) promoted the PLC-Ca^{2+} pathway (Laurent *et al.*, 1987; Allgeier *et al.*, 1994).

Activation of the cAMP dependent route by $G_s\alpha$ follows the classical G-protein signal transduction pathway (Figure 7B). After dissociation from the G-protein trimer, $G_s\alpha$ activates the adenylate cyclase which in turn initiates a rapid cAMP generation. The main downstream effectors are the PKA and the PI3K which regulate each other as well as many thyroid specific genes such as TSH-R, Na^+/I^- symporter (NIS), thyroid peroxidase (TPO) and Tg through cAMP response element binding protein and modulator (CREB and CREM, respectively) transcription factors (Kogai *et al.*, 1997; Uyttersprot *et al.*, 1999; Brunetti *et al.*, 2000; Nguyen *et al.*, 2000; De Gregorio *et al.*, 2007). cAMP also induces the activation of signal modulators such as an exchange protein activated by cAMP (Epac) and a small GTPase Rap1 which both enhance the PKA and PI3K signaling (Figure 7B; Tsygankova *et al.*, 2001; Hochbaum *et al.*, 2008). The prominent mitogenic Ras small GTPase is implicated in the thyroid follicular cell proliferation (Figure 7B). Ras activation is needed to promote PI3K signaling in the G0-G1 transition in the cell cycle while binding of Ras to one of its major substrates, Raf-1, was suppressed (Ciullo *et al.*, 2001; De Gregorio *et al.*, 2007). The Ras function is not limited to PI3K co-activation but it seems to regulate a downstream signaling cascade other than Braf-Erk, and a few studies have named the small GTPase Ral guanine nucleotide dissociation stimulator (GDS) as a candidate (al-Alawi *et al.*, 1995; Miller *et al.*, 1997; Miller *et al.*, 1998). However, the Ras-MAPK/Erk pathway may be activated independently of TSH-cAMP and contribute to the follicular cell proliferation (reviewed in Kimura *et al.*, 2001).

Studies with rat, dog and human thyroid have shown that insulin or IGF-I is necessary in parallel with TSH for the follicular cells to potentiate proliferation (Burikhanov *et al.*, 1996; reviewed in Deleu *et al.*, 1999; Ariga *et al.*, 2000; Saito *et al.*, 2001). The PI3K and PKA routes are apparently activated differentially so that PKA is directly under cAMP regulation and initial PI3K activation is at least in part dependent on insulin/IGF-I stimulation (Coulonval *et al.*, 2000; Saito *et al.*, 2001; Fukushima *et al.*, 2008). Of these two, the PI3K pathway is considered to be the principal mitogenic route, and it further promotes the established Akt/mammalian target of rapamycin (mTOR)/p70 ribosomal S6 protein kinase (S6K)1 pathway and several effectors such as cyclins E, D1, A and cyclin dependent kinases (Figure 7B; Saito *et al.*, 2001; Yeager *et al.*, 2008). However, controversial results exist suggesting that mTOR activation is Akt-independent (Cass *et al.*, 1999; Brewer *et al.*, 2007). Even though it is likely that more than one mitogenic signaling pathway contributes to thyroid follicular cell proliferation, their relative impacts have been difficult to

establish. Moreover, their very low proliferation rate under normal conditions *in vivo* raises the question whether the *in vitro* studies can fully reconstruct the relevant signaling events.

The second branch of the TSH-R, starting from the $G_q\alpha$ subunit, activates another classical GPCR pathway by promoting PLC and leading to generation of two second messengers, inositol-trisphosphate (IP3) and diacyl glycerol (DAG)(Figure 7C). IP3 then induces the release of Ca^{2+} from the endoplasmic reticulum into cytosol, and DAG directly activates PKC (Figure 7C). Increase in the intracellular Ca^{2+} concentration was shown to be necessary for follicular cell H_2O_2 production, the essential step in thyroid hormonogenesis (Deme *et al.*, 1985). However, the cell is protected from oxidative stress by a negative feedback mechanism in which the H_2O_2 inhibits further Ca^{2+} release (Tornquist *et al.*, 2000). Simultaneous proliferation and hormone synthesis would be unfavorable to the cell, and the regulation of the TSH concentration is one mechanism to avoid this. When TSH is high both the G_s and $G_q\alpha$ pathways could be activated but a second putative mechanism exists to downregulate cAMP/PKA pathway while PLC/Ca^{2+} pathway remains intact: the sphingosine 1-phosphate mediated inhibition of adenylyl cyclase which abrogates cAMP generation (Okajima *et al.*, 1997).

Ultimately the TSH-R activation stimulates the activity of dual function oxidase (Duox), the oxidase producing H_2O_2 for thyroid hormone synthesis (Figure 7C). In human thyroid Duox2 seems to be more prominently expressed than Duox1, and patients with mutated Duox2 may suffer from thyroid hormone defects (reviewed in Ohye and Sugawara, 2010; Moreno *et al.*, 2002; Pachucki *et al.*, 2004; Ameziane-El-Hassani *et al.*, 2005). In rats, the two enzymes likewise co-exist but differ in expression level in *in vivo* tissue and *in vitro* cell lines (Rigutto *et al.*, 2007). However, the significance of two similar enzymes co-expressed in the same tissue has not been elucidated. Duox1 and 2 share some regulatory features such as the EF-hand motif which binds the cytosolic Ca^{2+} as a cofactor (Deme *et al.*, 1985; Ameziane-El-Hassani *et al.*, 2005). Since then several individual regulatory events have been found. For instance, PKA was shown to phosphorylate Duox1 while Duox2 was activated by PKC (Rigutto *et al.*, 2009). They also have respective maturation factors, Duox activators (DuoxA) 1 and 2, which only allow the release of properly folded enzyme from the endoplasmic reticulum, and are transported to the plasmamembrane with the enzyme where they determine the type of generated ROS (Figure 7C; Grasberger and Refetoff, 2006; Grasberger *et al.*, 2007; Morand *et al.*, 2009). Matching Duox/DuoxA pair produces H_2O_2 but mismatching ones produce mainly $O_2^{-}\bullet$ (Morand *et al.*, 2009).

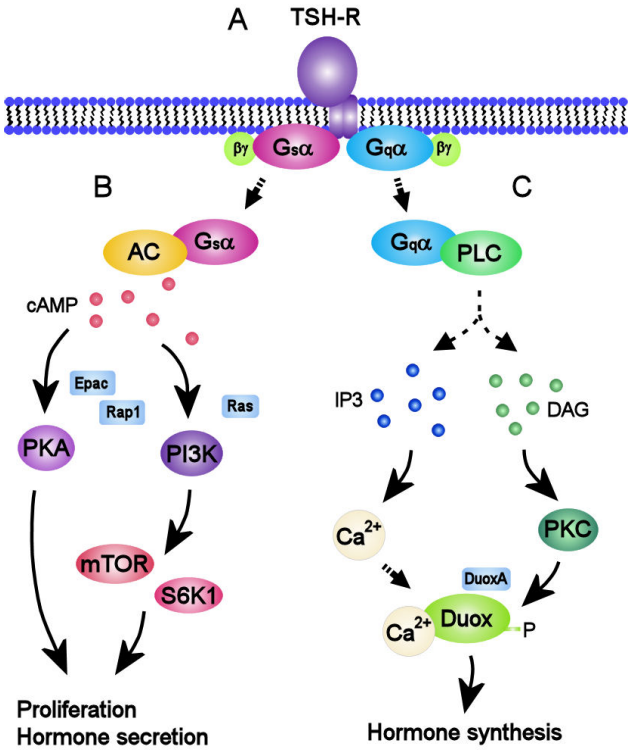

Figure 7. The TSH-receptor pathway. A) In human and rat thyroids the TSH-receptor (TSH-R) can bind and activate both $G_s\alpha$ and $G_q\alpha$ proteins. B) $G_s\alpha$ activates adenylate cyclase (AC) which in turn induces the activation of protein kinase A (PKA) and PI3K through cyclic AMP (cAMP). Mammalian target of rapamycin (mTOR) and ribosomal p70 S6 kinase (S6K) 1 mediate the proliferation of thyrocytes. C) $G_q\alpha$ activates phospholipase C (PLC) and the second messenger production (inositol-trisphosphate, IP3, and diacylglycerol, DAG). Ca^{2+} released from endoplasmic reticulum and activated protein kinase C (PKC) then induce dual oxidase (Duox)-derived H_2O_2 production essential for thyroid hormone synthesis. Both pathways are further modulated by various factors.

5.1.2 Thyroid hormone synthesis and secretion

Thyroid hormones are synthesized in response to TSH stimulation by the follicular cells which contain the necessary cellular machinery (Strum and Karnovsky, 1970; Tice and Wollman, 1974; Ofverholm and Ericson, 1984). The process is characterized by the production of H_2O_2 and therefore the cells are exposed to significant oxidative stress (Dupuy *et al.*, 1989; De Deken *et al.*, 2002). Additionally, they are relatively long-lived and their mitogenic activity is low which both increase the susceptibility to cumulative oxidative macromolecule damage (Coclet *et al.*, 1989; Saad *et al.*, 2006). The follicular cells use at least two mechanisms to avoid damage to macromolecules. Firstly, they express a variety of antioxidant enzymes and, secondly, they sequester the hormone synthesis in the follicle lumen by assembling the functional enzyme complex, termed thyroxisome, only on the outside surface of the cell membrane in microvilli (reviewed in Song *et al.*, 2007). The thyroid hormonogenesis is sensitive to oxidative stress which impairs the iodide uptake and processing by the gland (Nadolnik *et al.*, 2008). However, depletion of ROS induces downregulation of several thyroid specific proteins such as TPO, Duox, thyroglobulin (Tg) and pendrin anion transporter suggesting that a certain minimal level ROS production is needed for thyroid hormone synthesis (Poncin *et al.*, 2009).

The thyroid hormone synthesis begins by iodide organification. Iodide is efficiently transported from the blood stream into the follicular cells by NIS upon TSH receptor stimulation (Dai *et al.*, 1996). Iodide is then directly transported across the cell into the follicle lumen where it is oxidized by Duox2-derived H_2O_2 in a TPO catalyzed reaction (Taurog *et al.*, 1996; Royaux *et al.*, 2000). This active intermediate is covalently attached to thyroglobulin (Tg) to form mono- or diiodotyrosine (Lamas *et al.*, 1989; reviewed in Dunn and Dunn, 1999; Kessler *et al.*, 2008). In the next phase the TPO uses another molecule of H_2O_2 to oxidize a iodotyrosine into a radical form (Doerge *et al.*, 1994; Taurog *et al.*, 1994). Two activated iodotyrosines are then coupled in a condensation reaction, and form either T3 or T4 depending on how many iodide residues they contain.

Newly synthesized thyroid hormones are stored in the follicle lumen bound to a multimeric Tg (Berndorfer *et al.*, 1996). When the gland is stimulated by TSH the Tg-hormone complex is taken up into the cell by micropinocytosis and the vesicles are fused into endosomes (Bernier-Valentin *et al.*, 1990; Bernier-Valentin *et al.*, 1991). Specific endosomal proteases cleave the hormone molecules which are then transported into the blood stream, and the remaining Tg is degraded in lysosomes (Rousset *et al.*, 1989a; Rousset *et al.*, 1989b; Kostrouch *et al.*, 1991).

5.1.3 Thyroid antioxidant defense system

The main functional cells in the thyroid, the follicular cells, are highly susceptible and sensitive for oxidative stress during hormone synthesis, as discussed above. Indeed, even under physiological conditions the level of basal lipid peroxidation in thyroid is higher than in e.g. liver, kidney or heart (Nadolnik and Valentyukevich, 2007). Normal thyroid tissue has also markedly higher mutation rate than many other tissues which is probably due to the generally higher ROS production (Maier *et al.*, 2006). After stimulation of the oxidation process, however, the increase in the lipid peroxidation level was not as pronounced as in the other tissues suggesting the involvement of antioxidant enzymes (Nadolnik and Valentyukevich, 2007). Indeed, most of the Prx, Gpx and other enzymes relevant in the antioxidant defense system are expressed in thyroid (reviewed in Schweizer *et al.*, 2008).

As determined by enzyme activities, SOD1 was the principal form in the thyroids of the nine mammalian species studied whereas SOD2 and SOD3 varied more (Marklund, 1984a). For instance, in human, pig and rabbit thyroid activity of SOD3 was higher than that of SOD2, but in cow and dog thyroid it was lower; this study did not provide data for rat (Marklund, 1984a). However, total SOD and catalase activities were lower than in the liver, kidney or heart as compared to other antioxidant enzymes suggesting that they are not the principal defense mechanism in thyroid (Nadolnik and Valentyukevich, 2007). Despite this, the expression of SOD1 and 2 was enhanced by TSH stimulation indicating that they are needed to detoxify the

ROS produced during high cellular activity (Verma *et al.*, 1991; Nishida *et al.*, 1997; Singh *et al.*, 1997). Recently, the superoxide producing Nox4 oxidase was found to be expressed in normal thyroid tissue and in differentiated thyroid cancer tissue, and it was also responsive to TSH (Weyemi *et al.*, 2010). Therefore, the SODs could function to neutralize Nox4 produced O_2^{-}• upon receptor stimulation, but SOD1 and 2 are probably more relevant in this scheme than SOD3 since Nox4 was located inside the follicular cells (Weyemi *et al.*, 2010). It is not known if SODs participate in H_2O_2 production for hormone synthesis.

A wide variety of Gpx (1, 3 and 4) and Prx (1, 2, 3, 4 and 5) enzymes is expressed in thyroid gland either constitutively or TSH-dependently (Schweizer *et al.*, 2008). The Gpx-glutathione system seems to be especially important for the maintenance of basal redox homeostasis of the follicular cells (Nadolnik and Valentyukevich, 2007). The Gpx expression was shown to be in the same range with liver and heart, but glutathione reductase and reduced glutathione were both relatively high in thyroid indicating a significant role (Nadolnik and Valentyukevich, 2007). Some of these antioxidants, such as Gpx3, may also have a more pronounced role in the modulation of the H_2O_2 availability in thyroid hormone synthesis (Howie *et al.*, 1995). Prx1 and 2 are constitutively expressed in the follicular cells and have been shown to regulate the intracellular H_2O_2 levels and protect them from H_2O_2 induced apoptosis (Kim *et al.*, 2000).

5.2 Thyroid disorders

Multiple disorders with varying degrees of severity can occur in the thyroid gland. Clinically the symptoms may present as hyper- or hypothyroidism and benign or malignant growth of the gland. Characteristically, the symptoms develop relatively slowly over weeks or years, and affect the whole body due to the function of thyroid hormones as universal regulators of growth and metabolism.

5.2.1 Goiter, hypo- and hyperthyroidism

Goiter is a common symptom in different thyroid disorders and may be induced by a variety of genetic and environmental factors such as deficient or excess dietary iodide, defective iodide metabolism and hormone synthesis, as well as autoantibodies against TSH-R or TPO (Ieiri *et al.*, 1991; reviewed in Dumont *et al.*, 1992; Delange, 1994; Everett *et al.*, 1997; Kotani *et al.*, 1999; Santos *et al.*, 1999; Varela *et al.*, 2006). Additionally, female gender, increasing age and physiological state, e.g. pregnancy, can predispose to abnormal thyroid function and goiter (reviewed in Glinoer, 1997; Canaris *et al.*, 2000; Morganti *et al.*, 2005; Giles Senyurek *et al.*, 2008). In most cases the ultimate cause for characteristic follicular cell hyperproliferation is TSH-R pathway stimulation by excess TSH production, absence of negative T3 feedback, stimulation of the receptor directly by antibodies or activating mutations of the TSH-

R/cAMP cascade components (reviewed in Dumont *et al.*, 1992). Furthermore, iodide has an inhibitory effect on thyroid growth which is removed while the thyroidal iodide stores are depleted by enhanced hormone production or defective iodide trapping over the course of disease (reviewed in Dumont *et al.*, 1992).

