Regulation of the CNC and Small MAF Transcription Factors in Placental and Myometrial Cells

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Abstract

Members of the MAF (proto-) oncogene and CNC families of basic leucine zipper transcription factors (bZIP) play important roles in development, differentiation, mammalian gene expression and stress signalling. We analyzed the regulation of the human small MAF transcription factor family members, MAFF, MAFG and MAFK, as well as two CNC family members, NRF2 and NRF3, in human reproductive tissue. We found that *MAFF* expression was induced by the proinflammatory cytokines interleukin 1 beta (IL1B) and tumor necrosis factor (TNF) in PHM1-31 myometrial cells. It was particularly interesting that the proinflammatory cytokine Interleukin 6 not affect the expression of MAFF. Additionally, the transcript and protein levels of the highly homologous *MAFG* and *MAFK* genes were not modulated by these cytokines. Using electromobility shift assays (EMSA)s we showed that MAFF is capable of heterodimerzing with NRF3, and since they are both highly expressed in the placenta we investigated their role in more detail in this tissue. We found that *MAFF* and *NRF3* are expressed in the anchoring villi from 12 weeks to term. Furthermore, we demonstrated that *MAFF* and *NRF3* are highly expressed in primary placental cytotrophoblasts, but not in placental fibroblasts. This led us to examine whether NRF3 is also regulated by proinflammatory cytokines and we showed that NRF3, is also modulated by TNF in the human JAR placental cell line.

Parallel studies investigated the role *NRF2* plays in the antioxidant response of placental cells due to exposure to arsenic. We provided evidence for the involvement of *NRF2* by confirming the increase in binding of endogenous NRF2/small MAF heterodimers to Stress Response Element (StRE), and the upregulation of heme oxygenase-1 (HO1) expression, upon exposure of JAR cells to arsenic. Our results suggest a role for *MAFF*, *NRF3* and *NRF2* in proinflammatory cytokine control of myometrial and placental gene expression as well as arsenic mediated stress in placental cells.

Résumé

Les facteurs de transcription à domaine leucine basique zipper (bZip) appartenant aux familles Maf (proto-)oncogènes et Cap 'n' Collar (CNC) sont des régulateurs clés du développement, de la différenciation, de l'expression génique chez les mammifères et de la réponse au stress. Nous avons analysé la régulation des membres de la famille des facteurs de transcription des petits MAF (MAFF, MAFG et MAFK) et nous avons également analysé la régulation de deux membres de la famille CNC (NRF2 et NRF3) dans l'organe reproducteur humain. Nous avons trouvé que l'expression de *MAFF* est induit par l'interleukine 1 béta (IL1B), une cytokine proinflammatoire et par le facteur de nécrose tumorale (TNF) dans les cellules de myomètres. De manière intéressante, l'interleukine 6, une autre cytokine proinflammatoire, n'affecte pas l'expression de MAFF et les deux cytokines (IL1B et IL6) n'ont pas d'effet sur les niveaux de transcrits et de protéines des gènes homologues *MAFG* et *MAFK*. En utilisant la technique de retard sur gel (EMSA), nous avons montré que MAFF est capable de s'hétérodimériser avec NRF3, et comme les deux facteurs de transcription sont hautement exprimés dans le placenta nous avons étudié leurs rôles de manière plus approfondie. Nous avons trouvé que *MAFF* et *NRF3* sont exprimés dans la partie ancrée du villus dès la 12è semaine de grossesse et ce jusqu'à la naissance. De plus, nous avons démontré que *MAFF* et *NRF3* sont hautement exprimés dans le cytotrophoblaste placentaire primaire mais pas dans les fibroblastes placentaires. Ceci nous a mené à examiner si NRF3 est aussi régulé par les cytokines proinflammatoires et nous avons montré que NRF3 est également modulé par TNF dans la lignée JAR de cellules placentaires humaines.

En parallèle, nous avons étudié le rôle joué par *NRF2* dans la réponse antioxidante des cellules placentaires à une exposition à l'arsenic. Nous avons clairement montré l'implication de *NRF2* en confirmant l'augmentation de fixation des hétérodimères endogènes NRF2/petit MAF aux Eléments de Réponse au Stress (« Stress Response Element » : StRE), et l'augmentation de l'expression de l'hème oxygénase-1 (HO1), en réponse à l'arsenic dans les cellules JAR. Nos résultats suggèrent un rôle pour *MAFF*, *NRF3* et *NRF2* dans le contrôle des cytokines proinflammatoires de l'expression des gènes placentaires et myométriaux et également dans le stress induit par l'arsenic dans les cellules placentaires.

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Preface

In accordance with the "Guidelines for Thesis Preparation", the candidate has chosen to present the results of his thesis in classical form. A general introduction is presented in Chapter I and appears, in part, in the following journal articles following the subjection to the peer review process; an asterix (*) denotes publications for which the indicated authors contributed equally:

1. **Functional and Placental Expression Analysis of the Human NRF3 Transcription Factor**. Benoit Chenais*, Anna Derjuga*, Wael Massrieh*, Kristy Red-Horse, Valerie Bellingard, Susan J. Fisher, and Volker Blank. * [Co-First Authors]. *Molecular Endocrinology*. 2005 Jan;19(1):125-37. Epub 2004 Sep 23.

2. **Regulation of the MAFF Transcription Factor by Proinflammatory Cytokines in Myometrial Cells.** Massrieh W, Derjuga A, Doualla-Bell F, Ku CY, Sanborn BM, Blank V. *Biology of Reproduction*. 2006 Apr;74(4):699-705. 2005 Dec 21.

3. **Induction of Endogenous NRF2/small MAF Heterodimers by Arsenic-Mediated Stress in Placental Choriocarcinoma Cells.** Massrieh W, Derjuga A, Blank V. *Antioxidant and Redox Signalling*. 2006 Jan-Feb; 8(1-2):53-9.

Materials and methods for research presented in this document are detailed in chapter II. The results described in chapter III, IV, and V have been published, in part, in the following journal articles following the subjection to the peer review process; an asterix (*) denotes publications for which the indicated authors contributed equally:

4. **Functional and Placental Expression Analysis of the Human NRF3 Transcription Factor**. Benoit Chenais*, Anna Derjuga*, Wael Massrieh*, Kristy Red-Horse, Valerie Bellingard, Susan J. Fisher, and Volker Blank. * [Co-First Authors]. *Molecular Endocrinology*. 2005 Jan;19(1):125-37. Epub 2004 Sep 23.

5. **Regulation of the MAFF Transcription Factor by Proinflammatory Cytokines in Myometrial Cells.** Massrieh W, Derjuga A, Doualla-Bell F, Ku CY, Sanborn BM, Blank V. *Biology of Reproduction*. 2006 Apr;74(4):699-705. 2005 Dec 21.

6. **Induction of Endogenous NRF2/small MAF Heterodimers by Arsenic-Mediated Stress in Placental Choriocarcinoma Cells.** Massrieh W*, Derjuga A*, Blank V. * [Co-First Authors]. *Antioxidants and Redox Signalling*. 2006 Jan-Feb; 8(1-2):53-9.

Contribution of Authors:

All research presented in Chapter III was performed by the candidate, with the exception of these specific contributions:

- Anna Derjuga participated in the immunoblot analysis in Fig. 19, Fig. 21, and Fig. 22.

All research presented in Chapter IV was performed by the candidate, with the exception of these specific contributions:

- Kristy Red-Horse performed the isolation of the chorionic villi and the cytotrophoblast cells.

- Dr. Benoit Chenais performed all experiments for Fig. 23, and collaborated with the candidate for Fig. 24.
- Anna Derjuga was responsible for experiments presented in Fig. 25, Fig. 27 and Fig. 28.

The candidate carried out EMSA for Fig. 8B in Appendix A which appears in the Chapter VIII- Appendices and in the following journal article:

7. **Regulation of globin gene transcription by heme in erythroleukemia cells: analysis of putative heme regulatory motifs in the p45 NF-E2 transcription factor.** Moore A, Boudia MM, Lehalle D, Massrieh W, Derjuga A, Blank V. *Antioxidants and Redox Signalling*. 2006 Jan-Feb; 8(1-2):68-75.

Table of Contents

List of Figures and Tables

List of Designations and Abbreviations

Designations:

The following formatting has been used throughout the thesis in accordance to the "Guidelines for Human Gene Nomenclature". Hester M. Wain, Elspeth A. Bruford, Ruth C. Lovering, Michael J. Lush, Mathew W. Wright and Sue Povey. *Genomics* 79(4):464-470 (2002).

Mouse/Chicken

Gene:	First letter Upper case. Word italicized (e.g. *Nrf2*)
cDNA:	First letter Upper case. Word italicized (e.g. *Nrf2*)
RNA:	First letter Upper case. Word italicized (e.g. *Nrf2*)
Protein:	All Upper case letters. (e.g. NRF2)

Human

Gene:	All Upper case letters. Word italicized (e.g. *NRF2*)
cDNA:	All Upper case letters. Word italicized (e.g. *NRF2*)
RNA:	All Upper case letters. Word italicized (e.g. *NRF2*)
Protein:	All Upper case letters. (e.g. NRF2)

Abbreviations:

ACTB	Actin, beta
ALAS-E	5-Aminolevulinate Synthase
ARE	Antioxidant Response Element
As_2O_3	Arsenic Trioxide
ChIP	Chromatin Immunoprecipitation
CDNB	1-chloro-2,4-dinitrobenzene

CNC	Cap 'n Collar
CUL3	Cullin 3
DCFH-DA	Dichlorofluorescin diacetate
EHR	Extended Homology Region
EMSA	Electrophoretic Mobility Shift Assay
GCLC	Glutamate-Cysteine Ligase Catalytic Subunit
GCS	Glutamylcysteine Synthetase
GSH	Glutathione
GSS	Glutathione Synthetase
GST	Glutathione S-Transferase
H_2O_2	Hydrogen Peroxide.
HO1	Heme Oxygenase-1
HO2	Heme Oxygenase-2
IL1B	Interleukin1 beta
IL6	Interleukin 6
IVR	Intervening Region of KEAP1
LCR	Locus Control Region
MAF	V-maf Musculoaponeurotic Fibrosarcoma
MARE	MAF Recognition Element
NFE2	Nuclear Factor (erythroid-derived 2)
NRF1	NFE2 Related Factor 1
NRF2	NFE2 Related Factor 2
NRF3	NFE2 Related Factor 3

NF-IL6	Nuclear Factor Il6
NQO1	NAD(P)H: Quinine Oxidoreductase
OT	Oxytocin
OTR	Oxytocin Receptor
PBGD	Porphobilinogen Deaminase
PCR	Polymerase Chain Reaction
pCR-BluntII-TOPO	pCR-BluntII-TOPO Vector
PDTC	Pyrrolidinedithiocarbamate
PEITC	Phenylethylisothiocyanate
PIC	Preinitiation Complex
PTGS2	Prostaglandin Endoperoxide Synthase 2
ROS	Reactive Oxygen Species
RT	Reverse Transcription
STAT-3	Signal Transducer and Activator of Transcription-3
StRE	Stress Response Element
TNF	Tumor Necrosis Factor
TXNRD1	Thioredoxin Reductase 1 Gene

CHAPTER I

A. Transcription Factor Families

Gene regulation gives the cell control over structure and function, and is the basis for cellular differentiation, morphogenesis and the versatility and adaptability of any organism [1]. Initiation of messenger RNA (mRNA) synthesis by RNA polymerase II is a primary control point in the regulation of differential gene expression. It is clear that the frequency of initiation of mRNA synthesis depends ultimately on factors that interact with the specific "nuclear messengers" in gene promoters [2, 3]. The analysis of these nuclear messengers has shed light on the final steps in cellular signal transduction and has thus offered a novel approach to tracing the critical events by starting at the end and working backwards.

Clues to the biological processes in which these nuclear messengers participate may be obtained by determining their tissue expression patterns, elucidating their DNA binding specificities [4] or mapping their chromosomal sites of occupancy [5]. Ultimately, understanding the function of a transcriptional activator will require identification of the genes that it controls. This requires derivation of appropriate cell lines or animal models in which the protein can be expressed (or inactivated) in a regulated fashion. The consequences of its expression (or inactivation) on the transcription of specific genes is then monitored. In the most powerful version of this approach, target gene transcription can be assayed in parallel using high-density DNA microarrays [6].

Factors involved in the transcription of eukaryotic protein-coding genes by RNA polymerase II fall into two groups:

a) General transcription factors, which are involved in the formation of the preinitiation complex (PIC). The most common are abbreviated as TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. They are ubiquitous and along with RNA Polymerase II they interact with the core promoter region and specify the transcription start site. PIC formation usually begins with TFIID binding to the TATA box found in most core promoters, followed by the entry of other general transcription factors and RNA Polymerase II through either a sequential assembly or a preassembled RNA Polymerase II holoenzyme pathway. [3]

b) Inducible transcription factors (signal regulated transcription factors) bind upstream or downstream of the initiation site and require activation or inhibition to transmit regulatory signals between gene-specific activators and the general transcription machinery [3].

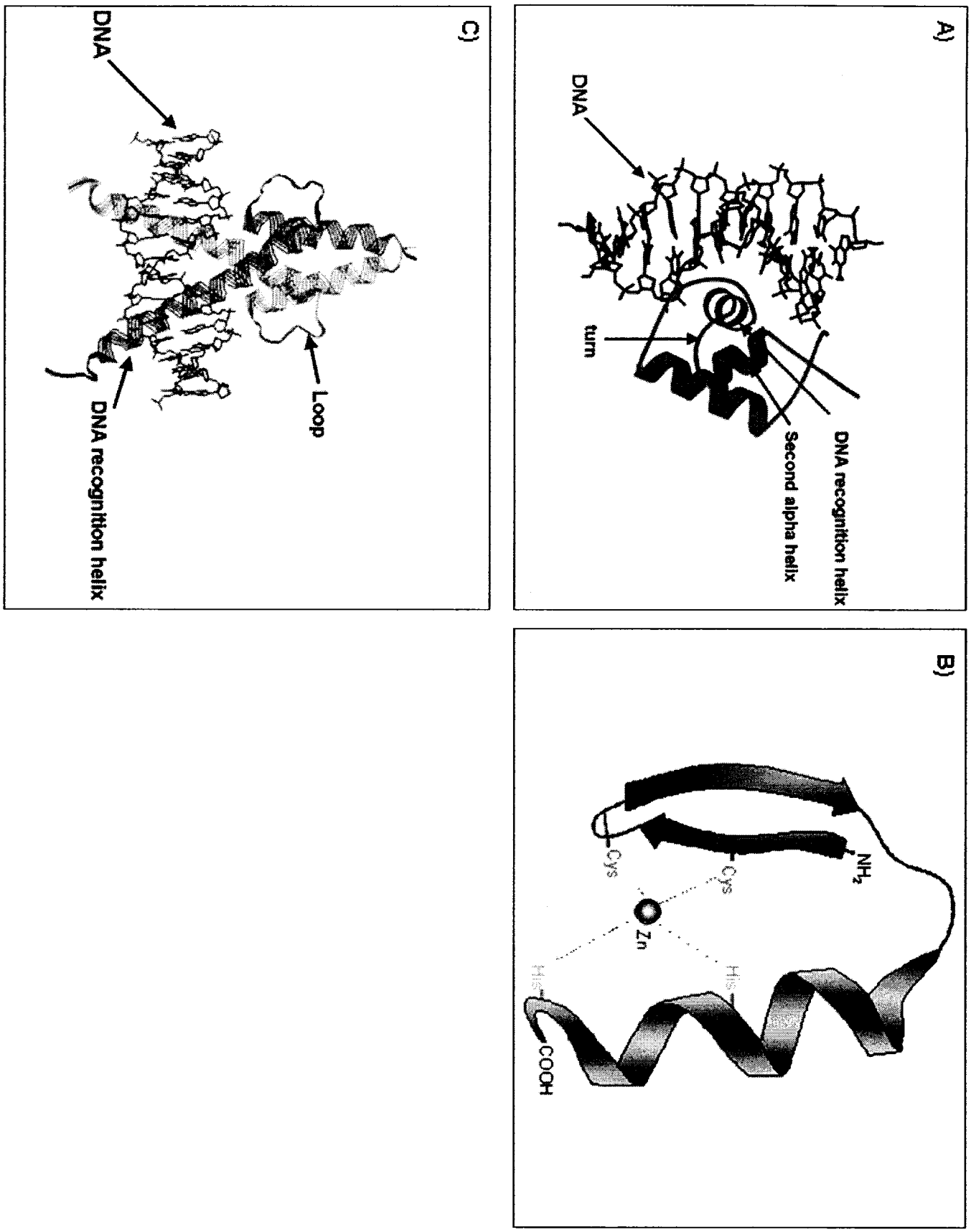
Transcription factors often are classified according to the type of DNA-binding domain they contain. Most of the structural classes of DNA-binding domains have characteristic consensus amino acid sequences. A few of the more common classes of DNA-binding domains whose three-dimensional structures have been determined are described here.

