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Stanniocalcin-1 in cell stress and differentiation

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Academic Dissertation

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"Fish – you've got to respect them. We were fish, long ago, before we were apes."

Clive Owen, *Closer*, 2004

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ABSTRACT

Stanniocalcin-1 (STC-1) is a 56 kD homodimeric protein which was originally identified in bony fish, where it regulates calcium/phosphate homeostasis and protects against toxic hypercalcemia. STC-1 was considered unique to fish until the cloning of cDNA for human *STC-1* in 1995 and mouse *Stc-1* in 1996. STC-1 is conserved through evolution with human and salmon STC-1 sharing 60% identity and 80% similarity. The surprisingly high homology between mammalian and fish STC-1 and the protective actions of STC-1 in terminally differentiated neurons, originally reported by my colleagues, prompted me to further study the role of STC-1 in cell stress and differentiation.

One purpose was to determine whether there is an inter-relationship between terminally differentiated cells and STC-1 expression. The study revealed an accumulation of STC-1 in mature megakaryocytes and adipocytes, i.e. postmitotic cells with limited or lost proliferative capacity. Still proliferating uninduced cells were negative for STC-1 mRNA and protein, whereas differentiating cells accumulated STC-1 in their cytoplasm. Interestingly, in liposarcomas the grade inversely correlated with STC-1 expression.

Another aim was to study how STC-1 gene expression is regulated. Given that IL-6 is a cytokine with neuroprotective actions, by unknown mechanisms, we examined whether IL-6 regulates STC-1 gene expression. Treatment of human neural Paju cells with IL-6 induced a dose-dependent upregulation of *STC-1* mRNA levels. This induction of STC-1 expression by IL-6 occurred mainly through the MAPK signaling pathway.

Furthermore, I studied the role of IL-6–mediated STC-1 expression as a mechanism of cytoprotection conferred by hypoxic preconditioning (HOPC) in brain and heart. My findings show that *Stc-1* was upregulated in brain after hypoxia treatment. In the brain of IL-6 deficient mice, however, no upregulation of *Stc-1* expression was evident. After induced brain injury the STC-1 response in brains of IL-6 transgenic mice, with IL-6 overexpression in astroglial cells, was stronger than in brains of WT mice. These results indicate that IL-6–mediated expression of STC-1 is one molecular mechanism of HOPC-induced tolerance to brain ischemia.

The protection conferred by HOPC in heart occurs during a bimodal time course comprising early and delayed preconditioning. Interestingly, my results show that the expression of *Stc-1* in heart was upregulated in a biphasic manner during HOPC. IL-6 deficient mice did not, however, show a similar biphasic manner of *Stc-1* upregulation as did WT mice. Instead, only an early upregulation of *Stc-1* expression was evident. The results suggest that the upregulation of *Stc-1* during the delayed preconditioning is IL-6–dependent. The upregulated expression of *Stc-1* during the early preconditioning, however, is only partly IL-6–dependent and possibly also directly mediated by HIF-1.

These findings suggest that STC-1 is a pro-survival protein for terminally differentiated cells and that STC-1 expression may in fact be regulated by stress. In addition, I show that STC-1 gene upregulation, mediated in part by IL-6, is a new mechanism of protection conferred by HOPC in brain and heart.

Because of its importance for fundamental biological processes, such as differentiation and cytoprotection, STC-1 may have therapeutic implications for management of stroke, neurodegenerative diseases, cancer, and obesity.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals I-IV.

I Serlachius M, Alitalo R, Olsen HS and Andersson LC. Expression of stanniocalcin-1 in megakaryocytes and platelets. *British Journal of Haematology* **119**: 359-363, 2002.

II Serlachius M and Andersson LC. Upregulated expression of stanniocalcin-1 during adipogenesis. *Experimental Cell Research* **296**: 256-264, 2004.

III Westberg JA*, Serlachius M*, Lankila P, Penkowa M, Hidalgo J and Andersson LC. Hypoxic preconditioning induces neuroprotective stanniocalcin-1 in brain via IL-6 signaling. *Stroke* **38**: 1025-1030, 2007.

IV Westberg JA*, Serlachius M*, Lankila P and Andersson LC. Hypoxic preconditioning induces elevated expression of stanniocalcin-1 in the heart. *American Journal of Physiology - Heart and Circulatory Physiology* **293**: H1766–H1771, 2007.

*) Equal contribution

ABBREVIATIONS AND CHEMICAL FORMULAS

AEC	3-amino-9-ethylcarbazole
ALS	Amyotrophic lateral sclerosis
Arg	Arginine
Asn	Asparagine
BCL-2	B-cell lymphoma 2
BRCA1	Breast cancer 1
C/EBPs	CCAAT-enhancer-binding proteins
Ca^{2+}	Calcium (divalent ion)
CDK	Cyclin-dependent kinase
CLC	Cardiotrophin-like cytokine
CNTF	Ciliary neurotrophic factor
Co^{2+}	Cobalt (divalent ion)
$CoCl_2$	Cobalt chloride
COOH	Carboxyl
CREB	cAMP respone element-binding
CS	Corpuscles of Stannius
CT-1	Cardiotrophin 1
Cu^{2+}	Copper (divalent ion)
Cys	Cysteine
DAB	3,3'-diamino-benzidin-tetrahydrochlorid
DD-RT-PCR	Differential display reverse transcription-polymerase chain reaction
DFO	Desferrioxamine
DRPLA	Dentatorubro-pallidoluysian atrophy
EGR-1	Early growth factor response 1
EST	Expressed sequence tag
FITC	Fluorescein isothiocyanate
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
HD	Huntington´s disease
HGF	Hepatocyte growth factor
HIF-1	Hypoxia inducible factor 1
HOPC	Hypoxic preconditioning
HRE	Hypoxia responsive elements
HrSTC-1	Human recombinant STC-1
HSP70	Heat shock protein 70
JAK	Janus-activated kinase
IL-6	Interleukin 6
Il-6$^{-/-}$	*Il-6* deficient mouse, *Il-6* knockout
IL-6Rα	Interleukin 6 receptor alpha
IPC	Ischemic preconditioning
LIF	Leukemia inhibitory factor
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
MD	Myotonic dystrophy
MEK	MAP (mitogen-activated protein) kinase kinase
mitoK_{ATP}	mitochondrial ATP-sensitive K^+-channel
MLC	Myosin light chain

MT-1	Metallothionein 1
MTF-1	metal-response transcription factor 1
NAIP	Neuronal apoptosis inhibiting protein
NF-IL6	Nuclear factor for IL-6
NFκB	Nuclear factor-kappa B
Ni^{2+}	Nickel (divalent ion)
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
OSM	Oncostatin M
p300/CBP	p300/CREB binding protein
P_i	Inorganic phosphate
PI3K	Phosphoinositide 3-kinase
PiT-1	Sodium-dependent phosphate transporter 1
PMA	Phorbol 12-myristate 13-acetate
PPARγ	Peroxisome proliferator-activated receptor gamma
ROS	Reactive oxygen species
SBMA	Spinobulbar muscular atrophy
SCA	Spinocerebellar ataxia
Ser	Serine
sgp130	Soluble form of gp130
sIL-6Rα	Soluble interleukin 6 receptor alpha
STAT	Signal transducer and activator of transcription (acute-phase response factor)
STC-1	Stanniocalcin-1
Stc-1$^{-/-}$	Stanniocalcin-1 deficient mouse, Stanniocalcin-1 knockout
STC-2	Stanniocalcin-2
STCrP	STC-related protein
Thr	Threonine
TICs	Thecal-interstitial cells
TNF-α	Tumor necrosis factor alpha
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
Zn^{2+}	Zinc (divalent ion)

INTRODUCTION

The cells of an adult multicellular organism can be divided into three broad classes of cell types in terms of proliferative capacity: labile cells, stable cells, and permanent cells. Permanent cells normally only divide in embryonic and fetal life. They are terminally differentiated and have irreversibly lost their ability to divide, i.e. they are not replaced when lost. This group includes cells that are designed to survive for 100 years, such as cardiac muscle cells, photoreceptors in the retina, and neurons. Terminally differentiated cells do not give rise to neoplasms. In other words, cell proliferation appears to be incompatible with the expression of a terminally differentiated program of gene expression. Thus, irreversible arrest of cell division and expression of the terminally differentiated phenotype are interdependent.

Terminal differentiation is essential for several fundamental biological processes including myotube formation, neural differentiation, bone formation, angiogenesis, and wound repair. Dedifferentiation and uncontrolled proliferation, however, are distinctive for cancer.

Postmitotic cells are especially prone to endo- and exogenic stress. During cell damage, i.e. hypoxia, they need to protect themselves against increasing intracellular Ca^{2+} concentration, i.e. hypercalcemia. Instead of renewing themselves, terminally differentiated cells survive with the help of proteins that are turned on during stress and act as survival factors. These factors are crucial for the maintenance of the integrity of especially postmitotic terminally differentiated cells.

Since terminal differentiation is obligatory for biological events such as those listed above, it is important to identify proteins responsible for the maintenance and protection of the terminally differentiated phenotype, in particular during stress. This study adds stanniocalcin-1 to the list of proteins essential for the survival of terminally differentiated cells and for the protection of these cells under stressful conditions.

REVIEW OF LITERATURE

1. STANNIOCALCIN

1.1 Fish STC

In 1839, at the University of Rostock in Germany, Professor H. Stannius (Figure 1) discovered paired glands adjacent to the fish kidney that he believed to be the adrenals (Stannius, 1839). These glands, named the Corpuscles of Stannius (CS) by Ecker in 1898 (Vincent, 1898), secrete a calcitonin-like hormone (Fontaine, 1964) that was later purified and namned Stanniocalcin (STC) after the CS (Lafeber et al, 1986). The CS was not, however, the equivalent of the adrenals, but an organ specific for fish located ventrally on the surface of the kidney or scattered throughout the kidney proper. STC had a calcitonin-like effect (inhibiting) on whole body calcium (Ca^{2+}). Removal of the CS produced hypercalcemia (Ishibashi & Imai, 2002). The main target organs for the anti-hypercalcemic effect of fish STC are the gills (Wagner et al, 1988) and gut (Sundell et al, 1992, Takagi et al, 1985). Not only does STC lower the Ca^{2+} intake, but it also stimulates the uptake (resorption) of inorganic phosphate (P_i) by the proximal tubule epithelium in the fish kidney (Lu et al, 1994).

Figure 1. The Corpuscles of Stannius are named after Professor H. Stannius, who discovered them in 1839.

The expression of fish STC was considered exclusive to the CS until *stc* mRNA was detected in piscine ovary, testis, and kidney (McCudden et al, 2001). The level of STC in these organs is however about 100-fold lower than in the CS, possibly explaining the late discovery of these expression sites. It is still unclear whether STC is systemically secreted (into the circulation) as an endocrine hormone, as from the CS, or whether it acts more locally in the ovary, testes, and kidney.

1.2 Mammalian STC-1

Since the Ca^{2+} concentration of the surrounding water is higher than that in cells, Ca^{2+} will enter the body by diffusion and the regulation of Ca^{2+} intake at the gills and intestine becomes of great importance in both seawater and freshwater fish. Mammalians do not run the risk of becoming spontaneously hypercalcemic like fish since their plasma Ca^{2+} concentrations increase only transiently after the ingestion of food containing Ca^{2+}. In response to such temporary hypercalcemia, calcitonin is secreted to facilitate Ca^{2+} transfer to bone and to normalize the concentration level of Ca^{2+} in plasma. Therefore, the need of an additional anti-hypercalcemic protein in mammalians was not obvious. In addition, since the CS have no counterpart in mammalians, STC was considered unique to holostean and teleostean fish. However, reports showing that fish STC injected into frogs, birds, and rats induced hypocalcemia suggested the presence of a functional STC receptor in other vertebrates (Madsen et al, 1998, Milet et al, 1984, Srivastav & Swarup, 1982). Later, a report showed that human kidney cells cross-reacted with salmon anti-STC antibodies (Wagner et al, 1995). It was not until 1995, however, when two laboratories (Chang et al, 1995, Olsen et al, 1996) independently cloned the cDNA for the human ortholog of fish *stc*. Because of its high degree of homology to fish *stc*, this human ortholog was also named *STC*. One year later also the mouse *Stc* cDNA was cloned (Chang et al, 1996) showing a high level of similarity to its human homolog.

When a second member of the gene family, *STC-2*, was identified in 1998 by screening for *STC* homologs in an expressed sequence tag (EST) database (Chang et al, 1998, DiMattia et al, 1998, Ishibashi et al, 1998, Moore et al, 1999), *STC* was renamed *STC-1*.

1.3 STC-2

Stanniocalcin-2, (STC-2), was initially identified as a stanniocalcin by virtue of its approximately 34% similarity to STC-1 (Chang & Reddel, 1998). The degree of sequence homology supports the fact that these two proteins have evolved from a common ancestor gene.

As for STC-1, little is known about the function(s) of STC-2, previously also called STC-related protein (STCrP) (DiMattia et al, 1998). It is tempting to assume that the function(s) of the two proteins overlap because of the similarity in amino acid sequence. There are, however, differences between these two proteins. Not only are there distinct differences in the carboxy terminus (COOH) of the amino acid sequences between STC-1 and STC-2, but the expression patterns of the two genes differ (Chang & Reddel, 1998, Chang et al, 2003, Ishibashi et al, 1998, Varghese et al, 1998), as well. Moreover, there are data showing that STC-2 inhibits phosphate uptake in a kidney cell line (Ishibashi et al, 1998) and that STC-2 is unable to displace STC-1 from its putative receptor (Luo et al, 2004, McCudden et al, 2002), suggesting opposite roles of STC-2 and STC-1.

1.4 The genes

1.4.1 Fish stc-1

The fish *stc-1* gene, isolated from the sockeye salmon (McCudden et al, 2001), is about 4 kb long and contains five exons. Exon 2 is highly conserved between fish and mammalians. Exon 3 in mammalians corresponds to exon 3-4 in fish, apparently resulting from a fusion of the fish exons 3 and 4 (Figure 2) (Chang et al, 2003).

1.4.2 Mammalian STC-1

The human *STC-1* gene is located on the short arm of chromosome 8, 8p11.2-p21. The mouse *Stc-1* gene is located on chromosome 14 D1. Both genes span about 13 kb with 83.5% nucleotide sequence identity in the coding region (cDNA). Additionally, there is high sequence conservation between humans and mice in the approximately 3 kb 3'-untranslated region (3'UTR). This high degree of homology in the 3'UTR region may indicate conservation of elements required for the stabilization of the mRNA (Varghese et al, 1998). The human *STC-1* 5'UTR region contains 4 short interrupted

blocks, each consisting of three to six CAG trinucleotide repeats, separated by 6-15 nucleotides (Chang et al, 1998). In addition, the 3'UTR consists of one block of six CAG repeats. Interestingly, CAG and other trinucleotide repeats (CGG and GAA) are associated with genes linked to at least seven inherited diseases, such as fragile X syndrome, myotonic dystrophy (MD), spinobulbar muscular atrophy (SBMA), spinocerebellar ataxia (SCA), dentatorubro-pallidoluysian atrophy (DRPLA), Friedreich´s ataxia, and Huntington´s disease (HD) (Sutherland & Richards, 1995, Warren, 1996). The significance of the CAG repeats in the human *STC-1* 5'UTR is, however, still unknown.