A goiter can be diffuse i.e. the whole gland is enlarged, or nodular with one or more hyperplastic nodules. If the follicular cells are functional and continue to produce thyroid hormone, the goiter is called toxic, and the nodules are referred to as "hot" in radioiodine imaging due to their ability to take up radioactive iodide (reviewed in Griggs and Divgi, 2008). These cells are still differentiated thyroid cells, and are rarely malignant (reviewed in Ashcraft and Van Herle, 1981). On the other hand, the downregulation of thyroid specific gene expression and subsequent deficient iodide uptake indicates a possible malignant transformation (Gerard *et al.*, 2003). In radioiodine imaging such nodules or areas of the gland appear "cold" (reviewed in Griggs and Divgi, 2008). A goiter without the symptoms of hyperthyroidism is referred to as nontoxic.

Hypothyroidism develops when the level of serum thyroid hormones is insufficient. Since they are positively regulating a number of body functions the characteristic symptoms are overall slowing down of the metabolism, fatigue, and defective function of the heart, respiration, digestion and the nervous system (reviewed in Guha *et al.*, 2002). Additionally, goiter is common in a number of hypothyroid disorders. Causes for hypothyroidism may be genetic mutations in the hormone synthesis machinery, blocking autoimmune antibodies for TSH-R or environmental, the most prominent worldwide being dietary iodine deficiency (reviewed in Topliss and Eastman, 2004). The symptoms in iodine deficiency-related hypothyroidism can be reversed with dietary iodide supplementation but e.g. autoimmune-related hypothyroidism requires medical thyroxine administration (reviewed in Guha *et al.*, 2002).

Hyperthyroidism involves excess thyroid hormone production. The symptoms are mostly opposite to those of hypothyroidism due to the stimulating effect of T3 and T4 on the cells and tissues of the body. They include e.g. increased heart and respiratory rate, muscle and bone wasting, fatigue and cardiac arrhythmias (reviewed in Cooper, 2003). Hyperthyroidism may be caused by various autoimmune thyroid disorders, activating mutations in TSH-R or downstream components of the TSH-R pathway, thyroiditis or, to a lesser extent, environmental factors which induce abnormally high thyroid hormone synthesis or secretion (reviewed in Topliss and Eastman, 2004). A goiter may also be present due to the TSH-R stimulation causing hyperplasia of follicular cells. In some cases the large number of follicular cells with normal level of hormonal activity may bring about hyperthyroidism (Akcurin *et al.*, 2008; Hebrant *et al.*, 2009). Hyperthyroidism is generally treated with surgical removal of a part of or the whole gland, destruction of hyperactive cells by radioactive iodide or thyroid function suppressing medication (reviewed in Cooper, 2003).

5.2.2 Benign hyperproliferation disorders: AA and FNAH

Autonomous adenoma (AA) and familial non-autoimmune hyperthyroidism (FNAH) represent variants of the same disorder: genetic hyperthyroidism (Hebrant *et al.*, 2010). A number of TSH-R mutations have been described in the two disorders, and approximately 24% of them are common to both (Figure 8, left; Hebrant *et al.*, 2009). The main difference is the nature of the mutation: in AA it is a sporadic event in a single follicular cell usually occurring post-natally whereas in FNAH it is a germline mutation affecting the whole gland (Parma *et al.*, 1997).

AA generally presents as a solitary encapsulated nodule which is able to efficiently metabolize iodide and secrete thyroid hormones without TSH stimulation, i.e. autonomously, more efficiently than normal thyroid tissue (Figure 8, left; Van Sande *et al.*, 1988; Deleu *et al.*, 2000). Therefore they appear as "hot" nodules and can be readily detected by radioiodine imaging. Other clinical characteristics are low serum TSH and normal or increased thyroid hormone levels. The tumors are relatively slow-growing but once they get large enough and are provided with sufficient iodide they can induce hyperthyroidism (Ermans and Camus, 1972; Deleu *et al.*, 2000). The proliferating cells have been located mainly to the periphery of the tumor which may account for the low growth-rate although there is very little apoptosis in the tumor (Deleu *et al.*, 2000). AA is most prevalent in iodide deficient areas and commonly occurs in women and elderly people (Bransom *et al.*, 1979; Parma *et al.*, 1997). AA incidence is highest in the sixth decade of life but some cases have also been reported in 9-16 year old children (Bransom *et al.*, 1979; Schwab *et al.*, 2009). The management strategy for AA varies depending on the extent of hyperthyroidism and the size of the nodule, and can include surgery, radioiodine treatment or thyroid suppressing drugs (reviewed in Delbridge, 2006).

Most AA nodules are of monoclonal origin and harbor genetic mutations in the TSH-R or in the downstream cAMP pathway components (Hicks *et al.*, 1990; Namba *et al.*, 1990a; Trulzsch *et al.*, 2001). Majority (appr. 60-80%) of the mutations occur in the TSH-R and a smaller proportion (appr. 5-25%) in the $G_s\alpha$ subunit (Russo *et al.*, 1995; Duprez *et al.*, 1997; Parma *et al.*, 1997; Trulzsch *et al.*, 2001). These mutations are usually activating and, therefore, result in elevated proliferation rate but are rarely malignant. Additionally, shut-down of negative feedback mechanisms controlling the proliferation have been suggested recently (van Staveren *et al.*, 2006). The PLC/Ca^{2+} pathway seems largely unaffected (Deleu *et al.*, 2000). In general, in AA the expression of many metabolism, inflammation, cell-cell contact and apoptosis-related genes is altered which may explain the increased proliferation, lower apoptosis and absence of inflammation (Wattel *et al.*, 2005; Hebrant *et al.*, 2009). Thyroid specific gene expression is altered variably with an increase in NIS and TPO in most adenomas which may be the cause for observed increased iodide metabolism, but there is no consistent effect on Tg or TSH-R which can be either up- or downregulated (Figure 8,

left; Deleu *et al.*, 2000). Telomere length was shown to be significantly smaller in adenomatous follicular cells than in normal cells indicating that in most adenomas the cells have a limited lifespan due to the absence of telomerase even though they are proliferating faster (De Deken *et al.*, 1998). Telomerase activity has been recently confirmed as a useful marker for thyroid malignancy when cytological examination gives ambiguous results (Lerma and Mora, 2005; Guerra *et al.*, 2006).

FNAH affects the entire thyroid gland due to an autosomal mutation, and the first symptoms usually appear during the first years of life, but a later onset is not rare (reviewed in Gozu *et al.*, 2010; Karges *et al.*, 2005). Furthermore, the disorder does not show sexual predominance (Hebrant *et al.*, 2009). Diffuse goiter is the major finding along with hyperthyroidism, and patients may develop also a multinodular goiter later in life (Figure 8, left; reviewed in Gozu *et al.*, 2010). Similarly to AA, serum TSH levels are generally low and thyroid hormones normal or elevated (Hebrant *et al.*, 2009). Follicular cells in FNAH goiter are more sensitive to TSH than in normal thyroid but do not show increased hormone synthesis (Hebrant *et al.*, 2009). Additionally, the development of goiter is more likely to result from decreased apoptosis and, therefore, the hyperthyroidism seems to be caused by the larger number of cells in the gland (Akcurin *et al.*, 2008; Hebrant *et al.*, 2009). Generally, the course and severity of the disorder varies considerably even within the family although the inflicted members share the same mutation referring to additional genetic, epigenetic and environmental factors (reviewed in Gozu *et al.*, 2010). Total or near total thyroidectomy or radioiodine ablation is often needed to control relapses to hyperthyroidism, but in milder disease anti-thyroid medication may suffice (reviewed in Hebrant *et al.*, 2011).

At least 17 different mutations of the TSH-R have been described in the FNAH families (reviewed in Gozu *et al.*, 2010). These mutations were shown to induce a 2-7 fold higher cAMP production in the follicular cells thus promoting their proliferation and survival; however, also contradictory results have been obtained showing lowered cAMP induction after TSH stimulation (Hebrant *et al.*, 2009). The involvement of $G_s\alpha$ subunit mutations is unknown. A microarray analysis with AA and FNAH tissue established a highly similar gene expression patterns in the two disorders with over 90% of genes regulated in the same way (Hebrant *et al.*, 2009). This was considered as evidence that AA and FNAH are variants of the same disorder, genetic hyperthyroidism. However, they share only few same TSH-R mutations, and it has been suggested that in AA the mutations are responsible for a more severe phenotype (Tonacchera *et al.*, 1996; Alberti *et al.*, 2001; Corvilain *et al.*, 2001). The close to normal iodide metabolism and H_2O_2 generation of follicular cells in FNAH indicate that the thyroid specific gene expression is not markedly altered with the exception of enhanced sensitivity to TSH stimulation (Figure 8, left; Hebrant *et al.*, 2009).

5.2.3 Thyroid cancers: PTC and ATC

Approximately 5% of thyroid nodules turn out to be malignant tumors (reviewed in Riesco-Eizaguirre and Santisteban, 2007). Endocrine gland cancers account for a minority of all cancers, but within this group over 90% of cancers are those of the thyroid gland (reviewed in Correa and Chen, 1995). The thyroid cancers are broadly divided into four groups based on the type of cells affected and the differentiation state: *1)* papillary carcinoma (PTC) is the most benign form with well-differentiated but non-functional follicular cells, *2)* follicular carcinoma (FTC) is rather similar to PTC but is more aggressive and may retain some iodide binding capacity, *3)* anaplastic thyroid carcinoma (ATC) is the most aggressive form characterized by undifferentiated cells with high capacity for metastasis formation, and *4)* medullary carcinoma (MTC) initiates from the parafollicular cells and is often metastatic but to a lesser extent than ATC. Additionally, there are several subtypes and mixed forms of these cancers.

PTC is the most common form (appr. 70%) followed by FTC (appr. 17,5%), MTC (2-5%) and ATC (2-3%)(reviewed in Correa and Chen, 1995). The PTC and FTC are more common in women but the MTC and ATC show no sexual predominance (reviewed in Correa and Chen, 1995). The age of onset is relatively low for PTC with the incidence rate rising sharply from the late teens onward and gradually declining later in life; the other carcinomas usually occur more often in the second half of life (reviewed in Correa and Chen, 1995). The treatments for thyroid tumors are essentially the same as for thyroid nodules including surgery, thyroid suppressing medication and radioiodine ablation. Problems arise from the iodide trapping deficiencies of cancer cells which impede the uptake of cytotoxic doses of radioiodine and complicate the detection of recurrent cancer as well as from inconclusive determination between adenomas and carcinomas in 15-20% of fine needle aspiration (FNA) samples, the most used cytological diagnostic technique (reviewed in Riesco-Eizaguirre and Santisteban, 2007). However, screening for mutations in the most common genes involved in thyroid cancer can be an efficient method to discriminate between benign and malignant neoplasms (Mathur *et al.*, 2010; Moses *et al.*, 2010). There are more than 70 putative thyroid cancer markers, including the traditional p53 and Ras, various growth factors and their receptors, anti-apoptotic Bcl-2 and the transcription factor Ets-1 in addition to the thyroid-specific genes, but these have not reach a significant use in clinical diagnostics due to variable sensitivities and specificities (reviewed in Gomez Saez, 2010).

One clear sign of malignancy in thyroid tissue is impaired thyroid hormone synthesis, i.e. the cancer tissues appear cold in radioiodide imaging. The main reason for this is defective Tg iodination due to absent or misregulated NIS, pendrin, TPO and the Duox enzymes (Figure 8, right; Gerard *et al.*, 2003). Thyroid cancers are frequently found to harbor mutations in the well-established oncogenes such as p53, Ras and Braf. However, they have some unique characteristics: the overall rate of

malignant transformation is very low despite the high incidence of benign nodules; the growth rate of a well-differentiated thyroid tumor is slower than in well-differentiated cancers of e.g. breast or lung; thyroid tumors have negligible apoptosis rate; and the patient survival rates remain high although the well-differentiated tumors frequently metastasize (reviewed in Riesco-Eizaguirre and Santisteban, 2007). Furthermore, the gradual accumulation of PI3K/Akt and Ras/MAPK mutations may convert the initially benign tumor into a more malignant form as is the case with ATC arising from PTC or FTC (Figure 8, right; Hou *et al.*, 2007; Wang *et al.*, 2007).

The main oncogenes responsible for thyroid cancers include rearranged during transfection (RET)/PTC, Ras and Braf, although RET/PTC can be found in adenomas as well (Figure 8, right; Ishizaka *et al.*, 1991; reviewed in Riesco-Eizaguirre and Santisteban, 2007). The TSH-R and $G_s\alpha$ mutations causing AA or FNAH hyperthyroidism usually present as benign neoplasms without cancerous characteristics and are believed to be resistant to malignant transformation (reviewed in Riesco-Eizaguirre and Santisteban, 2007); however, some autonomously functioning benign nodules have been found to contain PTC or other carcinomas (Tfayli *et al.*, 2010; Majima *et al.*, 2005; Bitterman *et al.*, 2006; Cerci *et al.*, 2007; Kim *et al.*, 2007; Nishida *et al.*, 2008; Uludag *et al.*, 2008).

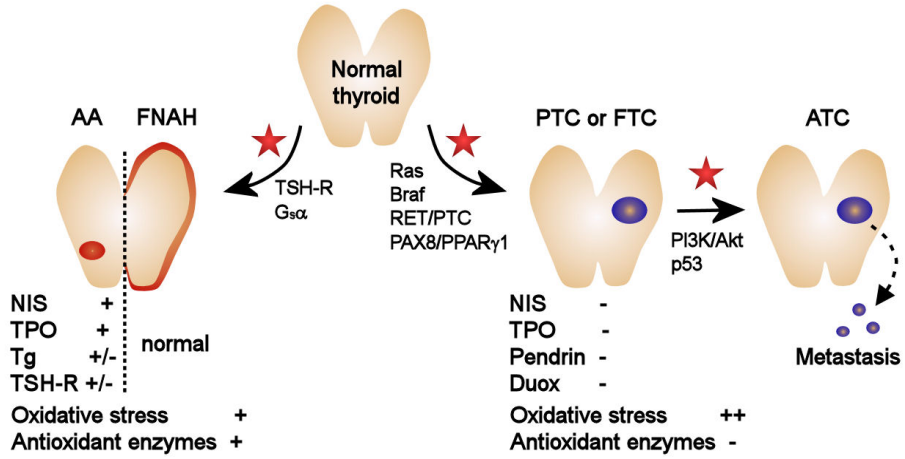

Figure 8. Thyroid hyperproliferation disorders. Left: Activating mutations in the thyrotropin receptor (TSH-R) or $G_s\alpha$ subunit induce autonomous adenoma (AA) or familial non-autoimmune hyperthyroidism (FNAH) characterized by a solitary nodule or diffuse goiter, respectively. In AA, the iodide is taken up and organified more efficiently than in normal tissue while in FNAH the large number of cells produce thyroid hormones in excess. Higher oxidative stress is counterbalanced by increased antioxidant enzyme expression. Right: Mutations in several oncogenes induce malignant transformation into papillary or follicular thyroid carcinomas (PTC or FTC, respectively). Accumulation of further mutations may promote the onset of poorly differentiated anaplastic thyroid carcinoma (ATC) with metastatic capacity. Many thyroid specific genes are downregulated in thyroid cancers which results in loss of the ability to take up and process iodide. Higher oxidative stress is further increased by inactivated antioxidant enzymes.