1. Transcription Factor DNA binding motifs

1.1 Helix-Turn-helix (HTH)

The HTH family of transcription factors DNA binding motif is composed of two α helices joined by a short strand of amino acids (Fig. 1A) [7]. In particular, recognition and binding to DNA is done by the two α helices, one occupying the N-terminal end of the motif, the other at

Fig.1 A) Helix-turn-helix transcription factors. Structure is composed of two alpha helices joined by a short strand of amino acids. The first helix contributes most to DNA recognition, and hence it is called the "recognition helix". The second α helix stabilizes the interaction between protein and DNA [8]. **B) Zinc finger transcription factors**. Structure consists of two antiparallel beta strands, and an alpha helix. A sub-set of zinc-fingers comprise a pair of cysteine residues in the beta sheets and two histidine residues in the alpha helix which are responsible for binding a zinc atom. The α-helix occurs at the C-terminal part of the finger, whilst the beta sheet occurs at the N-terminal part [9]. **C) Helix-loop-Helix transcription factors.** Structure is characterised by two alpha helices connected by a loop. The longer helix contains basic amino acid residues that facilitate DNA binding. The smaller helix typically allows dimerization by folding and packing against the longer helix [10].


A)
DNA
turn
Second alpha helix
DNA recognition helix
B)
Cys
Cys
Zn
His
His
NH2
COOH
C)
DNA
DNA recognition helix
Loop

the C-terminus [7]. In most cases, the first helix contributes most to DNA recognition and hence it is often called the "recognition helix".

1.2 Zinc Finger

The zinc finger family consists of two antiparallel β strands, and an α helix (Fig. 1B). They were originally identified as DNA binding structures in the RNA polymerase III transcription factor TFIIIA, which binds to the internal control region of the 5S RNA gene [11].

1.3 Helix-Loop-Helix (HLH)

This family of transcription factors is characterized by two α helices connected by a loop (Fig. 1C). One helix is typically smaller and due to the flexibility of the loop, allows dimerization by folding and packing against another helix [12].

1.4 Basic-Leucine Zippers (BZIP)

The 3D structure of bZIP proteins consists of three α–helices [13]. The most striking feature of this family of inducible transcription factors is the presence of the basic leucine zipper (bZIP) domain at the carboxyl terminus (Fig. 2A) [14]. One region of this domain facilitates dimerization and the other contacts DNA. The dimer-forming region, termed the "leucine zipper" is formed by heptad repeats of leucine residues (LLLMLL or LLLLYL) [15] (Fig. 2A). Since there are 3.6 residues/turn in an α-helix, this positions one leucine at every second turn, just short of being exactly under each other in the coil. These leucines were shown to form the hydrophobic core of a coiled coil [16] which extends away from the DNA in a direction perpendicular to the overall DNA helix axis [17].

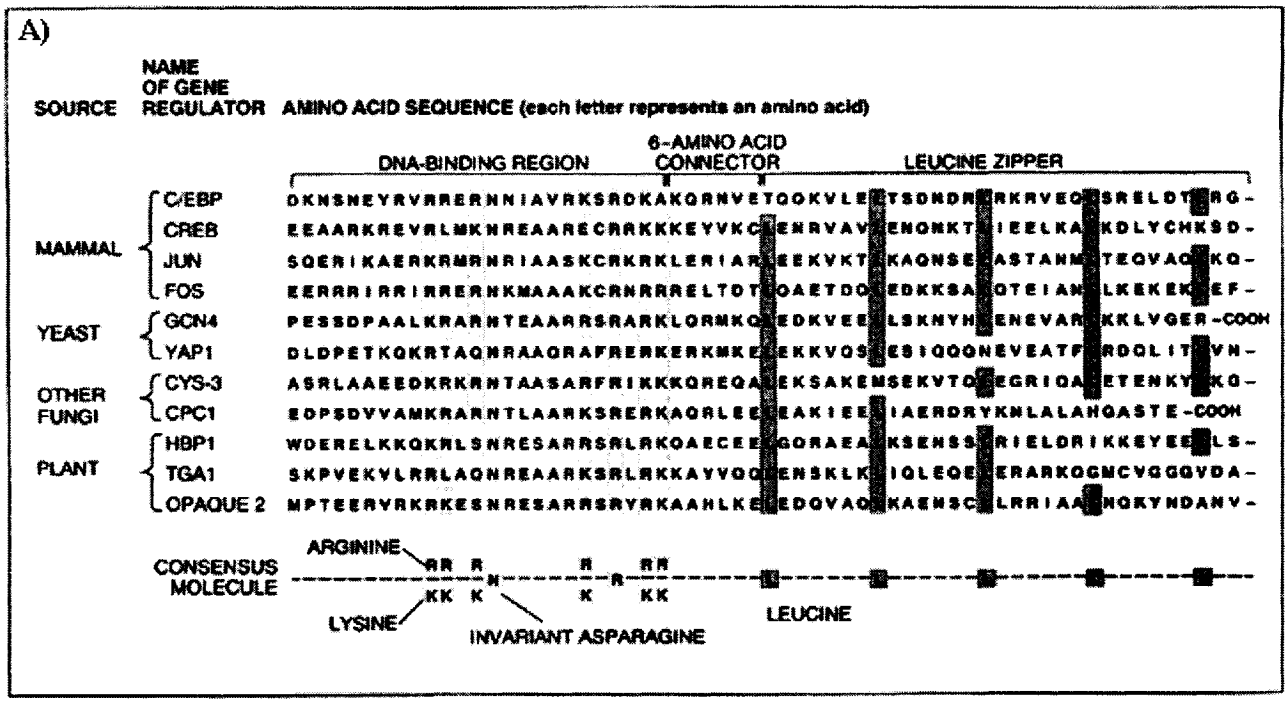
The DNA binding region, termed the basic region is immediately NH_2-terminal to the leucine zipper, and runs to opposite sides of the DNA double helix as continuous extensions from the leucine zipper and is embedded in the DNA major groove over a 13 or 14 bp region (Fig. 2B). This region has a characteristic sequence rich in basic amino acid residues and mediates DNA binding through hydrogen bonds and hydrophobic interactions between amino acid side chains and bases in the major groove [15]. These direct interactions recognize five conserved amino acid residues in the signature sequence of the bZIP basic regions, namely **N**XX**AA**XX**CR** (X represents variable amino acid residue type) [18].

The DNA-binding region of zipper proteins was once thought to be a continuous alpha helix and to protrude from the DNA (Fig. 2A) [19]. However, closer examination of the amino acid sequences of 11 proteins (Fig. 2A) showed that certain amino acids in the protrusion were conserved from molecule to molecule, suggesting that they aid binding and touch the DNA. Moreover, although the region was initially thought to be free of the helix-destabilizing residues (proline and glycine); asparagine, a more recently categorized potential helix breaker, was found to lie at a fixed point in the DNA-binding region of every protein. This would allow the protruding section to bend and thus contact the DNA continuously.

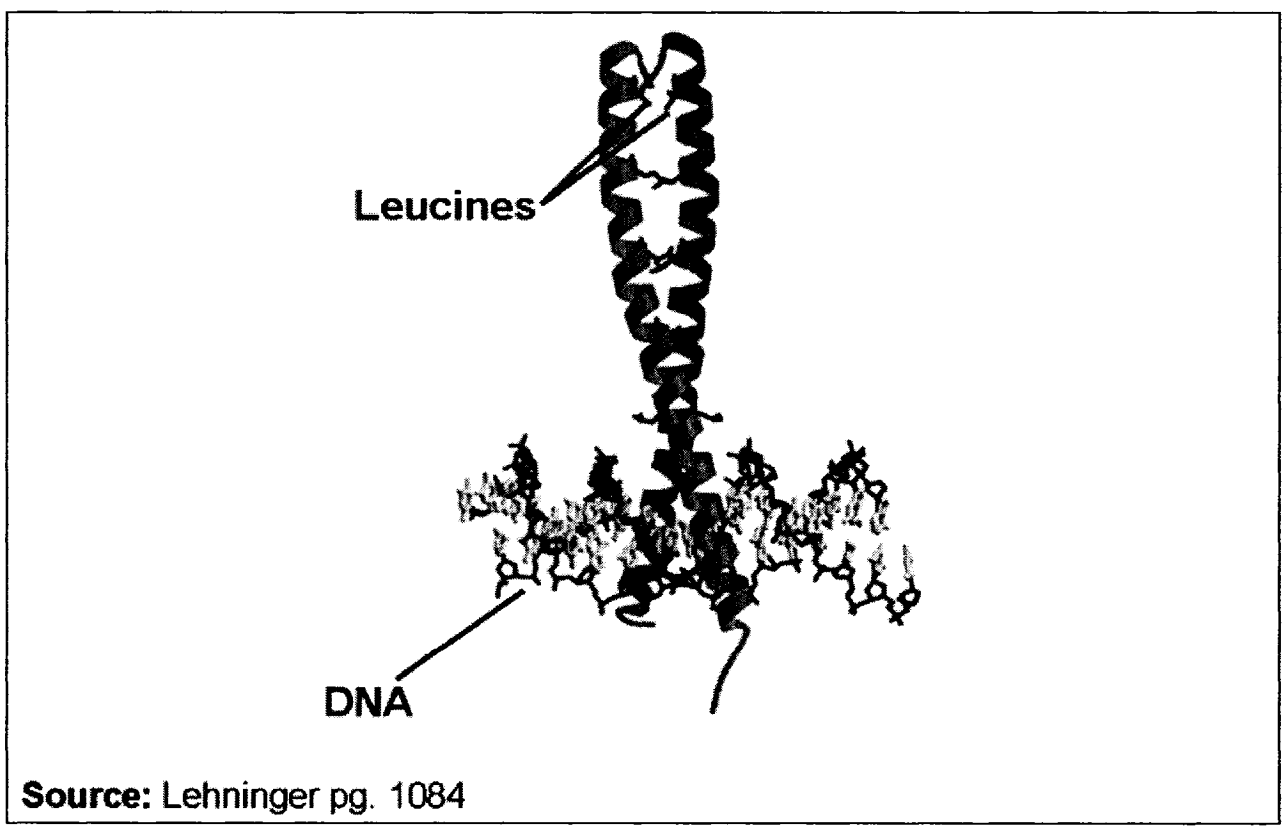
It is not surprising that the composition of the α-helices in both regions of the bZIP domain differ. In the basic domain, the α-helix surfaces are designed to repel one another whereas in the zipper region, they form an amphiphathic phase in which hydrophobic amino acid residues are regularly spaced alternately three or four positions apart in the sequence. As a result of this

Fig.2 A) Sequence alignment of various bZIP transcription factors. Conserved amino acids in the basic regions and heptad repeats of the leucine zippers are highlighted in grey. **B) BZIP transcription factor.** Structure consists of three alpha helices. BZIP domain is at the Carboxyl terminus. Leucine zipper is formed by heptad repeats of leucine zippers (LLLMLL or LLLLYL). These leucines form the hydrophobic core of a coiled coil which extends away from the DNA in a directions perpendicular to the overall DNA helix axis. The basic region is immediately NH_2-terminal to the leucine zipper is required for DNA binding through hydrogen bonds and hydrophobic interactions between amino acid side chains and bases in the major groove.

A)



B)



Source: Lehninger pg. 1084

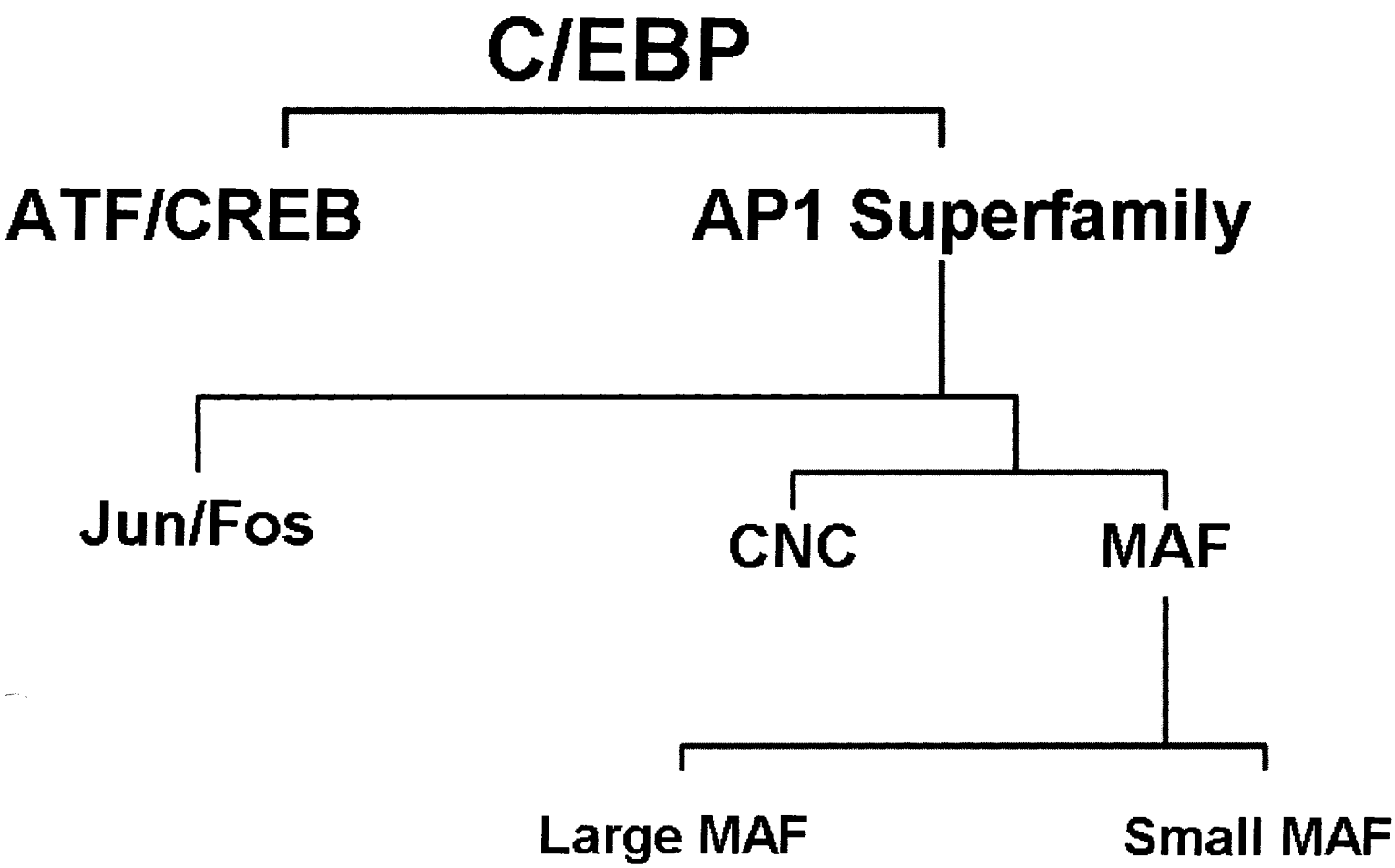
characteristic spacing, the hydrophobic side chains form a stripe down one side of the alpha helix. The hydrophobic stripes make up the interacting surfaces between the alpha-helical monomers in a coiled-coil dimer [14].

The activation domain which lies at the NH_2 terminus is rich in conserved acidic residues such as glutamic and aspartic acid, which confer its transactivation potential. This region may also be rich in proline, serine and threonine residues [15].

The first bZIP domain was discovered in 1988 as a heat-stable factor in rat liver nuclei that was capable of interacting with the CCAAT box motif present in several cellular gene promoters and certain viral enhancers. It was named CCAAT/Enhancer Binding Protein (C/EBP-α) [14]. An effort to find other proteins with similarities to the C/EBP family yielded the discovery of two other bZIP subfamilies: the Activating Transcription Factor/CRE-binding protein (ATF/CREB) family and the Activating Protein 1 (AP1) superfamily (Fig. 3). These two subfamilies were defined according to their ability to recognize specific binding sites. The AP1 superfamily controls gene expression by binding to TPA (12-*O*-tetradecanoylphorbol-13-acetate) response elements (TRE) and is responsible for transcriptional induction of a number of genes in response to activation of protein kinase C (PKC) pathway [20]. The ATF/CREB family regulates the cyclic AMP (cAMP) response element (CRE) and mediates cAMP-inducible genes in response to the activation of the PKA pathway [21].

2. AP1 Superfamily.

Fig.3 BZIP Family subdivisions. The ATF/CREB family regulate the CRE while the AP1 family control gene expression by binding to TRE. The CNC and MAF family are defined by unique conserved regions located NH_2 terminal to their basic DNA binding domain, called the CNC domain and the EHR. The small MAF family members differ from the large MAF in that they do not contain a conical activation domain.


C/EBP
ATF/CREB
AP1 Superfamily
Jun/Fos
CNC
MAF
Large MAF
Small MAF

The AP1 superfamily consists of a collection of structurally related transcription factors, which are further subdivided to either the JUN/FOS family or the CNC/MAF family (Fig. 3).