The human *STC-1* gene is organized into 4 exons sized 402 bp, 143 bp, 212 bp, and 3125 bp and into 3 introns sized 1.9 kb, 0.68 kb, and 6.1 kb. By comparison, the 4 exons in the mouse gene are 253 bp, 143 bp, 212 bp, and 3190 bp, and the introns are 1.83 kb, 0.6 kb, and 6.35 kb respectively (Figure 2). The exon-intron boundaries are identical in humans and mice.

The transcription initiation site is located 284 bp upstream of the translation start site (ATG) and a putative TATA box is located 32 bp upstream of the transcription start site (Chang et al, 1998).

1.4.3 Fish stc-2

As only *stc* was known in fish, finding a second gene for stanniocalcin in mammalians was surprising. Later, however, analyses of marine salmon and white suckers showed that a second *stc* also exists in fish (Marra et al, 1998, Wagner et al, 1998). Additionally, analysis of the well-studied genomes of pufferfish (www.fugubase.com) (Chang et al, 2003), *Danio rerio* (zebrafish) (Luo et al, 2004), and *Tetraodon nigroviridis* (http://hinvdb.ddbj.nig.ac.jp/) indicates that they contain an *stc-2* gene. These findings suggest that mammalian *STC-1* and *STC-2* derive from corresponding fish homologs.

1.4.4 Mammalian STC-2

The human *STC-2* gene is located on the long arm of chromosome 5, 5q33 or 5q35.2 (Moore et al, 1999, White et al, 1998). Like *STC-1*, it contains 4 exons (Ishibashi et al, 1998) (Figure 2), with the exon-intron boundaries conserved between *STC-1* and

STC-2. This indicates that *STC-1* and *STC-2* derive from a common ancestral gene. The mouse *Stc-2* gene is located on chromosome 11 A4.

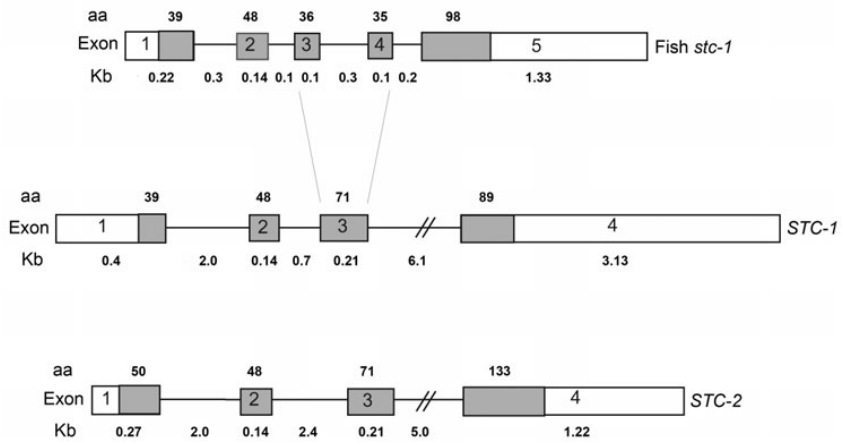

Figure 2. Genomic structure of fish *stc-1*, human *STC-1*, and *STC-2*. Exons are represented by boxes, introns by the intervening lines between exons, protein coding by shading, and UTRs by the open portion of the boxes. The sizes of exons and introns are shown in kilobases (kb). The number of amino acid residues (aa) is shown for each exon. Modified from (Chang et al, 2003).

1.5 The proteins

STC-1 is a 56 kD homodimeric glycoprotein. The *STC-1* cDNA of both human and mouse encode a 247 amino acid protein with only nine amino acid substitutions (Chang et al, 1996, Chang et al, 2003). The 204 first amino acids show 92% sequence similarity to salmon STC, with 118 identical residues (Chang et al, 2003). The last 43 residues at the C-terminus are, however, divergent, so that the whole human STC-1 shows approximately 55% identity with the 256 amino acid fish STC-1 (Ishibashi & Imai, 2002). The fact that the C-terminal region of human and mouse STC-1 differ from that of fish, suggests that the biological activity resides in the core and N-terminal domains (Gerritsen & Wagner, 2005).

Interestingly, the ovaries produce a number of high molecular weight STC variants, collectively referred to as big STC. Unlike the conventional 56 kD form of STC-1, or STC50, big STC comprises at least three proteins of 84, 112, and 135 kD (Paciga et al, 2002).

The human *STC-2* cDNA encodes a 302 amino acid protein (55 amino acids larger than human STC-1) that shows 34% identity to both human STC-1 and eel STC-1, with the N-terminal residues 24-101 being the most identical (50% identity and 73% amino acid homology). The amino acid sequence downstream of position 101 shows, however, less identity (23%) to human STC-1 with its 45 amino acids larger histidine rich COOH-terminal region (Moore et al, 1999). This cluster of histidines may interact with divalent metal ions such as Zn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+} (Ishibashi & Imai, 2002). In fact, this histidine cluster was used to purify STC-2 on a Ni^{2+} column (Moore et al, 1999). Despite their differences, both genes have identical exon-intron junctions (Ishibashi et al, 1998), suggesting that they were produced by gene duplication. In conclusion, as the level of similarity is greater between human STC-1 and eel STC-1 (53%), human STC-1 is more closely related to fish STC-1 than to human STC-2 (Figure 3) (Chang et al, 2003).

STCs have been considered to be glycosylated proteins that are secreted from the cell. They contain a conserved N-linked glycosylation site, Asn-X-Thr/Ser (N-X-T/S), around the residues 62-72 (Figure 3) (Chang et al, 2003). In addition, there is a signal peptide sequence of about 24 amino acids and a pro-sequence of about 15 amino acids in the N-terminus; these are further processed to yield the mature proteins (Moore et al, 1999). Furthermore, the STCs contain 11 conserved cysteine (Cys) residues. Ten cysteines participate in five intrachain disulfide bonds and the unpaired Cys at position 170 in mammalians (169 in fish) allows for a single disulfide linkage and for the homodimerization of the native protein (Chang et al, 2003, Gagliardi et al, 2005, Lafeber & Perry, 1988). More primitive fish such as the arawana and several other osteoglossiform species, however, express the monomeric form of the protein, with the Cys_{169} replaced by an arginine (Arg) (Figure 3) (Amemiya et al, 2002).

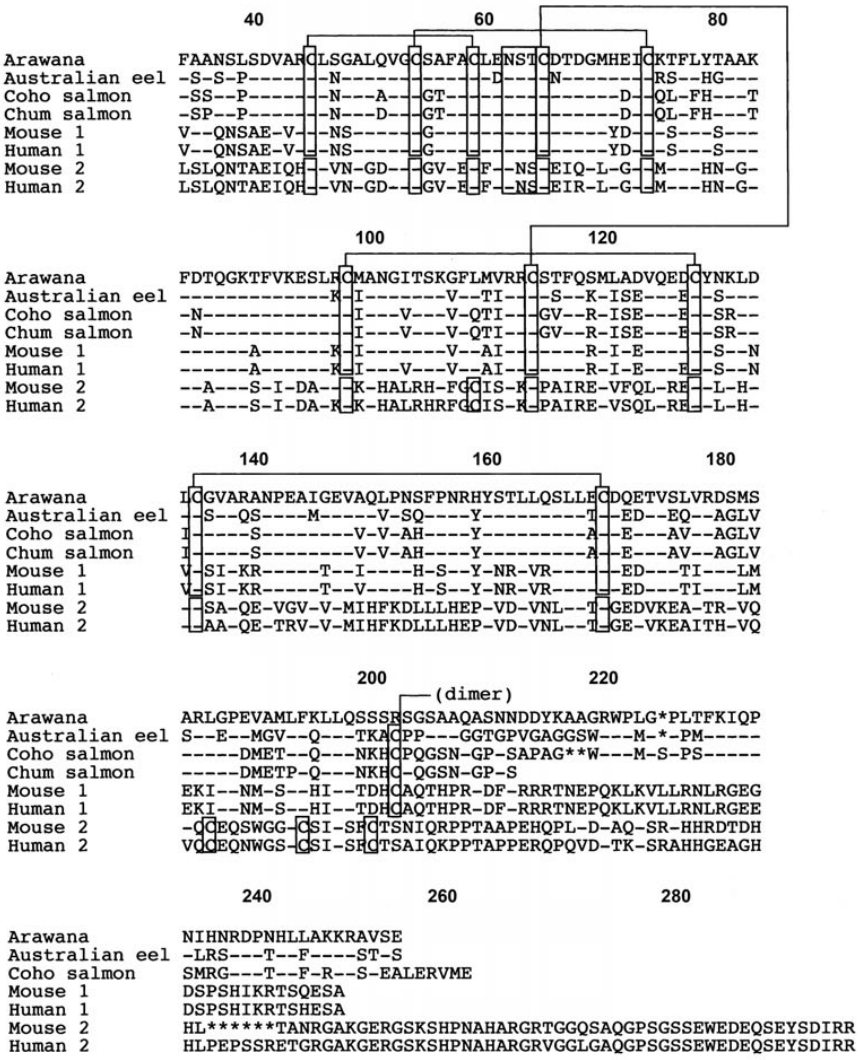

Figure 3. Amino acid sequence of Arawana STC-1 compared with STC-1 from Australian eel, Coho salmon, Chum salmon, mouse STC-1 (mouse 1), human STC-1 (human 1), mouse STC-2 (mouse 2), and human STC-2 (human 2). The sequence starts from position 34 of the human STC-1 protein sequence. Gaps (asterisks) are introduced to maximize the sequence identity. Dashes indicate amino acids identical to those of Arawana STC-1. A common glycosylation site (NST) at positions 62-64 is boxed. The positions of cysteine residues (C) are marked by boxes and the five common disulfide linkages are indicated. The site of inter-monomeric linkage is designated (dimer), but in Arawana the cysteine (C) is replaced by an arginine (R). Modified from (Amemiya et al, 2002).

1.6 Distribution and possible function

Whereas fish *stc-1* encodes for a 2 kb transcript, the mammalian *STC-1* is expressed as a predominant 4 kb transcript and an additional faint 2 kb transcript with the highest expression of STC-1 in the kidney, ovary, prostate, brain, bone, and thyroid gland (Varghese et al, 1998). The wide distribution of STC-1 in mammalians suggests that STC-1 has evolved into a local mediator of cell function rather than acting as an endocrine hormone. The fact that, in contrast to fish, circulating STC-1 is usually not detected in mammals, except for during pregnancy and lactation (Deol et al, 2000), supports this concept.

The physiological roles of mammalian STC-1 and STC-2 are only beginning to be elucidated. It is, nevertheless, clear that the role of STC-1 as an anti-hypercalcemic factor is somewhat conserved from fish to mammalians. Infusion of human recombinant STC-1 (hrSTC-1) to fish inhibits the transport of calcium through the gills (Olsen et al, 1996) and injection of hrSTC-1 into rats reduces renal phosphate excretion (Wagner et al, 1997). These findings suggest that mammalian STC-1, like its piscine counterpart, is a regulator of mineral homeostasis. Furthermore, hrSTC-1 decreases intestinal calcium uptake and simultaneously increases phosphate reabsorption in both swine and rat (Madsen et al, 1998). Transgenic mice overexpressing human STC-1 under the metallothionein-1 (*MT-1*) promoter show significantly higher serum phosphate levels (Varghese et al, 2002). Published data also suggest a role for STC-1 in the control of intracellular Ca^{2+} in rat cardiomyocytes (Sheikh-Hamad et al, 2003) and neurons (Zhang et al, 2000).

In contrast to fish, in which STC-1 is largely regulated by circulating Ca^{2+} levels, mammalian STC-1 appears to be regulated by additional factors that may or may not be directly linked to intracellular calcium/phosphate pathways, e.g. hypoxia (Lal et al, 2001, Yeung et al, 2005), vascular endothelial growth factor (VEGF) (Kahn et al, 2000, Wary et al, 2003), differentiation (Zhang et al, 1998), and D_3 vitamin (Honda et al, 1999). Therefore, it seems reasonable to assume that the role(s) of mammalian STC-1 is not restricted to the anti-hypercalcemic effects attributed to fish STC-1.

Disparities between *Stc-1* mRNA expression and protein distribution in several tissues suggest a paracrine/autocrine role for STC-1. This sort of cell-cell signaling is evident

in nephrons, where collecting duct cells produce STC-1 for targeting to upstream segments in the distal convoluted tubules (McCudden et al, 2002, Wong et al, 1998). During mouse urogenital development, a mesenchyme-epithelial signaling pathway is evident, where *Stc-1* mRNA is produced in the mesenchymal cells and the protein is sequestered by the adjacent epithelial cells (Stasko & Wagner, 2001). A similar mRNA-protein disparity was reported in the adult mouse ovary (Deol et al, 2000, Xiao et al, 2006). This discordant patterns of mRNA and protein distribution led to the formation of a sequestering hypothesis, whereby STC-1 appeared to be synthesized and released by one cell type and sequestered by its target cell (Wong et al, 1998).

A recent report demonstrates that *Stc-1* knockout mice (*Stc-1*$^{-/-}$) are normal at least as far as growth and reproduction are concerned (Chang et al, 2005). This is surprising, as one would assume that STC-1 plays a crucial role in many physiological processes, since it is such a widely expressed protein in mammalians and highly conserved from fish to man. One explanation is that the expression of the *Stc-2* gene might compensate for the loss of *Stc-1* function. No compensatory overexpression of STC-2 was, however, observed (Chang et al, 2005). To completely rule out this possibility, it will be necessary to generate mice with deletion of both *Stc-1* and *Stc-2* (double knockout). Another explanation would be that STC-1 is required only under stressful conditions.

1.7 Regulation of STC-1 expression

Consistent with its function as an anti-hypercalcemic hormone, fish STC-1 is induced by an increase in plasma Ca^{2+} levels (Aida et al, 1980, Hanssen et al, 1989, Hanssen et al, 1991, Lafeber & Perry, 1988, Lopez et al, 1984). Radman *et al*. (Radman et al, 2002) reported that a Ca^{2+} ion-sensing receptor mediates this induction. Furthermore, immortalized human fibroblasts show an almost 10-fold increase in *STC-1* mRNA levels by a 2.5-fold increase in medium Ca^{2+} concentration (Chang et al, 1995). Similarly, cultivation of neural cells in high Ca^{2+} (5.4 mM) induced STC-1 accumulation (Zhang et al, 2000).

Estradiol increases fish STC-1 and carbachol stimulates the production and release of STC-1 (Bonga, 1991). Treatment of female rats with calcitriol, the active metabolite

of vitamin D_3, induces a more than 3-fold *Stc-1* mRNA expression in kidney (Honda et al, 1999). Similarly, treatment with vitamin D_3 caused enhanced STC-1 expression in the apical membrane of distal nephron segments (Ookata et al, 2001). This increase may, however, be indirect, since vitamin D_3 induces hypercalcemia which in turn induces STC-1 expression (Srivastav et al, 1998, Srivastav et al, 1985). The regulation of STC-1 appears to be tissue-specific, since calcitriol caused an increase in STC-1 expression in the kidney, but not in the ovary (Honda et al, 1999).

Hypertonicity induces STC-1 expression in canine kidney (Sheikh-Hamad et al, 2000) and another study showed that dehydration stimulates STC-1 expression in rat kidney (Ishibashi & Imai, 2002). This effect of osmolarity on STC-1 expression needs not to be a kidney-specific phenomenon, since removal of the CS (stanniectomy) decreased plasma osmolarity (Bonga, 1991) and enhanced drinking in eels (van der Heijden et al, 1999). Several reports (Lal et al, 2001, Yeung et al, 2005, Zhang et al, 2000) show that hypoxia induces STC-1 expression. Furthermore, there is evidence for HIF-1 (hypoxia inducible factor 1)–regulated STC-1 expression in human cancer cells (Yeung et al, 2005). As for calcitriol and changes in osmolarity, the effect of hypoxia might be dependent on the Ca^{2+} concentration, suggesting a role for intracellular Ca^{2+} in STC-1 regulation.