Normally, the RET gene encodes a tyrosine kinase receptor and is functionally active in a proportion of neural crest derived cells (Takahashi and Cooper, 1987; Takahashi *et al.*, 1989; Nakamura *et al.*, 1994). It is an important signaling receptor in the nerve development and organogenesis during embryogenesis (Knight *et al.*; Pachnis *et al.*, 1993; Schuchardt *et al.*, 1994). In thyroid cancers it is found recombined into the N-terminal part of another gene, and there are several RET/PTC variants distinguished by the associated gene (Grieco *et al.*, 1990; Bongarzone *et al.*, 1993; Santoro *et al.*, 1994; Klugbauer *et al.*, 1996; Klugbauer *et al.*, 1998). RET/PTC1 and 3 are found in approximately 90% of PTC cases (reviewed in Tallini and Asa, 2001). The resulting fusion proteins are activated spontaneously by dimerization and induce several downstream signaling pathways, e.g. Ras-MAPK and PI3K-Akt, through different phosphorylated tyrosine residues (Arighi *et al.*, 1997; Murakami *et al.*, 1999a; Murakami *et al.*, 1999b; Melillo *et al.*, 2001b; reviewed in Tallini and Asa, 2001). These residues have been shown to act as docking sites for Grb7/10, PLCγ-PKC, insulin receptor substrate (IRS-) 1, and Shc-FRS2 which mediate the Ras-MAPK or PI3K-Akt cascade activation (Arighi *et al.*, 1997; Melillo *et al.*, 2001a; Melillo *et al.*, 2001b). However, RET/PTC is a relatively weak oncogene since it does not allow TSH-independent proliferation or induce genomic instability, and requires Ras/MAPK activation for dedifferentiation (Knauf *et al.*, 2003; Wang *et al.*, 2003).

The Ras/Braf/MEK/MAPK mitogenic pathway is active in 30% of all cancers and also very prominent in thyroid cancers (reviewed in Bos, 1989; reviewed in Riesco-Eizaguirre and Santisteban, 2007). Mutations of the three different Ras genes conferring constitutive activation have been found in all types of thyroid cancers, but more often in the poorly differentiated carcinomas and ATC (Lemoine *et al.*, 1988; Namba *et al.*, 1990b; Garcia-Rostan *et al.*, 2003; Vitagliano *et al.*, 2006). In general, Ras mutations induce cell hyperproliferation, but constitutive Ras overexpression has been found to promote apoptosis through e.g. the MAPKs or p53 (Chen *et al.*, 1998; Chen *et al.*, 2000). The situation is more complicated in thyroid cells which normally depend on TSH for growth. Mutated Ras in the presence of TSH first induces proliferation but later launches the apoptosis cascade via Erk and JNK; without TSH, however, the same Ras mutation inhibits apoptosis and allows growth independently of the hormone through new mutations induced by genomic instability (Saavedra *et al.*, 2000; Shirokawa *et al.*, 2000). Furthermore, Ras activation can also induce DNA damage and dedifferentiation of the follicular cells by inhibiting thyroid specific genes including Tg, TPO, NIS and Duox (Francis-Lang *et al.*, 1992; Saavedra *et al.*, 2000; De Vita *et al.*, 2005). Accordingly, it is evident that Ras can interfere with a number of cellular functions and thereby can strongly contribute to the aggressive phenotype.

Early Braf activation is very common in PTC but not in other well-differentiated thyroid cancers (Nikiforova *et al.*, 2003; Xing, 2005). It is also frequently found in PTC derived ATCs (Namba *et al.*, 1990b; Nikiforova *et al.*, 2003).

Constitutively active Braf can stimulate Erk activation independently of Ras, but there is discrepancy whether it can be considered an aggressive oncogene. Similarly to Ras, Braf mutations can induce dedifferentiation, downregulation of thyroid specific genes, genomic instability and apoptosis (Mitsutake *et al.*, 2005; Riesco-Eizaguirre *et al.*, 2006). However, they cannot permit TSH-independent growth (Mitsutake *et al.*, 2005). On the other hand, the genomic rearrangements promoted by mutated Braf may further contribute to the more aggressive phenotype and poorer prognosis of these tumors (Knauf *et al.*, 2003; Mitsutake *et al.*, 2005; Riesco-Eizaguirre *et al.*, 2006). Another possibility is that the higher preference of Braf to induce matrix metalloproteinase expression enhances the invasive phenotype of the cancer cells (Mesa *et al.*, 2006).

Some common genes associated with the thyroid tumorigenesis and tumor progression are shown in the figure 8 on the right. In addition to Ras, Braf and RET/PTC, the PI3K/Akt survival pathway is frequently inflicted and is constitutively active, or alternatively the Akt regulating PTEN lipid phosphatase is inactivated (Ringel *et al.*, 2001; Vasko *et al.*, 2004; Garcia-Rostan *et al.*, 2005; Wu *et al.*, 2005; Yeager *et al.*, 2007). Also the p53 tumor suppressor is impaired especially in the aggressive forms of thyroid cancers (Ito *et al.*, 1992; Fagin *et al.*, 1993). Thyroid specific gene expression may be disrupted by chromosomal translocation and fusion of the thyroidal transcription factor paired box gene (PAX) 8 gene and a nuclear receptor peroxisome proliferator-activated receptor (PPAR) γ1 (Kroll *et al.*, 2000). Further alterations in gene expression can be induced by epigenetic silencing by hypermethylation of genes involved in follicular cell function or tumor suppressing, and upregulation of a subset of microRNAs such as miR-146a and miR-21 (reviewed in Kouniavsky and Zeiger, 2010; reviewed in Riesco-Eizaguirre and Santisteban, 2007).

Suitable genetic mutations and genetic susceptibility are not the only factors contributing to the thyroid cancer development (reviewed in Krohn *et al.*, 2007). Deficiencies of important dietary minerals, such as iodide or selenium, or dietary goitrogens affect the thyroid and antioxidant enzyme functions. Pregnancy may induce hyperplasia of the follicular cells and goiter promoting the expansion of possible silent mutations. Tobacco smoke intensifies the oxidative stress and significantly contributes to DNA damage and subsequent mutagenesis. Lastly, age-related accumulation of mutations and weakening of cellular mechanisms for DNA repair and antioxidant defense expose the cells to transformation. In conclusion, in thyroid carcinogenesis a number of factors have to be combined in a single follicular cell before it can undergo malignant transformation, clonal expansion and form a tumor.

5.2.4 Oxidative stress and the antioxidant defenses in thyroid disorders

The origin of the first mutations ultimately resulting in thyroid tumors is unknown. Due to the nature of thyroid hormone synthesis and therefore the high rate of ROS generation, oxidative stress has been implied as one factor contributing to DNA damage and oncogene activation (reviewed in Krohn *et al.*, 2007). Even though H_2O_2 is rather inert towards DNA, a recent *in vitro* study suggested that it can still be a mutagenic factor in the follicular cells (Driessens *et al.*, 2009). Furthermore, the H_2O_2 metabolism is closely intertwined to that of iodine so that available iodine metabolites inhibit H_2O_2 generation by Duox enzymes resulting in decreased hormone synthesis and protection from hyperthyroidism (Corvilain *et al.*, 1988; Ohayon *et al.*, 1994; Panneels *et al.*, 1994; Cardoso *et al.*, 2001; Morand *et al.*, 2003). Iodine deficiency, on the other hand, promotes TSH signaling which in turn activates the iodine uptake and organification as well as thyrocyte proliferation with concomitant ROS generation (Uyttersprot *et al.*, 1997; Maier *et al.*, 2007). Accordingly, iodine deficiency can stimulate the development of oxidative stress and DNA damage in the follicular cells (Maier *et al.*, 2007). Therefore, it is not surprising that thyroid nodules and other malignancies have higher incidence in iodine deficient areas which include several European countries, as well (Belfiore *et al.*, 1987; Knudsen *et al.*, 2000; Vitti *et al.*, 2003).

Oxidative stress generally increases in iodine-deficiency induced goiter and toxic or non-toxic thyroid nodules as determined by the increase of oxidized lipids and DNA (Figure 8, left; Erdamar *et al.*, 2010; Maier *et al.*, 2007; Poncin *et al.*, 2008). However, there are also contradicting results showing no difference in the redox status between normal and goitrous or adenomatous thyroid tissues (Sadani and Nadkarni, 1996). Possible reasons for this discrepancy could be the natural variation in thyroid neoplasm function, sensitivity of the assay, medication given to thyroid disorder patients or the enhanced antioxidant defense which can counteract the ROS stress during goiter formation (Poncin *et al.*, 2008). As a response to heightened oxidative stress, the antioxidant enzymes, such as SOD3, SOD2, Gpx4, Prx3 and Prx5, are upregulated in goiter, but again some studies have found them downregulated in e.g. multinodular goiter (Figure 8, left; Erdamar *et al.*, 2010; Nishida *et al.*, 1993; Hasegawa *et al.*, 2002; Maier *et al.*, 2007; Poncin *et al.*, 2008). Maier *et al.* (2007) suggested that the antioxidant defense is an initial response during the acute phase (the first 8 weeks) of iodine deficiency to eliminate the oxidative stress, but later (3 months-1 year) the gland adapts to low iodine by upregulating TSH-R, TPO and NIS to ensure normal thyroid function. However, dietary deficiency of selenium, an important cofactor for several of the thyroidal antioxidant enzymes, impairs the ability of follicular cells to reduce the oxidative stress and, thus, become more susceptible to malignant transformation (reviewed in Duntas, 2006; reviewed in Schweizer *et al.*, 2008).

The data concerning oxidative stress in thyroid cancer is more consistent than in the case of goiter. PTC and FTC show distinctly higher oxidized lipid and DNA levels than the normal adjacent tissue (Figure 8, right; Erdamar *et al.*, 2010; Sadani and Nadkarni, 1996). The rarity of ATC complicates similar analysis, but the studies describing strongly reduced Gpx, cytosolic SOD1 and catalase in those tissues indicate oxidative stress (Figure 8, right; Hasegawa *et al.*, 2002; Hasegawa *et al.*, 2003). One candidate as a source of ROS in thyroid tumors is the Nox4 which was found robustly expressed in the cytosol of cancer cells (Weyemi *et al.*, 2010). The role of the Duox enzymes in tumor oxidative stress is still undetermined. Their expression was found to vary from tumor to tumor without a consistent pattern or correlation with the tumor type (Lacroix *et al.*, 2001; Gerard *et al.*, 2003). A histological analysis revealed their presence in the cytosol of the majority of the cancer cells instead of the normal apical cell membrane, and thus, provided that the enzymes are functional, could contribute to the oxidative stress (Lacroix *et al.*, 2001; Gerard *et al.*, 2003).

The antioxidant gene expression is impaired at variable levels in PTC, FTC and ATC resulting in inability to handle the ROS produced in metabolic events and, therefore, in increased oxidative stress (Figure 8, right). For instance, the expression levels of Gpx, catalase and SOD1 have been found to be weakly downregulated in the differentiated PTC and FTC but more strongly in ATC (Erdamar *et al.*, 2010; Hasegawa *et al.*, 2002; Hasegawa *et al.*, 2003). However, the expression of the mitochondrial SOD2 seems more ambiguous: one study showed low expression in the differentiated tumors while another showed higher expression as compared to normal tissue (Nishida *et al.*, 1993; Hasegawa *et al.*, 2003). The two studies agreed, though, that in ATC the SOD2 expression is close to that in normal tissue. These apparent reciprocal changes in tumor progress and their oxidative stress levels have lead to investigations whether the altered expression of antioxidant genes could be used as a biomarker for malignant transformation. For instance, Prx1 and Prx2 have been proposed as possible candidates since their expression was clearly and consistently decreased in the studied PTC samples (Yanagawa *et al.*, 1999; Brown *et al.*, 2006).

AIMS OF THE STUDY

SOD3 is an efficient antioxidant enzyme capable of attenuating the extent of O_2^{-}• derived tissue damage in various animal models of heart and lung ischemia or hyperoxia. However, there are a limited number of studies investigating its function in the skeletal muscle and in the thyroid gland. In previous studies, the focus has been in the antioxidative effect of SOD3 while fewer investigations have addressed its role in cellular signaling events although ROS are currently recognized as signaling agents. Local artificial elevation of SOD3 expression in the skeletal muscle could be beneficial for the recovery from ischemia where the ROS balance is disturbed. In thyroid gland, SOD3 has been studied very little despite its rather high expression which can indicate a functional role.

This study was carried out to fulfill the following aims:

I Characterization of the mechanisms by which exogenous SOD3 reduces the inflammatory reaction in a hind limb ischemia model.

II Characterization of the role of exogenous SOD3 in regulation of survival and apoptosis in a hind limb ischemia model.

III Characterization of the role and regulation of SOD3 in normal thyroid gland.

IV Determination of SOD3 expression changes in thyroid disorders.

MATERIALS AND METHODS

More detailed information on the use of materials and methods can be found in the original publications.

1 Cell lines and animals

Cell line	Description	Source	Used in
HEK 293T	Human embryonic kidney 293T	M. Santoro, Italy / ATCC	I
PC Cl3	Fischer rat wild type thyroid follicular cells, clone 3	M. Santoro, Italy / ATCC	III
PC RET/PTC1	PC Cl3 with stable RET/Papillary thyroid carcinoma oncogene transfection	A. Fusco, Italy	III
PC E1A	PC Cl3 with stable E1A oncogene transfection	A. Fusco, Italy	III
PCind RET/PTC1	PC Cl3 cells with doxicyclin inducible RET/PTC1 oncogene transfection	M. Santoro, Italy / ATCC	III
COS-7	Monkey CV-1 origin and carrying SV40 genetic material	M. Santoro, Italy / ATCC	III
NIH 3T3	Mouse embryonic fibroblast cell line 3T3	M. Santoro, Italy / ATCC	II
Animal	**Experiment**	**Source**	**Used in**
Balb/C mice ♀	Peritonitis	Local colony at the University of Turku	I
Sprague-Dawley rats ♂	PTU induced goiter	Local colony at the University of Turku	III
Fischer 344 rats ♂	Hind limb ischemia	Harlan	I,II

2 Virus and plasmid constructs

Construct	Description	Source/reference	Used in
Adenovirus SOD3	Replication deficient E1-partially E3-deleted AdBglII vector containing rabbit-Sod3	Laukkanen et al. 2000	I, II
Adenovirus LacZ	Replication deficient E1-partially E3-deleted AdBglII vector containing bacterial β-galactosidase	Laukkanen et al. 2000	I, II
Rabbit SOD3 plasmid	Rabbit Sod3 cDNA in pHHT631 expression vector under Elongation factor 1α promoter	Laukkanen et al. 2000	I
Human SOD3 plasmid	Human SOD3 cDNA in pcDNA3 expression vector	S. Marklund, Sweden (SOD3) / Life Technologies	III
pCEF-Gαs	Constitutively active form of Gαs in pCEF vector	S. Gutkind, USA	III
pCEF-Gαq	Constitutively active form of Gαq in pCEF vector	S. Gutkind, USA	III
pCEF-PKA	Active catalytic subunit of protein kinase A in pCEF vector	S. Gutkind, USA	III

3 Antibodies

Antibody	Application	Source	Used in
α-rat CD3	IHC	Serotec	I
α-rat CD68	IHC	Serotec	I
α-human IκB	WB	Santa Cruz	I
α-rat p-Erk1/2	WB	Cell Signaling	II
α-rat total-Erk1/2	WB	Cell Signaling	II
α-rat p-Akt	WB	Cell Signaling	II
α-rat total-Akt	WB	Cell Signaling	II
α-mouse p-FoxO3a (Ser318/321)	WB	Cell Signaling	II
α-rat p-FoxO3a (Thr32)	WB	Cell Signaling	II
α-rat caspase-3	WB	Cell Signaling	II
α-mouse SP-1	WB	Santa Cruz	II
α-rat alpha-tubulin	WB	Sigma	I, II