2.1 JUN and FOS Families.

The JUN (v-JUN, c-JUN, JUNB, JUND) and FOS (v-FOS, c-FOS, FOSB, FRA1, FRA2) subfamilies associate to form a variety of homo and heterodimers, all of which recognise TRE [20, 22, 23]. Like all other members of the bZIP family, the AP1 family members must dimerize prior to binding DNA. Unlike the JUN proteins which can form very stable homo- and heterodimers among themselves, the FOS proteins must heterodimerize with one of the JUN proteins since they can not form stable FOS-FOS homo- or heterodimers [24, 25]. On the basis of DNA sequence specificity and heterodimerization with JUN and FOS proteins, two other bZIP families of proteins have also been added to the AP1 superfamily (Fig. 3), the MAF and CNC families.

2.2 MAF and CNC Families

The MAF and CNC families are defined by unique conserved regions located NH_2 terminal to their basic DNA binding domain called the extended homology region (EHR) and the Cap'n'Collar (CNC) domain, respectively [26, 27].

3. The MAF Family

The founding member of the MAF protein family (v-MAF) was originally discovered as the transduced transforming component of avian musculoaponeurotic fibrosarcoma virus, AS42 [27]. Since the discovery of v-MAF, several other MAF genes were identified, including its cellular counterpart c-MAF. In general, viral oncogenes are modified and sometimes truncated versions of their cellular counterparts, and they usually exhibit stronger transforming activities than do their cellular counterparts [28]. For instance, even under the control of a strong retroviral promoter, the c-REL proto-oncogene exhibits no transforming activity [29]. Some of the cellular oncogenes, such as c-JUN, exhibit some transforming potential when they are overexpressed, but mutations seem to be necessary to convert them to fully transforming genes [30]. To this end, the overexpression of the c-MAF proto-oncogene was shown to cause cell transformation in chicken embryo fibroblast cells (CEF) [31].

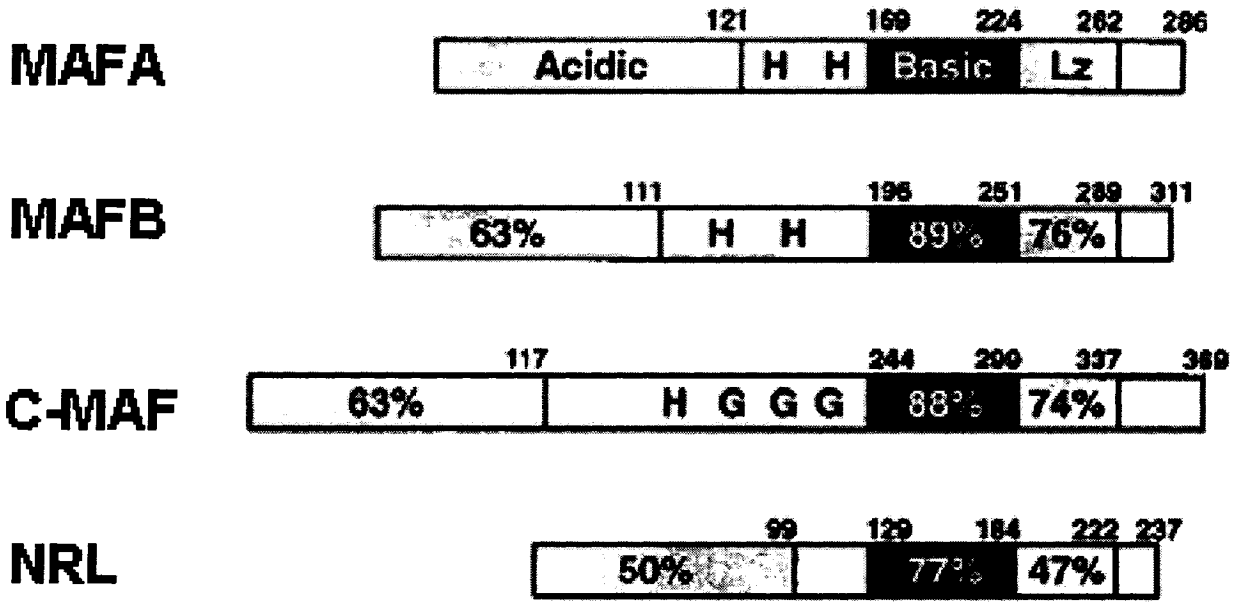
Using a *v-MAF* specific probe to screen a CEF cDNA library under relaxed hybridization conditions, the cDNAs coding two other members of the MAF family were isolated and named *MafK* and *MafF* [32]. Shortly after that, a third MAF, *MafG*, was identified using *MafK* as a probe to screen a chicken genomic library [33]. Early on these genes were noticed to share limited sequence similarity with the *v-MAF* gene. Also, their exon-intron structures were similar to each other but quite different from that of *c-MAF*. Furthermore, their putative gene products shared a high degree of structural similarity (65.4% identity), but they were only weakly related to the carboxyl terminus of the *v-MAF* protein [32]. During the same time, another MAF protein was identified as a retina specific gene and named neural retina leucine zipper (NRL) [34]. In contrast to the MAFK, MAFF and MAFG, the amino acid sequence of the NRL gene product shared significant homology with c-MAF. When compared, the amino

terminal portions of the c-MAF and NRL were both rich in acidic amino acids, as is often the case in the transactivator domain of transcription factors. MAFK, MAFF and MAFG lacked this putative transactivator domain. These findings ultimately led to division of the MAF family into two separate subfamilies, the large MAF and small MAF families (Fig. 3).

3.1 The Large MAF Family.

The large MAF family encompasses *c-MAF*, *NRL*, *MAFA* and *MAFB*. Since their discovery, the large MAF family members were shown to play important roles in the differentiation and development of a variety of hematopoietic and neural tissue. For instance, c-MAF has been shown to play a crucial role in lymphopoiesis. By binding to the MAF Response Element (MARE) in the proximal interleukin 4 (*IL4*) promoter, c-MAF, which is induced and expressed in T helper cells 2 but not T helper 1 clones, upregulates the expression of the *IL4* gene [35]. NRL, as mentioned earlier, is specifically expressed in retinal cells, including photoreceptors. In fact, its expression precedes that of rhodopsin during development [36]. Rhodopsin is the visual pigment of vertebrate rods, and its activation by light initiates the phototransduction process [37]. NRL has been shown to positively regulate the expression of Rhodopsin [38, 39]. MAFA, previously known as Lens-specific MAF (L-MAF), has also been shown to be an important regulator in eye development. More specifically, MAFA has been identified as a factor that binds to and regulates the αCE2 sequence, which has been previously identified as a lens-specific enhancer element in the chicken crystallin promoter [40, 41]. The upregulation of crystallin gene transcription is an important marker for the differentiation of the ectoderm into lens cells [42]. Thus, vertebrate lens induction and differentiation can be triggered by the activation of MAFA [43]. MAFA most closely resembles MAFB (Fig. 4). MAFB has been

Fig.4 Large MAFs. Schematic representation of MAFA and other large Mafs. Acidic, basic, and leucine zipper (Lz) domains are indicated; H and G denote histidine and glycine repeat regions, respectively. Numbers in each domain indicate percent identity to MAFA. Amino acid positions are numbered (adapted from Ongino et al., Science, 1998).


MAFA
121
169
224
262
286
Acidic
H H
Basic
Lz
MAFB
111
196
251
289
311
63%
H H
89%
76%
C-MAF
117
244
290
337
369
63%
H G G G
88%
74%
NRL
99
129
184
222
237
50%
77%
47%

identified as the affected gene in mice carrying the *Kreisler* (*kr*) mutation. The *kr* mutation was identified in an X-ray mutagenesis experiment, because mutant animals exhibited a hyperactive pattern of behaviour characterized by head-tossing and running in circles [44].

Kreis is the German word for circle and $kr^{-/-}$:$kr^{-/-}$ animals are deaf and cannot swim, a constellation of findings characteristic of mutations that affect inner ear function. The inner ear function is abnormal due to a defect in hindbrain development. Pattern formation in the hindbrain is governed by a segmentation process that provides the basis for the organisation of cranial motor nerves. This process subdivides the embryonic hindbrain into eight subunits, termed rhombomeres (r1-r8) [45]. A cascade of transcriptional activators, including the MAFB transcription factor, controls this segmentation process.

In kreisler mutants, r5 fails to form and this correlates with abnormalities in the neuroanatomical organisation of the hindbrain. Two independent studies have been performed, using molecular markers, to monitor changes in the *kreisler* hindbrain. One concluded that r5 and r6 are missing through a combination of changes in A-P identity and fate, followed by subsequent cell death [46]. The other study interpreted the data as the loss of overt segmentation of the hindbrain posterior to r4 leading to abnormal expression of markers in the region [47]. While these reports clearly demonstrate that the posterior hindbrain in *kreisler* mutants has an abnormal organization, they do not address which processes (formation, maintenance, or the identity of segments) are affected. A more recent study illustrates that kreisler has roles in regulating multiple steps of hindbrain patterning. The complete absence of r5 in kreisler mutants shows that it has an early role in the specification of this segment. The

formation of r6 independent of r5 argues against a strict prorhombomeric model for segmentation of this region of the hindbrain in mouse. In addition, through the direct regulation of HOX genes, kreisler participates in the control of segmental identity. Ectopic expression of kreisler in r3 leads to its transformation toward an r5 identity [48]. Therefore, rather than being restricted to a single aspect of patterning, it appears to be an emerging theme that genes involved in controlling segmental processes in the hindbrain can act at several different levels.

Additionally, all three large MAFs, MAFA, MAFB and c-MAF were apable of specifically binding to and activating the insulin C1 element within the nuclei of islet beta cells, hence clearly playing a role in regulation of the insulin gene [49]. In summary, large MAFs play important roles in the differentiation, development and gene regulation of a variety of hematopoietic, neural and endocrine tissue [15, 41, 50].

3.2 The Small MAF Family

The small MAF family consists of MAFK, MAFF and MAFG (Fig. 5B). Typical of the bZIP family that they belong to, the structure of the small MAF transcription factors is composed of a basic region rich in basic residues required for DNA binding and an adjacent leucine zipper region formed by a heptad repeat of hydrophobic residues, usually leucine, required for dimerization. The small MAFs differ in that they lack an obvious canonical activation domain [15]. As mentioned earlier, the distinguishing feature of the MAF transcription factors is the EHR domain, characterized by the presence of glycine and histidine clusters N-terminal to the basic domain (Fig. 5A & 5B) [41]. NMR spectroscopy provided an understanding of how the basic region recognizes the consensus sequence and also elucidated the mechanism of how the

EHR recognizes the flanking region. By drawing parallels to earlier findings that described how the SKN1 transcription factor binds to the TRE [51], the amino acid sequence NXXYAXXCR within the MAFG basic domain was proposed to make direct interaction with the specific residues in the TRE, as well as with the phosphate backbone of the DNA. Using chemical shift perturbation experiments, two amino acids (Val 34 and Arg 35) in the EHR were shown to enable MAFG to make a broader area of contact and to recognize longer DNA sequences in the flanking region. This was further supported in another experiment where the truncation of the NH_2-terminus was reported to result in loss of DNA-binding specificity.

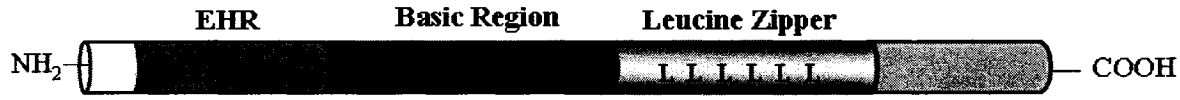
The first direct physiological role for the small MAF family emerged during the analysis of erythroid specific transcription, which has proven to be an excellent model system for deciphering the mechanisms governing tissue specific and temporal aspects of gene regulation. MAFK was identified as one of the subunits of nuclear factor erythroid (NFE2) [52] and in this process, the small MAF family of transcription factors merged with the stream of molecular biology dealing with erythroid specific gene regulation.

3.2.1 MAF Response Element (MARE)

The NFE2 binding site was originally identified in the promoter of the porphobilinogen deaminase (PBGD) gene [53]. It has also been subsequently identified in the promoter regions

Fig.5 The Small MAF family. A) The functional domains of the small MAF proteins. **B)** The amino acid sequence alignment of the murine MafF, MafG and MafK. Conserved leucine residues are shadowed in grey. Conserved amino acids are in grey.