Several studies report a role for STC-1 in atherosclerosis, angiogenesis, and in wound repair. Lysophosphatidylcholine, a pro-atherogenic compound present in atherosclerotic lesions, upregulated STC-1 expression (Sato et al, 1998). Others, on the other hand, have shown that STC-1 is highly induced during angiogenesis (Kahn et al, 2000) and during capillary morphogenesis (Bell et al, 2001). The fact that VEGF and HGF (hepatocyte growth factor) induce STC-1 expression supports these findings (Kahn et al, 2000, Wary et al, 2003, Zlot et al, 2003). Serum-stimulated fibroblasts upregulated their STC-1 expression after 2 hours of stimulation (Iyer et al, 1999), suggesting a role for STC-1 in wound healing.

1.8 STC-1 in bone

Like parathyroid hormone, extracts of CS can stimulate osteoclastic resorption of embryonic mouse bone *in vitro* (Lafeber et al, 1986). Yoshiko *et al*. provided direct

evidence for the role of STC-1 in bone physiology as they detected *Stc-1* mRNA in neonatal mouse calvaria, primary cultures of mouse osteoblasts, and human and mouse osteoblastc cell lines (Yoshiko et al, 1999). More recent studies indicate that STC-1 retards longitudinal bone growth directly at the growth plate (Wu et al, 2006).

Of the cells in the normal skeletal system, osteoblasts, i.e. the cells responsible for the production of bone, are a major source of STC-1 (Yoshiko et al, 2002, Yoshiko et al, 2003). Yoshiko and colleagues demonstrated that rhSTC-1 stimulated bone mineralization by increasing phosphate uptake in rat calvaria cell cultures, a mechanism involving upregulation of PiT-1, a sodium-dependent phosphate transporter (Yoshiko et al, 2002). *Stc-1* mRNA is expressed in osteoblasts and chondrocytes, but not in osteoclasts (Yoshiko et al, 1999). Recent evidence, however, shows that STC-1 affects all bone cells, including osteoclasts. Transgenic mice, overexpressing human STC-1 under the muscle-specific myosin-light chain promoter (MLC-hSTC-1), show decreased bone length and increased cartilage matrix synthesis. Moreover, the rate of bone formation, but not of bone mineralization, is decreased. Abnormal bone thickening and increase in trabecular bone number, density, and thickness are indicative of suppressed osteoclast activity (Filvaroff et al, 2002). One explanation for the reduction in skeletal growth in *Stc-1* transgenic mice is that STC-1 accelerates osteogenic maturation/differentiation. The fact that STC-1 stimulates osteoblast differentiation (Yoshiko et al, 2003) supports this assumption further.

1.9 STC-1 in muscle and cell metabolism

STC-1 protein is evident in cardiomyocytes of the developing mouse heart and at all stages of differentiation from myoblasts to myotube formation in developing skeletal muscle (Jiang et al, 2000). This is supportive of a role for STC-1 in myocyte function.

Although *Stc-1* mRNA levels are relatively low in skeletal muscle, injected radiolabeled hrSTC-1 accumulates here (De Niu et al, 2000). A differential staining pattern for STC-1 is evident during embryonic myogenesis, i.e. it appears during myotomal condensation and increases in intensity as myotubes align for fusion and myotube formation (Jiang et al, 2000). Interestingly, the muscles of MLC-hSTC-1 transgenic mice are smaller, in actual weight and as a proportion of overall body mass, than age-matched control mice (Filvaroff et al, 2002). One explanation for this

is that STC-1 might stimulate premature muscle differentiation, as is the case in osteogenic maturation, and thereby accelerate myotube formation.

Mitochondria are enlarged in otherwise ultrastructurally normal muscles of MLC-hSTC-1 mice (Filvaroff et al, 2002). Moreover, these mice show increased food and oxygen consumption and faster glucose clearance than the control animals, suggesting a role for STC-1 in cellular metabolism. The discovery of high-affinity receptor-like binding of STC-1 in mitochondria (McCudden et al, 2002) supports this hypothesis.

As for skeletal muscle, STC-1 expression in the heart appears to be relatively low (De Niu et al, 2000). Treatment of cultured rat cardiomyocytes with hrSTC-1, however, slowed their endogenous beating rate and decreased the rise in intracellular calcium with each contraction (Sheikh-Hamad et al, 2003). This regulation of Ca^{2+} currents occurred at least in part through L-channels in the heart muscle. Furthermore, STC-1 was markedly upregulated in the failing heart, and its expression decreased again after mechanical unloading, suggesting a cardioprotective role for STC-1 (Sheikh-Hamad et al, 2003).

1.10 STC-1 in reproduction

The presence of *Stc-1* mRNA in gonadal tissues in both fish and mammalians (McCudden et al, 2001, Varghese et al, 1998) suggests that STC-1 may have a role in reproduction. Transgenic mice overexpressing STC-1 under the *MT-1* promoter show compromised female reproduction and deleterious effects on maternal lactation/nursing behaviour (Varghese et al, 2002).

As mentioned earlier, the ovaries produce high molecular weight STC variants, collectively referred to as big STC. The reason for this variation in size, which does not appear to be due to differential glycosylation (Paciga et al, 2002), remains to be elucidated. The higher molecular weight variants might represent splicing variants or post-transcriptional modifications.

The ovaries express high levels of *Stc-1* mRNA, with increased expression during pregnancy and lactation (Deol et al, 2000). Almost all *Stc-1* mRNA is confined to the steroid-producing thecal-interstitial cells (TICs) (Deol et al, 2000, Varghese et al,

1998). The protein, on the other hand, targets to the oocytes and to the cholesterol lipid droplets of nearby corpus luteal cells to suppress progesterone synthesis (Paciga et al, 2003, Varghese et al, 1998). Thus, the ovary has both STC-1 producing and STC-1 sequestering cells, suggesting a paracrine cell-cell signaling role for STC-1. Big STC and its receptors both appear on the lipid storage droplets of small and large luteal cells (Paciga et al, 2003).

Circulating STC-1 is not detected in mammalians, except during pregnancy and lactation. This may be due to a rapid clearance of STC-1 from the circulation by erythrocytes claimed to carry large numbers of high-affinity binding sites for STC-1 (James et al, 2005). During pregnancy and lactation, however, ovarian big STC production increases, and the hormone is released into the circulation. During lactation, ovarian big STC is highly dependent on the suckling stimulus and has a regulatory effect on the lactating mammary gland (Deol et al, 2000, Hasilo et al, 2005). During the virgin state, mammary glands express high levels of STC-1 together with microsomal- and mitochondria-associated STC-1 receptors. During pregnancy and lactation, however, there is a progressive decline in these receptors and a simultaneous rise in nuclear receptors, specifically on milk-producing alveolar cells. The endogenous STC-1 expression of the mammary gland falls dramatically and instead an exogenous, blood-borne form of STC-1 (ovarian big STC) targets the mammary gland (Hasilo et al, 2005).

Both STC-1 and STC-2 are involved in blastocyst implantation and stromal cell decidualization (i.e. transformation of the endometrial stroma into a dense cellular matrix) in rat uterus. While STC-1 appears to be involved in the entire decidualization process, STC-2 seems to participate mainly in the primary decidualization (Xiao et al, 2006).

1.11 STC-1 and cancer

STC-1 was originally cloned in the search for cancer-related genes (Chang et al, 1995) and is differentially expressed in several cancers, as compared to normal tissues. *STC-1* mRNA was present in the bone marrow and blood of breast cancer patients, whereas no *STC-1* mRNA was evident in healthy volunteers (Wascher et al, 2003). The authors therefore suggested STC-1 as a novel molecular marker for human breast

cancer. Furthermore, elevated levels of *STC-1* mRNA in the vasculature of breast adenocarcinomas and other tumors were reported (Kahn et al, 2000). In another study, both STC-1 and STC-2 expression were evident in a subset of estrogen receptor-positive tumors (Bouras et al, 2002). A 10-fold upregulation of STC-1 in colon tumors was primarily due to expression of STC-1 in the tumor vasculature (Gerritsen et al, 2002). Increased STC-1 expression was evident in more than 75% of the tumor samples of human hepatocellular carcinomas (Okabe et al, 2001). A decrease in STC-1 expression was, however, evident in breast and ovarian cancer (Welcsh et al, 2002). The expression correlated with the expression of the tumor suppressor protein BRCA1 (breast cancer 1) in breast, but not in ovarian cancer. The authors concluded that loss of STC-1 expression occurs during early breast tumorigenesis. A similar downregulation of STC-1 in ovarian tumors was evident (Ismail et al, 2000).

The precise role of STC-1 in carcinogenesis is still unclear. In particular, the involvement of cancer cells in the differential STC-1 expression has to be determined. Tumor vasculature may be responsive for the increased expression of STC-1 (Gerritsen et al, 2002, Kahn et al, 2000). The fact that VEGF induces STC-1 expression supports this assumption (Liu et al, 2003, Wary et al, 2003).

HIF-1, a key regulator in the cellular responses to oxygen deprivation, i.e. hypoxia, is involved in cancer progression (Akakura et al, 2001, Carmeliet et al, 1998, Jiang et al, 1997, Maxwell et al, 1997, Ravi et al, 2000). HIF-1 activates STC-1 in human cancer cells suggesting a role for STC-1 in the hypoxia-induced Warburg effect (Yeung et al, 2005). The Warburg effect, i.e. the reprogramming of tumor metabolism from an oxidative to a more glycolytic pathway, in which HIF-1 plays a key role, is one of the most universal characteristics of solid tumors (Chang et al, 2003). As HIF-1 induces expression of both STC-1 (Yeung et al, 2005) and VEGF (Forsythe et al, 1996), and VEGF induces expression of STC-1 (Liu et al, 2003, Wary et al, 2003), STC-1 appears to be involved in the vascularization of tumors, induced by the hypoxic environment.

1.12 STC-1: a pro-survival factor for differentiated cells?

In a study mature fibroblasts downregulated their STC-1 expression after immortalization (Chang et al, 1995). In addition, STC-1 expression is downregulated

in several human cancers (Ismail et al, 2000, Welcsh et al, 2002), which might be due to the acquired proliferative capacity of cancer cells. Furthermore, since STC-1 expression is induced by serum-stimulation of fibroblasts (Iyer et al, 1999) and during angiogenesis (Bell et al, 2001, Kahn et al, 2000) one might speculate that STC-1 acts as a pro-survival factor at the tissue level during wound healing.

Transgenic mice overexpressing STC-1 under the mouse *MT-1* or the rat *MLC* promoter show dwarfism. Bone and muscle growth retardation (Filvaroff et al, 2002, Varghese et al, 2002) may be due to the overexpressing STC-1, accelerating bone and muscle maturation/differentiation. Similarly, hrSTC-1 accelerates osteogenic development in a fetal rat calvaria cell culture, whereas *Stc-1* antisense oligonucleotides retard the development (Yoshiko et al, 2003).

Short-lived cells or cells with proliferative potential do not generally express STC-1. Chang and colleagues were unable to detect *STC-1* mRNA in liver (Chang et al, 1995) although a high level of receptor-like activity is evident in a subset of hepatocytes (McCudden et al, 2002). Neither do mature lymphocytes that retain their proliferative potential express STC-1. Cells with limited proliferative capacity, however, such as oocytes (Deol et al, 2000), osteoblasts (Yoshiko et al, 2002, Yoshiko et al, 2003), chondrocytes (Yoshiko et al, 1999), cardiomyocytes (Sheikh-Hamad et al, 2003), striated muscle (Jiang et al, 2000), and brain neurons (Zhang et al, 2000, Zhang et al, 1998) express STC-1.

1.12.1 STC-1 in neural differentiation

Our laboratory became interested in mammalian STC-1, in an attempt to identify changes in gene expression during neural differentiation. We have worked with a model system for several years consisting of the Paju cell line. Paju is a robust human cell line, growing in monolayer as polygonic cells, with a slight tendency to spontaneous sprouting when reaching confluency, and can be genetically manipulated by transfection with cDNA constructs. This cell line was originally established in our laboratory from the pleural fluid of a teenaged girl with a widely metastasized neural-crest-derived neoplasia (Zhang et al, 1998).

Paju cells respond to various stimuli by activating a program of neural differentiation. Treatment with phorbol 12-myristate 13-acetate (PMA) induces vigorous neural sprouting and cessation of cell proliferation, mimicking the terminal neural differentiation in the CNS (Zhang et al, 1998).

To identify changes in gene expression during induced terminal neural differentiation, our laboratory analyzed mRNA extracted from Paju cells, before and after PMA-induced neural differentiation (Zhang et al, 1998). A differential display reverse transcription-polymerase chain reaction (DD-RT-PCR) assay was performed for a number of genes. *STC-1* was one of the genes, showing a strongly upregulated expression after induced differentiation. Northern blotting revealed that the upregulation of *STC-1* expression in Paju cells, after treatment with PMA, precedes the terminal morphological differentiation. Immunohistochemical staining of uninduced Paju cells with rabbit antibodies to STC-1 showed no or very weak STC-1 reactivity. Staining of cells, treated for three days with PMA, however, revealed perinuclear cytoplasmic staining for STC-1 (Zhang et al, 1998).

This finding prompted our laboratory to study the expression of STC-1 in mammalian brain tissue (Franzen et al, 2000). Immunohistochemical staining of sections from different parts of normal human brain disclosed the presence of STC-1 in neurons, while no staining of glial structures was evident. In addition to the neurons, endothelial cells of brain vessels, as well as the epithelium of the choroid plexus stained for STC-1. A particularly strong staining was evident in large cortical neurons, in the cerebellar Purkinje cells, and in large neurons of basal brain nuclei. Similarly, the pigmented neurons of Substantia nigra showed a strong cytoplasmic reactivity for STC-1. Most of the immunoreactive STC-1 located to the neural soma in a slightly granular pattern, or in co-distribution with Nissl bodies. Some larger neurons frequently showed staining also in the nucleus, suggesting nuclear import of STC-1 (Zhang et al, 1998).

Zhang *et al*. examined whether the expression of STC-1 in neurons *in vivo* was similarly regulated by cell differentiation. No expression of STC-1 in fetal brain, and only a weak staining in large brain neurons of newborn and one-week old mice was evident. Terminally differentiated brain neurons of adult mice and rats, however,

displayed a robust staining for STC-1, similar to that observed in human brain. The onset of the expression of STC-1 in brain neurons, during rat development, was confirmed by *in situ* hybridization. A strong signal of *Stc-1* message was evident in the brain neurons in postnatal animals, but not in fetal brain (Zhang et al, 1998).

STC-1 increases the resorption of inorganic phosphate in fish kidney (Lu et al, 1994). Given that differentiated Paju cells, and cells transfected with *STC-1* cDNA, release STC-1 to the medium, Zhang *et al*. treated normal Paju cells with recombinant STC-1 *in vitro*. They observed that treatment of Paju cells with STC-1 increased the rate of uptake of $KH_2{}^{32}PO_4$ (Zhang et al, 2000). This observation indicates that STC-1 has retained at least some of its regulatory influence on the calcium-phosphate homeostasis, from fish to man, when studied in cell culture conditions.