4 Quantitative PCR primers

Primer	Sequence	Tm (C°)	Used in
rat TNF fw	AGA TGT GGA ACT GGC AGA GG	60	I
rat TNF rev	CCC ATT TGG GAA CTT CTC CT		
rat IL-1α fw	TCG GGA GGA GAC GAC TCT AA	60	I
rat IL-1α rev	GAA AGC TGC GGA TGT GAA GT		
rat IL-6 fw	CCG GAG AGG AGA CTT CAC AG	55	I
rat IL-6 rev	ACA GTG CAT CAT CGC TGT TC		
rat MCP-1 fw	CTC ACC TGC TGC TAC TCA TTC ACT	55	I
rat MCP-1 rev	TGC TGC TGG TGA TTC TCT TGT AGT		
rat MIP2 fw	ATC CAG AGC TTG ACG GTG AC	55	I
rat MIP2 rev	GGA CTT GCC GCT CTT CAG TA		
rat ICAM fw	AGG TAT CCA TCC ATC CCA CA	55	I
rat ICAM rev	GCC ACA GTT CTC AAA GCA CA		
rat VCAM fw	TGA CAT CTC CCC TGG ATC TC	55	I
rat VCAM rev	CTC CAG TTT CCT TCG CTG AC		
rat PSEL fw	TTC CCA CAC TTC CTT CTG CT	60	I
rat PSEL rev	CAC GCT GTA GTC GGG GTA TT		
rat ESEL fw	TTT TTG GCA CGG TAT GTG AA	60	I
rat ESEL rev	AGG TTG CTG CCA CAG AGA GT		

(Primers continue)

Primer	Sequence	Tm (C°)	Used in
human SOD1 fw	AGG GCA TCA TCA ATT TCG AG	60	III
human SOD1 rev	ACA TTG CCC AAG TCT CCA AC		
human SOD2 fw	TTG GCC AAG GGA GAT GTT AC	60	III
human SOD2 rev	AGT CAC GTT TGA TGG CTT CC		
human SOD3 fw	CTT CGC CTC TGC TGA AGT CT	60	II, III
human SOD3 rev	GGG TGT TTC GGT ACA AAT GG		
rat Sod1 fw	GTC GTC TCC TTG CTT TTT GC	60	III
rat Sod1 rev	TGC TCG CCT TCA GTT AAT CC		
rat Sod2 fw	AAG GAG CAA GGT CGC TTA CA	60	III
rat Sod2 rev	TGG CTA ACA TTC TCC CAG TTG		
rat Sod3 fw	GAC CTG GAG ATC TGG ATG GA	60	II, III
rat Sod3 rev	GTG GTT GGA GGT GTT CTG CT		
AdSOD3 fw	GTT GCG TGA GCG GAA AGA TG	60	II
AdSOD3 rev	GTG AGC GCC TGC CAG ATC TC		
rat NOX2 fw	TTG TTG CAG GAG TGC TCA TC	60	II
rat NOX2 rev	CTG CCA GCA GGT AGA TCA CA		
rat Ets-1 fw	GAA ATG ATG TCC CAG GCA CT	60	II
rat Ets-1 rev	CTT TAC CCA GGG CAC ACA GT		
rat Elk-1 fw	AGC GGC CAG AAG TTT GTC TA	60	II
rat Elk-1 rev	CTG TCA TTC CTG CAC CCT TT		
rat Bim fw	ATC TCA GTG CAA TGG CTT CCA	60	II
rat Bim rev	GCT CCT GTG CGA TCC GTA TC		
rat β-actin fw	TCG TGC GTG ACA TTA AGG AG	55	I, II, III
rat β-actin rev	GTC AGG CAG CTC GTA GCT CT		
hu β-actin fw	TGC GTG ACA TTA AGG AGA AG	55	II, III
hu β-actin rev	GCT CGT AGC TCT TCT CCA		

5 Miscellaneous reagents

Reagent	Application	Source	Used in
Fentanyl fluanisone	Anesthesia	Janssen Pharmaceutica	I, II
Midazolame	Anesthesia	Roche	I, II
Proteose peptone	Peritonitis	BD Difco	I
IL-1β	Peritonitis	R&D Systems	I
Propylthiouracil (PTU)	Goiter induction	Sigma	III

(Miscellaneous reagents continue)

Reagent	**Application**	**Source**	**Used in**
Heparin	Leukocyte collection	Løvens Kemiske Fabrik	I
Reastain Diff-Quick	Leukocyte staining	Reagena	I
pNFκB-Luc reporter plasmid	Luciferase assay	Stratagene	I
Fugene6	Transfection	Roche	II, III
Polyfect	Transfection	Sigma	I, III
TSH	PC Cl3 stimulation	Sigma	III
Forskolin	PC Cl3 stimulation	Sigma	III
H89	PC Cl3 stimulation	Calbiochem	III
Thapsigargin	Ca2+ uptake assay	Research Biochemicals International	III
Kdalert GAPDH assay kit	RNA interference optimization	Ambion	III
OnTargetplus SMART pool oligos	RNA intereference	Dharmacon	III
Optimem	RNA intereference	Life Technologies	III
BrdU Labeling and Detection Kit I	BrdU proliferation assay	Roche	III
Hoechst	BrdU proliferation assay	Sigma	III
Diogenes reagent	Superoxide detection	National Diagnostics	III
H_2DCFDA	H_2O_2 detection	Invitrogen	III
NE-PER cell fractionation kit	cell fractionation	Thermo Scientific	II
miScript Primer Assay, miR-21	miR-21 detection	Qiagen	II
miScript Primer Assay, RNU5	miR-21 quantification	Qiagen	II
Optimal cutting temperature compound	Histology	Tissue-Tek	I, II, III
Hematoxylin	IHC	Sigma	I, II, III
Eosin	IHC	Sigma	I, II, III
Lysis buffer	Cell/tissue lysis for WB	Sigma	I, II, III
Tri reagent	Cell/tissue lysis for qPCR	Sigma	I, II, III
Revert-Aid M-MuLV	First strand synthesis	Fermentas	I, II
QuantiTect RT	First strand synthesis	Qiagen	III
SYBR Green PCR Master mix	qPCR	Applied Biosystems	I, II, III

6 Methods

Method (in vivo)	**Used in**
Rat hindlimb ischemia	I,II
Mouse peritonitis	I
Rat PTU induced goiter	III
Method (in vitro)	**Used in**
Luciferase reporter assay	I
Affymetrix DNA array	III
HEEBO DNA array	III
Calcium uptake assay	III
RNA interference	III
BrdU proliferation assay	III
Chemiluminescence assay for superoxide detection	III
Fluorimetric homovanillic acid-based assay for H_2O_2 detection	III
Fluorimetric H2DCFDA assay for H_2O_2 detection	III
Cell fractionation	II
Cell culturing, incubations and/or transfections	I, II, III
Immunohistochemistry, IHC	I, II, III
Western blot analysis, WB	I, II, III
Quantitative Real-Time PCR, qPCR	I, II, III

7 Statistics

Method	**Used in**
Student's t-test	I,III
One-way Anova with Tukey-Kramer multiple comparison post-analysis test	II

RESULTS

1 SOD3 in the ischemic injury of the muscle

1.1 SOD3 administration attenuated tissue damage in ischemic muscle (I,II)

Several studies have shown how SOD3 overexpression or protein administration has relieved the consequences of hyperoxia or ischemic injury in e.g. myocardium and lungs, attenuated inflammation and reduced tissue injury (Folz *et al.*, 1999; Li *et al.*, 2001; reviewed in Qin *et al.*, 2008). We studied the role of SOD3 in an ischemic hind limb injury using an *in vivo* rat model (Laurila *et al.*, 2009). The injury was induced by surgical ligation of the distal and proximal femoral artery under anesthesia, and resulted in a relatively mild chronic ischemia affecting the thigh muscles as well as the lower limb. Immediately after the ligation, SOD3 or control β-galactosidase (LacZ) genes in an adenoviral vector ($0.5x10^9$ pfu) were injected i.m. into the thigh muscles. The animals were then followed for 3, 7 and 10 days after which the muscle tissues were collected and prepared for analyses. The successful expression of SOD3 and LacZ vectors has been verified in our earlier study (Laurila *et al.*, 2009).

Ischemic tissue develops the characteristic signs of inflammation including necrosis and apoptosis, initial infiltration of leukocytes and connective tissue formation in the later phases. CD68+ macrophages are one of the earliest leukocyte subpopulations that arrive at the site of tissue damage (reviewed in Bosco *et al.*, 2008). We determined the area of injury in SOD3 and LacZ treated tissues in the histological cryosections in two separate experiments following these criteria. We stained the histological sections with hematoxylin-eosin only or in addition with CD68 specific antibody. The inflammatory area according to that possessed by CD68+ macrophages in LacZ animals was approximately 30% of the whole section area while in SOD3 animals it was reduced to 10% indicating a 3-fold decrease ($p< 0.001$) after three days. At the following 7- and 10-day time-points the difference grew larger reaching 12-fold ($p< 0.01$) reduction in the SOD3 animals. In both groups the areas became progressively smaller during the 10-day follow-up period.

Next, we determined the injury area according to the visible morphological changes such as fibrosis and fragmentation of the hematoxylin-eosin stained muscle thin sections. In this analysis, the injury areas in SOD3 animals were approximately 53% ($p< 0.001$) and 40% ($p< 0.05$) lower at 3- and 7-day time-points, respectively, than in the control groups. A similar trend as in the CD68+-based analysis was seen after 10 days: the injury areas were smaller in both groups, but as compared to each other the difference was not statistically significant. These results are in line with each other and demonstrate the beneficial role of SOD3 in limiting the extent of tissue damage in ischemic injury. Additionally, they indicate that the resolution of the injury

begins already during the first 10 days after induction which is in accordance with the work of Paoni *et al.* (2002).

Apoptotic cell death is a common finding in ischemic injuries at the core of the ischemia and at the peri-ischemic area (Abbate *et al.*, 2006). Since we observed reduced injured areas in the muscle histological sections, we determined the apoptotic rate by western blotting the protein preparations from the rat tissues and detecting the level of cleaved caspase-3. Caspase-3 is one of the effector caspases, and after it is cleaved the cascade is destined to proceed (Faleiro *et al.*, 1997; Woo *et al.*, 1998). The blot revealed a faint caspase-3 band in SOD3 animals and a strong expression in LacZ animals 3 and 7 days after the injury induction. At 10-day time-point the cleaved caspase-3 expression in LacZ group was reduced approximately to the same level as in SOD3 group. According to this caspase-3 analysis, there is less apoptosis in SOD3 treated animals as compared to controls, thus further pronouncing its pro-survival effect.

1.2 Migration of inflammatory cells was reduced by SOD3 treatment (I)

Reduced accumulation of inflammatory cells to the site of injury caused by ischemia-reperfusion in SOD3 overexpressing tissues has been shown in previous studies (Laukkanen *et al.*, 2002; Dewald *et al.*, 2003). We confirmed the same phenomenon in our rat model by counting the infiltrated CD68+ macrophages and CD3+ lymphocytes from the histological thin sections. Throughout the follow-up period the number of CD68+ cells remained 3-5-fold lower in SOD3 animals (3-day, $p< 0.05$; 7-day, $p< 0.01$; 10-day, $p< 0.05$) than in the LacZ control group. Macrophage infiltration was maximal on day 7 in the LacZ group, but was decreased by half by the 10-day time-point, while in the SOD3 group the levels were decreasing steadily over time. This clear reduction can also in part explain the smaller injury areas in the muscle sections since neutrophils and macrophages contribute to oxidative tissue damage by releasing large amounts of ROS in the oxidative burst (Nathan, 1987a).

The number of CD3+ lymphocytes counted was distinctly lower than that of macrophages. At 3-day time-point they were nearly the same in both groups, but their number increased by 60% in LacZ group 10 days after induction of ischemia while in the SOD3 group it remained at the same level as on day 3 (SOD3 vs. LacZ, $p< 0.05$). This was expected since macrophages secrete lymphocyte attracting chemokines, and when they are less in number it follows that lymphocytes are not stimulated efficiently enough to infiltrate in high numbers (reviewed in Schutyser *et al.*, 2003; Bosco *et al.*, 2006).

In order to compare the efficacy of SOD3 to anti-inflammatory drugs, we used a mouse peritonitis model suitable for such studies due to the easy i.p. administration of adenoviral vectors and medical substances. AdLacZ or AdSOD3 ($0.5x10^9$ pfu) were

diluted in PBS to ensure a more even distribution in the peritoneal cavity and then injected i.p. approximately 72 hours before the induction of peritonitis. The potent anti-inflammatory glucocorticoid drug Dexamethasone was given i.p. in 50 mg/kg doze 30 minutes before the induction of peritonitis. Peritonitis was induced by injection of 5% proteose-peptone/IL-1β in 1 ml of PBS, and after 18 hours the peritoneal cavity was lavaged and the leukocyte subpopulations were analyzed. AdSOD3 administration reduced the total leukocyte count by 40% ($p < 0.01$), and had the most prominent effect on macrophages with 67% ($p < 0.001$) reduction. Additionally, neutrophil (p= n.s.) and lymphocyte ($p < 0.05$) counts were smaller in SOD3 treated animals. As compared to PBS control, Dexamethasone decreased the total leukocyte count by 20% ($p < 0.05$) with similar distinct effect on the macrophages (60%, $p < 0.01$). No effect on the neutrophil subpopulation was seen, and the reduction in lymphocyte count did not reach statistical significance. The peritonitis model showed that SOD3 can effectively reduce leukocyte infiltration with special emphasis on macrophages. The adenoviral vector, however, likely worsens the inflammatory response since the overall leukocyte number was relatively high in SOD3 and LacZ treated mice.

2 SOD3 in the thyroid gland

2.1 SOD3 was differentially expressed in the normal human and rat thyroid glands (III)

Our second tissue type under investigation was the thyroid gland where oxidative stress is continuously present due to the mechanisms of thyroid hormone synthesis and, therefore, creates an interesting environment to study an antioxidant enzyme (reviewed in Song *et al.*, 2007). Furthermore, the several available cell lines, animal models and human samples of normal and pathological thyroid tissue offered us a chance to study the role of SOD3 in cell proliferation more thoroughly.

In several mammalian species the thyroid gland is one of the tissues with high SOD3 activity (Marklund, 1984a). However, this study did not include rat thyroid and, therefore, we first determined the expression level of SOD3 in rat thyroid with a quantitative PCR. As control rat tissues we chose liver and heart in which the SOD3 activity is close to the mean level, and kidney and aorta which have higher than the mean level of SOD3 activity (Marklund, 1984a; Laukkanen *et al.*, 2002). Additionally, to understand better the relative amounts of the three enzymes, we included SOD1 and SOD2 into the same experiment.

In rat, SOD3 was the most prominent of the three enzymes in kidney, aorta and thyroid, but in liver and heart SOD1 was most abundant. When compared to heart, thyroidal SOD3 expression was 2.5-fold higher, whereas in kidney and aorta it was

approximately 4-fold higher. The expression levels of SOD2 varied in all tissues studied. In general, these results were in line with previous studies on the SOD activities in various mammalian tissues (Marklund, 1984a). However, some discrepancies between the expression levels of the SODs and their respective activities in liver and kidney indicate that mRNA expression and enzymatic activity may not correlate (Marklund, 1984a).

We also determined the SOD expressions in the rat thyroid cell line PC clone 3 (PC Cl3) which was used in most of the *in vitro* experiments. The cell line is derived from Fisher 344 rat strain, and represents relatively normal functional thyroid follicular cells, but is immortalized (Fusco *et al.*, 1987; reviewed in Kimura *et al.*, 2001). The SOD3 was clearly the most prominent ($p< 0.01$) of the three enzymes in PC Cl3 cells as compared to SOD1 and SOD2, respectively. The pattern was similar to that *in vivo* with SOD1 as the least expressed and SOD3 as the most highly expressed enzyme, although the relative expression level was drastically higher in the cell line. This may be due to the sensitivity of the quantitative PCR resulting in frequently occurring differences between separate experiments. However, the cell line can be considered suitable for *in vitro* studies on the role of SOD3 in the rat thyroid gland.