A



B

```
                                                    ←—— EHR ——→ ←——BASIC
MafF  MSVDPLSSKALKIKRELSENTPHLSDEALMGLSVRELNRHLRGLSAEEVTRLKQRRRTLK
MafG  MTTPNKGNKALKVKREPGENGTSLTDEELVTMSVRELNQHLRGLSKEEIVQLKQRRRTLK
MafK  MTTNPKPNKALKVKKEAGENAPVLSDDELVSMSVRELNQHLRGLTKEEVTRLKQRRRTLK

      REGION ——→  ←————————LEUCINE ZIPPER————————→
MafF  NRGYAASCRVKRVCQKEELQKQKSELEREVDKLARENAAMRLELDALRGKCEALQGFARS
MafG  NRGYAASCRVKRVTQKEELEKQKAELQQEVEKLASENASMKLELDALRSKYEALQTFAR-
MafK  NRGYAASCRIKRVTQKEELERQRVELQQEVEKLARENSSMRLELDALRSKYEALQTFAR-

MafF  VAAARGPATLVAPASVITIVKSTPGSGSGPAHGPDPAHGPASCS
MafG  -TVARSPVAPARGPLAAGLGPLVPGKVAATSVITIVKSKTDARS
MafK  -TVARGPVAPSKVATTSVITIVKSTELSSTSVPFSAAS------
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of several erythroid specific genes such as ferrochelatase [54] and 5-Aminolevulinate synthase (ALAS-E) [55] as well as in the Locus Control Region (LCR) of the Globin gene [56]. The NFE2 consensus binding site was determined to be [TGCTGACTCAC]. The core of its motif bore a striking resemblance to that of the TRE. Further investigation into the binding sites of MAF homodimers showed that they bound either a 13 bp palindromic sequence which had the TRE core or a 14 bp palindromic sequence with the CRE core. The former sequence was called TRE-type MAF Response Element (T-MARE) [TGCTGACTCAGCA]. The latter was called CRE-type MARE (C-MARE) [TGCTGACGTCAGCA] (Fig. 6). The specific flanking regions [TGC] and [GCA] at either end of the core TRE/CRE were shown to be crucial for recognition of the MARE by MAF proteins [57] and are what distinguishes the MAF family proteins from members of the AP1 or CREB/ATF family [13, 34, 57].

Using Surface Plasmon Resonance microarray studies, it was concluded that MAREs create a huge diversity in the regulation of gene expression through single base alterations. Such subtle changes in the MARE sequences are sufficient to alter rather drastically the binding specificity for any given transcription factor dimer and thereby impart enormous diversity to gene regulatory networks formed by the MAF/CNC families of transcription factors [58].

In effect, the resemblance between the NFE2 binding site and the T-MARE sequence was actually what provided the initial insight into the possible role that the small MAF proteins might be an essential subunit of transcription factor NFE2. The striking similarity between the MARE and the Antioxidant Response Element (ARE) sequence led researchers in a different direction. This crucial observation opened up the field to investigate the role of the small MAF heterodimers in the regulation of cytoprotective genes. These genes, such as, glutathione S-

Fig.6 MAF Response Element (MARE). Consensus sequences recognized by bZIP transcription factors. TRE and CRE are included within T-MARE and C-MARE, respectively, and establish the core sequence of the MARE (black). Three bases on each side of the core form the flanking sequence (in grey), which is critical for recognition of the MARE by Maf family proteins [50].

MARE
- TRE type (T-MARE)
- cAMP type (C-MARE)

TPA-Responsive Element

cAMP Responsive Element

transferase (GST), heme oxygenase-1 (HO1) and NAD(P)H:quinone oxidoreductase 1 (NQO1) [59], give cells the ability to adapt to oxidative stress or to xenobiotic exposure. It has long been known that the genes encoding these enzymes are often coordinately regulated through AREs in their gene-regulatory regions [60, 61].

3.2.2 Small MAF gene targeting.

The role of the small MAF members in ARE-dependent gene regulation became apparent when *MafG* $^{-/-}$:: *MafK* $^{-/-}$:: *MafF* $^{-/-}$ fibroblasts lacking all three small MAF proteins were established and examined (Table 1). After exposure to 1-chloro-2,4-dinitrobenzene (CDNB), which is known to act as a strong oxidative stress agent, a complete loss of mRNA induction was observed in almost all of the ARE- dependent genes examined, i.e. GST, NQO1, HO1, glutamate-cysteine ligase catalytic subunit (GCLC) and thioredoxin reductase 1 gene (TXNRD1) [62]. Interestingly, when *MafG* $^{-/-}$:: *MafF* $^{-/-}$ mice were examined none of the above tested genes, other than NQO1 and TXNRD1, achieved any significant loss of induction. This implies that each single small MAF protein is more or less capable of supporting ARE-mediated transcriptional activation. This was the first report that genetically demonstrated the ability of each small MAF protein to contribute to ARE-dependent gene activation and the essential roles of the family of small MAF proteins in the activation [62]. Hence, functional redundancy has made it difficult to assign specific *in vivo* roles to each of the small MAF transcription factors. In reality, prior to the triple knock-outs, null mutation of all three small MAF genes did not greatly clarify *in vivo* functions of this extensively interacting regulatory network. Those studies showed that *MafK* and *MafF* loss of function resulted in no discernable phenotype in homozygous mutant animals [63, 64] (Table 1). *MafG* null mice were also viable,

but exhibited mildly impaired platelet formation, as well as behavioral abnormalities [65] (Table 1). The neurological abnormality was exacerbated in *MafG* $^{-/-}$:: *MafK* $^{-/-}$ compound mutant mice, further supporting the argument that MAFG and MAFK share compensatory functions in the central nervous system [66]. Present findings showing that baroreceptor stimulation by phenylephrine can induce MAFG expression in cardiovascular control sites involved in the central processing of baroreceptor input, further supporting that MAFG may play critical functions in signal transduction in the central nervous system [67].

3.2.3 Small MAFs Tissue Distribution Analysis.

In order to shed light on the physiological functions of the small MAF members, the tissue distribution of their mRNAs in chicken, mice and humans were examined. In chickens, the levels of *MafK* transcripts were less variable among the tissues analysed and were relatively high in lung, mesentary and bursa of Fabricius. The *MafF* gene was found to be expressed in brain, heart, mesentary and ovary [32].

In mice, the mRNA expression pattern of all three small MAF was compared using a quantitative fluorescence PCR assay. Not unexpectedly, each of the small MAF mRNAs displayed a unique expression pattern: although *MafK* and *MafF* were most abundantly expressed in the lung, *MafG* appeared to be most abundant in the heart. *MafG* transcripts differed by no more than 10-fold in any of the tissues examined, whereas *MafF* levels in different tissues varied by more than 65-fold [64]. Examination of *MafK* during murine development led to discovery of two different promoters. More specifically, murine *MafK* was shown to be composed of four exons. The two first two exons, I_M and I_N are independent of

Table 1. Small MAFs gene targeting phenotypes.

MafK and *MafF* loss of function resulted in no discernable phenotype in homozygous mutant animals [63, 64]. *MafG* null mice were also viable, but exhibited mildly impaired platelet formation, as well as behavioral abnormalities [65]. The neurological abnormality was exacerbated in *MafG* $^{-/-}$:: *MafK* $^{-/-}$ compound mutant mice [66]. The triple knock out die in midgestation [62].

Knock Out	Phenotype
MafF	Normal
MafK	Normal
MafG	Decrease in Platelets, Mild motor disorder
MafG/MafK	Platelets strongly reduced, Motor disorder earlier, Perinatal lethality
MafG/MafF	Compromised expression of a subset of ARE-dependent genes
MafF/MafK	Fertile and Healthy
MafG/MafF/MafK	Embryonic Lethality at midgestation

each other and encode two distinct 5' UTRs for expression in either the mesodermal or neuronal tissues, respectively. The second and the third exons which encode the entire protein coding sequence appear to be used in common by either *MafK* transcripts. [68]. In similar fashion, the murine *MafF* locus is organized with two coding exons. However, it is regulated by three different promoters which, are alternatively spliced to generate three *MafF* mRNAs that encode the same protein product [64].

In human cells, *MAFK* and *MAFG* mRNA expression were examined by Northern blot analysis. The *MAFK* mRNA was abundantly expressed in heart, skeletal muscle and placenta, whereas *MAFG* mRNA was expressed at a high level in skeletal muscle and only moderately in heart and brain. The *MAFF* transcript was also observed in the placenta as well as term myometrium and kidney [69]. Large size transcripts of *MAFK* and *MAFG* were found in all tissues, which became apparent upon longer exposure of the Northern blot filters. In particular, the large size transcript of *MAFK* was quite strong in placenta [70]. These transcripts may reflect alternative RNA processing as seen in mice.

4. The CNC Family

Over the last several years, three groups of small MAF interacting molecules, JUN, FOS and CNC families, which can form either productive or unproductive complexes, have been described [71]. The most physiologically important of these partner molecules are probably the vertebrate CNC transcription factors, so named originally for their sequence homology to the Drosophila Cap'n'Collar protein [72] (Fig. 7). The CNC domain spans 43 amino acids immediately amino terminal to the basic domain and is highly conserved among the family

members. This family includes the *Drosophila* CNC, *C. elegans* SKN1 and vertebrate p45, NRF1, NRF2, BACH1 and BACH2 proteins.

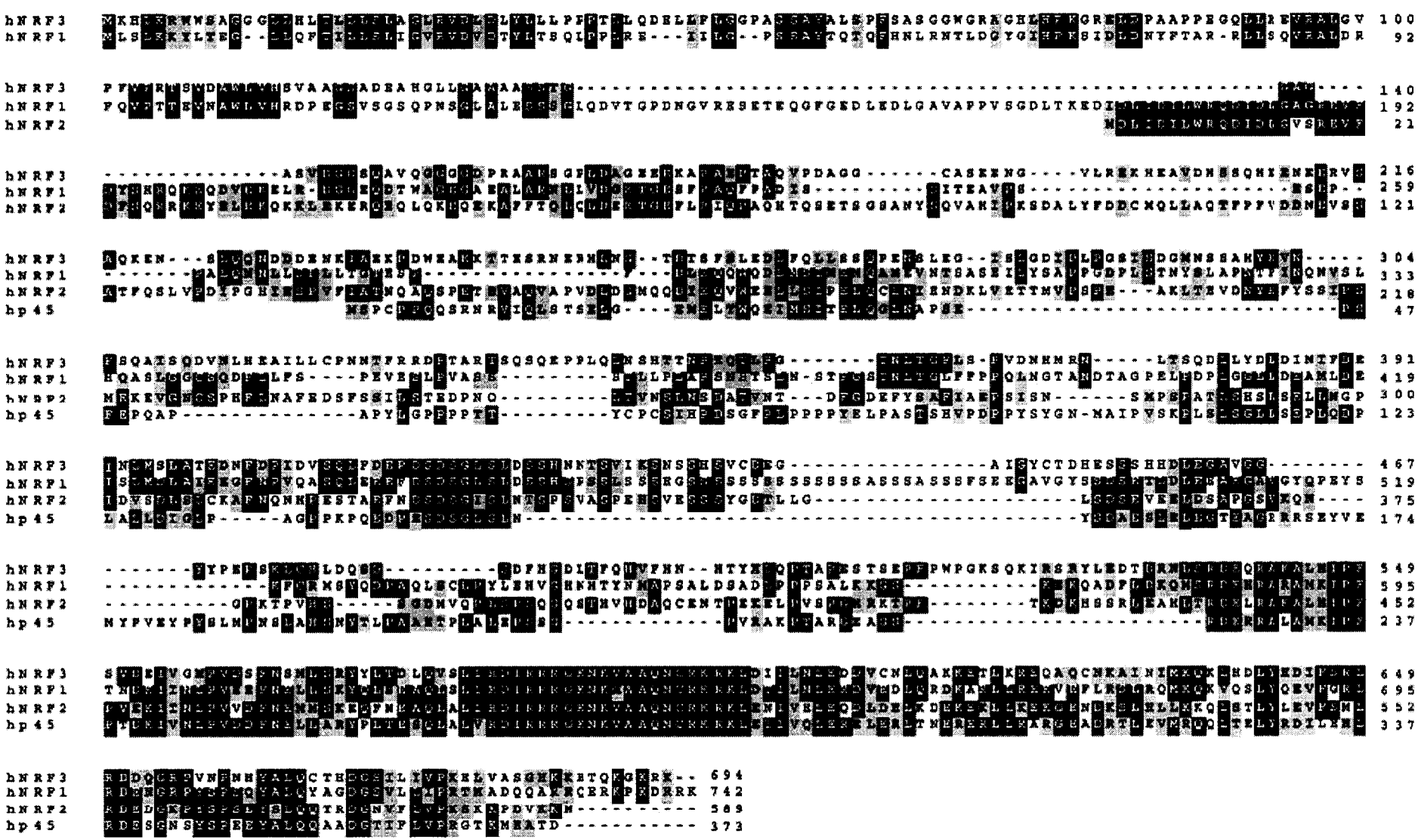
4.1 P45

As mentioned earlier, this was the first identified partner molecule of MAFK. Together they forme the erythroid specific transactivator NFE2. NFE2 was purified from mouse erythroleukemia (MEL) cells as a novel bZIP transcription factor composed of a 45 kDa subunit (p45) and an 18 kDa subunit (MAFK) [52]. Surprisingly, p45 null mutant mice exhibited normal erythropoiesis and instead showed high mortality due to severe hemorrhaging resulting from the absence of circulating platelets [73]. Further investigation indicated that proplatelet formation, a pathway of platelet production from megakaryocytes, was significantly inhibited in p45 null megakaryocytes [74].

4.2 NRF1

NRF1 was isolated from a cDNA expression library using a probe containing the tandem MARE of the β-globin LCR [75, 76]. It is strongly expressed in muscle, kidney, liver, lung, heart and ovary [75]. *Nrf1* null mice were found to die of anemia at late gestation [77]. However, embryonic stem (ES) cells with a disrupted *Nrf1* were still able to contribute efficiently to blood formation in chimeric animals [77]. This unexpected result indicates that the defective erythropoiesis in *Nrf1* null mice is non-cell autonomous, i,e, the wild-type cells surrounding the mutant cell can rescue the mutant phenotype. To further exploit the availability of cells from *Nrf1* null mice, the involvement of NRF1 in the regulation of antioxidant gene expression was also examined. Results indicated the NRF1 plays a role in the

FIG.7 The CNC family. Alignment of human CNC proteins NRF3, NRF1, NRF2 and p45. Identical residues among proteins are in black. Conserved amino acids are shaded.



regulation of Glutathione (GSH) synthesis via the expression of its two subunits γ-glutamylcysteine synthetase (GCS) and glutathione synthetase (GSS) [78].

However, important new findings in adult hepatocytes obtained from chimeric NRF1 deficient embryonic stem cells show that expression of these genes (GCS and GSS) were not affected. Loss of NRF1 in these cells was associated with a significant increase in intracellular ROS levels that was coupled with elevated lipid and DNA oxidation. Furthermore, chimeric mice generated with NRF1-deficient embryonic stem cells showed widespread apoptosis in fetal livers at late gestation, demonstrating a cell autonomous role of NRF1 in the survival of hepatocytes. Although the basis of this discrepancy is not known, it may be attributed to that only a subset of ARE-dependent genes depends on NRF1 in adult hepatocytes [79].

4.3 BACH1 and BACH2

BACH1 and BACH2 were discovered by yeast two hybrid screening [80]. The BACH family proteins harbor a protein-protein interaction domain (called a BTB domain, for broad complex tramtrack bric a brac, also known as a POZ domain, for pox and zinc finger) in addition to the characteristic CNC-type b-Zip structural elements. BACH1 is a ubiquitous factor and is highly expressed in hematopoietic organs, such as the bone marrow and fetal liver [81], while BACH2 is most abundant in the brain and B lymphocytes [80]. Similar to the small MAF family members, the BACH factors do not contain a canonical transcriptional activation domain and repress transcription when expressed transiently in fibroblasts. In fact, BACH2 has been shown to function as an inhibitory factor, which represses a set of genes during oxidative stress. In response to oxidative stress, BACH2 translocates into the nucleus, and by competing with NRF2 for MAF family partner proteins it targets certain ARE-dependent antioxidant genes