Influx of calcium is a common initiator of terminal cell damage. Since exposure to elevated concentrations of calcium triggers upregulated expression of STC-1, the functional role of STC-1 in Paju cells transfected with *STC-1* cDNA was investigated. Paju cells overexpressing STC-1 displayed increased resistance to treatment with cobalt chloride ($CoCl_2$), which mimics hypoxia. Paju cells overexpressing STC-1 were also more resistant to treatment with thapsigargin, which inhibits Ca^{2+} ATPases and releases calcium from intracellular stores, resulting in elevated concentrations of intracellular free calcium (Zhang et al, 2000).

Further evidence for a neuroprotective role of STC-1 *in vivo* derives from studies on experimental and clinical ischemic brain damage. *In situ* hybridization revealed activated *Stc-1* transcription, and immunohistochemistry showed an elevated and redistiributed expression of STC-1 protein in the neurons of the penumbra of the induced brain infarct (Zhang et al, 2000). Correspondingly, upregulated and redistributed expression of STC-1 was observed in the neurons of the penumbra, surrounding the infarct area of a patient who died within 15 hours after onset of an ischemic stroke (Zhang et al, 2000). Long *et al*. similarly observed elevated levels of STC-1 expression in response to traumatic brain damage in mouse hippocampus (Long et al, 2003).

Human brain neurons can survive for over 100 years without renewing cell divisions. It is conceivable that such cells are endowed with different mechanisms to maintain their integrity. The findings of Zhang *et al*. suggested that STC-1 might play a role as a survival factor for postmitotically differentiated neurons. In addition to STC-1, terminally differentiated neurons also display high expression of anti-apoptotic proteins like B-cell lymphoma 2 (BCL-2) (Zhang et al, 1996) and neuronal apoptosis inhibiting protein (NAIP) (Simons et al, 1999, Xu et al, 1997). Interestingly, the distribution of NAIP expression in human brain largely coincides with that of STC-1, with highest levels in large neurons and in the epithelium of the choroid plexus.

Enhanced uptake of P_i, in response to elevated STC-1 expression, may contribute to the neuroprotective role. P_i influx stimulates ATP synthesis and enhances energy charge in cultivated fetal rat neurons (Glinn et al, 1998). Furthermore, neurons, pre-exposed to P_i, showed higher steady state concentrations of ATP and displayed improved survival under exitotoxic conditions.

The ultimate mechanism by which STC-1 confers cytoprotection is largely unknown. McCudden *et al*. showed that STC-1 binds to the inner mitochondrial membrane and demonstrated that STC-1 has a concentration-dependent stimulatory effect on electron transfer in isolated sub-mitochondrial particles (McCudden et al, 2002). Taken together, STC-1 might act as a maintenance factor for terminally differentiated neurons and may increase the efficiency of energy synthesis under stressful conditions. Rather than being secreted, STC-1 may in fact protect the cell in which it is produced.

2. MEGAKARYOCYTE DIFFERENTIATION

The renewal of blood cells, i.e. hematopoiesis, represents a fine tuned concerted action between cell proliferation and cell differentiation. While mature lymphocytes retain their proliferative potential, granulocytes undergo postmitotic differentiation and mammalian red cells even expel their nuclei.

The megakaryocytes, fragmenting into platelets, are sessile cells in the hematopoietic tissue, and are rarely found in the circulation. The megakaryocytes originate from a precursor cell, the megakaryocyte-erythroid progenitor (MEP), shared with the

erythroid cell lineage (Figure 4). During megakaryocytopoiesis, the differentiating cells undergo endomitotic polyploidisation. This process is associated with an increase in cytoplasmic volume and, thus, indirectly regulates platelet production. Endomitosis only occurs during terminal differentiation of the megakaryocyte (Vitrat et al, 1998). The endogenous cell cycle-blocker, the cyclin-dependent kinase inhibitor $p21^{Cip1/Waf1}$, plays an important role in the exit from the endomitotic cell cycle, and in the coupling of the cell cycle arrest to terminal differentiation (Baccini et al, 2001).

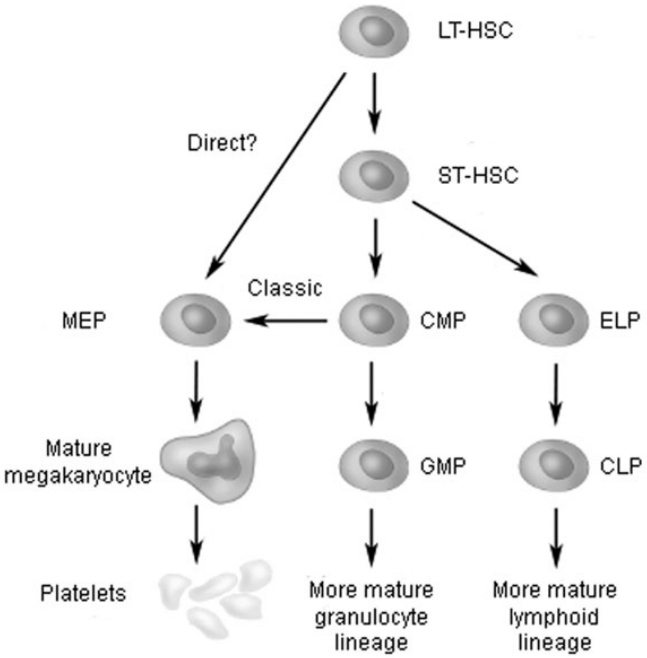

Figure 4. Megakaryocytopoiesis pathways. The figure extends from the HSC to platelets and offers a combination of the more "classical" pathway, leading to the common megakaryocyte-erythroid progenitor (MEP), and a proposed "direct" route from the HSC. LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; CMP, common myeloid progenitor; ELP, early lymphoid progenitor; GMP, granulocyte/monocyte progenitor; CLP, common lymphoid progenitor. Modified from (Pang et al, 2005).

Cytokines are important for megakaryocyte differentiation. As determined by its effects on megakaryocyte size, number, and ploidy, one of the most powerful cytokines for megakaryocyte maturation is considered to be IL-6 (Imai et al, 1991,

Zauli and Catani, 1995). *In vivo* injection of IL-6 significantly increases platelet production and transgenic mice carrying human IL-6 have an increased number of MK in their bone marrow (Suematsu et al, 1989).

K562 is a pluripotent human erythroleukemia cell line with an ability to differentiate along a megakaryocytic, erythroid, or, to a lesser extent, monocytic lineage (Alitalo, 1990). Phorbol esters (PMA) induce differentiation into megakaryocytes, with concomitant loss of monocyte- and erythroid-specific markers (Long et al, 1990). This induction stimulates the MAPK pathway, demonstrated to be responsible for the differentiation of K562 cells along the megakaryocyte-restricted pathway. Interestingly, the same pathway suppresses erythroid differentiation (Whalen et al, 1997).

3. ADIPOCYTE DIFFERENTIATION

When food intake chronically exceeds energy expenditure, most of the surplus energy accumulates as triacylglycerols, and leads to an increased volume of fat tissue. Accumulation of adipose tissue involves both hypertrophy, i.e. increase in the size of individual mature fat cells, and hyperplasia, i.e. recruitment of new adipocytes (Brook et al, 1972).

Mature fat cells in adult individuals are terminally differentiated and do not proliferate. The perivascular and stromal areas of adipose tissue contain precursor cells, i.e. preadipocytes, which have mitotic capability, and are committed to adipocyte differentiation (Sorisky et al, 2000) (Figure 5). Adipocyte differentiation includes mitotic clonal expansion, growth arrest and, differentiation, a process characterized by subsequent appearance of early, intermediate, and late protein markers, and of triglyceride accumulation (Bernlohr et al, 1985, Greenberg et al, 1993, Gregoire et al, 1998, MacDougald & Lane, 1995).

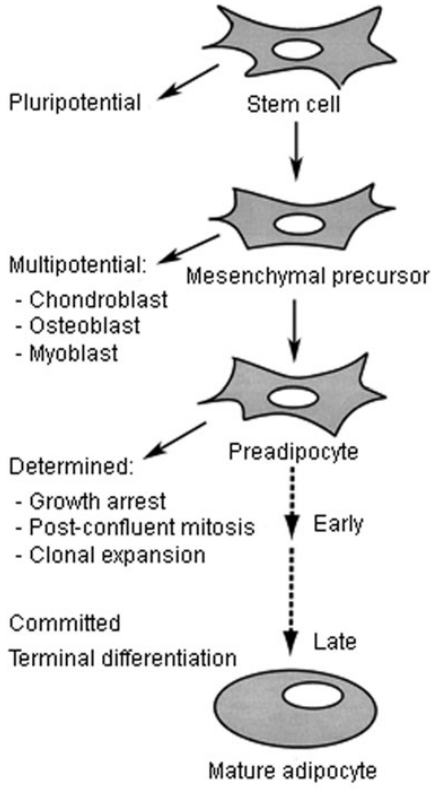

Figure 5. Overview of stages in adipocyte differentiation. Our current understanding of adipocyte differentiation is that a pluripotent stem cell precursor gives rise to a mesenchymal precursor cell with the potential to differentiate along mesodermal lineages of chondroblasts, osteoblasts, myoblasts, and adipocytes. Given appropriate environmental and gene expression cues, preadipocytes undergo clonal expansion and subsequent terminal differentiation. Modified from (Gregoire et al, 1998).

Terminal maturation of fat cells is dependent on a cross-talk between the CCAAT/enhancer binding proteins (C/EBPs) and the peroxisome proliferator-activated receptor gamma (PPARγ), which is a member of the nuclear receptor gene family (Shao & Lazar, 1997, Wu et al, 1995). The synergistic action of C/EBPs and PPARγ induces a gene expression cascade, which results in the acquisition of the functional phenotype of mature fat cells. This cascade includes the induction of genes regulating lipid metabolism (glycerophosphate dehydrogenase, fatty acid synthase,

acetyl CoA carboxylase, malic enzyme, glucose transporter 4, the insulin receptor, and fatty acid binding protein), and the activation of the expression of endogenous inhibitors of cyclin-dependent kinases (p18, p21, and p27), allowing the maturing adipocytes to exit the cell cycle (Morrison & Farmer, 1999, Spiegelman et al, 1993). Moreover, analysis of gene expression during induced fat cell maturation has revealed an altered expression of genes associated with extended survival of the postmitotically differentiated adipocytes. These include upregulated expression of the anti-apoptotic protein BCL-2 and NAIP (Magun et al, 1998) and downregulated expression of the pro-apoptotic activity of DNAse-1 (Sorisky et al, 2000). This study adds *STC-1* to the list of survival genes, which display upregulated expression during terminal adipocyte differentiation.

Of several *in vitro* models, established to study the cellular and molecular events in adipogenesis, one of the best well-characterized models is the murine 3T3-L1 preadipocyte cell line (Green & Kehinde, 1975). This cell line grows as fibroblasts, but upon treatment with an adipogenic cocktail containing methylisobutylxanthine, dexamethasone, and insulin, the cells differentiate synchronously into mature adipocytes (Green & Kehinde, 1979).

4. IL-6

The cytokines of the interleukin-6 (IL-6) family comprises IL-6, IL-11, LIF (leukemia inhibitory factor), OSM (oncostatin M), CNTF (ciliary neurotrophic factor), CT-1 (cardiotrophin-1), and CLC (cardiotrophin-like cytokine) (Heinrich et al, 2003). IL-6 is a multifunctional cytokine with major roles in the immune, hematopoietic, and nervous systems (Kamimura et al, 2003, Van Wagoner & Benveniste, 1999). In addition, IL-6 modulates bone metabolism, cell proliferation, differentiation, and apoptosis (Kamimura et al, 2003).

The receptor-complex involved in the recognition of IL-6 comprises the non-signaling subtype-specific receptor IL-6Rα (R refers to the receptor) and the common signal transducing receptor gp130. The binding of IL-6 to the α-receptor is specific and only this complex is able to efficiently recruit the signaling receptor gp130. Although gp130 is ubiquitously expressed, the number of cells that respond to IL-6 is restricted

to the expression of the α-receptor. Cells lacking the α-receptor may, however, respond to IL-6 stimulation with the help of soluble α-receptors (sIL-6Rα), formed by shedding of membrane-bound receptors or by alternative splicing (Lust et al, 1992, Muller-Newen et al, 1996). Although this IL-6–sIL-6Rα complex acts agonistically with the IL-6–IL-6Rα complex, the naturally occuring combination of sIL-6Rα and a soluble form of gp130 (sgp130), sIL-6Rα–sgp130, exerts antagonistic activity on IL-6 responses (Muller-Newen et al, 1998).

Upon stimulation by an IL-6–IL-6Rα or IL-6–sIL-6Rα complex, the gp130, acting as a homodimer, associates with the JAKs (Janus-activated kinases) and becomes tyrosine phosphorylated. This either activates the STAT (signal transducer and activator of transcription), MAPK (mitogen-activated protein kinase), or the PI3K (phosphoinositide 3-kinase) pathway acting downstream on the regulation of gene expression (Heinrich et al, 2003).

Both *in vivo* and *in vitro* studies indicate that IL-6 mediates neuroprotective activity (Gadient & Otten, 1997). Treatment with IL-6 increased the survival of retinal ganglion cells *in vitro* (Mendonca Torres & de Araujo, 2001) and protected cerebellar granule cells (Peng et al, 2005) and neuroblastoma cells in culture (Bissonnette et al, 2004) against glutamate-induced toxicity and oxidative damage. Moreover, injection of IL-6 reduced the volume of induced brain infarcts in rats and protected against N-methyl-D-aspartate–induced toxicity in cortical, striatal, and retinal neurons (Ali et al, 2000). Inhibition of IL-6 signaling by treatment with monoclonal antibodies, however, aggravated ischemic cerebral injury in mice (Yamashita et al, 2005).

The low level of IL-6 normally present in the CNS rises rapidly in response to mechanical, ischemic, or excitotoxic injury. These increased amounts of IL-6 in the injured brain originate mainly from local production in neuro-glial and endothelial cells (Van Wagoner & Benveniste, 1999). Penkowa *et al*. previously reported that mice with transgenic IL-6 overexpression in the astroglial cells, under the control of the *GFAP* (glial fibrillary acidic protein) gene promoter (GFAP-IL6 mice), displayed elevated resistance to neuronal damage and apoptotic cell death after brain injury (Penkowa et al, 2003) and pellagra neurotoxicity (Penkowa et al, 2003). On the other

hand, excitotoxic stress (Penkowa et al, 2001) and brain cryoinjury induced increased degeneration and apoptotic cell death in brains of *Il-6* deficient mice (*Il-6*$^{-/-}$), as compared to WT controls (Penkowa et al, 2000).

In addition to its neuroprotective role, IL-6 shows cardioprotective activity with the role of an obligatory mediator of delayed ischemic preconditioning in heart (Dawn et al, 2004). Furthermore, hypoxic stress, a triggering factor in preconditioning, induces IL-6 in cardiomyocytes (Yamauchi-Takihara et al, 1995).