A difference was seen between rat tissues and normal human thyroid tissue. Similar quantitative PCR experiment with mRNA from a pool of human tissue showed that SOD3 expression level is only approximately 20% from SOD1 level and SOD2 level was even lower. This result is well in concert with the observed activities of the SODs in human thyroid (Marklund, 1984a). According to the study by Marklund (1984a) the mean SOD3 activity in human tissues is 10-fold higher than in rat which suggests that the observed seemingly low mRNA expression level in human thyroid does not refer to overall low SOD3 protein expression in human thyroid in comparison to rat thyroid. Altogether, these results from human and rat thyroid tissues indicate a role for SOD3 in thyroid gland function.

2.2 SOD3 expression was increased in rat goiter, but not in human goiter (III)

The thyroid gland is prone to high oxidative stress even under normal conditions, and therefore it is plausible that the primary function of SOD enzymes in thyroid is to act as antioxidants (reviewed in Song *et al.*, 2007). The main ROS causing oxidative stress is H_2O_2 which is produced by the Duox2 during the thyroid hormone synthesis driven by TSH (reviewed in Ohye and Sugawara, 2010). However, the only substrate for SOD enzymes is $O_2^-\bullet$ (Klug *et al.*, 1972; Marklund, 1982). There are at least two $O_2^-\bullet$ sources in the thyroid gland. Firstly, the Duox enzymes may leak $O_2^-\bullet$ when inappropriately complexed with a mismatching DuoxA maturation factor (Morand *et al.*, 2009). The second source of $O_2^-\bullet$ is the Nox4 which was recently found to respond to TSH stimulation (Weyemi *et al.*, 2010). The oxidative stress levels as indicated by lipid peroxidation measurements are elevated in goitrous gland creating a need for

upregulation of the antioxidant defenses (Erdamar *et al.*, 2010; Maier *et al.*, 2007; Poncin *et al.*, 2008). We therefore investigated how the SOD3 expression changed in goitrous thyroid tissues of rat and human.

We induced goiter formation in rats by administering 0.25% propylthiouracile (PTU) in their drinking water *ad libitum*. PTU is a known goitrogen which increases TSH level and thus follicular cell proliferation by inhibiting thyroid hormone formation. After two weeks the animals were sacrificed and the thyroid glands were collected. PTU treated rats had on average 4-fold heavier glands than the control group on normal water. There was large variation in the weights of the glands, but it can be attributed to the method of PTU administration (Levey, 1963). Additionally, a histological examination revealed the characteristic morphological signs of goiter: reduced colloid and hyperproliferation of follicular cells. Similar results have been reported previously with PTU treated rats (Laezza *et al.*, 2006). Quantitative PCR of pooled mRNA from control and PTU thyroid glands revealed a 10-fold ($p< 0.01$) increase in SOD3 expression in the goiter suggesting that it is needed in the hyperproliferative gland.

Next, we analyzed normal human thyroid tissue as well as pooled AA and FNAH samples with HEEBO oligonucleotide microarray to see if SOD3 behaved similarly as in the rat tissue. AA and FNAH are genetic hyperthyroidism disorders characterized by dominant activating mutations of the TSH-R, and nodular or diffuse goiter formation, respectively (Deleu *et al.*, 2000; Trulzsch *et al.*, 2001; Hebrant *et al.*, 2009). No difference was observed between the tissues analyzed. Only slight, non-significant downregulation of SOD3 was seen in the FNAH samples. This suggests that the phenotype of the human disorder could be milder than that induced by PTU in rats, but also that there are likely to be species specific differences.

3 Regulation of cellular signaling pathways by SOD3 in muscle

3.1 SOD3 overexpression rescued the redox balance after ischemia (II)

Skeletal muscle is known to express low level of SOD3 activity in human and medium level in rat, as compared to the average of the respective species (Marklund, 1984a). An injury causing oxidative stress is likely to alter this balance due to the expression of inflammatory cytokines and other factors to which SOD3 is sensitive (Marklund, 1992; Stralin and Marklund, 2000; Stralin and Marklund, 2001). We studied the change in rat endogenous SOD3 mRNA expression by quantitative PCR. The SOD3 expression was first increased ($p<$ ns.) at 3-day time-point, but then decreased by 2-fold ($p< 0.05$) and further by 10-fold ($p< 0.001$) at 7- and 10-day time-points, respectively, in the LacZ animals as compared to the uninjured muscle. The SOD3

thus seems to be progressively downregulated in the acute phase of the ischemic injury.

The deficient endogenous SOD3 was successfully corrected by introduction of SOD3 in an adenoviral vector into the mucle tissue. Already on the day 3 there was a 13-fold increase in exogenous SOD3 expression. By the day 10 it was reduced close to the normal level of endogenous SOD3. Thus, the vector gives a transient expression of SOD3 in the muscle tissues for the duration of the early acute phase of the ischemic injury.

At the same time, expression of endogenous rat Nox2, which is the principal Nox enzyme in phagocytic leukocytes (reviewed in Lambeth, 2004), and iNOS, which is induced in activated phagocytes (reviewed in Guzik *et al.*, 2003), were both strongly upregulated ($p < 0.001$) in 3-day LacZ animals as compared to the uninjured tissue. This is likely due to the flux of macrophages and other phagocytes into the injury site, and signified a change in the overall redox balance of the tissue towards oxidative stress. In the SOD3 treated muscles the Nox2 and iNOS were downregulated ($p < 0.01$ and $p < 0.001$, respectively) as compared to the LacZ tissues, further underlining the impact SOD3 had on the phagocyte population and thereby on the redox balance of the tissue. However, the Nox2 and iNOS expressions were not suppressed to the same level with the normal tissue indicating residual elevated oxidative stress.

3.2 NFκB, inflammatory cytokines and adhesion molecules were downregulated after SOD3 treatment (I)

Endothelial cell and mast cell activation after ischemic tissue injury initiates the chemokine and cytokine cascades that are ultimately responsible for the leukocyte transmigration via the adhesion molecules (Frangogiannis *et al.*, 1998; reviewed in Alom-Ruiz *et al.*, 2008). One of the key transcription factors regulating their expression is the ROS-responsive, pro-inflammatory NFκB (reviewed in Pahl, 1999). It is sequestered in the cytoplasm by an inhibitor of κB (IκB) until the cell receives a suitable signal that induces degradation of IκB allowing nuclear translocation of NFκB (reviewed in Kabe *et al.*, 2005). We started to investigate the mechanism by which SOD3 could achieve the observed significant reduction in the leukocyte accumulation by first determining the activation of NFκB with an *in vitro* luciferase assay. In cells transiently transfected with SOD3 the NFκB activity was 50% ($p < 0.01$) lower than in the mock transfected cells. This finding was supported by an observation that the IκB was increased in the rat tissue protein preparations. Therefore, SOD3 seems to be able to suppress NFκB activation possibly by promoting its retention in the cytoplasm through IκB stabilization.

Since NFκB is an important signal transducer which regulates the expression of many chemokines, cytokines and adhesion molecules (reviewed in Pahl, 1999), we

next investigated how SOD3 or LacZ affected the expression levels of a set of these factors with quantitative PCR. We chose to investigate the tissues collected at the 3-day time-point because the transgene expression is maximal at that time (our observations and Laukkanen *et al.*, 2001b). In SOD3 animals, the pro-inflammatory cytokines TNF-α, IL-1α and IL-6, as well as chemokines MIP-2 and MCP-1 were markedly downregulated to only 10-30% ($p < 0.05$ or 0.01) of the level in LacZ animals. Similar strong decrease of the main endothelial cell adhesion molecules ICAM, VCAM, E-selectin and P-selectin was observed in the same tissues ($p < 0.05$ or 0.01). These findings could explain the evident effect of SOD3 on the leukocyte trafficking. Firstly, the lower amount of pro-inflammatory cytokines and, consequently, adhesion molecules on the endothelial surface effectively inhibits their transmigration. Secondly, MIP-2 and MCP-1 are important neutrophil and macrophage attractants (reviewed in Bosco *et al.*, 2008), and therefore, their amount generated in the tissue affects the number of accumulated leukocytes.

3.3 SOD3 activated the kinases Erk1/2 and Akt simultaneously (II)

We have shown that SOD3 can act as a mitogenic regulatory factor functioning through the well-known Ras-Erk1/2 pathway after adenoviral gene transfer (Laurila *et al.*, 2009). We next confirmed this earlier finding by determining the endogenous rat Erk1/2 activation in the normal uninjured, and SOD3 and LacZ treated tissues with western blotting and band densitometric analysis. The injury itself induced some Erk1/2 activation as suggested by the slightly increased values in LacZ treated animals. As expected, we saw higher Erk1/2 phosphorylation ($p < 0.001$) in the SOD3 treated tissues already on day 3 after the ischemic injury, and it was still elevated on the day 10 ($p < 0.05$) as compared to the LacZ treated animals. This early activation coincides with the SOD3 expression peak at the day 3 after transduction indicating cooperation between these factors (our observations and Laukkanen *et al.*, 2001b).

Our previous studies indicated that SOD3 overexpression can also activate *in vitro* the Akt survival pathway (Laurila *et al.*, 2009). PI3 kinase and Akt become activated in response to a number of stress stimuli as well as growth factors, and function in cell survival, proliferation and angiogenesis (reviewed in Datta *et al.*, 1999). We analyzed the activity of Akt by western blotting and densitometric analysis to see if it is upregulated in our model. Akt phosphorylation was induced by the injury in the LacZ group on the days 3 and 7, but in the SOD3 group it was markedly increased ($p < 0.001$) 3 days after the induction of ischemia. However, phospho-Akt was reduced to the same level with the uninjured control animals by the 10-day time-point. Two important survival pathways are thus activated by SOD3 during the first few days of ischemic injury, but only Erk1/2 activation is maintained for a longer period of time.

3.4 Key transcription factor FoxO3a was transiently inactivated by SOD3 (II)

The transcription factor FoxO3a is directly inactivated by Akt-mediated phosphorylation which allows transcription of Akt target genes and thereby progression of the cell cycle and inhibition of apoptosis (Brunet *et al.*, 1999; reviewed in Calnan and Brunet, 2008). However, FoxO3a can also be inactivated by Erk1/2 which demonstrates the importance of this factor in cell fate determination as a key transcription factor regulated by several upstream signaling cascades (Yang *et al.*, 2008). Here, as determined by western blotting and densitometric analysis, FoxO3a phosphorylation in LacZ animals showed a tendency to increase during the follow-up period, but the introduction of SOD3 into the tissue caused relatively strong phosphorylation ($p < 0.001$) on the day 3 which then declined to the same level with the LacZ animals on days 7 and 10.

We determined the amount of phospho-FoxO3a in the nuclear and cytosolic fractions of SOD3 or control vector transfected NIH 3T3 cells by western blotting and densitometric analysis to confirm FoxO3a inactivation. There was a statistically significant increase ($p < 0.05$) in the amount of total phospho-FoxO3a. However, the level of phosphorylated FoxO3a was increased ($p < 0.001$) only in the cytosolic fraction of SOD3 transfected cells as compared to the control cell cytosolic fraction. The amount of phospho-FoxO3a in the nucleus was not significantly different in the SOD3 transfected and control cells. These findings further suggest that SOD3 can induce the inactivation of FoxO3a.

Additionally, we measured by quantitative PCR the expression levels of SOD3 and FoxO3a mRNA in the same transfected cells to see whether SOD3 affects the FoxO3a expression and thus the total FoxO3a pool in the cell. The transfection successfully increased SOD3 expression ($p < 0.001$), but there was no significant change in the FoxO3a expression. Thus, it appears that SOD3 affects mainly the phosphorylation status of FoxO3a.

3.5 SOD3 regulated both pro- and anti-apoptotic factors to promote survival (II)

We next concentrated on the SOD3 induced survival and anti-apoptotic signaling through Erk1/2, Akt and FoxO3a. Two transcription factors under Ras-Erk1/2 regulation, v-ets erythroblastosis virus E26 oncogene homologue (Ets-) 1 and Elk-1, are both members of the Ets family of MAPK responsive transcription factors (reviewed in Wasylyk *et al.*, 1998). On the 7-day time-point both Ets-1 ($p < 0.001$) and Elk-1 ($p < 0.01$) mRNA expressions had increased to a higher level in the SOD3 group than in the uninjured control or LacZ groups. However, the increase in LacZ group as compared to control was statistically significant only in the case of Ets-1, although Elk-1 had a similar tendency. These findings provide further evidence for the activation of Ras-Erk1/2 cascade by SOD3.

FoxO3a has recently been shown to repress miR-21 transcription as one mechanism of its growth inhibitory function (Wang and Li, 2010). MiR-21 suppresses many apoptosis-related genes, and its high expression has been associated with e.g. protection of myocardium after infarction and an aggressive phenotype of cancer cells (Asangani *et al.*, 2008; Dong *et al.*, 2009). We therefore analyzed the expression levels of miR-21 in our experimental animals by quantitative PCR, and found that it was strongly increased in the SOD3 group after 3 days of ischemia as compared to LacZ ($p< 0.01$) or uninjured control ($p< 0.001$) animals. The injury itself induced a statistically significant increase ($p< 0.05$) in the LacZ group.

Erk1/2 regulates a number of downstream targets including transcription factors, kinases, phosphatases, cytoskeletal proteins, signaling proteins, and apoptotic proteins and proteinases (reviewed in Yoon and Seger, 2006). We studied more specifically the pro-apoptotic Bcl-2 protein Bim which is negatively regulated by Erk1/2 (reviewed in Ewings *et al.*, 2007b). Furthermore, when active in nucleus, FoxO3a can target the Bim promoter and drive the cell towards apoptosis by upregulating Bim expression (Dijkers *et al.*, 2000). In line with our results showing that Erk1/2 was activated and FoxO3a inactivated, we found lower ($p< 0.01$) Bim expression in the SOD3 treated animals as compared to the LacZ group at the 3-day time point. The injury itself induced a several-fold increase in the Bim expression ($p< 0.01$) as compared to the normal tissue, while SOD3 suppressed it close to the normal level. These results indicate that SOD3 may utilize miR-21 and Bim in its anti-apoptotic signaling.

4 SOD3 function and signaling in the thyroid gland

4.1 TSH-R cAMP and PLC pathways induced SOD3 expression (III)

The observation that SOD3 is a mitogenic factor and was induced in rat goiter led us to experiment whether it is directly responsive to TSH-R activation, the main driving force for the follicular cell proliferation and thyroid enlargement during goiter. TSH-R activates two G-proteins: $G_s\alpha$ which leads to follicular cell proliferation, differentiation and hormone secretion via cyclic AMP (cAMP) and PKA, and $G_q\alpha$ which induces hormone synthesis via PLC. After TSH incubation the SOD3 expression was increased 3-fold ($p< 0.01$) in PC Cl3 cells. It was downregulated by a specific PKA inhibitor H89 ($p< 0.05$), but not completely blocked which would indicate that also other signaling routes may affect SOD3 expression in the follicular cells. Transfection of PC Cl3 cells to overexpress $G_s\alpha$ or PKA induced 5.5-fold ($p< 0.01$) or 6-fold ($p< 0.001$) increases in SOD3 expression, respectively. Additionally, incubation with Forskolin, an adenylate cyclase activator, resulted in 6-fold ($p< 0.05$) SOD3 expression. The responsiveness of SOD3 to the $G_q\alpha$ pathway was studied by transfecting the $G_q\alpha$ into the same cells or incubation with thapsigargin, an agent that

increases the cytosolic Ca^{2+} which is a cofactor for Duox, thus stimulating the PLC-dependent pathway (Kimura *et al.*, 1995). Both treatments had a similar positive effect on SOD3 expression showing 3-fold ($p< 0.01$ for $G_q\alpha$ and 0.05 for thapsigargin) upregulation. These results demonstrate that SOD3 expression could indeed be regulated by TSH-mediated TSH-R activation which would indicate that it is acting similarly to the thyroid specific genes marking a certain level of differentiation.