[82]. It has also more recently been shown, via its negative effects on proliferation and differentiation of neuronal cells, to serve as a gatekeeper of the differentiated status of these cells [83].

BACH1 has also been linked to oxidative stress and plays a pivotal role in setting the levels of both constitutive and inducible expression of HO1, whose byproducts mediate anti-inflammatory and anti-oxidant actions. Myocardial expression of HO1 protein in *Bach1*$^{-/-}$ mice was constitutively up-regulated when these mice were subjected to myocardial ischemia/reperfusion (I/R) injury *in vivo*. While myocardial I/R induced HO1 protein in ischemic myocytes in both strains of mice, the extent of induction was significantly greater in *Bach1*$^{-/-}$ mice compared to *Bach1*$^{+/+}$ mice. Also, myocardial infarction was markedly reduced in size in *Bach1*$^{-/-}$ mice, further supporting BACH1 as a key player in the mechanism controlling the activation level of the cytoprotective program involving HO1 [84].

BACH1 and BACH2 also possess a BTB domain [80]. In addition to enabling heterodimer formation with MAFK through their respective zipper motifs, it was demonstrated that BACH1-MAFK heterodimers interact with each other through the BTB domain, generating a multimeric and multivalent DNA binding complex [81]. Through this ability to form multimers, BACH1 is believed to mediate interactions between distant, multiple MAREs, and thus may serve as an architectural transcription factor. With the identification of BACH family proteins, it was revealed that small MAF proteins have a wider choice of partner molecules for binding to MARE. Through the choice of dimeric partner, small MAF members can switch transcriptional activity from repression to activation.

4.4 NRF3

The function of the mammalian CNC factors has been extensively studied, but only a small amount of information is available on NRF3, the most recently identified member [85]. *NRF3* transcripts are primarily expressed in the placenta and at lower levels in heart, brain, lung, pancreas and kidney [85]. It was hypothesized that gene targeting of the mouse *Nrf3* locus would indicate a role for NRF3 in the placenta, however *Nrf3* null mice develop normally and exhibit no obvious phenotypic differences when compared to wild-type animals [86]. To test whether *Nrf3* null mice are still capable of giving normal birth, knock-out females and males were bred and it was found that both sexes were fertile and that females were able to bear normal-sized litters. Thus, although *Nrf3* is highly expressed in the placenta and testis, and at medium levels in the uterus, its function can be compensated by other CNC members in these tissues. It was previously proposed that NRF3, in conjunction with NRF2, may have a role in keratinocyte function [87], but no skin abnormalities were observed in *Nrf3* $^{-/-}$ animals. In fact, contrary to NRF2, NRF3 was found to negatively regulate ARE-mediated expression and antioxidant induction of NQO1 [88]. The complexity of CNC transcription factors was revealed by finding that the absence of NRF3 does not appear to cause additional lethality in compound *Nrf3/Nrf2* and *Nrf3/p45* deficient mice [86]. Furthermore, the first description of *in situ* hybridization of *Nrf3* in chick embryo failed to indicate any role for this transcription factor in hindbrain HOX gene regulation. Instead, the surprising expression in mesodermal embryogenesis implies an earlier more specific role for NRF3 in gene regulation [89].

4.5 NRF2

Similar to *NRF3* knock-out mice, the *NRF2* null mice developed normally and were fertile and initially yielded no obvious defective phenotype. Furthermore, they were not anemic, again indicating that erythropoiesis was not significantly affected by loss of NRF2 [90, 91]. However, because NRF2 is most prominently expressed in detoxification organs, such as liver and kidney, and organs exposed to the external environment, such as skin and lung [92], it was chosen as a prime candidate with which to study the possible interaction of CNC members with the ARE. Those studies led to a new paradigm in the field of molecular toxicology. NRF2 was identified as a major player in the antioxidant response. Results indicated that the expression of a specific subset of antioxidant enzymes, known as the phase II enzymes, was remarkably induced in the liver and was markedly diminished in *Nrf2* null mice [91]. This initial recognition of the involvement of NRF2 in the antioxidant response sparked a substantial amount of research in this field. It was found that insufficient induction of cytoprotective enzymes brings about an increased susceptibility of cells to toxic xenobiotics including acetaminophen, butylated hydroxytoluene, and diesel exhaust [93-96]. In addition to this deficient induction profile, cancer chemoprevention mechanisms are also abolished in mice deficient in NRF2 [97]. Using *in vivo* analysis, a point mutation detected in the promoter region of the *Nrf2* gene in C57Bl/6J mice attributed the susceptibility of a strain of mice to hyperoxia [98]. This study demonstrated that NRF2 is fundamental to the defense against reactive oxygen species (ROS) and implied that hyperoxia is linked to the *NRF2* locus. Thus, protection against electrophiles and ROS share a common mechanism, with NRF2 functioning as the central mediator.

5. CNC/MAFF Networking

The CNC and BACH family of transcription factors require small MAF partners in order to bind to the MARE sequence. The advantage in this scheme of compulsory heterodimeric interactions between the CNC and small MAF family members is that subtle variations in DNA binding specificity or in trans-activation or trans-repression potential can be achieved through dimer formation of only a limited number of b-Zip transcription factor partners. In this regard, it should be noted again that the small MAFs do not possess any canonical transactivation domain [15], and hence function as bidirectional regulators of transcription, i.e. repressors in their small MAF homodimers state and activators when in heterodimeric state with CNC family members. Thus, a deficiency in small MAFs would impair the function of CNC factors yet an excess of small MAFs is also predicted to increase small MAF homodimer formation and lead to repressed transcription. Indeed, it has been demonstrated that regulation from MARE sites can be turned on and off in living cells by experimentally manipulating the balance between the small MAF proteins and partner molecules that contain trans-activation domains [99-101]. When these findings were extended into an animal model, embryos overexpressing transgene-derived-MAFK died of anaemia. Additionally, it was discovered that megakaryocytes obtained from these mice exhibited reduced proplatelet formation and MARE-dependent transcription, a similar phenotypes to *MafG* -null megakaryocytes. When the *MafG* null mutants were bred to MAFK overexpressing animals, both loss- and gain-of-function phenotypes were reversed. This result provided direct *in vivo* evidence that the quantitative balance between the small MAFs and their heterodimeric partner molecules serves as a molecular switch for gene expression [102]. Nagai et al. went further to demonstrate that MAFK homodimers can suppress transcription, not only by competition for the DNA binding

site, but also by direct inhibition of transcription. Using domain analysis they showed that MAFK possesses a suppression domain in its basic region-leucine zipper [101].

More recent evidence provides yet another example in which one of the small MAFs, MAFG, achieves repression not through simple passive repression but through sumoylation, which then mediates transcriptional repression through recruitment of a histone deacetylase containing complex [103]. This is particularly interesting when taken together with earlier findings that small MAF proteins co-localize with heterochromatin prior to induction by DMSO, but co-localize with p45 after induction [104]. Upon induction, relocation of small MAF proteins coincides with movement of the β-globin locus from heterochromatin to foci containing p45. Thus, one can speculate that the cue for differentiation is first bestowed upon small MAF proteins, which initially sequester the β-globin locus within heterochromatin. Subsequently, the small MAFs bring the β-globin locus to the p45 foci where genes are actively transcribed.

These lines of evidence have introduced a new concept to the understanding of regulatory mechanisms directed by bZIP transcription factors. Small MAFs, as homodimers or as part of repressive heterodimers, repress transcription by either passively competing with transcriptional activators for the MARE or actively recruiting a repression complex. In addition, small MAFs act as transcriptional repressors by sequestering gene loci within heterochromatin. Alteration in subnuclear localization has thus emerged as a potentially important mechanism in the regulatory system directed by small MAF and CNC proteins.

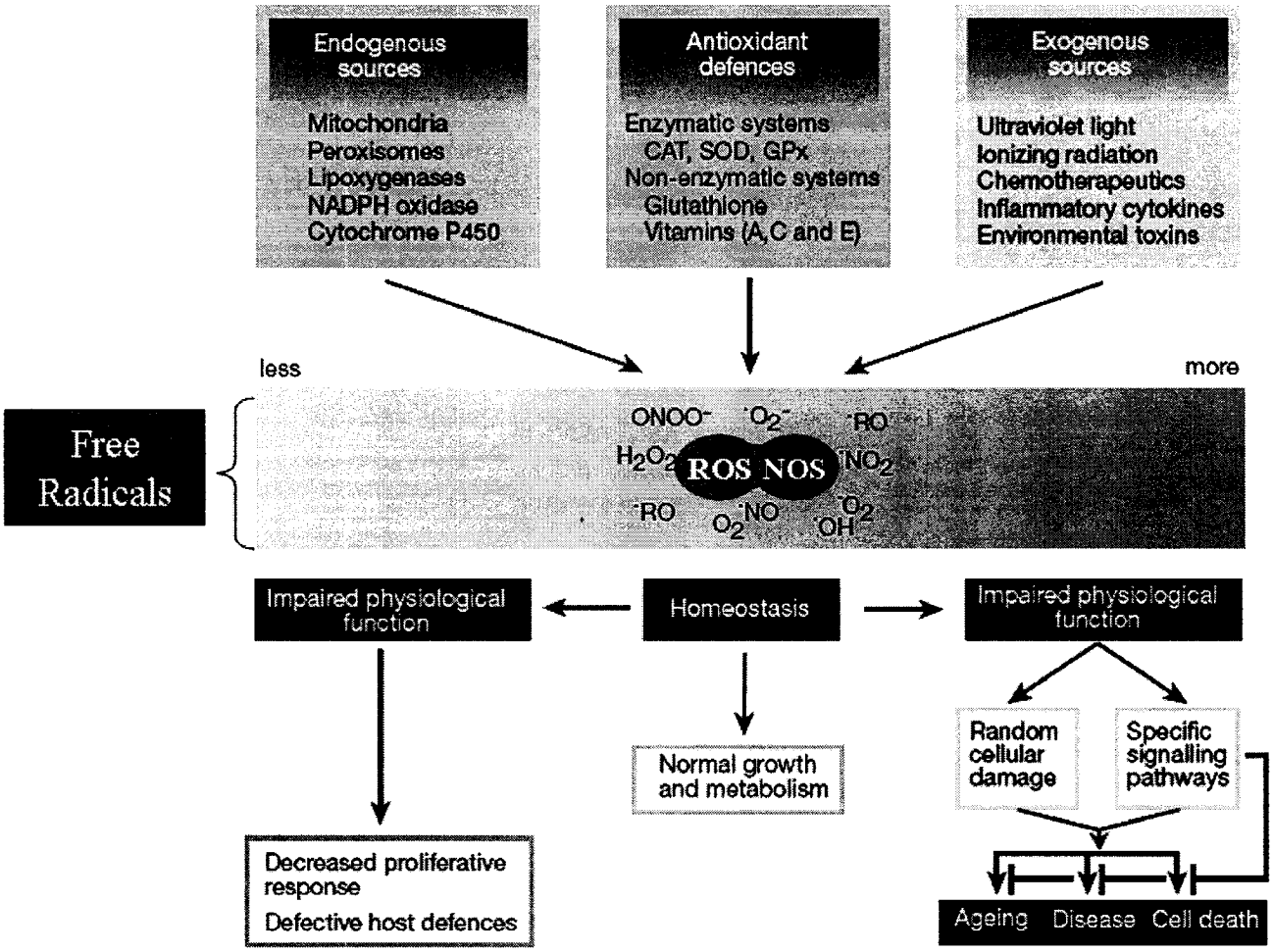
B. Oxidative Stress and the Female Reproductive System

Although NRF2 may have sparked an enormous interest in oxidative stress and the mechanisms in which this process is controlled in the cell, the concept of oxidative damage is not a novel one.

1. Oxidative stress

Oxidative stress is imposed on cells as a result of one of three factors: 1) an increase in oxidant generation, 2) a decrease in antioxidant protection, or 3) a failure to repair oxidative damage. Cell damage is induced by free radical species which are unstable and highly reactive [105]. Free radicals are defined as any species with one or more unpaired electrons in the outer orbit. They become stable by acquiring electrons from nucleic acids, lipids, proteins, carbohydrates or any nearby molecule causing a cascade of chain reactions resulting in cellular damage and disease [106, 107](Fig. 8). The generation of the highly reactive free radicals is an inherent feature of normal cellular metabolism [108]. There are two major types of free radical species: reactive oxygen species (ROS) and reactive nitrogen species (NOS). NOS have been associated with asthma, septic shock and **artheroclerosis**. The two common examples are nitric oxide (NO) and nitrogen dioxide (NO_2). ROS on the other hand, are either reactive anions containing oxygen atoms or molecules containing oxygen atoms that can either produce free radicals or are chemically activated by them. Examples are hydroxyl radical (HO•), superoxide anions (O_2^-), and hydrogen peroxide (H_2O_2). Most reactive oxygen species come from endogenous sources as byproducts of normal and essential metabolic reactions, such as energy generation from mitochondria or the detoxification reactions involving the liver cytochrome P-450 enzyme

Fig.8 Free radicals and Oxidative Stress. There are two major types of free radical species: reactive oxygen species (ROS) and reactive nitrogen species (NOS). Oxidants are generated as a result of normal intracellular metabolism in mitochondria and peroxisomes, as well as from a variety of cytosolic enzyme systems. In addition, a number of external agents can trigger ROS production. A sophisticated enzymatic and non-enzymatic antioxidant defence system including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) counteracts and regulates overall ROS levels to maintain physiological homeostasis [109].


Endogenous sources
Mitochondria
Peroxisomes
Lipoxygenases
NADPH oxidase
Cytochrome P450
Antioxidant defences
Enzymatic systems
CAT, SOD, GPx
Non-enzymatic systems
Glutathione
Vitamins (A, C and E)
Exogenous sources
Ultraviolet light
Ionizing radiation
Chemotherapeutics
Inflammatory cytokines
Environmental toxins
less
more
Free Radicals
ONOO⁻
$^{\cdot}O_2^-$
$^{\cdot}RO$
H_2O_2
ROS NOS
$^{\cdot}NO_2$
$^{\cdot}RO$
O_2 $^{\cdot}NO$
$^{\cdot}OH$
Impaired physiological function
Homeostasis
Impaired physiological function
Random cellular damage
Specific signalling pathways
Normal growth and metabolism
Decreased proliferative response
Defective host defences
Ageing
Disease
Cell death

system. Exogenous sources include exposure to cigarette smoke, environmental pollutants such as emission from automobiles and industries, consumption of alcohol in excess, asbestos, exposure to ionizing radiation, and bacterial, fungal or viral infections.

Under normal conditions, scavenging molecules known as antioxidants convert ROS to H_2O to prevent overproduction of ROS. There are two types of antioxidants in the human body: non-enzymatic antioxidants and enzymatic antioxidants.

Non-enzymatic antioxidants are also known as synthetic antioxidants or dietary supplements e.g. vitamin C, vitamin E, selenium, zinc, taurine, hypotaurine, glutathione, beta carotene, and carotene [110]. A good example of how one of these non-enzymatic antioxidants functions is vitamin C. It is a chain breaking antioxidant that stops the propagation of the peroxidative process and also helps recycle oxidized vitamin E and glutathione [111].