5. HYPOXIC PRECONDITIONING

Hypoxia is defined as a decrease in tissue or ambient tissue oxygen concentration below normal. Animals exposed for a few hours to hypoxia (8% oxygen) are relatively protected for several days against subsequent ischemic damage. This phenomenon, studied in a number of organs, such as brain, heart, retina, and other organs (Brucklacher et al, 2002, Gage & Stanton, 1996, Gidday et al, 1994, Moolman et al, 1994, Neckar et al, 2002a, Samoilov et al, 2003, Tajima et al, 1994) is known as hypoxic preconditioning (HOPC). HOPC is not to be confused with ischemic preconditioning (IPC), first described by Kitagawa *et al*. (Kitagawa et al, 1990a, Kitagawa et al, 1990b), Kato *et al*. (Kato et al, 1991), and Kirino *et al*. (Kirino et al, 1991). IPC is defined as a "prophylactic" transient decrease in local blood flow to a tissue (circulatory hypoxia), preventing oxygen and nutrient supply, leading to a long-lasting adaptive response to subsequent severe ischemia.

5.1 HOPC in brain

Gidday *et al*. first observed that HOPC alone (8% oxygen for 3 hours) protected neonatal rat pups from global ischemia 1 day later (Gidday et al, 1994, Gidday et al, 1999), as other studies later confirmed (Bergeron et al, 2000, Ota et al, 1998, Vannucci et al, 1998). More recently, HOPC was also shown to protect adult mice against focal transient cerebral ischemia (Bernaudin et al, 2002a, Miller et al, 2001). HOPC requires activation of the cell genome and *de novo* protein synthesis, since protein synthesis inhibitors block the effect of HOPC (Gidday et al, 1999). Interestingly, NMDA (N-methyl-D-aspartate) receptor antagonists block the effect of IPC (Kato et al, 1992), but not that of HOPC in the brain (Gage & Stanton, 1996). Inhibitors of RNA and protein synthesis, on the other hand, prevent HOPC (Gidday et

al, 1999). These findings suggest that increased transcription and translation are necessary for hypoxia-induced tolerance.

5.2 HOPC in heart

Murry and colleagues introduced the term "preconditioning" into cardiovascular biology (Murry et al, 1986). The protection conferred by HOPC or IPC in heart occurs in a bimodal time course comprise of early and delayed preconditioning.

A distinct feature of the early preconditioning is that the protection is short-lived. The tolerance develops within minutes after the sublethal injury and lasts for a few hours. A delayed form of adaptaion occurs after 12-24 hours and lasts for 3 to 4 days. This form of preconditioning, independently described by two groups in 1993 (Kuzuya et al, 1993, Marber et al, 1993), is known as "delayed preconditioning", "late preconditioning", or "second window of protection".

Several studies show that high-altitude hypoxia might have cardioprotective effects against ischemic injury similar to those observed in ischemic preconditioning. Indeed, HOPC protects the myocardium by increasing coronary circulation and angiogenesis (Zhong et al, 2002) and by reducing incidence of arrhythmias (Asemu et al, 1999). Mitochondrial ATP-sensitive K^+ (mitoK_{ATP}) channels are critical for this phenomenon in both rat (Asemu et al, 1999, Neckar et al, 2002b, Yue et al, 2002) and rabbit (Baker et al, 1997a, Baker et al, 1997b).

Hypoxic stress increases the production of IL-6 in cardiac myocytes (Yamauchi-Takihara et al, 1995). Since the protective effects of ischemic preconditioning are absent in *Il-6*$^{-/-}$ mice, IL-6 obviously plays a key role in the activation of the late preconditioning phase (Dawn et al, 2004).

5.3 HIF-1

The transcription factor HIF-1α, a protein present in its inactive form in most normoxic cells, is activated with the onset of hypoxia (Ratcliffe et al, 1998). Desferrioxamine (DFO) and $CoCl_2$, agents known to mimick hypoxia by inhibiting prolyl hydroxylases, both activate HIF-1α (Bergeron et al, 2000). This active form of

HIF-1α, together with HIF-β, becomes phosphorylated; this stabilizes both proteins and promotes their dimerization. The dimer, in concert with p300/CBP (p300/CREB binding protein), acts on hypoxia-responsive elements (HRE) in the promoters of a variety of hypoxia-responsive genes (O'Rourke et al, 1997, Ratcliffe et al, 1998, Semenza et al, 1996, Vaux et al, 2001). Genes with HREs are involved in a number of cellular events, such as vasomotor control (adrenomedullin, *iNOS*, β-adrenergic receptor and endothelin), angiogenesis (*VEGF* and *FLT1*), erythropoiesis (*EPO*), iron metabolism (transferrin and transferrin receptor), cell cycle (*p21*, *IGF2*, and *IGFBP1*,*2*,*3*), cell death (*NIP3* and *NIX*), and energy metabolism (glucose transporters 1 and 3, *PFK*, *LDH* aldolases, and enolase) (reviewed in (Sharp & Bernaudin, 2004). These genes act to increase blood flow, increase glucose and lactate delivery to cells, and promote rapid glycolysis during hypoxia allowing the cells to survive.

Although HIF-1α is clearly important, there are other transcription factors than HIF-1α, such as EGR-1, MTF-1, NFκB, and CREB that mediate HOPC. These transcription factors are involved in cellular responses to stimuli such as stress. For example, EGR-1, induced by hypoxia, regulates the expression of *VEGF* by binding to its promoter. The fact that both HIF-1α and EGR-1 activate *VEGF*, apparently increases its response to HOPC (Yan et al, 2000). Furthermore, the transcription factor MTF-1 is involved in neuroprotection by modulating the hypoxia-induced expression of *MT-1*, a potentially protective gene in cerebral ischemia (Bernaudin et al, 2002a, Emerson et al, 2000, Trendelenburg et al, 2002) (Figure 6). In addition, there are about a dozen genes induced by HOPC that are not regulated by any of the factors mentioned above (Bernaudin et al, 2002b). This study adds IL-6–mediated STC-1 to the list of hypoxia-responsive target genes leading to cytoprotection (Figure 7).

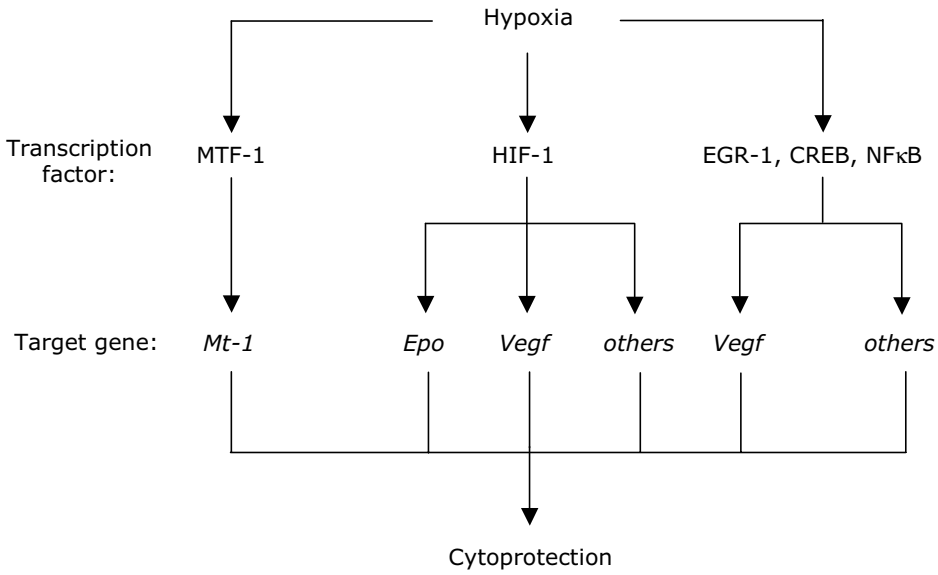

Figure 6. Hypoxia acts on several transcription factors to induce hypoxia-responsive target genes. The summed induction of these genes is suggested to lead to cytoprotection. Modified from (Ran et al, 2005, Sharp & Bernaudin, 2004).

Some studies show that hypoxia, by damaging both nuclear and mitochondrial DNA, stimulates DNA repair (Englander et al, 1999, Lee et al, 2002, Wang et al, 2000). Whether this DNA repair-response to hypoxia is important for cellular protection in HOPC is unclear.

A surprising characteristic of both IPC and HOPC is that knocking down of one gene can totally block the effect of preconditioning, even though a dozen or so genes are induced in both IPC and HOPC (Shimizu et al, 2001, Wick et al, 2002). For example, knocking down VEGF or nitric oxide (NO) abrogates HOPC (Gidday et al, 1999, Wick et al, 2002). This suggests that several signaling cascades act together rather than in parallel, initiated by a common mechanism by reactive oxygen species (ROS) (McLaughlin et al, 2003, Sharp et al, 1999) or converging on a common protective protein such as heat shock protein 70 (HSP70).

Instead of renewing themselves, postmitotic cells must survive stress with the help of "pro-survival" proteins. Interestingly, stress might even be beneficial – "What doesn´t kill you makes you stronger". This study will clarify whether STC-1 is a pro-survival protein necessary during cell stress and differentiation.

AIMS OF THE STUDY

The aims of the present study were to answer the following questions:

1) Is there an inter-relationship between terminally differentiated cells and STC-1 expression?

2) Does STC-1 confer protection in mammalian cells?

3) How is the STC-1 gene expression regulated under hypoxic stress?

MATERIALS AND METHODS

1. CELL CULTURE (I-IV)

All cultures were maintained in a 5% CO_2 atmosphere at 37°C.

1.1 K562 cells (I)

The human erythroleukemia cell line, K562, was cultured in suspension in supplemented RPMI 1640.

1.2 3T3-L1 fibroblasts (II)

The murine preadipocyte cell line, 3T3-L1 was cultured surface-adherent in supplemented Dulbecco's modified Eagle's medium (DMEM).

1.3 Paju cells (III)

Paju cells, a human neural-crest-derived tumor cell line, were cultured surface adherent in supplemented RPMI 1640. For signaling pathway experiments, we preincubated cells with the respective inhibitor (Calbiochem) at 50 μM (AG490), 25 μM (PD98059), or 200 nM (Wortmannin) before activation with 2.5 ng/ml IL-6 (R&D Systems). Cells were collected and RNA isolated by use of TRIZOL® Reagent (Invitrogen).

1.4 HL-1 cardiomyocytes (IV)

The murine cardiomyocyte cell line HL-1 (a kind gift from Dr. W. Claycomb, New Orleans) was cultured surface adherent in supplemented Claycomb media (Claycomb et al, 1998) in the absence of norepinephrine, due to the ability of norepinephrine to induce IL-6 (Yamauchi-Takihara et al, 1995). Cells were treated with 5 ng/ml IL-6 to induce STC-1 expression.

1.4.1 Hypoxia treatment

HL-1 cells were exposed to hypoxic conditions for 6 hours (as described in Keira et al, 2004). Cultures were aerated and returned to normal conditions for an additional 17 or 41 hours. Total RNA was isolated by use of TRIZOL® Reagent (Invitrogen).

2. INDUCTION OF DIFFERENTIATION

2.1 K562 cells (I)

K562 cells were induced to megakaryocytic differentiation by treatment with 20 nM PMA (Sigma) and to erythroid differentiation by treatment either with 2 mM sodium butyrate (Sigma) (Andersson et al, 1979) or 30 μM hemin (Sigma) (Rutherford et al, 1979). Cells were harvested after 0, 1, 2, 3, and 4 days for Northern blotting, Western blotting, and immunohistochemistry.

2.2 3T3-L1 fibroblasts (II)

One-day post-confluent 3T3-L1 cells were induced to adipocyte differentiation by the addition of an adipogenic cocktail, containing 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 1 μM dexamethasone (Sigma), and 1.7 μM insulin (Orion Pharma). After 48 h, the medium was replaced with fresh DMEM containing 1.7 μM insulin, and the cells were allowed to differentiate for an additional 5 days without any further manipulation. Typical areas were photographed with phase optics, and the cells were harvested for Oil Red-O staining, Northern blotting, Western blotting, and immunohistochemistry.

3. OIL RED-O STAINING (II)

Oil Red-O is a fat-soluble dye used for staining of neutral triglycerides and lipids. Uninduced and induced 3T3-L1 cells, cultured on glass chamber slides, were subjected to Oil Red-O staining. Slides were stained with hematoxylin, washed with distilled water, and mounted in Aquamount (BDH).

4. NORTHERN BLOTTING (I, II)

Equalized aliquots (10 μg) of total RNA (extracted using the RNAWIZ^{TM} kit according to the manufacturer's instructions, Ambion) were electrophoresed, transferred to Hybond-N membranes (Amersham), hybridized with 1×10^6 cpm/ml of [α^{32}P] human *STC-1* cDNA and subjected to autoradiography. Membranes were stripped and re-hybridized with [α^{32}P] human glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) (I) or [α^{32}P] actin (II) control cDNA (Clonetech).

5. WESTERN BLOTTING (I, II)

Platelets isolated from buffy coats of blood units were either lysed directly in boiling Laemmli sample buffer or first extracted for 10 minutes on ice with a lysis containing a proteinase inhibitor cocktail (Roche) and then boiled in Laemmli sample buffer (I). K562 cells were collected before and after induced differentiation at indicated time points and lysed in boiling Laemmli sample buffer. Both uninduced and induced 3T3-L1 cells were extracted on ice with lysis buffer (II). Equal amounts of protein of each sample (20 μg for I/10 μg for II) were separated by SDS-PAGE (12%) and electrophoretically transferred to nitrocellulose membranes (Bio-Rad Protein Assay, Bio-Rad Laboratories). HrSTC-1 protein served as positive control. Filters were blocked and blotted with polyclonal antibodies against either rabbit anti-STC-1 (1:2000) followed by peroxidase conjugated anti-rabbit IgG (DAKO) (I,II) or guinea pig anti-perilipin (1:2000; Progen) followed by biotinylated anti-guinea pig IgG (Zymed) (II) and a streptavidin peroxidase complex (Amersham). Blots were visualized by enhanced chemiluminescence (ECL, Amersham). HrSTC-1 and rabbit antiserum against hSTC-1 were prepared as described (Olsen et al, 1996).

6. TISSUE PROCESSING AND IMMUNOHISTOCHEMISTRY

6.1 Material (I, II, III, IV)

For STC-1 immunohistochemistry on megakaryocytes, sections from formalin-fixed core biopsies of normal human bone marrow and sections from spleen and lymph nodes from a patient with extramedullary myelopoiesis, were collected. Additionally, sections from adult mouse spleen and fetal (17d) rat liver were stained. Air-dried smears from normal human bone marrow and cytocentrifuged preparations of K562 cells were likewise stained for STC-1 (I).

For STC-1 immunohistochemistry on adipocytes, induced and uninduced 3T3-L1 cells and sections of formalin-fixed normal fat tissue, brown fat from benign hibernomas, and liposarcomas of grade 1-4 (Table 1), were collected (II).

For STC-1 immunohistochemistry on GFAP-IL6 transgenic mice, we transcardially perfused deeply anesthetized mice with heparinized 0.9% saline for 1 minute

followed by Zamboni's fixative (1.6% paraformaldehyde, 19 mM KH_2PO_4, and 100 mM $Na_2HPO_4 \cdot 7H_2O$ in 240 ml saturated picric acid-1600 ml H_2O, adjusted to pH 7.4 with HCl) for 8-10 minutes. Brains were removed and embedded in paraffin. Pair-wise mounted 4-µm sections from brains of control and GFAP-IL6 mice on the same slides were subjected to STC-1 immunohistochemistry. Staining with pre-immune rabbit sera served as control (III).

Cytocentrifuged preparations of HL-1 cells, before and after 6 hours of hypoxia treatment, were immunohistochemically stained for STC-1 (IV).