4.2 SOD3 participated in follicular cell proliferation, but not in thyroid hormone synthesis (III)

TSH is responsible for follicular cell proliferation, differentiation by promoting thyroid specific gene expression, and subsequent hormone synthesis and secretion. Since we found SOD3 to be induced by TSH we wanted to study further whether it has a function in the thyroid gland. H_2O_2, a mitogenic substance at subtoxic levels in many cell types but also an essential factor in thyroid hormone synthesis (reviewed in Stone and Yang, 2006; reviewed in Song *et al.*, 2007), is also the product of SOD3. The next step was therefore to study whether SOD3 participated in the follicular cell proliferation or thyroid hormone synthesis.

Overexpression of SOD3 did not affect the PC Cl3 proliferation rate presumably due to the already high endogenous SOD3 expression level (BrDU data not shown), and we therefore used the RNA interference technique. A quantitative PCR analysis revealed a 95% ($p< 0.001$) decrease in the expression of SOD3 mRNA as compared to the control cells 48 hours after the transfection of siSOD3. Next, we stimulated the PC Cl3 cells transfected with either control siRNA or siSOD3 with TSH and determined their proliferation rates with a BrdU assay. In the control cells stimulated by TSH the proportion of BrdU positive nuclei was 33% higher when compared to unstimulated cells, but this effect was abrogated by SOD3 knock-down resulting in only 15% ($p< 0.05$) higher BrdU incorporation. This confirmed that thyroid follicular cells need SOD3 in order to proliferate properly under TSH stimulation.

Since Duox2-derived H_2O_2 is required in the thyroid hormone synthesis in large amounts we tested if SOD3 could produce it at sufficient levels and thereby support the Duox2 (Corvilain *et al.*, 1991; De Deken *et al.*, 2000). The experiment was done with non-thyroidal COS-7 cell line in order to specifically examine the two enzymes without interference by factors that could affect their behavior in thyroid cells. Firstly, we confirmed that the enzymes were functional by measuring $O_2^-\bullet$ generation by a chemiluminescence assay from Duox2/mismatching DuoxA1 co-transfected cells and Duox2/DuoxA1/SOD3 co-transfected cells. Duox2 alone is not functional, but requires a maturation factor for H_2O_2 synthesis (Morand *et al.*, 2009). However, if the factor is mismatching the Duox2 will leak $O_2^-\bullet$ (Morand *et al.*, 2009), and this was indeed observed in the experiment. With SOD3 present the amount of $O_2^-\bullet$ produced

was significantly lower ($p< 0.001$) suggesting that transfected SOD3 can successfully dismutate the ROS. We then measured the H_2O_2 generation by a fluorimetric assay from Duox2/matching DuoxA2 and SOD3 transfected COS-7 cells to evaluate their relative efficiencies. The H_2O_2 amount derived from Duox2/DuoxA2 expressing cells was 28-fold higher ($p< 0.05$) than that from SOD3 expressing cells which indicates that SOD3 is not able to compete with Duox2. Thus, it seems that SOD3 and its product are more likely to play a role in follicular cell proliferation/differentiation than in thyroid hormone synthesis.

4.3 SOD3 expression was downregulated in thyroid cancer cells and tissues (III)

The ability of the TSH-R downstream pathways to increase SOD3 expression and its apparent function in cell proliferation led us to investigate its expression pattern in thyroid cancer cells which, as most other transformed cells, proliferate rapidly and lose their differentiation markers (Gerard *et al.*, 2003; reviewed in Riesco-Eizaguirre and Santisteban, 2007). We compared the SOD3 expression in normal rat PC Cl3 cells and oncogenic PC Cl3-derived cell lines stably transformed with Braf, RET/PTC1 and E1A. A quantitative PCR analysis showed markedly downregulated SOD3 expression: by 50% in PC Braf ($p< 0.05$), by 60% in PC RET/PTC1 ($p< 0.01$) and nearly undetectable in PC E1A ($p< 0.001$) cell lines. Accordingly, also the H_2O_2 production was significantly decreased in the PC RET/PTC1 ($p< 0.001$) and PC E1A ($p< 0.01$) cells as compared to normal PC Cl3 cells, possibly reflecting the reduced SOD3 expression. This suggests that SOD3 gene expression follows that of the other thyroid specific genes, e.g. TPO and Tg, which have been shown to be lost after malignant transformation (Gerard *et al.*, 2003). Furthermore, the results may indicate that SOD3 expression is also dependent on the specific oncogene.

Then, to extend our study of rat tissues to human thyroid cancers we analyzed patient samples of PTC and ATC comparing them with normal thyroid tissue by Affymetrix DNA array and quantitative PCR for SOD1, 2 and 3 expressions. Both methods showed marked downregulation of SOD3 in PTC and ATC, thus resembling the rat cell lines. However, SOD1 and 2 were more variable. SOD1 was upregulated in PTC but downregulated in ATC, while SOD2 was regulated in the opposite way. These data with both rat cells and human tissues suggest that SOD3 could be considered as a differentiation marker of malignant transformation since it is consistently downregulated, unlike SOD1 and 2 which varied considerably in human tissues. Several previous studies have found similar markedly altered SOD1 and 2 expression levels in thyroid cancers (Nishida *et al.*, 1993; Mano *et al.*, 1997; Hasegawa *et al.*, 2003), but to our knowledge there are no previous reports on SOD3 in thyroid cancer.

4.4 Oncogene dose affected SOD3 expression in a thyroid cancer cell line (III)

The different expression levels of SOD3 in the various cancer cell lines may indicate specific oncogene-driven regulation of the SOD3. Previous studies have associated e.g. Braf mutations with dedifferentiation and lower expression of TPO, NIS and other thyroid specific genes (Di Cristofaro *et al.*, 2006; Durante *et al.*, 2007; Romei *et al.*, 2008). We therefore utilized the inducible PC RET/PTC1 cell line, activated by doxicyclin, to determine the effect of this oncogene on the magnitude of SOD3 expression. An analysis by quantitative PCR revealed a steady increase in the RET/PTC1 mRNA expression 24 (50-fold, $p < 0.05$) and 72 hours (120-fold, $p < 0.05$) after doxicyclin administration. Simultaneously, the SOD3 mRNA expression was significantly downregulated by 40% at 24 hours ($p < 0.05$) and by 70% at 72 hours ($p < 0.001$). According to these results, it seems that the amount of the oncogene can have a profound inhibitory effect on the SOD3 expression.

DISCUSSION

1 SOD3-mediated effects in skeletal muscle and thyroid gland

SOD3 has been studied as a candidate for potential antioxidant therapies for various conditions involving oxidative stress, inflammation and cancer alongside the basic research to reveal its molecular properties. Mainly, it has been considered to function through attenuation of oxidative stress in normal and pathological tissues (Carlsson *et al.*, 1995; Gongora *et al.*, 2008; van Deel *et al.*, 2008). This is well-grounded since the ROS are known to cause and worsen tissue damage by oxidizing cellular macromolecules and antioxidant enzymes such as Prx and Gpx, disrupt the cell membranes and disturb cellular signaling events causing metabolic dysfunction (reviewed in Poli *et al.*, 2004; reviewed in Valko *et al.*, 2006). Cancer studies have shown that transformation disrupts the normal antioxidant system of the cell and that the increased O_2^{-}• can promote the aggressive phenotype by enhancing the migratory properties (Khanzode *et al.*, 2004; Teoh *et al.*, 2007; Chaiswing *et al.*, 2008; Kumar *et al.*, 2008; Sinha *et al.*, 2009). However, the findings after SOD3 administration or overexpression in various experimental settings of ischemia include less apoptosis, signs of inflammation and inflammatory cell accumulation, and in cancer the development and invasion of tumors are inhibited (Folz *et al.*, 1999; Laukkanen *et al.*, 2002; Park *et al.*, 2005; Teoh *et al.*, 2007; Chaiswing *et al.*, 2008). Studies with SOD3 gene knock-down but otherwise normal tissues have further shown that it is also essential in the maintenance of normal redox balance (Gongora *et al.*, 2008; Kliment *et al.*, 2009).

1.1 SOD3 improves muscle recovery from ischemic injury

In our rat hind limb ischemia model the ligation of distal and proximal femoral artery results in a relatively mild acute ischemia in the thigh area that becomes chronic in the absence of reperfusion (reviewed in Blaisdell, 2002). The existing collateral arteries are likely to provide the muscle tissue with sufficient blood supply that prevents wide-scale necrosis. However, the ischemic injury is still able to induce an inflammatory response and an alteration in the tissue redox balance as indicated by inflammatory cell accumulation and increased Nox2 and iNOS expressions (I, II; Laurila *et al.*, 2009). In a similar mouse study the lower limb muscles that should be prone to more severe ischemia after femoral artery occlusion were starting to heal around the day 10 (Paoni *et al.*, 2002), and therefore we expected also the thigh muscles to be in a state of recovery by then.

Our analyses of the rat ischemic skeletal muscle revealed significantly smaller areas of tissue inflammation and damage in the SOD3 treated animals as compared to the control group during the inflammatory phase i.e. the first 10 days after the injury

(I, Figure 1; II, Figure 1A, B). Similarly, the numbers of infiltrated neutrophils, CD68+ monocyte/macrophages and CD3+ T-lymphocytes were reduced after SOD3 administration in both muscle tissue and the mouse peritonitis model (I, Figure 1, 2, 3), being in line with the earlier findings suggesting that SOD3 can attenuate tissue inflammation and damage. The lowering number of inflammatory cells towards the 10-day time-point also indicated that the injury was beginning to heal. Our study revealed a new aspect of SOD3-mediated regulation of leukocyte migration: the mouse peritonitis model showed that SOD3 may have an especially strong effect on the monocyte/macrophage subpopulation (I, Figure 3B). Macrophages are a versatile population of leukocytes with various subtypes that are enriched at different stages of the inflammatory response. The first infiltrating population, the CD68+/ED1+ macrophages, is generally pro-inflammatory and orchestrates the initial responses by promoting the cytokine expression as well as phagocytoses cell debris and generates ROS in an oxidative burst (Honda *et al.*, 1990). Later, the anti-inflammatory CD163+/ED2+ macrophages take over and organize the healing process (Honda *et al.*, 1990). Therefore, it seems that the anti-inflammatory effect of SOD3 is mediated in part by inhibition of the pro-inflammatory monocyte/macrophage accumulation.

Previous studies have indicated that SOD3 overexpression induced by an adenoviral vector, similar to the one used here, is maximal around the day 3 after infection (our observations and Laukkanen *et al.*, 2001b). In our hind limb ischemia setting this delay indicates that the early neutrophil population can infiltrate the tissue before the SOD3 takes effect, but that the macrophages which arrive over a period of a few days respond to SOD3. In the peritonitis model, the amount of neutrophils was slightly reduced (I, Figure 3B), however, indicating that SOD3 may also affect their trafficking provided that its expression has reached an effective level. The lymphocyte population appears at the site of inflammation simultaneously with other inflammatory cells, and they are partly dependent on the presence of macrophages for their accumulation (reviewed in Gillitzer, 2001; Linfert *et al.*, 2009). Thus, the weak macrophage-derived stimuli may in part account for the modest lymphocyte response seen here. Accordingly, in the muscle tissue the amount of CD3+ T-lymphocytes was nearly similar after 3 days of SOD3 or control gene injection, but as the inflammation proceeded the numbers increased by 60% by day 10 in the control animals while in SOD3 treated animals it remained at the same level with the earlier time point (I, Figure 2). The role of lymphocytes in ischemic injury and muscle tissue healing is not clear, although it has been suggested that they modulate e.g. arteriogenesis in injured tissue (Stabile *et al.*, 2003; Stabile *et al.*, 2006).

The total amount of peritoneal leukocytes in LacZ control mice was nearly twice as large as in the PBS treated mice (I, Figure 3A, 4A). The SOD3 overexpression, however, was able to suppress them to the same level with the PBS treated mice. The increase was mainly due to higher amounts of neutrophils and

macrophages while the lymphocyte accumulation was not affected (I, Figure 3B, 4B). Most likely, the adenoviral vector itself contributed to the apparently more severe inflammation indicating that the method of gene administration should be improved or changed. The used adenoviral vector was replication deficient, and the excess viral particles were likely cleared from the tissues by neutrophils and macrophages during the acute inflammation which results in relatively short period of tissue exposure to the vector and, thus, does not evoke the adaptive immunity and attract masses of lymphocytes. We also compared the effect of SOD3 to a clinical glucocorticoid drug Dexamethasone in the mouse peritonitis model to see whether SOD3 could have potential in therapeutic use. The SOD3 was able to reduce the accumulation of monocyte and lymphocyte populations to the same level as Dexamethasone thereby also negating the adverse effects of the vector (I, Figure 3, 4). The neutrophil population remained unaffected by the drug. Accordingly, SOD3 showed a clear therapeutic effect in the muscle ischemia model, but the viral vector may not be the best option for administration.

1.2 SOD3 and the blockade of the cytokine/adhesion molecule cascade

The distinct effect of SOD3 administration on the monocyte/macrophage population and the previous study showing that SOD3 has gene regulatory properties (Laurila *et al.*, 2009), lead us to speculate that it could also regulate the factors participating in the early inflammatory response after ischemic injury. We therefore investigated the 3-day time-point rat tissue for expression of some central cytokines, chemokines and cell adhesion molecules which are responsible for the transmigration of leukocytes to the site of inflammation. The early time point was chosen for maximal SOD3 expression and presence of acute inflammation after femoral artery ligation (Paoni *et al.*, 2002; Laurila *et al.*, 2009). TNF-α, IL-1α and IL-1β are pro-inflammatory cytokines that are released from e.g. tissue-resident mast cells, macrophages, muscle cells, fibroblasts and endothelial cells upon a variety of stimuli indicating physiological stress, and induce a number of signaling pathways leading to e.g. upregulation of other chemokines and cell adhesion molecules (reviewed in Kleinbongard *et al.*, 2011; reviewed in Kuno and Matsushima, 1994). IL-6 is expressed in the muscle after exercise or cytokine stimulation, but is also produced by leukocytes in response to TNF-α, and it regulates e.g. the inflammatory reaction and adhesion molecule expression (Youker *et al.*, 1992; Frangogiannis *et al.*, 1998; Nagaraju *et al.*, 1998; Sawa *et al.*, 1998; reviewed in Febbraio and Pedersen, 2002). MIP-2 and MCP-1 are chemokines secreted by many cell types and are especially important in recruiting neutrophils and macrophages (reviewed in Frangogiannis, 2007). We found that all of these factors were significantly downregulated in SOD3 treated rat tissues (I, Figure 5B). Similarly, the few most common cell adhesion molecules we studied - ICAM, VCAM, E-selectin and P-selectin - were strongly downregulated in the same tissue samples (I, Figure 5C). When the expression of these factors is low the leukocytes will not receive the cues to activate and

orient themselves towards the injury site or be able to cross the vascular wall thus explaining the reduced leukocyte counts (Lefer *et al.*, 1996; Zhang *et al.*, 2001a; Zhang *et al.*, 2001b; Shireman *et al.*, 2007).