Enzymatic antioxidants are grouped into Phase I and Phase II enzymes. Phase I enzymes (e.g. cytochrome P-450, peroxidases, oxidases, lipoxygenases and hydroxylases) activate the harmful xenobiotics, drugs and carcinogens; while Phase II enzymes (e.g. NQO1, GST, superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase) detoxify them [112]. These enzymes are constitutively expressed at a low level under physiological conditions. However, their expression is increased in cells exposed to a diverse range of compounds, including oxidants and electrophilic agents that can also undergo redox cycling to produce excess reactive oxygen species. Central to the induction of these enzymes is

the transcription factor NRF2, which acts through the ARE to activate gene transcription [113, 114]

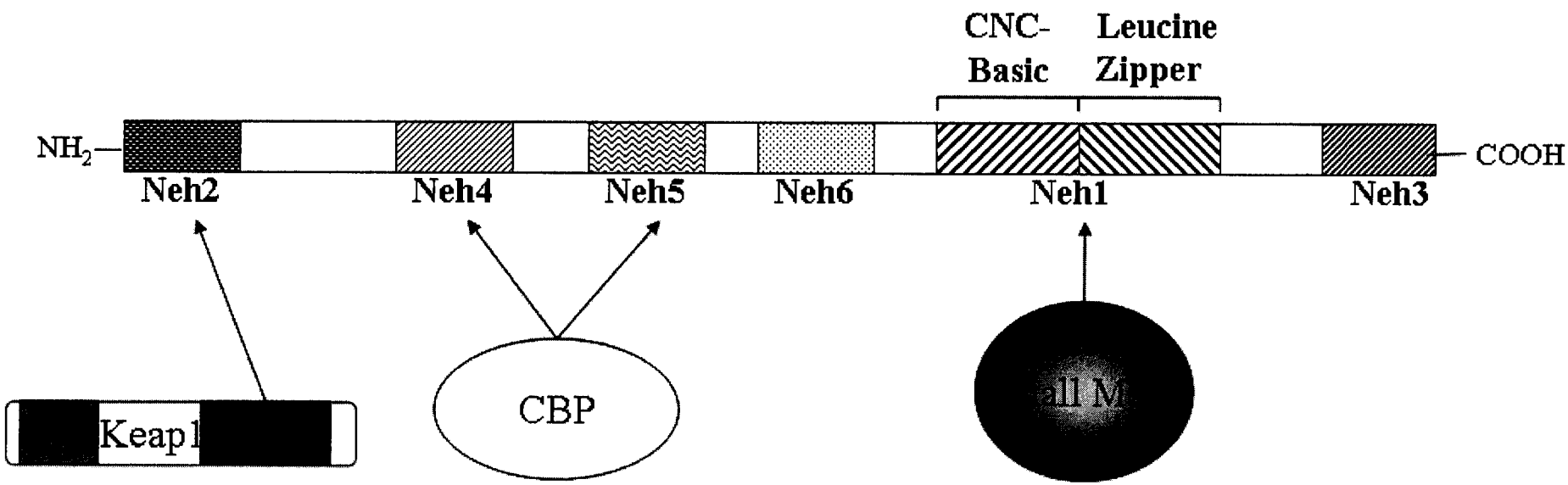
1.1 NRF2 Regulation

The involvement of NRF2 in both the constitutive and the inducible expression of ARE-dependent genes has been well established and documented in numerous *in vitro* and *in vivo* studies [91, 95, 115-118]. This sparked a great interest in investigating the molecular mechanisms and signalling pathways that regulate the activation of NRF2.

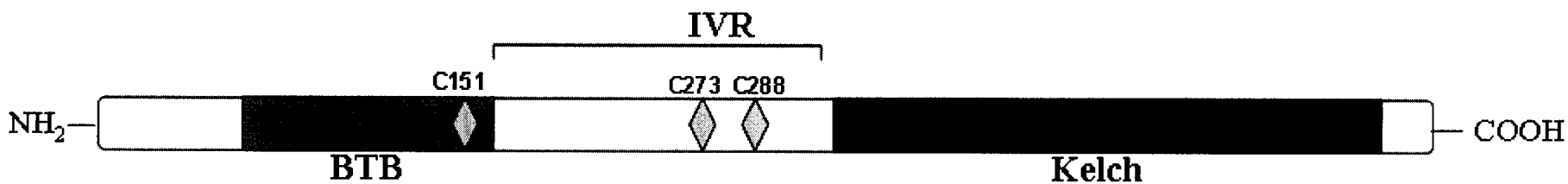
In comparing the human NRF2 and chicken ECH amino acid sequences, six highly conserved regions were found and named homology domains Neh1 to Neh6 (NRF2-ECH homology) (Fig. 9A). The first conserved domain, Neh1, contains the CNC homology region and basic-leucine zipper domain. The amino and carboxyl termini of the proteins are also highly conserved, and are referred to as Neh2 and Neh3, respectively. Additionally, there are two conserved acidic domains (Neh4 and Neh5) as well as a serine-rich conserved region (Neh6). Elucidation of the relationship between the structural domains of NRF2 and their function showed that deletion of the N-terminal Neh2 domain enhances the transcriptional activity of NRF2 [119]. This suggested that the N-terminus is necessary for recruiting a negative regulator of NRF2. To identify the negative regulator of NRF2, GAL4 – Neh2 was used as bait in the yeast two-hybrid system. Inspection of the newly cloned cDNA revealed the presence of two canonical protein interaction motifs: a BTB domain [80] and a double glycine repeat (DGR) module [120] in the middle and at the carboxy-terminal end of the protein, respectively (Fig. 9B). Database searches also revealed that this unusual combination of motifs is characteristic of the drosophila cytoskeleton binding protein, Kelch [121]; hence this

Fig.9. A) Functional domains identified in NRF2. Six functional Neh units were identified in NRF2. KEAP1 interacts with the Neh2 domain and represses the activity of NRF2. Neh4 and Neh5 interact with CBP and synergistically contribute to the strong transcriptional activation exerted by NRF2. Neh1 corresponds to the bZIP motif, mediating DNA binding and dimerization with small Maf proteins. **B) Domain structure of KEAP1**. KEAP1 is divided into 3 domains: BTB, IVR (intervening region), double glycine repeat; also called Kelch domain. The DGR/Kelch domain is important for NRF2 binding and interaction with the actin skeleton. The IVR is important for KEAP1 reactivity to electrophilic and oxidative stimuli. Two of the cysteine residues in the IVR are crucial for the repressive activity of KEAP1 on NRF2. The BTB domain is thought to be involved in dimer formation.

A)


CNC-
Basic
Leucine
Zipper
NH2
COOH
Neh2
Neh4
Neh5
Neh6
Neh1
Neh3
Keap1
CBP
sMaf

B)


IVR
C151
C273
C288
NH2
COOH
BTB
Kelch

newly characterized protein was named Kelch-like ECH-associated protein 1 (KEAP1) [119]. It is agreed that KEAP1, acts by directly interacting with NRF2 and preventing its nuclear accumulation. However, the mechanism by which NRF2 translocates and accumulates in the nucleus is still in question. One model, "Static Model", proposes that NRF2 is retained in the cytoplasm by interaction with two molecules of KEAP1, which are dimerized through their BTB domains and anchored to the actin cytoskeleton via the Kelch or DGR region. Inducers of the phase II response interact with cysteine thiol groups in the intervening region (IVR) of KEAP1, causing the formation of disulfide bonds. This results in a conformational change that renders KEAP1 unable to bind to NRF2, which then translocates to the nucleus (Fig. 10B) [122]. The other model, "Cycling model", provides evidence to dispute the static model and demonstrate that KEAP1 does not passively sequester NRF2 in the cytoplasm but actively targets NRF2 for ubiquitination and degradation by the proteosome under basal culture conditions. Several reports support this model in which NRF2 is degraded by the proteosome in a KEAP1-dependent manner [93, 114, 123] (Fig. 10A). It was demonstrated that KEAP1 associates with Cullin 3 (CUL3), one of the components of ubiquitin ligase, to form a functional E3 ubiquitin ligase complex that targets multiple lysine residues located in the N-terminal Neh2 domain of NRF2 for ubiquitin conjugation both *in vivo* and *in vitro* [124]. Inhibition of KEAP1-dependent ubiquitination of NRF2 correlates with decreased association of KEAP1 with CUL3, not through the disruption of association between KEAP1 and NRF2. This implies that the ability of KEAP1 to assemble into a functional E3 ubiquitin ligase complex is the critical determinant that controls steady-state levels of NRF2 in response to oxidative stress.

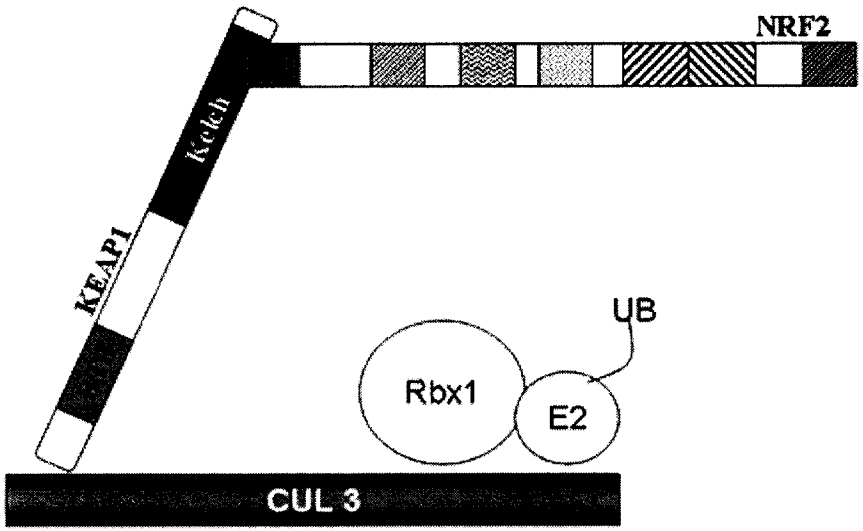
1.2 CNC/MAF and Oxidative Stress

NRF2 as well as the other molecules in the CNC family of transcription factors do not bind to ARE as homodimers or heterodimers [115]. Hence, it has been assumed that the involvement of CNC factors in ARE-mediated transcription obviates the involvement of the small MAF partners in order to bind to the ARE sequence. Indeed, data from several studies suggest that CNC/small MAF heterodimers are involved in the regulation of detoxifying enzyme genes and in the response to oxidative stress [91, 116, 117, 125-127]. Some studies report enhanced transcriptional activation when low amounts of small MAF proteins were cotransfected with the activating CNC factor [99, 116]. In all, however, increasing levels of small MAF inhibited both basal and CNC protein-inducible promoter activity, a result attributed by several to a putative shift towards MAF-MAF homodimer formation [99, 116, 128].

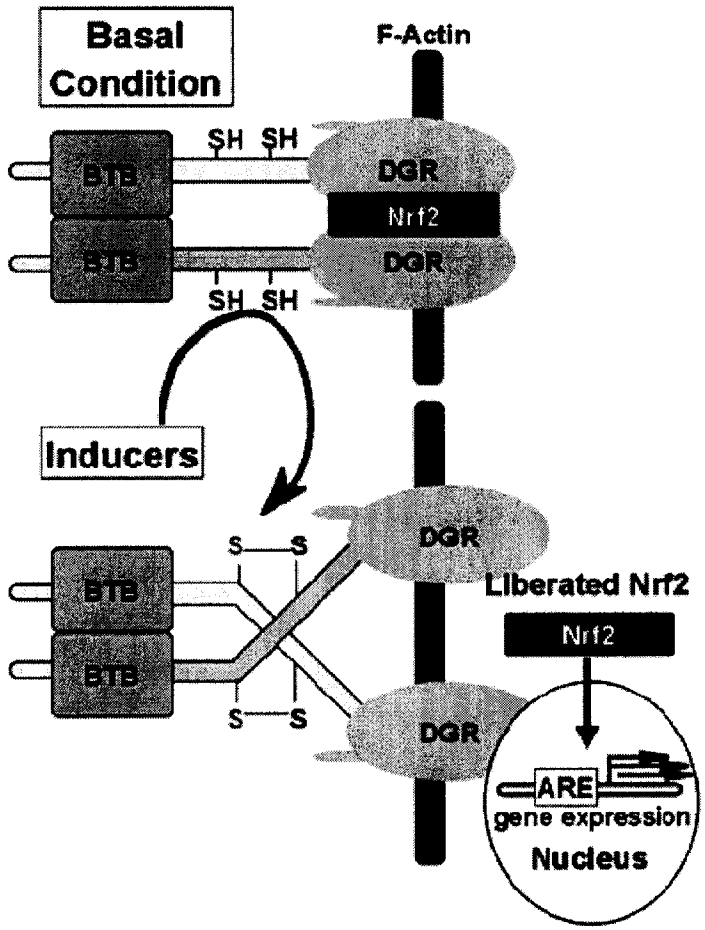
Although the ability of small MAF proteins to dimerize with CNC factors and bind to DNA-recognition elements has been demonstrated by numerous researchers in various experimental systems *in vitro*, little information is available on the dimerization partners of the small MAF proteins *in situ*. Nevertheless, small MAFs play a role in the cellular response to hydrogen peroxide, heavy metals and electrophiles [129, 130]. For instance, increased expression of the hamster MAFG homologue (*Adapt66*) has been reported in HA-1 hamster fibroblasts following exposure to H_2O_2 [131]. Furthermore, increase in all small MAF expression in HepG2 cells exposed to pyrrolidinedithiocarbamate (PDTC) and phenylethyl isothiocyanate (PEITC), two commonly used electrophilic agents, have been reported [130]. More recently, small MAF proteins have been shown to be involved in the cellular

Fig.10 NRF2 Regulation A) Cycled model of NRF2 regulation. KEAP1 associates with CUL3 and Rbx1 to form a functional E3 ubiquitin ligase complex that targets multiple lysine residues located in the N-terminal Neh2 domain of NRF2 for ubiquitin conjugation. **B) Static model of NRF2 regulation.** NRF2 is retained in the cytoplasm by interaction with two molecules of KEAP1, which are dimerized through their BTB domains and anchored to the actin cytoskeleton via the DGR region. Inducers of the phase II response interact with cysteine thiol groups in the intervening region (IVR) of KEAP1, causing the formation of disulfide bonds. This results in conformational change that renders KEAP1 unable to bind to NRF2, which then translocates to the nucleus [122].

A)


NRF2
Kelch
KEAP1
UB
Rbx1
E2
CUL 3

B)


Basal Condition
F-Actin
SH SH
BTB
BTB
DGR
Nrf2
DGR
SH SH
Inducers
S—S
BTB
BTB
DGR
Liberated Nrf2
Nrf2
S—S
DGR
ARE
gene expression
Nucleus

stress response, based on the observations that heavy metals such as cadmium, zinc and arsenite induce expression of small MAFs in human HeLa cells [129].

1.3 Oxidative Stress and Gestational Disorders

Oxidative stress has been implicated in the etiology of multiple diseases that range from cardiovascular diseases such as atherosclerosis [132], to nervous system disorders such Parkinson's and Alzheimer's [133], to inflammatory-immune injury such as rheumatoid arthritis [134] and several kinds of gestational disorders [135]. As more research is done focusing on the reactive oxygen species in human diseases, there is new light being shed on the role of these radicals in the physiological functions of the female reproductive tract. There is an increased interest to examine the role of oxidative stress in female reproduction, because it may be a major link in the infertility puzzle as well as in some reproductive organ diseases. Oxygen radicals and reactive oxygen species are a double edged sword, because they play a dual role in the female reproductive tract. On one hand, they can affect the physiologic processes involved in implantation to oocyte maturation to fertilization to blastocyst development [136-138]. On the other hand, they are capable of causing abortions [139], preeclampsia [140], free radical-induced birth defects [141], and preterm labour [142].

Preeclampsia is associated with serious health complications for the mother and severe fetal morbidity and fetal mortality. Overall preeclampsia complicates 5% of all pregnancies and 11% of all first pregnancies. Preterm birth is defined as birth that occurs before 37 weeks of gestation. Preterm labour occurs in 5 to 10 percent of all pregnancies and accounts for 75 percent of neonatal mortality and morbidity, including long-term handicap. The cost of caring

for preterm babies in the United States has been estimated at around $8 billion annually [143], which offers yet another reason why it is vital that research in this area uncovers the molecular mechanisms involved in the birth process, and to use that information to develop better diagnostic indicators and improve methods of therapeutic management.

These gestational disorders can also be caused by external environmental agents that induce oxidative stress in the placenta. Perhaps one of the most notorious of these contaminants is arsenic. Arsenic has become a major public health concern worldwide because millions of people are at risk of drinking water contaminated with arsenic [144].

Arsenic has been shown to cross the placenta in both animals and humans. Experimental studies support a role for arsenic as a developmental toxicant leading to spontaneous abortions, birth defects and embryonic lethality [145-147]. Placental levels of arsenic can be significantly increased in humans living in regions with high environmental contamination [148]. These data support the notion that placental cells are exposed to extensive oxidative stress during pregnancy, implicating oxidative stress in the pathogenesis of preeclampsia and miscarriage [149].

As mentioned earlier, arsenic has been already established to induce oxidative stress [150, 151] and NRF2 is a key player in the cellular oxidative stress response [152]. One conventional view predicts that the interaction of KEAP1 with electrophilic or oxidant inducers through its thiol groups triggers the dissociation of NRF2 from the NRF2 /KEAP1/CUL3 complex in the cytoplasm, thus keeping NRF2 from ubiquitination by KEAP1/CUL3. Alternatively,

oxidative/electrophilic signals inhibit the ubiquitination activity of KEAP1/CUL3, but not the association of KEAP1 with NRF2, thereby allowing newly-synthesized NRF2 to bypass KEAP1/CUL3 and accumulate in the nucleus [153].

2. Reproductive Organs

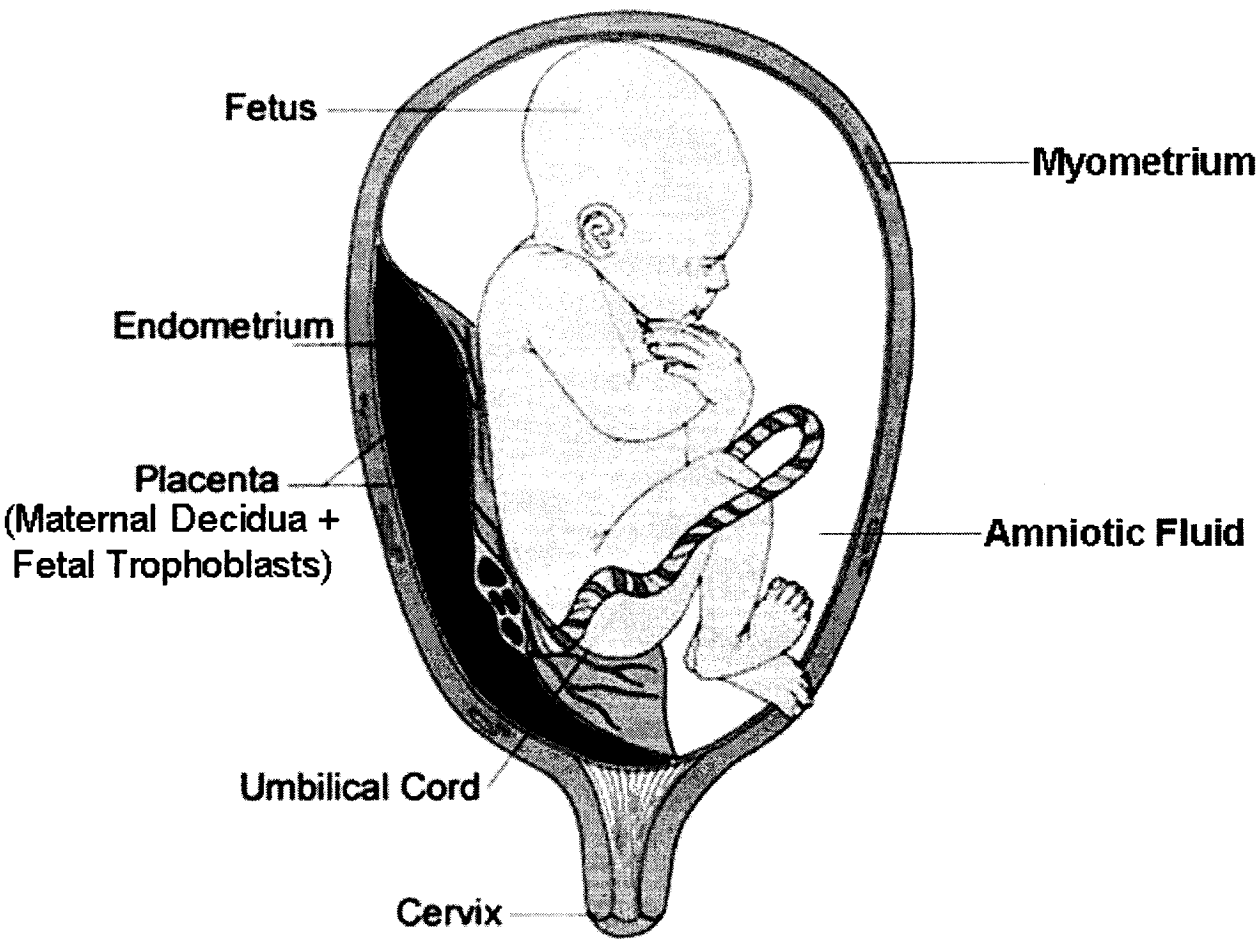
The two major organs affected by oxidative stress in the female reproductive system are the myometrium and the placenta (Fig. 11)

2.1 Myometrium

The myometrium is the outermost part of the uterus. This smooth muscle organ consists of bundles of myometrial cells embedded in a matrix of collagen. Human myometrial cells have a complex cytoskeletal structure that is necessary not only for generating force within each cell, but also for transmitting contractile forces through a linkage of groups of cells and coordinating information to the connective tissue collagen fibers[154].