6.2 Staining procedure (I, II)

The sections were processed in a microwave oven (2 × 5 minutes in 900W) for antigen retrieval and treated with a methanol-perhydrol solution to block endogenous peroxidase activity. Immunohistochemical staining was performed using a commercial Elite ABC Kit (Vectastain, Vector Laboratories). Sections/smears/cytocentrifuged preparations were blocked with CAS-Block solution (Zymed), and incubated with a rabbit anti-STC-1 antibody (Olsen et al, 1996), diluted 1:2000 in Chem Mate (Dako). Normal rabbit serum served as primary antibody in control stainings. The peroxidase staining was visualized by 3-amino-9-ethylcarbazole (AEC) solution (0.2 mg/ml in 0.05 M acetate buffer containing 0.03% perhydrol; pH 5). Slides were counterstained with hematoxylin, washed with distilled water, and mounted in Aquamount (BDH).

6.3 Double immunohistochemistry (II)

Normal fat tissue, liposarcomas, and hormonally induced 3T3-L1 cells were subjected to co-staining with anti-STC-1 and anti-perilipin antibodies. Briefly, deparaffinized and rehydrated sections of formalin-fixed samples were processed in a microwave oven (4 × 5 minutes in 620W) for antigen retrieval, treated with a methanol-perhydrol solution to block endogenous peroxidase activity, and blocked with CAS-Block solution (Zymed). Slides were incubated with the guinea pig anti-perilipin antibody (Progen), diluted 1:150 in Chem Mate (Dako). This reagent proved to be necessary for successful staining. The staining proceeded according to the commercially available Histostain-Plus Kit (Zymed). The perilipin staining was visualized by 3,3'-

diamino-benzidin-tetrahydrochlorid (DAB). Slides were rinsed in distilled water after which the staining proceeded according to the DAKO EnVision Doublestain kit (DAKO EnVision Doublestain kit), starting from doublestain blocking. Slides were incubated with rabbit anti-STC-1 antibody, diluted 1:1000 in Chem Mate (Dako) and visualized by Fast Red. Normal rabbit serum (1:1000) and normal guinea pig serum (1:150) served as primary antibodies in all control stainings. Slides were stained with hematoxylin, washed with distilled water, and mounted in Aquamount (BDH).

7. CO-LOCALIZATION OF MITOCHONDRIA AND STC-1 (IV)

HL-1 cells, cultured on coverslips, were transfected with a FLAG-*STC-1* construct using Lipofectamine 2000 according to the manufaturer's instructions (Invitrogen). MitoTracker Red CMXRos (Molecular Probes) was used for the mitochondrial staining of living transfected cells. After fixation, permeabilization, and blocking, FLAG-tagged STC-1 was detected with a monoclonal FLAG antibody (5 μg/ml; Sigma). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Dako) served as secondary antibody.

8. IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY (I)

Smears from human bone marrow were fixed, permeabilised and blocked. Immunofluorescence was performed with a STC-1 antibody diluted 1:100 and a FITC-conjugated swine anti-rabbit immunoglobulin (Dako) diluted 1:50. Confocal microscopic images were obtained using a Leica SP2 laser-scanning microscope (Jena).

9. FLOW-CYTOMETRY (I)

K562 cells were treated with PMA for 0, 1, 2, 3, and 4 days and stained with a monoclonal antibody which recognises the megakaryocytic differentiation marker GPIIb/IIIa, followed by FITC-conjugated goat anti-mouse immunoglobulin (Dako). Expression of surface GPIIb/IIIa was analysed by a FACScan flow cytometer (BD Biosciences).

10. cDNA SYNTHESIS AND QUANTITATIVE REAL-TIME PCR (III, IV)

We prepared cDNA with the cloned AMV first-strand synthesis kit (Invitrogen) and performed quantitative real-time PCR with the Roche LightCycler instrument and the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I kit (Roche). Human *STC-1* primers were 5'-ACAGCAAGCTGAATGTGTGC-3' and 5'-CAGGCTTCGG-ACAAGTCTGT-3'. Mouse primers were: *Stc-1*; 5'-ATGCTCCAAAA-CTCAGCAGTGATTC-3' and 5'-CAGGCTTCGGAC-AAGTCTGT-3', *Il-6*; 5'-CTTCCCTACTTCACAAGTCC-3' and 5'-GCCACTCCTTCTGTGACTC-3', *Epo*; 5'-ACCCTGCTGCTTTTACTCTC-3' and 5'-ATGAAGCTGAAGGGTCTCTG-3', and *Vegf-a*; 5'-GAACTTTCTGCT-CTCTTGGG-3' and 5'-TGATGTTGCTC-TCTGACGTG-3'. *STC-1*, *Il-6*, *Epo*, and *Vegf-a* mRNA were normalized against levels of mouse beta-2-microglobulin (*ß2m*) or human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Primers were: *ß2m*; 5'-GCTATCCAGAAAACCCCTCA-3' and 5'-ATGTCTCGATCCCAGTAGAC-3', *GAPDH*; 5'-GGTGAAGGTCGG-AGTCAAC-3' and 5'-CAAATGAGCCCCAGCCTTC-3'. All primers were from Proligo LLC.

11. EXPERIMENTAL ANIMALS (III, IV)

Table 1. Experimental animals used in publications III and IV.

Publication III	Mouse type Sample number	Treatment	Organ/Gene studied
Figure 1	WT n = 32	No hypoxia/ 2,4,6h hypoxia + 6,12,24,48h recovery	Brain/*Stc-1* and *Epo*
Figure 4	WT n = 8 GFAP-IL-6 n = 8	No injury/ Focal brain injury No injury/ Focal brain injury	Brain/*Stc-1* Brain/*Stc-1*
Figure 5A	WT n = 20	No hypoxia/ 2,4,6h hypoxia + 6h recovery	Brain/*Il-6*
Figures 5B,C	*Il-6*$^{-/-}$ n = 17	No hypoxia/ 2,4,6h hypoxia + 48h recovery	Brain/*Stc-1* and *Epo*

Publication IV	Mouse type Sample number	Treatment	Organ/Gene studied
Figure 1	WT n = 40	No hypoxia/ 2,4,6h hypoxia + 6,12,24,48,72h recovery	Heart/*Stc-1* and *Vegf*
Figure 2	*Il-6*$^{-/-}$ n = 20	No hypoxia/ 2,4,6h hypoxia + 48h recovery	Heart/*Stc-1* and *Vegf*

All procedures involving experimental animals were performed according to institutional and local guidelines. All efforts were made to minimise animal distress and to reduce the number of animals used.

Wild-type mice were of strain C57BL/6129SvJ. The construction and characterization of GFAP-IL6 transgenic mice, with a mixed C57B6×SJL genetic background, is previously described (Campbell et al, 1993). Age and gender littermate WT mice served as controls. *Il-6*$^{-/-}$ mice (B6;129S2-*Il6*^{tm1Kopf}/J) were from the Jackson Laboratory (Stock# 002254).

12. HYPOXIA TREATMENT (III, IV)

We subjected mice to hypoxia in a controlled-environment chamber perfused with 8% v/v oxygen in nitrogen. Mice were killed at indicated time points, and collected brain and heart tissue were immediately snapfrozen in liquid nitrogen for extraction of total RNA as described above.

13. INDUCTION OF BRAIN LESIONS (III)

For induction of brain lesions the cryolesion method was used (Penkowa et al, 2000, Penkowa et al, 2003). Mice were lesioned under tribromethanol anesthesia and the skull over the right fronto-parietal cortex was exposed. Application of pellets of dry ice of consistent size during 60 seconds to the surface of the skull produced a focal cryoinjury. The mice were killed 24 hours later for extraction of total RNA.

14. *IN SITU* HYBRIDIZATION (III, IV)

We generated single-stranded antisense and sense RNA probes of a 356-bp mouse *Stc-1* cDNA (position 147-502) fragment cloned into the pSPT18 plasmid

(Invitrogen). Probes were labeled with digoxigenin-uridine triphosphate by *in vitro* transcription with SP6 and T7 RNA polymerases according to the manufacturer's instructions (Roche). A sense probe was used as a negative control. Automated hybridization was carried out with a Ventana Discovery Slide Stainer (Ventana Medical Systems). Sections were incubated with a monoclonal biotinylated anti-digoxin antibody (Jackson ImmunoResearch Laboratories) diluted 1:2000. The probe was detected with the Ventana Blue Map kit (Ventana Medical Systems).

15. STATISTICAL ANALYSIS (III, IV)

Data are expressed as means ± s.d. We examined normality of the data and performed all analyses with the Student *t*-test (III, IV) or two-way ANOVA (III). $P < 0.05$ was regarded as statistically significant.

Table 2. List of methods used.

Method	Publication
Cell culture	I, II, III, IV
Immunohistochemistry	I, II, III, IV
Induction of differentiation	I, II
Northern blotting	I, II
Western blotting	I, II
Flow-cytometry	I
Immunofluorescence	I
Confocal microscopy	I
Oil Red-O staining	II
Double immunohistochemistry	II
cDNA synthesis and quantitative real-time PCR	III, IV
Hypoxia on mice	III, IV
In situ hybridization	III, IV
Statistics	III, IV
Cryolesion	III
Transfection	IV
Mitochondrial staining	IV
Hypoxia on cells	IV

RESULTS AND DISCUSSION

1. UPREGULATED STC-1 EXPRESSION DURING MEGAKARYOCYTOPOIESIS (I)

There are no reports of STC-1 expression in hematopoietic cells. A fast turnover rate is characteristic for most hematopoietic cells. The half-life of megakaryocytes, however, is relatively long (Schwartz et al, 2003). This prolonged survival makes the megakaryocytes especially interesting as far as terminal differentiation is concerned. We therefore examined the expression of STC-1 in mature megakaryocytes and platelets of human, mouse, and rat. To investigate the kinetics of STC-1 expression during megakaryocytopoiesis, we used the K562 erythroleukemia cell line. This human cell line can be induced either to erythroid differentiation, by treatment with sodium butyrate (Andersson et al, 1979) or hemin (Rutherford et al, 1979), or to megakaryocytic differentiation, by treatment with PMA (Long et al, 1990).

1.1 Megakaryocytes and platelets express STC-1

We performed immunohistochemical staining of hematopoietic tissues with antibodies to STC-1. The only hematopoietic cells showing strong expression of STC-1 were mature megakaryocytes and platelets. In addition to normal human bone marrow, the megakaryocytes in foci of extramedullary hematopoiesis in the spleen of patients with myelofibrosis, stained for STC-1. Similarly, rodent megakaryocytes in mouse bone marrow and spleen, and in rat fetal liver, stained for STC-1.

In smears of human bone marrow a strong STC-1 expression was restricted to mature megakaryocytes and platelets. Expression of STC-1 in platelets was confirmed by Western blotting when platelets were directly lysed in boiling Laemmli sample buffer whereas extracts obtained with lysis buffer appeared negative.

1.2 STC-1 shows a perinuclear expression with a Golgi-like distribution

Confocal microscopy of megakaryocytes revealed a perinuclear expression of STC-1 with a Golgi-like distribution. Strongly stained aggregates were obvious inside the cell close to the plasma membrane, apparently representing platelets that are to be shed from the cell. Recently shed platelets were also evident in the close vicinity of the megakaryocyte.

1.3 K562 cells induced to megakaryocytic differentiation show a robust accumulation of STC-1

Treatment of K562 cells with PMA induced megakaryocytic differentiation as shown by the expression of the cell surface marker GPIIb/IIIa, while treatment of K562 cells with hemin or sodium butyrate induced erythroid differentiation detected by intracytoplasmic accumulation of fetal hemoglobin (data not shown).

Western blotting revealed accumulation of STC-1 protein in lysates of K562 cells treated with PMA but not in lysates of cells treated with hemin or sodium butyrate. Immunohistochemical staining confirmed the presence of STC-1 in the cytoplasm of K562 cells treated with PMA.

1.4 Kinetics of STC-1 expression after treatment with PMA

Northern blot analysis revealed an up-regulation of a 4 kb and a 2 kb *STC-1* transcript in K562 cells already after one day of PMA treatment and increased expression of *STC-1* mRNA until day 3. No expression of *STC-1* mRNA in K562 cells treated with hemin or sodium butyrate was evident.

We report for the first time a strongly upregulated expression of STC-1 during megakaryocytic differentiation in a leukemia cell line and a robust accumulation of STC-1 protein in normal megakaryocytes and platelets *in vivo* (Serlachius et al, 2004).

Megakaryocytes undergo endomitotic polyploidisation during their terminal differentiation (Vitrat et al, 1998). It is, however, not clear whether cell differentiation *per se* or a cell cycle block/exit triggers STC-1 expression. Our observation is in favour of the latter possibility, i.e. that introduction of a cell cycle block in G1-S by treatment of Paju cells with hydroxyurea, triggers STC-1 expression without any morphological signs of neural sprouting (data not shown). Given this, it is interesting that the endogenous cell cycle blocker, the cyclin-dependent kinase (CDK) inhibitor $p21^{Cip1/Waf1}$ plays a pivotal role in the polyploidisation and differentiation of megakaryocytes (Baccini et al, 2001). It remains to be determined whether the Cip/Kip family of CDK inhibitors is directly involved in the regulation of STC-1 expression.

In comparison with other hematopoietic cells, megakaryocytes have a rather slow turnover rate. Infusion of [$^2H_2$]-glucose to label hematopoietic precursors showed that the half-life of human CD41a positive cells *in vivo* ranged from 5 to 21 days, while the replacement half-life for glycophorin A-positive erythroid precursors, was 2 to 6 days (Schwartz et al, 2003). Expression of survival factors may contribute to this extended survival of megakaryocyes. In fact, the anti-apoptotic protein BCL-xL is upregulated during megakaryopoiesis (Kaluzhny et al, 2002). It is tempting to speculate that the upregulated expression of STC-1 also has bearing on the survival of postmitotically differentiated megakaryocytes.

2. UPREGULATED STC-1 EXPRESSION DURING ADIPOGENESIS (II)

As megakaryocytes, mature adipocytes are postmitotic cells that have undergone growth arrest and subsequent terminal differentiation. It was therefore interesting to examine the expression of STC-1 in both white and brown fat tissue as well as in liposarcomas of different grades. To investigate the kinetics of STC-1 expression during adipogenesis, we used the 3T3-L1 preadipocyte cell line. This murine cell line can be induced to differentiate into mature adipocytes by treatment with an adipogenic cocktail (Green & Kehinde, 1979).

2.1 Both white and brown fat express STC-1

Immunohistochemical staining of sections from normal human white fat tissue revealed a strong expression of STC-1 in the scanty cytoplasm of mature adipocytes. Immunostaining of brown fat from benign hibernomas, likewise showed a strong positivity for STC-1. Co-staining of white fat tissue with antibodies to STC-1 and to the adipocyte marker perilipin, confirmed the localization of the STC-1 expression to mature adipocytes.

2.2 STC-1 expression in liposarcomas

Postmitotic adipocytes of mature fat tissue do not give rise to neoplasms, but the precursor cells, endowed with a capability of adipocyte differentiation, are considered the clonogenic pool of lipomas and liposarcomas. Immunostaining of sections from lipomas and from low-grade (grades 1 and 2) liposarcomas showed expression of STC-1. Polynucleated giant cells, frequently appearing in grade 3 liposarcomas also

showed immunoreactivity for STC-1, while the surrounding sarcomatous spindle cells stained only weakly for STC-1. STC-1-expressing cells occurred only at a low frequency among the highly proliferative cells of grade 4 liposarcomas.