The inflammatory response is a complex phenomenon characterized by involvement of a number of cell populations, both local and transmigrated from the circulation, and an exchange of a vast amount of signals with both synergistic and antagonistic effects on their functions. The signaling events are induced by e.g. ROS, cytokines, the complement cascade and Toll-like receptors responding to the tissue- and leukocyte-derived stimuli (reviewed in Frangogiannis, 2007). To find a common nominator for the SOD3 and the cytokines, chemokines and adhesion molecules studied here, we decided to determine the impact of SOD3 on the NFκB, a ROS responsive transcription factor which is one of the central regulators of the inflammatory reaction (reviewed in Kabe *et al.*, 2005). For instance, it is necessary for the expression of TNF-α, several interleukins, ICAM and VCAM (reviewed in Pahl, 1999). An *in vitro* luciferase assay showed that SOD3 can markedly suppress NFκB activity, and induce a modest increase in the inhibitory IκB in SOD3 treated muscle tissue (I, Figure 5A). Additionally, our observation that the rat phagocytic Nox2 was markedly increased in control tissues but downregulated after SOD3 overexpression (II, Figure 1F) further supported the involvement of these factors in the SOD3 mediated anti-inflammatory mechanism. Namely, both of them are ROS-responsive: NFκB activity is known to be enhanced by Nox-derived O_2^{-}• and diminished by administration of antioxidants such as SOD (Fan *et al.*, 2002; Lin *et al.*, 2007; Li *et al.*, 2009b), and the IκB is marked for degradation upon increased oxidative stress leading to subsequent NFκB activation (Fan *et al.*, 2003). Altogether, our findings suggest that SOD3 attenuates leukocyte migration and tissue inflammation in a ROS-dependent manner through the NFκB/IκB complex and subsequent downregulation of essential cytokines, signaling factors and adhesion molecules.

1.3 Increase in SOD3 upon TSH-R stimulation indicates a role in thyroid physiology

The thyroid gland is constantly under oxidative stress because the thyroid hormone synthesis requires H_2O_2 generation, and due to this it has mounted potent antioxidant defenses (reviewed in Song *et al.*, 2007). SOD enzyme activities in normal thyroid gland are relatively high in several mammalian species which suggests a functional role in this organ (Marklund, 1984a). The mitogenic properties of H_2O_2 in general and SOD3 in skeletal muscle (reviewed in Stone and Yang, 2006; Laurila *et al.*, 2009) as well as the H_2O_2 dependent hormone synthesis gave a starting point for our thyroid studies.

The study by Marklund *et al.* (1984a) did not include the SOD activity levels in the rat thyroid, and therefore we determined the expression levels of all SOD isoforms

in this and control tissues. SOD3 was the most abundant isoform in rat kidney, aorta and thyroid while SOD1 was the major isoform in liver and heart, but SOD2 expression was inconsistent (III, Figure 1A). However, the expression levels determined by us and the given activities (Marklund, 1984a) in the tissues were in contrast: in rat liver, kidney and heart the SOD3 activity is lowest (19-345 U/g tissue), SOD1 very high (>10600 U/g tissue) and SOD2 between these two (1070-2830 U/g tissue). These discrepancies are likely to be a consequence of the strict regulation of SOD activity by various post-translational modifications (Enghild *et al.*, 1999; Bowler *et al.*, 2002; Brown *et al.*, 2004; Olsen *et al.*, 2004). On the other hand, in human thyroid gland the activity of SOD1 was similarly very high, SOD3 approximately 4-fold higher than SOD2, and SOD2 the lowest (Marklund, 1984a) which were in line with our expression studies (III, Figure 1G). In general, it seems that it is not feasible to correlate SOD activity directly with gene expression since even small quantities of this enzyme can function at relatively high rate and produce the tissue effect. Secondly, the SOD3 may not be needed continuously since most ROS generating reactions take place well-guarded in the follicular lumen, but could serve a more special role in the thyroid gland which includes its activation only on certain occasions.

In order to evaluate the participation of SOD3 in the thyroid gland physiology we started by investigating the changes in its expression level *in vivo* after PTU administration. PTU inhibits thyroid hormone synthesis that consequently upregulates TSH secretion from the pituitary gland leading to continuous TSH-R activation and thyroid hypertrophy, goiter. Accordingly, we observed an increase in thyroid weights as well as typical morphological changes in the PTU treated rats when compared to control rats (III, Figure 1B-D). Also the expression level of SOD3 was several fold upregulated (III, Figure 1E) which is in line with a previous study of murine goiter (Maier *et al.*, 2007). We then extended our studies to human tissues, but failed to see any difference between normal and hypertrophic AA and FNAH tissues (III, Figure 1F) which was unexpected since TSH has previously been shown to induce both SOD1 and SOD2 expression (Verma *et al.*, 1991; Nishida *et al.*, 1997; Singh *et al.*, 1997).

In all these cases of thyroid hypertrophy the growth was caused mainly by increased TSH-R activity by either endogenous TSH (PTU) or mutations rendering it constitutively active (AA, FNAH; Hebrant *et al.*, 2009). However, the species-related difference and/or the mode of TSH-R activation may have caused the observed results. The species difference aspect was supported by the prominent role of SOD3 in rat tissue while in human tissue it was SOD1 as well as by the overall lower level of SOD activity in rats (III, Figure 1A, G, H; Marklund, 1984a). Additionally, rats are more susceptible to hypothyroidism and increased TSH-mediated hypertrophy due to the lack of thyroid hormone binding globulin while in human circulation majority of the

hormones are stored in bound form and released upon demand (reviewed in Wu and Farrelly, 2006). TSH may also promote the generation of O_2^-• from thyroidal Nox increasing the need for specific antioxidant enzymes (Weyemi *et al.,* 2010; Pomerance *et al.*, 2000). These physiological features could induce a more rapid and profound effect on the rat follicular cells after thyroid hormone deprivation. Moreover, AA and FNAH primary cultures have been found to respond to TSH nearly the same way as normal tissue (Hebrant *et al.*, 2009) while the *in vivo* hyperthyroidism induces repression of TSH production that could thereby even out the differences in TSH-responsive genes between normal thyroid and these patient samples.

1.4 SOD3 is involved in thyroid follicular cell proliferation

TSH receptor is one of the most important regulators of thyroid gland function and determines e.g. whether the follicular cell will proliferate or synthesize thyroid hormones (Allgeier *et al.*, 1994; Corvilain *et al.*, 1994).We first confirmed the strong SOD3 expression in the rat PC Clone 3 thyroid cell line which we used in these experiments and found out that the expression is similar to that *in vivo* (III, Figure 1H). Significantly increased SOD3 expression after stimulation of the two signaling cascades downstream of TSH-R (III, Figure 2) suggested that SOD3 is needed in the follicular cell physiology, either in proliferation/secretion (G_sα-cAMP pathway) or hormone synthesis (G_qα-PLC pathway)(reviewed in Song *et al.*, 2007). At least in humans the activation of these two pathways by TSH-R stimulation has been confirmed, and research on rat cell lines has indicated similar functions (Laurent *et al.*, 1987; reviewed in Kimura *et al.*, 2001). However, the rat cell lines seem to require markedly higher TSH concentration for this to occur which has raised questions about the reliability of these models (reviewed in Kimura *et al.*, 2001). Here, the overexpression of G_qα should enable proper downstream signal transduction to overcome the need for large dose of TSH. Furthermore, TSH-R is responsible for the maintenance of differentiation in follicular cells i.e. expression of the thyroid specific genes such as TPO, Tg and NIS (Avvedimento *et al.*, 1984; Magnusson and Rapoport, 1985; Nagayama *et al.*, 1989b; Levy *et al.*, 1997). The relatively high expression and TSH-R responsiveness would therefore indicate that SOD3 could also function as a thyroid differentiation marker.

The increased SOD3 expression in goitrous rat tissue and after TSH-R stimulation prompted us to investigate whether it is related to follicular cell proliferation or hormone synthesis. To accomplish this, we knocked down SOD3 with specific siRNA in PC Clone 3 cells or studied the relative efficiencies of SOD3 and Duox2 in H_2O_2 generation in a heterologous COS-7 *in vitro* model. In the absence of SOD3 the TSH-mediated upregulation in proliferation rate was significantly reduced (III, Figure 3A, B) indicating that SOD3 can participate in follicular cell growth. The COS-7 cells do not express thyroid specific genes naturally and thus we were able to

compare the two enzymes. SOD3 transfection successfully diminished the O_2^-• production by mismatched Duox2/DuoxA1 in these cells thereby confirming the functionality of both of these enzymes (III, Figure 3C). However, the H_2O_2 production capacity of SOD3 was minimal as compared to matching Duox2/DuoxA2 (III, Figure 3D) suggesting that it cannot take over or compensate for the Duox enzymes in thyroid hormone synthesis. The previous studies pointing out a role for SOD3 in mitogenesis (Laurila *et al.*, 2009) can thus be extended to thyroid gland. Our result may also refer to the general protective role of SOD3 against ROS at its expression sites (Gongora *et al.*, 2008) since increased proliferation is often associated with increased ROS generation (Pani *et al.*, 2000; Bello *et al.*, 2003; Duval *et al.*, 2003). However, more research is needed to determine the molecular mechanism behind this since SOD3 mediated proliferative/survival effect has been closely associated with the Ras-Erk1/2 pathway (Laurila *et al.*, 2009) which does not seem to be prominent or is even inhibited during the proliferation of normal thyrocytes (al-Alawi *et al.*, 1995; Ciullo *et al.*, 2001). A more recent study, on the other hand, stated that Ras-Braf-Erk1/2 cascade is stimulated by TSH via PKA (Vuchak *et al.*, 2009). Furthermore, thyroid cancers are frequently harboring hyperactive Ras and Raf mutations which provide the cells with immortality (reviewed in Riesco-Eizaguirre and Santisteban, 2007).

2 SOD3 in the cellular signaling events

SOD3 is an extracellular enzyme, and its only known substrate is O_2^-• which it converts into H_2O_2, the only known product (McCord and Fridovich, 1969; Klug *et al.*, 1972); nowadays both of these ROS are recognized as signaling agents (reviewed in Thannickal and Fanburg, 2000). Due to its location, SOD3 cannot act on the ROS generated by intracellular sources and thus its signaling mechanism is dependent solely on its ability to alter the relative proportions of these two ROS outside the cell. The most likely route for SOD3-mediated signaling is the diffusion of H_2O_2 through the cell membrane into the cell interior where it can reach the ROS-sensitive signaling molecules. A recent study offered support for this by showing that SOD3 derived H_2O_2 can inhibit cellular phosphatases and thereby promote VEGF expression (Oshikawa *et al.*, 2010). Naturally, in tissue environment there are a number of other pro- and antioxidants which bring more variation to the overall redox balance, but the final impact of SOD3 on the signaling events depends on its expression and activity levels.

The expression, activity and binding affinity of SOD3 must be heavily regulated, since a sudden increase in H_2O_2 generation at the wrong time and place could have fatal consequences for the surrounding cells. This leads to tissue-specific SOD3 activity which is not always comparable to the expression level of the enzyme. However, SOD3 is an efficient enzyme in terms of kinetics, and therefore even

relatively small amounts of active enzyme should produce the physiological effects (Marklund, 1982). Moreover, its long half-life, up to 85 hours, in muscle tissue after administration extends its operating window (Karlsson *et al.*, 1994). We used here the same rat hind limb model as previously, and therefore expected the SOD3 activity to increase approximately 2-fold in the muscle tissue as compared to the control gene treated tissues (Laurila *et al.*, 2009). Several previous studies have indeed shown that cells *in vitro* and *in vivo* respond to only a modest increase in SOD3 concentration (Hatori *et al.*, 1992; Sjoquist and Marklund, 1992; Laukkanen *et al.*, 2002).

2.1 SOD3 in the determination of cell fate – apoptosis or survival?

Anti-apoptotic effects of SOD3 administration have been mentioned in several studies involving tissue injuries (Laukkanen *et al.*, 2001b; Laukkanen *et al.*, 2002; Ozumi *et al.*, 2005; Kliment *et al.*, 2009). Apoptosis is directly caused by ischemia and oxidative stress, and when they are limited also the extent of apoptosis is reduced leading to improved recovery (reviewed in Abbate *et al.*, 2006). The ROS-derived tissue damage is partly caused by the inflammatory cells which are capable of generating large amounts of ROS in the oxidative burst (Nathan, 1987a; reviewed in Nathan, 1987b), and therefore the presence of SOD3 leading to their suppressed migration can affect the overall oxidative stress levels as suggested by the lowered Nox2 and iNOS mRNA expression (II, Figure 1F, G). However, there is little knowledge on SOD3 mediated cellular signaling other than related to attenuation of oxidative stress, although SOD3 has been shown to possess signaling properties (Laurila *et al.*, 2009).

We began to characterize the role of SOD3 in signaling by first determining the expression of endogenous SOD3 in rat tissues. SOD3 is known to be upregulated by various inflammatory cytokines and downregulated by many growth factors (Marklund, 1992; Stralin and Marklund, 2000; Stralin and Marklund, 2001). Furthermore, it is induced during the first few days after acute injury (Horiuchi *et al.*, 2004; Shvedova *et al.*, 2007). Indeed, we observed a small increase 3 days after ischemia induction but progressive decrease at the 7- and 10-day time-points (II, Figure 1E). However, SOD3 transgene expression compensated well for the loss of endogenous SOD3 expression (II, Figure 1E). Although the enzyme activity was not determined, according to our previous study, it is feasible to expect the amount of functional SOD3 enzyme to have increased as well (Laurila *et al.*, 2009). The induced ischemia in this model is not very severe in the thigh muscles since characteristically after femoral artery closure the calf and lower parts of the leg are the most severely inflicted areas (reviewed in Blaisdell, 2002). Therefore, the initial endogenous SOD3 upregulation may have remained relatively low in the absence of more severe inflammation while downregulation at the later time points could have resulted from increasing growth factor expression, to which SOD3 is sensitive, as the inflammation

began to resolve. The more advanced tissue damage and inflammation would still suggest that the healing process was delayed in our control animals (I, Figure 1; II, Figure 1A, B, C), and therefore the SOD3 administration with early high expression and possible long retention in tissue enabling continuous removal of ROS could be of benefit.

Cleaved caspase-3 expression is a highly specific marker for apoptosis due to its role as a key caspase in the process (Faleiro *et al.*, 1997; Woo *et al.*, 1998; reviewed in Porter and Janicke, 1999). We used cleaved caspase-3 specific antibodies to determine the relative levels of apoptosis in the ischemic rat tissues. The markedly higher caspase-3 expression at 3- and 7-day time-points in control animals in contrast to very low expression in the SOD3 animals supported the view that SOD3 is effectively preventing apoptosis already at an early phase (II, Figure 1D).

Several inflammatory cytokines can sensitize or directly induce the cell to apoptosis in injured tissues where a proportion of cells is inevitably going to die. Unlike necrosis, where the cell bursts and releases its contents into the extracellular space, programmed and well-organized apoptosis will cause less additional damage than has already been inflicted by the injury itself, and will thus inhibit further exacerbation of the inflammatory reaction. The central pro-inflammatory cytokine TNF-α has pro-apoptotic properties among the more conventional ones related to e.g. inflammatory cell activation and migration (Hashimoto *et al.*, 1994; Adanali *et al.*, 2001; Zhang *et al.*, 2001b). Its binding to the TNF-receptor activates under appropriate circumstances the signaling cascade leading eventually to the cleavage of caspase-3 and commitment to apoptosis (Natoli *et al.*, 1998; Jiang *et al.*, 2009). Thus, the SOD3-driven downregulation of TNF-α could be one mechanism that reduced the cleaved caspase-3 in our experimental setting (I, Figure 5B; II, Figure 1D).