Myometrial contractions occur when phosphorylated myosin interacts with actin to form actomyosin. The subsequent hydrolysis of the phosphorylated protein-protein complex provides the energy for contraction. At the time of labor, the myometrium becomes excitable, spontaneously active and develops high frequency, high-amplitude contractions. The transition from a quiescent to active state has been termed activationafter which the myometrium can undergo stimulation in response to endogenous and/or exogenous agonists [155].

Fig.11 Gestational Tissue. The myometrium is the outer most area of the uterus, comprised of smooth muscle that undergoe contractions during labor. The placenta is formed by two components the maternal wall of the uterus (decidua) and the (tropoblast) cells from the fetus.


Fetus
Myometrium
Endometrium
Placenta
(Maternal Decidua +
Fetal Trophoblasts)
Amniotic Fluid
Umbilical Cord
Cervix

Prior to myometrial contractions, the fetus is almost entirely dependent on the mother. At a functional level, the mother must integrate maternal and fetal physiology, immune system, and endocrine systems, a very delicate and vital task executed by the placenta.

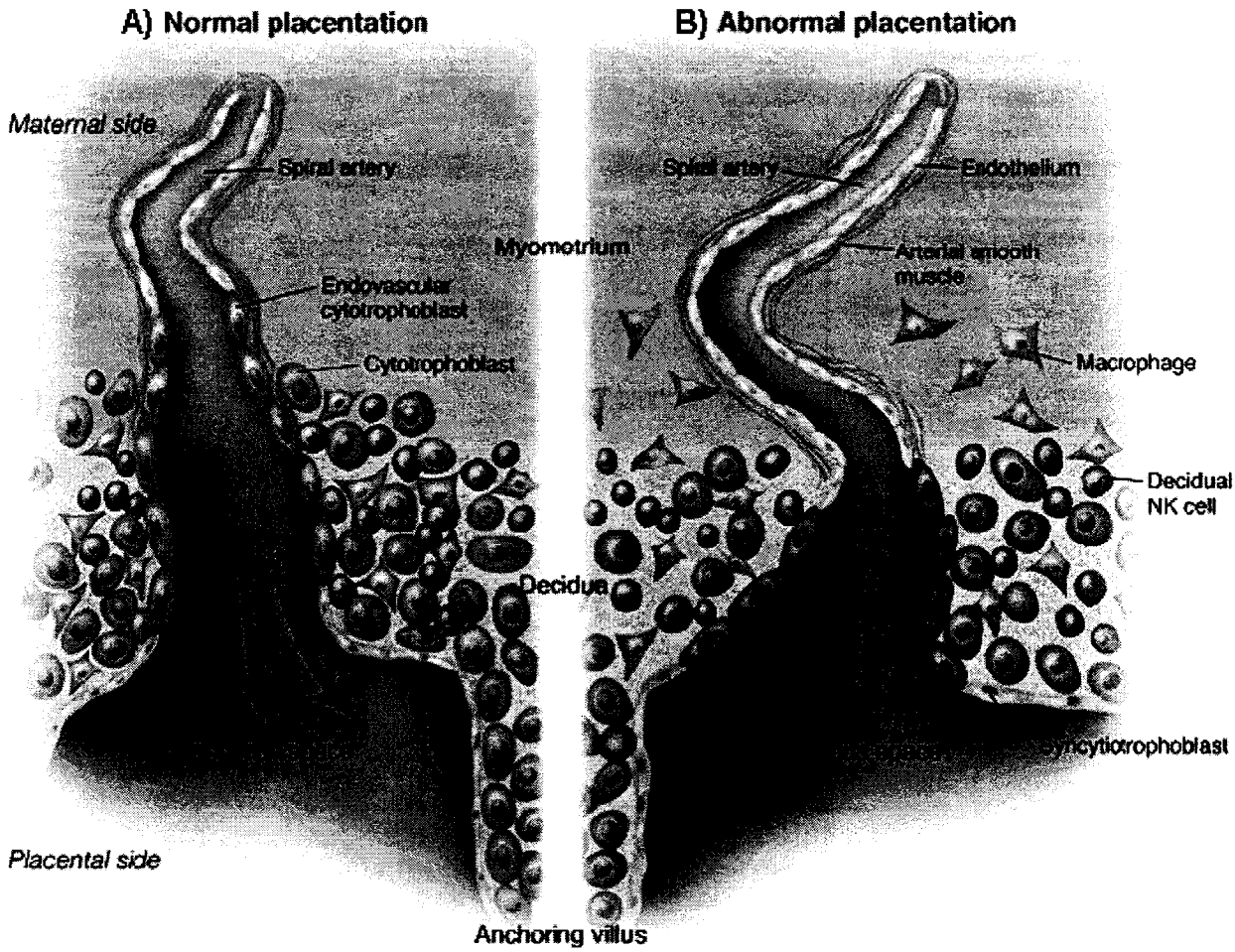
2.2 Placenta

The human placenta is a unique, transient organ, and possesses, through its capacity to proliferate and to invade maternal tissues, qualities that are usually found in malignant tumors. However, growth and invasion at the fetal-maternal interface is under tight control and malignant placental tumors are rare [156]. What is known is that profuse and steady maternal blood flow to the placenta is clearly required to support fetal growth during the second and third trimesters. What is observed, however, is that the transition in the maternal placental circulation at 10 to 12 weeks is a potentially dangerous one [157]. Without sufficient changes in the uterine vasculature in early pregnancy, the placenta is susceptible to the development of focal regions of hypoxia. Hence, careful orchestration is required to prevent overwhelming oxidative stress to the placenta, which may contribute to pregnancy failure.

Plugging/unplugging of the spiral arteries appears to be related to successful invasion of the extravillous trophoblast cells. This process involves the penetration of the arterial walls and replacing part of the maternal endothelium such that the smooth muscle is lost and the artery dilates. In normal pregnancies trophoblast invasion is complete, unplugging of the vessels is orderly, and the arteries are fully converted (Fig. 12A). If trophoblast invasion is less complete, the vessels may retain some of their vasoreactivity and the unplugging of the vessels may be

Fig.12 Trophoblast Invasion of Maternal Decidua (Placentation)

A) Normal placentation at 15 to 16 weeks of pregnancy. During normal placentation, cytotrophoblasts cross these placental-maternal bridges and invade the maternal decidua and adjacent spiral arteries. They penetrate the walls of the arteries and replace part of the maternal endothelium, stimulating remodeling of the arterial wall such that the smooth muscle is lost and the artery dilates. During normal pregnancy, these immune cells facilitate deep invasion of cytotrophoblasts into the myometrial segments and promote extensive spiral artery remodelling. **B) Poor placentation at 15 to 16 weeks of pregnancy.** In the preclinical stage of preeclampsia, invasion is restricted with impaired arterial remodelling [158].


A) Normal placentation
B) Abnormal placentation
Maternal side
Spiral artery
Myometrium
Endovascular cytotrophoblast
Cytotrophoblast
Spiral artery
Endothelium
Arterial smooth muscle
Macrophage
Decidual NK cell
Decidua
Syncytiotrophoblast
Placental side
Anchoring villus

premature and disorganised (Fig. 12B), leading to a greater degree of intermittent perfusion of the intervillous space and placental oxidative stress, thereby predisposing the mother to preeclampsia or pregnancy failure [159]. In turn, reduced oxygen tension in the placental tissue may lead to the production and secretion of cytotoxic factors that have been postulated to affect the maternal vasculature [160]. Indeed, it has been reported that the normal human placenta can produce more inflammatory cytokines, when incubated under low oxygen tension [161]. Proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1b (IL1B) can produce endothelial dysfunction [162], and synthesis of these cytokines as well as IL6 has been documented in the human placenta [163, 164]. Proinflammatory cytokines are notorious for affecting the endothelium in a fashion similar to events reported in preeclamptic women [162], and TNF levels are higher in the circulation of women with preeclampsia [165].

There are increasing amounts of evidence suggesting an important role for the proinflammatory cytokines IL1B, IL6 and TNF in the events that lead to preterm labor, causing morbidity and mortality of the fetus and serious health complications for the mother [166, 167]. Furthermore, it has been reported that expression of the oxytocin receptor is regulated by IL1B, IL6 and TNF in uterine smooth muscle cells [167-169]. Hence, there appears to be a link between the presence of proinflammatory cytokines in the uterus or amnion and the induction of labor.

3. Cytokines

Cytokines are small, nonstructural proteins with molecular weights ranging from 8 to 40,000 daltons. Originally called lymphokines and monokines to indicate their cellular sources, it

became clear that the term "cytokine" is the best description, because nearly all nucleated cells are capable of synthesizing these proteins and, in turn, of responding to them. The concept that some cytokines function primarily to induce inflammation, while others suppress inflammation is fundamental to cytokine biology and also to clinical medicine. The concept is based on the genes coding for the synthesis of small mediator molecules that are up-regulated during inflammation. An example of genes that are proinflammatory are phospholipase A2, prostaglandin-endoperoxide synthase 2 (PTGS2, previously known as COX2), and inducible NO synthase. These genes encode for enzymes that increase the synthesis of platelet-activating factor and leukotrienes, prostanoids, and NO.

While these genes may be essential to warding off the harmful effects of infection, they can also result in injury to the host from an over-exuberant inflammatory response that may exceed the damage caused by the inflicting agent itself. Examples of this phenomenon of self-injury include septic shock, inflammatory bowel disease, and certain autoimmune diseases such as rheumatoid arthritis [170, 171]. Proinflammatory cytokines include cytokines such as TNF, IL1B and IL6.

3.1 IL6

IL6 is secreted by T cells and macrophages to stimulate immune response to trauma, especially burns or other tissue damage leading to inflammation. It is one of the most important mediators of fever and of the acute phase response and is elevated in response to muscle contraction [172].

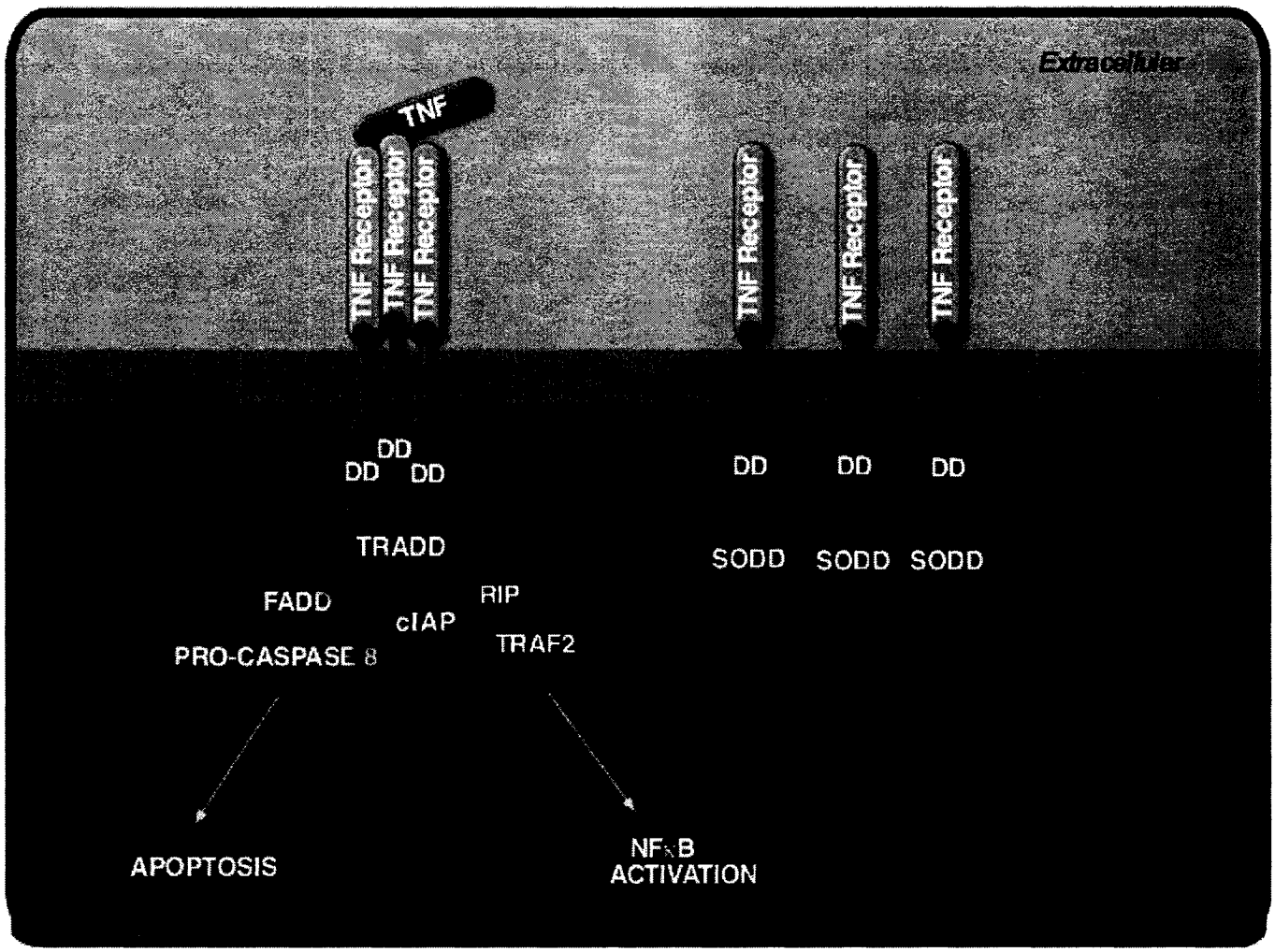
IL6 signals through a specific interleukin-6 receptor (IL6R). In view of the fact that the IL6R lacks a tyrosine kinase domain, it requires an accessory signal transducer named gp130 [173]. There are two possible mechanisms of interaction of IL6 with its receptor. Cells with the IL6R may bind IL6 directly inducing homodimerization of gp130 leading to signal transduction. Alternatively, the outer domain of the IL6R may be shed after proteolytic cleavage by protein kinase C to form soluble IL6R, which may also bind IL6. The formed complex can then cause homodimerization of gp130 and initiate the signal transduction cascade by the tyrosine-specific phosphorylation and subsequent activation of Janus kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs) [174, 175].

3.2 TNF

Another proinflammatory cytokine involved in labour, TNF, transduces growth regulatory signals into the cell through the TNF receptor (TNFR). TNF stimulates proliferation in normal cells; however, it initiates programmed cell death (PCD) or apoptosis in transformed cells causing DNA fragmentation and cytolysis [176, 177].

Upon ligation of TNF, the TNFR trimerizes and induces the association of the death domains (DD)s. Through functional studies the DD was identified as a conserved protein-protein interaction motif that is necessary for transmission of the apoptotic signal [178]. This

Fig.13 TNF Signalling. Once bound, TNF trimerizes the TNFR and induces the association of their DD. This aggregation recruits the adapter protein TRADD which in turn promotes the recruitment of the DD-containing cytoplasmic proteins FADD, TRAF2 and RIP to form an active TNF RI signaling complex.


Extracellular
TNF
TNF Receptor
TNF Receptor
TNF Receptor
TNF Receptor
TNF Receptor
TNF Receptor
DD
DD
DD
DD
DD
DD
TRADD
SODD
SODD
SODD
FADD
RIP
cIAP
TRAF2
PRO-CASPASE 8
APOPTOSIS
NF-κB
ACTIVATION

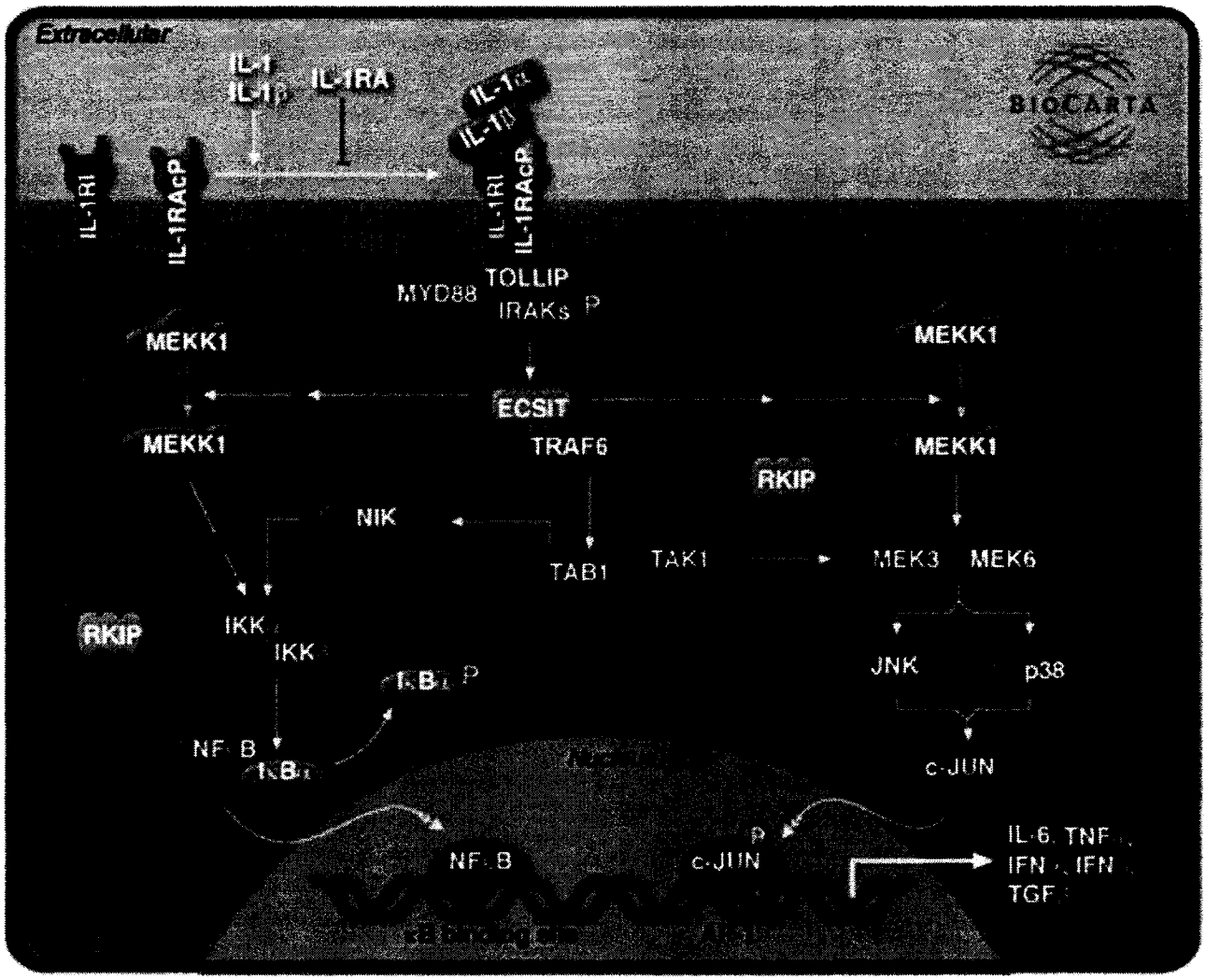
aggregation of the DDs of TNFR recruits the adapter protein TNFR associated death domain (TRADD) [179], which in turn promotes the recruitment of DD-containing cytoplasmic proteins FAS-associated death domain (FADD), TNFR associated factor 2 (TRAF2) and receptor interactive protein (RIP) to form an active TNFR signaling complex (Fig. 13) [180].

The TNF-induced survival pathway is mediated by the transcription factor NFκB. Activation of NFκB occurs via phosphorylation of IKB at Ser^{32} and Ser^{36}, resulting in the dissociation and subsequent nuclear localization of active NFκB. Recent studies have demonstrated that cells in which the NFκB signaling pathway is blocked are more likely to undergo apoptosis in response to TNF [181]. Therefore, the availability of NFκB may play a critical role in the ability of TNF to act as an apoptosis-inducer and anti-tumor agent.

3.3 IL1B

A third inflammatory cytokine involved in labour is IL1B. IL1B signals primarily through the type 1 IL1 receptor (IL1R1). IL1R1 binds IL1B, but requires the IL1B receptor accessory protein (IL1RAcP) to transduce a signal (Fig. 14) [182]. IL1B binding causes activation of two kinases, IRAK-1 and IRAK-2, associated with the IL1R1 complex. IRAK-1 (IL1 Receptor Associated Kinase) activates and recruits TRAF6 to the IL1R1 complex. TRAF6 activates two pathways, one leading to NF-kB activation and another leading to c-JUN activation. The TRAF associated protein ECSIT leads to c-JUN activation through the Map kinase/JNK signaling system. TRAF6 also signals through the TAB1/TAK1 kinases to trigger the degradation of IκB, and activation of NFκB.

Fig.14 IL1B signal transduction through IL1R1. IL1R1 binds IL1B utilizing IL1RAcP. IL1B binding causes activation of two kinases, IRAK-1 and IRAK-2, associated with the IL1R1 complex. IRAK-1 activates and recruits TRAF6 to the IL1R1 complex. TRAF6 activates two pathways, one leading to NF-kB activation and another leading to cJUN activation. The TRAF associated protein ECSIT leads to c-Jun activation through the Map kinase/JNK signaling system. TRAF6 also signals through the TAB1/TAK1 kinases to trigger the degradation of IκB, and activation of NFκB.


Extracellular
IL-1
IL-1RA
BIOCARTA
IL-1RI
IL-1RAcP
MYD88
TOLLIP
IRAKs
P
MEKK1
ECSIT
TRAF6
RKIP
NIK
TAB1
TAK1
MEK3
MEK6
IKK
JNK
p38
c-JUN
NF-κB
IκB
IL-6, TNF
IFN
TGF

From the above descriptions of IL1B signal transduction, it can be seen that many of these pathways are shared with TNF. Although the receptors for TNF and IL1B are clearly different, the postreceptor events are amazingly similar. Thus, the finding that IL1B and TNF activate the same portfolio of genes is not surprising. However, given the same cell and given the same array of activated genes, IL1B does not result in programmed cell death, whereas TNF does. Furthermore unlike IL1B, the receptors for TNF are homodimers and trimers, and, hence, the recruitment of kinases is somewhat different.

The involvement of proinflammatory cytokines in the placenta and gestational membranes has been extensively investigated in the context of both normal and abnormal pregnancy and delivery. Patterns of expression of proinflammatory cytokines in the fetal membranes and decidua suggest that inflammatory activation occurs modestly with term labour, but much more robustly in preterm delivery, particularly in the presence of intrauterine infection [183].

The mechanism by which infection and/or inflammation may lead to labor are postulated to involve infection-induced release of proinflammatory cytokines, which in turn may stimulate prostaglandin synthesis by gestational tissues, thereby causing uterine contractions [184].

Therefore, anti-cytokine therapy which has already emerged as an effective treatment in several human diseases such as arthritis, cancer and asthma, may prove a useful tool for the prevention of gestational disorders caused by infectious and inflammatory responses.

Specific Research Aims