2.3 *Stc-1* mRNA appears as the majority of the 3T3-L1 fibroblasts acquire adipocyte morphology

To study the kinetics of STC-1 expression in relation to adipocyte differentiation, we used 3T3-L1 cells. When confluent monolayers of these cells were treated with an adipogenic cocktail for 48 hours, and then transferred to fresh medium containing insulin, the cells started to accumulate lipid droplets that ultimately occupied most of the cytoplasm. After 7 days of induction, the majority of the cells displayed the phenotype of differentiated adipocytes, as shown by cytoplasmic triglyceride accumulation (Oil Red-O staining). Northern blot data on the kinetics of 3T3-L1 differentiation revealed an *Stc-1* transcript 4 days after the induction of adipocyte differentiation.

Northern blot analysis of 3T3-L1 cells, induced to adipocyte differentiation, and of normal fat tissue, revealed a 4-kb and a faint 2-kb *Stc-1* transcript. Uninduced 3T3-L1 cells, on the other hand, did not express detectable amounts of *Stc-1* mRNA. Western blotting and immunohistochemical staining confirmed the expression of both STC-1 and perilipin in the induced 3T3-L1 cells. STC-1 showed a perinuclear staining, whereas perilipin coated the surface of the lipid droplets

These results are in some respect contradictory to previous results. Paciga *et al*. reported the production of high molecular weight STC (big STC) in the theca and interstitial cells of mouse and bovine ovary (Paciga et al, 2002). In addition, they found evidence for sequestration of high molecular weight STC into luteal cells through a receptor-dependent process (Varghese et al, 1998). Like ovarian luteal cells, adrenocortical cells and adipocytes contain cholesterol/lipid storage droplets. Indeed, the characterization of big STC variants in both adipocytes and adrenocortical cells was recently reported (Paciga et al, 2005). In my study, I was not able to detect the variant sized STC. I have, however, merely determined the size of the STC-1 protein in 3T3-L1 cells, not in normal white or brown fat tissue. It is plausible that fat contains different splice variants of STC-1 *in vivo*. STC-1 is indeed expressed as a 4

kb and/or a 2 kb transcript in several tissues (Varghese et al, 1998). These might represent different splice variants of STC-1 as the case above indicates.

The expression of STC-1 during adipogenesis closely resembles the pattern of STC-1 expression, which was originally reported in neural cells (Zhang et al, 1998). During proliferation, fetal and embryonic neural cells displayed low or undetectable levels of STC-1, and a strong accumulation of STC-1 protein appeared during terminal neural differentiation *in vivo* and *in vitro*. Here I report high constitutive expression of STC-1 in hormonally induced 3T3-L1 cells as well as in mature white and brown fat tissue. The co-localization of STC-1 and perilipin confirms the fact that STC-1 is expressed in adipocytes. Only a minority of proliferating cells in high-grade liposarcomas, on the other hand, expressed of STC-1.

The transcriptional regulation of STC-1 expression during adipocyte differentiation remains to be clarified. There are, however, observations suggesting that environmental stress and/or a cell cycle block, rather than differentiation *per se*, upregulates STC-1 expression. Treatment of the neuronal Paju cells with hydroxyurea upregulated STC-1 expression in the absence of morphological evidence of differentiation (data not shown). Serum starvation (my own unpublished observations) and exposure of cells *in vitro* to high concentrations of calcium, hypoxia (Zhang et al, 2000) or extreme pH values (Bumke et al, 2003) also initiated STC-1 expression in neural cells or fibroblasts.

I have also reported that megakaryocytes are the only hematopoietic cells with a high expression of STC-1 (Serlachius et al, 2004). The accumulation of STC-1 occurs concomitantly with the endomitotic polyploidisation, leading to a cell cycle arrest during megakaryopoiesis. It is tempting to suggest that similar mechanisms may be involved in the upregulated expression of STC-1 in the polyploidic cells in grade 3 liposarcomas, as shown in this study.

Not only is IL-6 important for megakaryocytopoiesis, but also for adipogenesis. Adipose tissue is a major source of circulating IL-6. This cytokine, a marker of adipocyte differentiation, is hormonally stimulated by catecholamines and insulin (Vicennati et al, 2002). Our adipogenic cocktail contained insulin, which may

subsequently have stimulated IL-6 production. Whether IL-6 in turn stimulates STC-1 expression in adipocytes needs to be investigated.

The expression of STC-1 in mature adipocytes also correlates with increased resistance to apoptosis of mature fat cells as compared with preadipocytes. Particularly under catabolic conditions, tumor necrosis factor alpha (TNF-α) induces apoptosis of preadipocytes, while terminally differentiated adipocytes appear to be more resistant to apoptosis induced by growth factor deprivation (Sorisky et al, 2000). Although this effect may preferentially be mediated by the upregulated expression of BCL-2 and NAIP, the contribution of STC-1 as an inhibitor of toxic calcium fluxes cannot be excluded.

In summary, our findings show that STC-1 is strongly upregulated during terminal adipocyte maturation. In analogy with other tissues containing terminally differentiated cells, we suggest that STC-1 contributes to the survival of mature fat cells, which have lost their capacity of renewal. The expression of STC-1 may have bearings on attempts to therapeutically manipulate the homeostasis of fat tissue.

3. IL-6–MEDIATED STC-1 EXPRESSION DURING HYPOXIC PRECONDITIONING IN BRAIN (III)

Animals exposed for a few hours to hypoxia (8% oxygen) are relatively protected for several days against ischemic brain damage. This phenomenon, known as HOPC, depends on new RNA and protein synthesis, but its molecular mechanism is poorly understood.

The cytokine IL-6 is evident, particularly in the lungs of animals subjected to HOPC (Yan et al, 1995). In addition, HOPC-induced IL-6 mediates cardioprotection (Dawn et al, 2004, Yamauchi-Takihara et al, 1995). These findings prompted us to investigate whether IL-6 mediates the expression of the neuroprotective STC-1 in HOPC.

3.1 Hypoxic preconditioning induces *Stc-1* expression in brain

We exposed adult mice to 8% oxygen for 6 hours. After various times of re-oxygenation in normal air, we collected brain hemispheres and quantified *Stc-1* mRNA expression. Upregulated expression of *Stc-1* mRNA was evident already during the hypoxic period and declined gradually during 48 hours. The kinetics of induced *Stc-1* expression followed that of erythropoietin (*Epo*), a well-known HOPC-responsive gene (Prass et al, 2003). *In situ* hybridization confirmed the previously observed (Zhang et al, 1998) neuronal localization of *Stc-1* expression in hypoxic brain.

3.2 Treatment of Paju cells with IL-6 upregulates *STC-1* expression

Given that IL-6 mediates neuroprotective activity, and elevated levels of IL-6 are evident in animals exposed to HOPC, we investigated the role of IL-6 in the induction of *STC-1* expression. The human neural cell line Paju was treated with recombinant IL-6, which induced dose-dependent upregulation of *STC-1* mRNA levels with a maximum at 9 hours, followed by a decline to background levels at 24 hours. The concentration of IL-6 giving maximum stimulation was 2.5 ng/ml, i.e. a concentration comparable to serum levels of IL-6, recorded in an *in vivo* experimental mouse CNS ischemia model (Clark et al, 1999). Notably, exposure of Paju cells to hypoxia overnight did not, however, directly induce increased *STC-1* expression (data not shown).

3.3 IL-6 activates *STC-1* through the MAPK pathway

Cytokines of the IL-6 family bind to their subtype-specific soluble or membrane-associated α-receptor. The complex that is formed interacts with the common transmembrane β-receptor protein gp130, which signals through the STAT3, MAPK, or PI3K pathway, or via a combination of these (Kamimura et al, 2003). Treatment with the MAP kinase kinase (MEK) inhibitor PD98059 completely blocked the IL-6–induced *STC-1* response in Paju cells. AG490, an inhibitor of JAK2 that blocks activation of STAT3, showed an intermediate inhibitory effect on the upregulation of *STC-1* by IL-6. This may be explained by the suggested crosstalk occurring between the STAT3 and MAP kinase pathways (Heinrich et al, 2003). The PI3K inhibitor Wortmannin failed to influence the increase of *STC-1* mRNA levels by IL-6. These

findings show that IL-6–induced *STC-1* expression in Paju cells is dependent on the MAPK signaling pathway, but that some degree of regulation is also directed through the STAT3 pathway.

3.4 Stronger STC-1 response after induced brain injury in GFAP-IL6 mice

To study the interplay between IL-6 and STC-1 expression *in vivo*, we used mice with transgenic overexpression of IL-6 targeted to the CNS astroglia under the control of the GFAP gene promoter (GFAP-IL6 mice). We extracted mRNA from brains of GFAP-IL6 mice and WT controls before and 24 hours after induction of a focal brain injury. Brains of GFAP-IL6 mice showed a higher constitutive expression of *Stc-1* mRNA than did the brains of WT mice. Both GFAP-IL6 and WT mice upregulated brain *Stc-1* mRNA 24 hours after induced focal brain injury, but this response was strongly enhanced in the GFAP-IL6 mice. When sections from a GFAP-IL6 mouse brain and from a control (WT) mouse brain, mounted on the same slide, were immunohistochemically stained for STC-1, the GFAP-IL6 mice revealed a stronger staining for STC-1, particularly in the large pyramidal neurons of the parafrontal cortex.

3.5 Lack of enhanced *Stc-1* induction in IL-6 deficient mice

Our results show a transient *Il-6* mRNA increase in WT mouse brains after exposure to hypoxia. To further substantiate the regulatory influence of IL-6 on *Stc-1* expression, we exposed *Il-6*$^{-/-}$ mice to HOPC and quantified the induction of *Stc-1* mRNA in the brain. HOPC that induced elevated *Stc-1* mRNA in the brains of WT mice did not elicit a measurable *Stc-1* response in the brains of the *Il-6*$^{-/-}$ mice. Brains from both WT mice and *Il-6*$^{-/-}$ mice, not exposed to hypoxia, however, showed similar levels of *Stc-1* mRNA, indicating that IL-6 is not required for the constitutive expression of STC-1 in brain neurons. The *Epo* response in *Il-6*$^{-/-}$ mice was similar to that measured in WT mice, confirming that the two groups experienced similar hypoxic stress.

Although the molecular pathways by which IL-6 mediates neuroprotection have received much attention, the molecular mechanism still remains incompletely understood. Here we show that IL-6–mediated expression of STC-1 is a molecular

mechanism of HOPC-induced tolerance to brain ischemia. Interestingly, our recent results (data not shown) show that the damaged area in the brain of WT mice is reduced if the mice are exposed to HOPC before middle cerebral artery occlusion/reperfusion (MCAO). Interestingly, HOPC does not reduce the size of damage caused by MCAO in the brain of *Stc-1*$^{-/-}$ mice. This finding confirms the fact that STC-1 indeed is one of the proteins responsible for the protective actions of HOPC.

Activation of HIF-1 is a well-established response to HOPC (Semenza, 2000). This transcription factor induces *Stc-1* expression in nasopharyngeal cancer cells subjected to oxygen deprivation (Yeung et al, 2005). Other hypoxia-inducible transcription factors include nuclear factor for IL-6 (NF-IL6), the transcription factor for IL-6 (Cummins & Taylor, 2005). Enhanced activity of NF-IL6, in response to hypoxia, occurs in heart, lung, and kidney, but not in the liver (Yan et al, 1995), suggesting tissue specific regulation. Our present data show that induction of *Stc-1* expression in the brain, following HOPC, is under the control of IL-6 since hypoxia fails to upregulate *Stc-1* in brains of *Il-6*$^{-/-}$ mice under conditions in which transcription of the HIF-1 target gene *Epo* is fully activated (Prass et al, 2003).

Astrocytes produce IL-6 under hypoxic conditions *in vitro* (Lau & Yu, 2001, Maeda et al, 1994). We show here that a transient increase in *Il-6* mRNA is evident in brains of WT mice after exposure to hypoxia. Moreover, an accumulation of IL-6 in the lungs of mice kept in low oxygen is evident (Yan et al, 1995). As IL-6 is known to penetrate the blood-brain barrier (Banks et al, 1994), the lungs may represent an additional source of IL-6 that upregulates the *Stc-1* expression in the brain and also in other organs sensitive to ischemic damage.

Our findings may have therapeutic implications for the management of stroke. It is noteworthy that physical exercise, inducing elevated serum levels of IL-6 also confers protection against neurodegenerative disorders like Alzheimer's disease (Larson et al, 2006, Rovio et al, 2005). Regular exercise also has a beneficial effect on disease progression in a mouse model of amyotrophic lateral sclerosis (ALS) (Kaspar et al, 2005). Induction of elevated expression of STC-1 in neurons may be a mediator in these events.

4. HYPOXIC PRECONDITIONING INDUCES ELEVATED EXPRESSION OF *STC-1* IN THE HEART (IV)

HOPC induces cardioprotective effects that occur in a biphasic manner; the first phase developing within minutes and lasting for up to a few hours (early preconditioning), and the second phase being delayed for 12-24 hours and lasting for 3 to 4 days (late preconditioning) (Kuzuya et al, 1993, Marber et al, 1993). Since our recent data shows that *Stc-1* expression in brain is upregulated through IL-6 signaling (publication III) after hypoxia, we attempted to clarify whether IL-6–mediated STC-1 is involved in the protective effects of HOPC in heart, as well.

STC-1 is physiologically expressed in the mammalian myocardium (Sheikh-Hamad et al, 2003). Given that hypoxic stress induces IL-6 (Yamauchi-Takihara et al, 1995), we treated HL-1 cardiomyocyte cells with hypoxia or IL-6 and measured the induction of *Stc-1* mRNA. Wild-type mice and IL-6 deficient mice were kept in hypoxic conditions (8% O_2) for 6 hours. Myocardial *Stc-1* mRNA expression was quantified during hypoxia and after recovery for up to 48 hours.

4.1 Exposure of mice to hypoxia induces upregulated expression of *Stc-1* in the heart

We exposed mice to 8% oxygen for 6 hours. After various times of reoxygenation in normal air, we collected heart tissue and quantified *Stc-1* mRNA expression. Upregulated expression of *Stc-1* mRNA was evident immediately during the period of hypoxia, with a rapid decline to background levels within 6 hours of recovery in normoxia. Thereafter a second phase of increased *Stc-1* expression occurred, reaching a maximum at 48 hours post-hypoxia, and declining to control levels at 72 hours. To put *Stc-1* into context with other known hypoxia-responsive genes, we analyzed the kinetics of *Vegf* expression in the same samples. Similarly to *Stc-1*, *Vegf* showed a biphasic response with an immediate early peak followed by a delayed late response. Furthermore, *in situ* hybridization localized the *Stc-1* expression mainly to cardiomyocytes.

4.2 Reduced primary and lack of secondary *Stc-1* induction by HOPC in *Il-6*$^{-/-}$ mice

IL-6 is a mediator of the late HOPC effect observed in myocardium (Dawn et al, 2004). To investigate the role of IL-6 for the *in vivo* upregulation of *Stc-1* in the heart following HOPC, we exposed *Il-6*$^{-/-}$ mice to 8% oxygen for 6 hours. The expression of *Stc-1* mRNA was quantified at the time points that gave maximum responses in WT mice (during hypoxia, and 48 hours after reoxygenation). HOPC induced an early *Stc-1* response in the hearts of *Il-6*$^{-/-}$ mice. The *Stc-1* expression, however, remained at a lower level than that observed in the hearts of WT mice, indicating a partial requirement for IL-6 signaling in the primary induction of *Stc-1*. A similarly altered response of *Vegf* to HOPC was observed in *Il-6*$^{-/-}$ mice. The second peak of *Stc-1* induction at 48 hours was absent, as was the *Vegf* response, in the hearts of the *Il-6*$^{-/-}$ mice, indicating the requirement for IL-6 in the late phase of *Stc-1* induction by HOPC .