2.2 SOD3 activates two survival/mitogenic pathways

The reduced damage and lower caspase-3 expression led us to investigate the effect of SOD3 on survival pathways that counteract the apoptosis pathways in tissue injuries. Our previous study has already established the activation of Ras-Erk1/2 cascade and Akt in this same rat model and therefore we now concentrated on their temporal regulation and the factors activated or inhibited by these two kinases (Laurila *et al.*, 2009). They both are important players in the pro-survival and anti-apoptotic signaling due to the large number of substrates they control as well their central position in many signaling networks (reviewed in Yoon and Seger, 2006; reviewed in Mullonkal and Toledo-Pereyra, 2007). In several studies they have been implicated in e.g. cardioprotection after infarction (Yang *et al.*, 2011; Aikawa *et al.*, 1997; Miki *et al.*, 2007). Under oxidative stress Akt activation and inactivation is likely to be related to the tissue redox balance since e.g. PTEN-mediated Akt regulation can be modulated by H_2O_2 (reviewed in Rhee *et al.*, 2005b). However, the H_2O_2 dependent activation

mechanism of Erk1/2 remains unknown, although similar mechanism involving H_2O_2 mediated inhibition of PTPs and subsequent RTK activation is possible as suggested by a study showing SOD3-mediated stimulation of VEGF receptor (Oshikawa *et al.*, 2010). We found that both Erk1/2 and Akt were activated in SOD3 treated tissues already at the 3-day time-point, but from there on differed so that Erk1/2 remained active while Akt activity returned to the same level as in uninjured control tissues by the day 10 (II, Figure 2A, D). This indicates that SOD3-Erk1/2 axis could play a more prominent role in the response to tissue injury, although the contribution of Akt pathway to prevention of apoptosis cannot be excluded based on our data.

The results from myocardium and skeletal muscle studies have proposed slightly different roles and dynamic co-operation for Erk1/2 and Akt in muscle regeneration: Erk1/2 is thought to mediate cell proliferation while Akt promotes myocyte differentiation in response to growth factors (Rommel *et al.*, 1999; Tiffin *et al.*, 2004; De Alvaro *et al.*, 2008). Thus, in the very early phase of injury response both the factors could induce the anti-apoptotic effect but then the Erk1/2 would first take over to allow replacement of the necrotic tissue mass. Then later, after 10-14 days, the Akt pathway would become more active and induce the differentiation of myofibroblasts into mature myocytes. Evidence for such a course of events has been obtained before in a study with a mouse ischemia model similar to ours (Paoni *et al.*, 2002). However, since we focused on the early events of the inflammatory response the possible late switch between Erk1/2 and Akt activities was not confirmed in these studies, although the Erk1/2-mediated signaling observed by us is in accordance with these studies.

Both Erk1/2 and Akt can phosphorylate the ROS-responsive transcription factor FoxO3a which is one of the key factors in the determination of cell fate (Liu *et al.*, 2005; reviewed in Calnan and Brunet, 2008; Yang *et al.*, 2008), although Akt has probably been reported more often in this context. Various upstream stress signals can induce FoxO3a signaling and result in either cell cycle arrest and stress response or apoptosis (Liu *et al.*, 2005). Phosphorylation by e.g. survival kinases inactivates FoxO3a and induces its cytoplasmic translocation thus preventing the transcription of negative cell cycle regulators and pro-apoptotic factors (Brunet *et al.*, 1999; Yang *et al.*, 2008). We found increased phospho-FoxO3a at the 3-day SOD3 rat tissues which was then lowered to the same level with the LacZ control tissue at the 7- and 10-day time-points (II, Figure 3A). Furthermore, SOD3 transfection into NIH 3T3 cells was able to increase the amount of cytoplasmic phospho-FoxO3a without affecting the FoxO3a expression level (II, Figure 3B, C, D). In these experimental settings we could not distinguish how much each of these kinases contributed to the observed FoxO3a regulation, but the results suggest that SOD3 can nevertheless induce its inactivation at an early time point. This provided more support for the anti-apoptotic influence of SOD3 administration in the tissues in addition to the caspase-3 analysis. However, the

trend showing progressive increase in phospho-FoxO3a at later time-points implies that mechanisms other than Erk1/2 and Akt activation may further regulate the established reduction in apoptosis.

2.3 Erk1/2 is the principal mediator of SOD3 effects in inflammation

2.3.1 Ets-1 and Elk-1

The direct Erk1/2 downstream transcription factors Ets-1 and Elk-1 are expressed in several tissues with slightly different sets of target genes. Ets-1 promotes e.g. survival, vascular cell proliferation and invasive phenotype of tumor cells (reviewed in Dittmer, 2003; Hashiya *et al.*, 2004). Moreover, ischemic models of e.g. skeletal muscle and retina have shown an improvement in angiogenesis upon Ets-1 activation (Taniyama *et al.*, 2001; Watanabe *et al.*, 2004). Elk-1 in turn has been found to promote c-Fos expression and to contribute to e.g. myoblast proliferation and anti-apoptotic signaling (Booy *et al.*; Gille *et al.*, 1995; De Alvaro *et al.*, 2008). To stress the importance of Erk1/2 activation we determined the expression of these two factors, and found them to be markedly upregulated in the SOD3 rat tissues (II, Figure 2B, C). The high expression supported the recovery-related functions of these transcription factors in tissues. This also further supported our view that the SOD3-Erk1/2 cascade could be the more significant one in attenuation of ischemic damage. Moreover, our previous study showed activation of AP-1, a Fos-Jun dimer, *in vitro* after SOD3 transfection (Laurila *et al.*, 2009), and therefore the SOD3-mediated Elk-1 upregulation may have a role in the AP-1 driven proliferation.

2.3.2 MicroRNA-21

To further elucidate the signaling mechanism and agents utilized by SOD3 we chose some downstream effectors of FoxO3a that have been shown to have a role in tissue ischemia and apoptosis regulation. The miR-21 is expressed in response to e.g. an EGFR-family RTK, found in breast cancer cells, via Erk1/2-Ets-1 and is a direct target of FoxO3a suppression (Wang and Li, 2010; Huang *et al.*, 2009). It has been associated with cardioprotection but also cell proliferation and tumor development in various cardiac disease and cancer models (reviewed in Cheng and Zhang, 2010; Asangani *et al.*, 2008; Dong *et al.*, 2009). Additionally, upregulation of miR-21 expression in cardiomyocytes *in vitro* after incubation with subtoxic H_2O_2 concentration indicated that this factor is ROS sensitive (Cheng *et al.*, 2009). We found markedly increased miR-21 expression in the SOD3 treated animals (II, Figure 3E) which suggests that it may be a prominent factor in the skeletal muscle recovery, and that SOD3 is able to enhance its expression. This effect of SOD3 is supported by a recent study showing that ischemic preconditioning, known to induce antioxidant

enzyme expression, promoted miR-21 expression after myocardial infarction (Dong *et al.*, 2009).

2.3.3 Bim

FoxO3a is one of the transcriptional regulators of Bim, a crucial pro-apoptotic factor functioning through mitochondrial Mcl-1/Bak/Bax cascade and caspase-3 (Gilley *et al.*, 2003; Liu *et al.*, 2005; Yang *et al.*, 2008). Expression of Bim is upregulated when Akt is inactivated enabling FoxO3a translocation to the nucleus (Zhu *et al.*, 2008). Ischemia has also been shown to upregulate Bim in animal models of myocardial or brain ischemia (Bhuiyan *et al.*, 2008; Li *et al.*, 2009a). Since we found FoxO3a to be inactivated in SOD3 animals soon after the ischemic injury was induced (II, Figure 3A), we determined the expression level of Bim from the rat tissues to explain the reduced apoptosis. Significantly downregulated Bim expression was well in line with this result (II, Figure 3F). Active Erk1/2 and Akt can phosphorylate Bim directly leading to abrogation of Bim function (Wiggins *et al.*; Luciano *et al.*, 2003; Ley *et al.*, 2004; Qi *et al.*, 2006) which in the light of our findings indicates that SOD3 can induce a Bim-dependent anti-apoptotic effect by two mechanisms: Akt-FoxO3a mediated suppression of transcription, and Erk1/2 and Akt mediated phosphorylation. Similar Erk1/2-Bim interaction providing tolerance against oxidative stress has been shown in a study with neuronal ischemia (Meller *et al.*, 2006). Additionally, the latter mechanism could account for the Erk1/2-driven anti-apoptotic signaling later in the inflammatory reaction when FoxO3a phosphorylation is no longer prominent due to downregulation of Akt activity, but the possible sustained reduction in Bim expression in the later time-points was not addressed.

2.4 The differential expression of SOD3 in thyroid cancer

These studies have shown that SOD3 is able to promote cell survival over apoptosis in inflammation by activating Erk1/2-dependent signaling cascades, and to participate in thyroid follicular cell proliferation by a yet unknown mechanism. Accordingly, adequate SOD3 expression can be considered to benefit both normal and injured tissue while its downregulation can exacerbate the damage. However, activating Ras and Raf mutations are very common in various cancers, including thyroid cancers, where they induce hyperproliferation of the transformed cells and prevent apoptosis independently of outside signals (reviewed in Riesco-Eizaguirre and Santisteban, 2007). Simultaneously, the expression of cell type specific genes is downregulated, and the cells thus dedifferentiate losing their characteristic functions (Francis-Lang *et al.*, 1992; Gerard *et al.*, 2003; De Vita *et al.*, 2005). We studied the SOD3 expression from three transformed rat thyroid cell lines derived from the PC Clone 3 – PC Braf, PC RET/PTC1 and PC E1A – to see how it changes in hyperproliferative environment (Fusco *et al.*, 1987; Santoro *et al.*, 1993). In all of them the SOD3 expression was

significantly lower than in the parental cell line, and accordingly the H_2O_2 generation was reduced (III, Figure 4A, D). These oncogenic cell lines have previously been determined to have lost the iodide uptake and organification ability along with the expression of Tg and NIS as well as dependency on TSH stimulation as an indication of their dedifferentiation (Santoro *et al.*, 1993; Trapasso *et al.*, 1999). This, together with the observed TSH responsiveness (III, Figure 2), suggests that SOD3 behaves similarly to these thyroid specific genes, and thus could serve as a marker for transformation.

The differential expression of SOD3 in the different oncogene transformants (III, Figure 4A) cought our interest and we investigated whether it could be dependent on the gene dose. For this purpose we used a doxycyclin-inducible RET/PTC1 oncogene transformed rat PC cell line. The results showed a progressive reduction in the SOD3 expression with simultaneous increase in RET/PTC1 expression (III, Figure 4B, C). Thus, it appears that strong oncogene upregulation signals the cells to downregulate SOD3, but the mechanism remains enigmatic still. Co-operation between Ras or Raf and the RET/PTC1 is one option: RET/PTC rearrangement has been shown to induce activation of the Ras-MAPK and PI3K cascades (Ouyang *et al.*, 2006; Henderson *et al.*, 2007; Lodyga *et al.*, 2009), the two of which can suppress the thyroid specific gene expression (Garcia and Santisteban, 2002; De Vita *et al.*, 2005). Similar connection has been found between E1A and Erk1/2 (Kukushkin *et al.*, 2002). Moreover, the viral or other oncogenes used to transform the original PC Clone 3 cells induce variable levels of malignancy alone, but require co-expression of e.g. Ras or Raf for a full cancer cell phenotype (Berlingieri *et al.*, 1993; Santoro *et al.*, 1993). We have previously found a possible positive feedback loop between Ras-Erk1/2 and SOD3 (Laurila *et al.*, 2009), and in this light it seems that at some threshold level the positive feedback switches to negative. These findings indicate that Ras/Raf overexpression could induce SOD3 downregulation. Another recent study described notable downregulation of FoxO3a activity in PTC (Karger *et al.*, 2009), regardless of higher than average oxidative stress in cancer tissue and, since FoxO3a regulates the expression of many antioxidant genes including SOD3 (Li *et al.*, 2006; Tothova *et al.*, 2007), this could be one mechanism behind SOD3 downregulation. However, further studies are needed to establish how much SOD3 actually participates in the differentiation/dedifferentiation and tumorigenesis in thyroid follicular cells. In addition, the nature of SOD3 co-operation with the Ras-Erk1/2 pathway in cancer cells remains elusive.

When we finally determined the SOD isoform expression from human PTC and ATC patient samples, we found significantly reduced SOD3 in both cancer types (III, Figure 4E, F). This supports the results we obtained from the rat cell line studies suggesting SOD3 as a differentiation marker for thyroid malignancy, and widens the perspective to human cancers, as well. In these samples, there were no notable

differences in the SOD3 expression between the cancer types, but the SOD1 and SOD2 were somewhat variably expressed rendering them unsuitable as such markers. However, this variance may be caused in part by the methods used since quantitative PCR and DNA array may have different sensitivities. Similar downregulation of antioxidant enzymes after transformation have been demonstrated in several other studies (Erdamar *et al.*; Yanagawa *et al.*, 1999; Hasegawa *et al.*, 2002; Hasegawa *et al.*, 2003). This phenomenon exposes the cells to redox unbalance and generally higher oxidative stress which can increase the DNA mutation rate, genetic instability and alter their behavior by e.g. suppressing differentiation thus resulting in a more malignant cell (reviewed in Szatrowski and Nathan, 1991; Laurent *et al.*, 2005; Valko *et al.*, 2006). Furthermore, although high ROS concentration is toxic to any cell, both $O_2^{-}\bullet$ and H_2O_2 have been found to promote an invasive and metastatic phenotype of tumor cells (Szatrowski and Nathan, 1991; Laurent *et al.*, 2005; Teoh *et al.*, 2007). The particular function of SOD3 downregulation in these cells remains obscure, but an applicable theory has been presented by Laurent *et al.* (2005) who suggest that a lower H_2O_2 generation rate induced by inhibition of relevant enzymes can be pro-tumorigenic in cancer cells. Thus, lower endogenous SOD3 function and consequently lower H_2O_2 concentration could offer them an advantage in proliferation and invasion.

CONCLUSIONS

Superoxide dismutases have been keenly studied for their antioxidant properties, and most research has concentrated on the SOD1 and SOD2 which were found earlier than SOD3. All three SODs have shown potential in attenuating oxidative stress and inflammation-related tissue damage in various *in vivo* models apparently by reducing the harmful O_2^-• content but also by regulating the expression of certain inflammatory factors. Early on, oxidative stress and altered SOD enzyme activity have also been associated with transformation and various properties of tumor cells. Furthermore, SOD3 has been recently assigned a role in cell signaling. This dissertation studies the signaling mechanisms behind the beneficial effects of SOD3 in ischemic tissue injury and begins to elucidate its role in thyroid gland.

Our results from the rat hind limb ischemia model demonstrate that while endogenous SOD3 was downregulated, exogenously administered SOD3 could inhibit leukocyte trafficking by reducing the expression of central pro-inflammatory cytokines and adhesion molecules resulting in smaller injured areas. Furthermore, we showed that SOD3 promoted the activation of both Erk1/2 and Akt survival kinases in the early phase of ischemia, but only the Erk1/2 was kept active later in the acute phase. The brief Akt and long-term Erk1/2 downstream signaling repressed the apoptosis-inducing factors and upregulated Erk1/2-dependent gene transcription. In the rat thyroid gland SOD3 expression was induced by TSH-R stimulation and further upregulated in goiter characterized by hyperplasia, whereas inhibition of SOD3 impaired rat follicular cell proliferation *in vitro*. Moreover, in rat and human thyroid cancer cell lines it was markedly downregulated in a gene dose-dependent manner.

These studies have revealed new aspects of SOD3-mediated signaling in normal cells/tissues and under pathological conditions. We conclude that SOD3 appears to participate in the functions of normal skeletal muscle and thyroid tissues by protecting them from basal level ROS and participating in cell proliferation. SOD3 is downregulated in tissue injuries and pathological conditions with different outcomes depending on the incident. In ischemia, reduced SOD3 enables the worsening of inflammation and apoptosis, but its administration restores the activity of Erk1/2 and Akt survival pathways and prevents phagocyte-derived oxidative stress. In cancer, absence of SOD3 can be a selective event providing the tumor cells with a growth advantage. Moreover, the consistent lack of SOD3 expression in human thyroid cancer, confirmed by our rat studies, indicates that the enzyme could be utilized as a differentiation marker for malignant transformation.

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