4.3 Oxygen deprivation induces *Stc-1* in cardiac myocytes *in vitro*

To investigate whether expression of *Stc-1* is directly regulated by hypoxia in myocardial cells, we cultured the cardiomyocyte cell line HL-1 in a 99.5% nitrogen atmosphere for 6 hours, after which the cells were returned to normal culture conditions. We observed an increased *Stc-1* expression within 6 hours, followed by a return to control levels at 17 hours. The kinetics of the *Stc-1* response followed the pattern of *Vegf* expression, another classical hypoxia responsive gene. The *Stc-1* and *Vegf* responses followed identical time courses, suggesting similar mechanisms of regulation. Immunohistochemical staining of HL-1 cells with rabbit antibodies to STC-1 showed that hypoxia induced an increased cytoplasmic accumulation of STC-1 protein with a granular distribution.

4.4. IL-6 induces elevated *Stc-1* expression in HL-1 cardiomyocytes

Treatment of HL-1 cells with IL-6 (5 ng/ml) induced increased *Stc-1* expression that reached a maximum at 6 hours and gradually declined during the following 48 hours.

4.5 Mitochondrial localization of STC-1

To further characterize the subcellular localization of STC-1, we transfected HL-1 cells with *STC-1* cDNA containing a carboxy terminus FLAG sequence. Co-staining

of the transfected cells with a monoclonal antibody to FLAG and with MitoTracker revealed a co-distribution, demonstrating mitochondrial targeting of expressed STC-1.

I present here findings demonstrating that HOPC induces elevated expression of *Stc-1* in the heart with IL-6 as a key-signaling molecule. The initial *Stc-1* response that occurred within a few hours of hypoxic stress appeared to be partially dependent on IL-6 signaling, since an *Stc-1* response of lower magnitude but with similar timing as in WT mice was observed in the hearts of *Il-6*$^{-/-}$ mice. Exposure of HL-1 cells to hypoxia *in vitro* also induced a rapid *Stc-1* response without measurable induction of IL-6 (data not shown). This indicates that other signaling pathways contribute to the induction of rapid *Stc-1* responses during hypoxia. There is a HIF-dependent increase in *Stc-1* expression in nasopharyngeal cancer cells (Yeung et al, 2005). Hence it is tempting to suggest that HIF-dependent pathways may contribute to the early *Stc-1* response to hypoxia in cardiomyocytes, as well. The later *Stc-1* response to HOPC occurred after 24-48 hours. This delayed peak of *Stc-1* response was nevertheless absent from the hearts of *Il-6*$^{-/-}$ mice exposed to HOPC, and therefore appears to depend on IL-6 signaling. Cultures of rat cardiomyocytes were subjected to 15 minutes of ischemia followed by reperfusion (Chandrasekar et al, 1999). An upregulated expression of IL-6 and IL-6R within one hour of reperfusion was evident, and the levels remained elevated for up to 6 hours. This time course coincides with the first window of protection associated with preconditioning. In addition, 30 minutes of ischemia followed by reperfusion of rat myocardial cells induced elevated IL-6 gene expression on days two and three after reperfusion (Roy et al, 2006). This coincides with the second window of protection following HOPC. These results are in agreement with our findings, indicating a key role of IL-6 for both windows of protection by HOPC.

Hypoxic stress induces IL-6 expression also in extracardiac organs and tissues, i.e. in lung and kidney. One might speculate that the contribution of IL-6 to the early *Stc-1* response occurs in an auto- or paracrine manner, while circulating IL-6 induces the later response. However, the relative contributions of the different sources of IL-6 to the induction of *Stc-1* expression after HOPC need to be established.

In summary, the induction of cardiac STC-1 expression by HOPC described here represents a novel molecular pathway that may be exploited for the prevention and management of ischemic heart damage.

This study adds IL-6–mediated STC-1 to the mechanisms behind hypoxia-induced cytoprotection. As shown in Figure 7, we suggest that IL-6, activated by the transcription factors NF-IL6, NFκB or HIF-1 in response to hypoxia, induces cytoprotection, mediated by STC-1. Alternatively, STC-1–mediated cytoprotection may be directly induced by HIF-1.

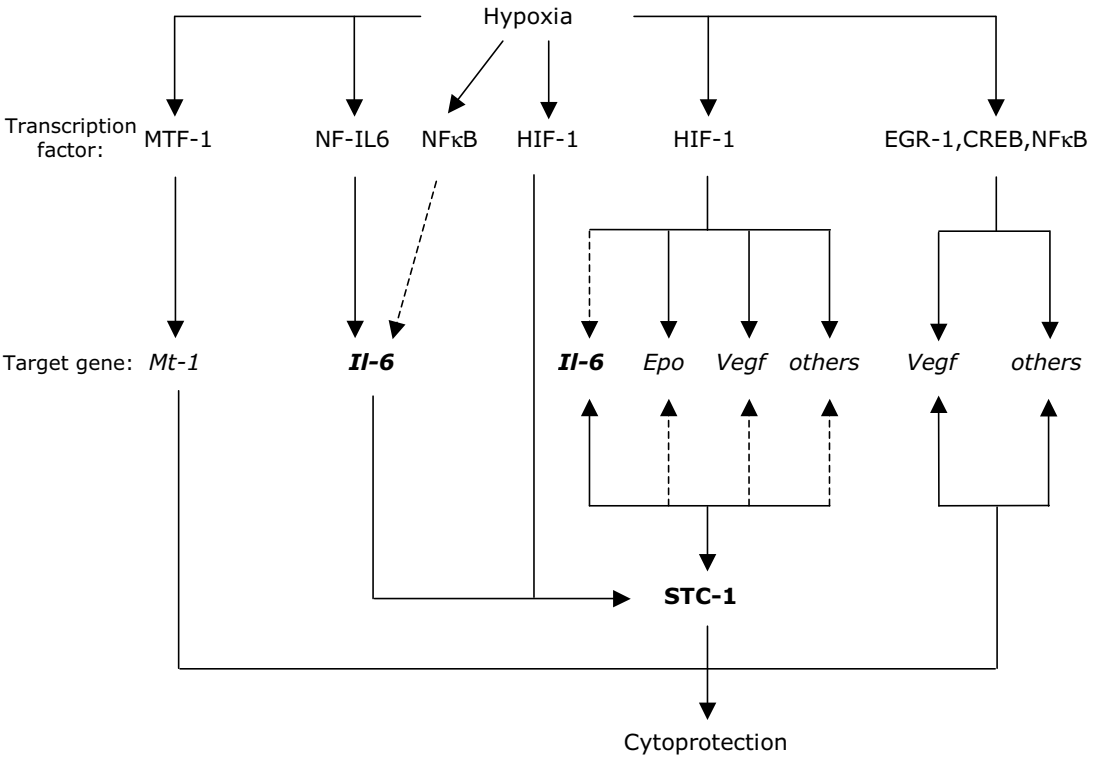

Figure 7. Hypothetical role of IL-6 and STC-1 in hypoxia-induced cytoprotection. Our findings are in bold. Modified from (Ran et al, 2005, Sharp & Bernaudin, 2004).

CONCLUDING REMARKS

Here I present novel data showing that there is an inter-relationship between terminally differentiated cells and STC-1 expression. In addition, my results reveal that STC-1 gene expression is regulated by IL-6 under hypoxic stress, possibly through the MAPK signaling pathway. With these results at hand it is tempting to speculate that IL-6 regulates STC-1 expression in postmitotic megakaryocytes and adipocytes, as well. Indeed, IL-6 plays an important role in both megakaryocyte (Baatout, 1996) and adipocyte differentiation (Harkins et al, 2004).

My results play the groundwork to now address the mechanism by which STC-1 confers cytoprotection. Although STC-1 deficient mice do not show an abnormal phenotype (Chang et al, 2005), accumulated data nevertheless indicate that STC-1 plays a cytoprotective role in postmitotic cells and during induced stress, such as hypercalcemia and hypoxia. My colleagues have previously found that upregulated expression of STC-1 increases the resistance to hypoxic and oxidative stress, and protects against toxicity induced by mobilization of intracellular calcium with thapsigargin (Zhang et al, 2000). Moreover, human neural Paju cells transfected with *STC-1* cDNA maintain an increased ATP-synthesis in the presence of $CoCl_2$ and show increased survival as compared to mock-transfected cells (Zhang et al, 2000).

Treatment of cultured rat cardiomyocytes with STC-1 slowed their beating rate and lowered the rise in intracellular calcium, indicating that STC-1 acts as a reversible blocker of transmembrane calcium currents through L-type channels (Sheikh-Hamad et al, 2003). A recent report showed that pre-treatment of neonatal rat cardiomyocytes with IL-6 caused a significant decrease in the amplitude of their Ca^{2+} oscillations (Smart et al, 2006). In the light of our present findings, it appears that this effect might be mediated through IL-6–induced STC-1 expression. Taken together, these findings indicate that through evolution, from fish to man, STC-1 has preserved some of its role as a regulator of calcium homeostasis.

McCudden *et al*. first reported binding of STC-1 to the inner membrane of mitochondria. They also presented evidence that treatment with recombinant STC-1 protein induced enhanced electron transport in sub-mitochondrial particles

(McCudden et al, 2002). We have previously shown that a high expression of STC-1 may confer increased resistance of the mitochondrial membrane potential to bacterial toxins, mediating potassium ionophore effects (Teplova et al, 2004). Here I demonstrate that most of the ectopic expression of STC-1 in HL-1 cells is targeted at mitochondria. Pre-treatment of cultured rat cardiomyocytes with IL-6 induced functional resistance to hypoxic stress in the mitochondria (Smart et al, 2006). Given that IL-6 strongly induces STC-1 expression, this is of particular interest since treatment of mitochondria with recombinant STC-1 protein induces mild uncoupling (Ellard et al, 2007). Altered mitochondrial metabolism, and in particular uncoupling, has been implicated as a central functional mechanism behind HOPC-induced cardioprotection (Chen et al, 2007, Sack, 2006). Taken together, these observations suggest that STC-1 may improve cell survival under stressful conditions by contributing to the maintenance of the mitochondrial function i.e. the energy synthesis, and/or by inducing the respiratory uncoupling effects suggested to contribute to the protection induced by HOPC (Minners et al, 2001).

I present here an intracellular STC-1 cytoprotection model (Figure 8) suggesting that STC-1 exerts its cytoprotective action in the cell in which it is synthesized rather than as an autocrine/paracrine hormone as suggested in the sequestering hypothesis by Wong and colleagues (Wong et al, 1998). This does not rule out the possibility of STC-1 being secreted and acting in an autocrine/paracrine fashion, but nevertheless, requires the cloning of the STC-1 receptor.

We have identified STC-1 as a protein essential for the maintenance and protection of terminally differentiated cells. This is of great importance for many fundamental biological processes. IL-6–mediated STC-1 expression represents a novel molecular pathway, which is an important area for future research and may lead to the development of novel therapeutic approaches to stroke, cancer, obesity, and metabolic disorders, to name a few.

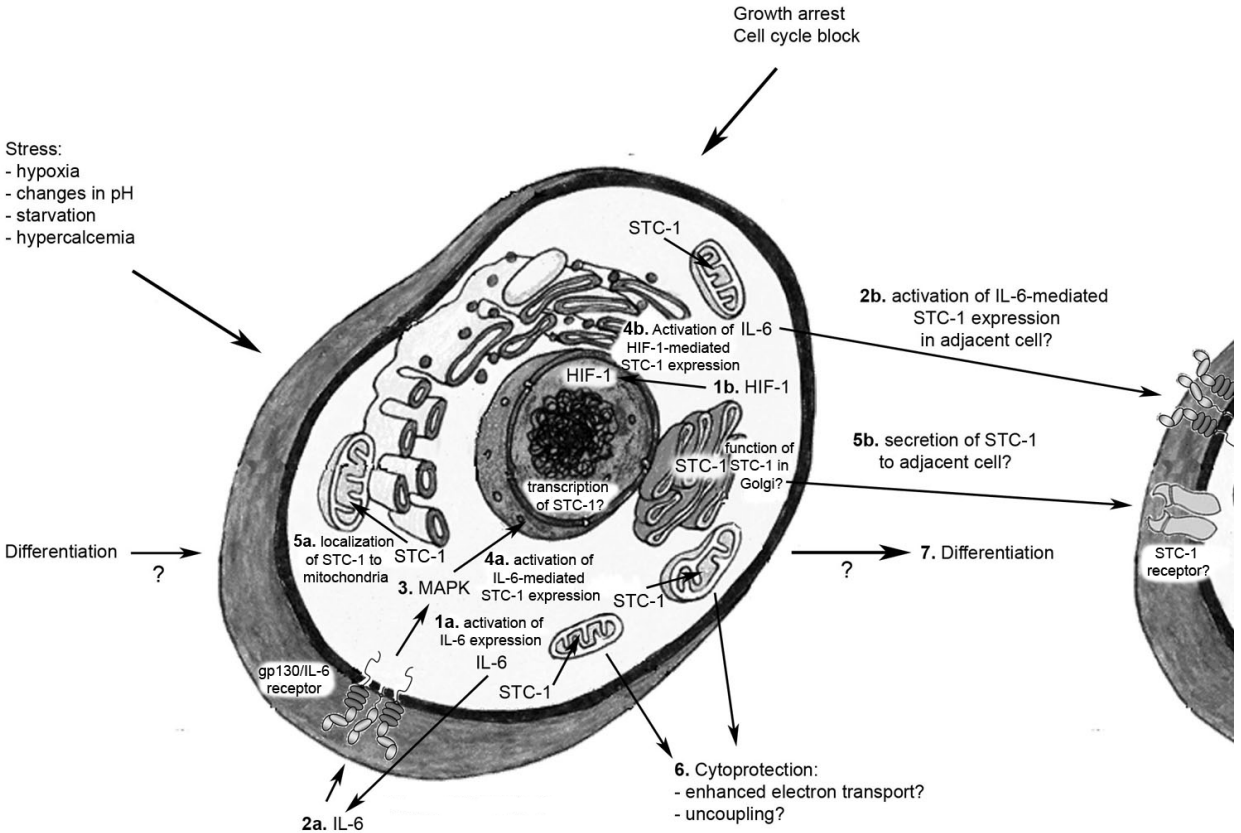

Figure 8. Presentation of the intracellular STC-1 cytoprotection model. Stressful conditions, cell cycle block, growth arrest, or possibly differentiation induce STC-1 expression, leading to cytoprotection. In response to any of the stimuli mentioned above IL-6 expression is induced (1a). IL-6 is either activating the same cell (2a) or the adjacent (2b) through its receptor complex. The signaling occurs mainly through the MAPK pathway (3) and induces STC-1 expression (4a). Alternatively STC-1 expression may be mediated by HIF-1 (4b). STC-1 is localized to mitochondria (5a) and/or secreted from the cell excerting its actions through a putative STC-1 receptor (5b) in an autocrine/paracrine manner. Eventually STC-1 is conferring cytoprotection (6) by mechanisms suggested to involve the mitochondria. In addition, STC-1 expression might trigger the process of terminal differentiation (7). The animal cell is modified from www.biologycorner.com/bio1/cell.html.

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