The objective of this research was to investigate the involvement of the CNC family members, NRF2 and NRF3, and the small MAFs in arsenic or proinflammatory cytokines mediated stress in gestational organs, the placenta and myometrium. We explored if NRF2 may be involved in the antioxidant response to arsenic mediated stress in placental cells. Subsequent experiments investigated the DNA binding of endogenous NRF2/Small MAF heterodimers and one of the downstream target genes of NRF2.

In light of data also suggesting an involvement of NRF3 and MAFF in placental function we examined if they might be expressed in a specific subset of differentiated cells in placenta villi and also if their transcript levels are regulated during different periods of gestation. These findings prompted further experiments aimed at characterising the activation domain of these transcription factors.

Due to the increasing literature on proinflammatory cytokine participation in gestational disorders we also examined the regulation of NRF3 and MAFF in placental and myometrial cells.

Chapter II

MATERIALS AND METHODS

1. Cell Culture and Reagents.

1.1 Primary Cells and Cell Lines

Primary myometrial cells were cultured in RPMI 1640 with 10% FBS. Human embryonic kidney 293T (HEK293T) cells were cultured in minimal essential medium alpha (MEM-α) and 10% bovine growth serum. The human choriocarcinoma JAR cell line (American Type Culture Collection, Manassas, VA, U.S.A.) was maintained in RPMI-1640 medium plus 1 mM sodium pyruvate, 10 mM HEPES and 10% fetal bovine serum. The human choriocarcinoma cell lines BeWo was maintained in F12K medium 10% heat-inactivated fetal bovine serum. The PHM1-31 myometrial cells were maintained in high-glucose DMEM containing 0.1 mg/ml Geneticin, 10% fetal bovine serum, 2 mM L-glutamine. All media contained L-glutamine and were supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin.

1.2 Preparation of primary myometrial cells

Primary myometrial cells were derived from 10-30g of myometrium dissected from a patient after a caesarian delivery. Prior institutional approval for the protocol was obtained. Procedure was adapted from bovine protocol cited in [185]. The tissue was placed in a 50 ml falcon tube, rinsed with 1xHBSS (136.9 mM NaCl, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5.5 mM Dextrose, 5.0 mM HEPES, pH adjusted to 7.3) plus 10,000 units penicillin/streptomycin and cut into smaller pieces. The sample was then rinsed with 1xHBSS/PenStrep four times and subsequently a filter sterilized enzyme mix (0.17 mg/ml Trypsin, 0.425 mg/ml Collagenase II, 0.105 mg/ml Dnase II) was added to the sample in a 50 ml falcon tube. The tissue and enzyme

mix was then incubated for 1hr at 37°C in 95% air with 5% CO_2. 10 ml of FBS were then added to terminate the enzymatic reaction and the tissue was filtered through sterile gauze. The eluate was centrifuged at 1500g for 12 minutes. The pellet was washed 3 times with 1xHBSS/PenStrep and plated into 6 well plates.

1.3 Derivation of PHM1-31 cells

PHM1-31 myometrial cells were derived from the immortalization mixture used to derive PHM1-41 cells [186]. Briefly, myometrium was removed from the upper edge of the uterine segment at the time of caesarian section in a term pregnant patient who was not in labor, using a protocol that had received prior institutional approval (University of Texas Health Sciences Centre in Houston). Myometrial cells were isolated by enzymatic digestion, infected at passage 2 with replication-defective adenovirus vector pLXSN16E6E7 (J.K. McDougal, University of Washington, Seattle, WA) expressing the E6/E7 proteins of human papilloma virus 16 and the neomycin resistance gene and selected with the neomycin analog Geneticin (Invitrogen) [186]. PHM1-31 cells were recovered by subcloning foci and reach confluency 3-4 days after being cultured at $2x10^5$ cells/100mm dish and have been used in this study up to passage 25. The cells maintain smooth muscle α-Actin expression at passage 27 and retain the ability to respond to oxytocin and thapsigargin with an increase in intracellular calcium.

1.4 Isolation of Chorionic Villi

Informed consent was obtained from all patients. Placentas from elective terminations of normal pregnancies (12 to 22 weeks) or from normal term deliveries (34 to 40 weeks) were collected immediately after delivery, washed thoroughly in phosphate-buffered saline (PBS)

with antibiotics, and placed on ice. Chorionic villi were dissected free from the rest of the placenta and lysed immediately in TRIzol (Invitrogen).

1.5 Isolation of Cytotrophoblasts and Placental fibroblasts

Cytotrophoblast progenitors were isolated from pooled first- or second-trimester human placentas by published methods [187, 188]. Briefly, placentas were subjected to a series of enzymatic digests, which detached cytotrophoblast progenitors from the stromal cores of the chorionic villi. Once detached, the cells were purified over a Percoll gradient (Collaborative Biomedical Products, Bedford, MA). Cytotrophoblast cell islands, visualized by using a dissecting microscope, were dissected from the surface of early gestation placentas [189]. Fibroblasts were isolated from first trimester placentas and passaged as previously described [188]. The lines were used after the third passage to ensure that contaminating cells were no longer present.

1.6 Induction studies

PHM1-31 cells reaching 80-90% confluency were FBS starved for 16 to 24 hours. They were then treated with TNF (100 ng/ml, Medicorp), IL1B (0.005 to 100 ng/ml, Research Diagnostics) or IL6 (100 ng/ml, RDI) or combinations of these cytokines. Induction time was as indicated in the figures. For the inhibition experiments the cells were starved for 16 to 24 hours and then actinomycin D (5 μg/ml) dissolved in DMSO (0.5%) or DMSO alone (0.5%) was added to the cells 1 hour prior to beginning the 3 hours treatment with IL1B (1 ng/ml). The analysis of primary myometrial cells was carried out in the presence of FBS. JAR cells at 70-

90% confluency were incubated with various concentrations of arsenic trioxide (As_2O_3) (Sigma) or TNF for the times indicated.

2. Plasmid constructs

2.1 Expression Vectors

All cDNA fragments were recovered by RT-PCR using their respective oligonucleotides. NRF1: hNRF1-F_{new} (5'-GGTCCTTCAGCAATGCTTTCTCTG-3'), hNRF1-R_{new} (5'-CCCTTCTTCCCCAGGCTCACTTT-3'), NRF2: hNrf2-F_{new} (5'-CCCAGCAGGACATGGATTTGAT-3') and hNrf2-R_{new} 5'-GGTCAAATCCTCCTAAATCTAG-3'). NRF3: Full-length NRF3 (694 amino acids): hNRF3-F_{new} (5'-GCGATGAAGCACCTGAAGCGGT-3') and NRF3-R_{new} (5'-CTCACTTTCTCTTTCCCTTTTGGG-3'). NRF3 (607 amino acids): NRF3-F_{607} (5'-GCCATGGAGGGCCAGCTGCTCCGGGAG-3') and NRF3-R_{new} (5'-CTCACTTTCTCTTTCCCTTTTGGG-3'). NRF3 (525 amino acids): NRF3-MT2F1 (5-CGCGAATTCGCCATGGAGAAGGCACCCGCGGAACCG-3') and NRF3-GSTR1 (5'-CACGAATTCTCACTTTCTCTTTCCCTTTTG-3'). NFE2: The pMT2 NF-E2 expression plasmid has been described previously [190]. MAFF: MAFF-F2 (5'-GGGCACCTTCTGCAAACATGT-3') and MAFF-R2 5'-GAGGCGGCGCTCAGGCACTTT-3'). MAFK: hMAFK-F1 (5'-CGTGCCCGGGTTATGACGACT-3') and hMAFK-R1 (5'-GGCCGGCACTAGGATGCAGC-3'). MAFG: The full length MAFG pMT2 expression plasmid has been described previously [190]. MAFG (407 bp) cDNA fragment with the primers MAFG611F (5'-CCGATCGTAGGGACGCGCGT-3') and MAFG1018R (5'-

CCACTCGGGAGTGGAGGGAA-3'). PTGS2: COX-2 F1 (5'-CTGCCCGCCGCTGCGATGCT-3') and COX-2 R1 (5'- CTACAGTTCAGTCGAACGTTC-3'). All resulting amplicons were cloned into pCR-BluntII-TOPO vector (Invitrogen). Subsequently, all cDNA inserts with the exception of NRF2 and the NRF3, were digested with EcoRI and subcloned into the pMT2 expression vector. As the NRF2 and NRF3 cDNA fragments comprise internal EcoRI sites, the inserts were recovered from the pCR-BluntII-TOPO plasmid by a partial EcoRI digest before being subcloned into the EcoRI site of the pMT2 expression vector.

2.2 NRF3 promoter Constructs.

To map the NRF3 transactivation domain, we used PCR generated fragments using oligonucleotides with BamHI restriction sites, to clone full-length (694 amino acids) and shorter versions of human NRF3 into the BamHI site of plasmid pSG424 in frame with the GAL4 DNA-binding domain [191]. For the pSG-NRF3$_{1-297}$ construct we used the EcoRI site present in the NRF3 cDNA to clone the fragment into the EcoRI site of pSG424. The (Gal4)$_5$-TK/luciferase reporter construct (a kind gift from Dr. Rongtuan Lin) was generated by inserting a 140 bp HindIII/XbaI fragment from the (Gal4)$_5$-TK/CAT vector [192] treated with Klenow enzyme cloned into the SmaI site of the TK/pGL3 vector. As a transfection control we used the pRL-TK vector (Promega). Transient transfections of HEK293T cells for reporter assays were performed in 24 well plates using the calcium-phosphate co-precipitation procedure [193]. 10^4 HEK293T cells were plated per well and transfected with 100 ng of (Gal4)$_5$-TK/luciferase reporter, 5 ng of pRL-TK and 500ng of either of the various pSG-NRF3 constructs.

3. Transfections

Transient transfections of HEK293T cells were performed using the calcium-phosphate co-precipitation procedure [193]. HEK293T cells at 30-50% confluency (100mm dish) with 10μg of the expression plasmids. In the case of the full-length NRF3 (694 amino acids) expression vector we used 30μg of the plasmid. Where mentioned transfection using FuGene 6 reagent (Roche) was performed according to manufacturer's specifications.

4. Immunoblot analysis

4.1 Protein Extraction.

To prepare whole cell extracts (WCE): the cells were scraped using 1xPBS, centrifuged and the pellets were then resuspended in NB buffer (250 mM sucrose, 420 mM NaCl, 10 mM Tris/HCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Triton X-100) and protease inhibitors (0.5 mM dithiothreitol, 0.2 mM PMSF, 1X protease inhibitor cocktail ("Complete", Roche). After incubation on ice for 10 minutes, samples were briefly centrifuged and the supernatant was collected. Cytoplasmic extracts were generated using the same procedure as for WCE except that NaCl in buffer was omitted. Nuclear extracts were prepared by resuspending the pellet from the cytoplasmic extract preparation in NB buffer with 420 mM NaCl, centrifuging and collecting the supernatant. The protein concentrations were determined using a protein assay kit (BioRad, Hercules, CA).

4.2 Western Analysis

20-30μg of the lysates were electrophoresed in 4-12% NuPage Novex Bis-Tris (Invitrogen) or 10-12% SDS-polyacrylamide gels. Resolved proteins were transferred electrophoretically to

PVDF membranes (Millipore). After transfer, the membrane was blocked for 1 hour at room temperature (RT) or overnight at 4°C in 1X Tris-buffered saline (TBS, 25 mM Tris-base pH 7.5, 150 mM NaCl) plus 5% milk and was subsequently incubated overnight with polyclonal sera in 1X TBS plus 5% milk, 0.05% Tween-20 and 350 mM NaCl. Following washing of the primary antisera, the membrane was incubated for 1 hour at RT with secondary goat anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase (Pierce). The proteins were detected using the Super-Signal West Pico chemiluminescent reagent (Pierce) following the manufacturer's instructions. For competition experiments, the peptide antigen was added to the antiserum mixture at a concentration of 50mM before incubation of the blot.

5. Antisera

For immunoblot and/or EMSA analyses we used antisera specific for various human proteins. The NRF1, NRF2, MafK (Santa Cruz), HO1 (Stressgen), Acitn Beta (ACTB) (SIGMA) antisera, IκB (H-4) sc-1643 (Santa Cruz) were obtained commercially. Generation of MafG antiserum has been described previously [194]. The following dilutions where used for the antisera: ACTB (1:10,000) (Sigma #A-5441), human MAFF (1:200), human MAFG (1:200) [194] human MAFK (1μg/ml) (Santa Cruz), NRF2 (1:1000), GAPDH (0.8:1000), NRF2 (1:1000).

5.1 Generation of MAFF antiserum

To construct the GST-MAFF fusion protein vector we generated a human *MAFF* cDNA using the following primer pairs Bam-MAFF (5'-GTCGGATCCATGTCTGTGGATCCCCTATCC-3') and MAFF-Eco (5'-GCAGAATTCTAGGAGCAGGAGGCCGGGCC-3'), and digested the

amplicon with EcoRI and BamHI. Subsequently, the product was cloned into the EcoRI and BamHI sites of the pGEX-2TK fusion vector (Amersham). Rabbits at Pocono Rabbit Farm & Laboratory, Inc. were immunized with the purified GST fusion protein comprising the entire 164 amino acids coding region of human *MAFF*. Pre-immune serum was used to confirm the specificity of the generated antiserum to MAFF.

5.2 Generation of NRF3 antisera

To facilitate biochemical studies of NRF3 complexes, we raised a high-titer polyclonal antiserum against a GST-NRF3 fusion protein. To this end, we generated a partial human NRF3 cDNA by PCR using the following oligonucleotides: FMG-GSTF3 (5'-CTCGGATCCAGCCAGGCTATAAGTCAGGAT-3') and FMG-GSTR2 (5'-CGCGAATTCAGTTTCTATCTGTGTCTTCAAG-3'). The amplicon was digested with EcoRI and BamHI and cloned into the corresponding sites of the pGEX-2TK vector (Amersham). Female New Zealand White rabbits were immunized with the GST fusion protein containing amino acids 306-534 of human NRF3. We chose the center portion of NRF3 as antigen to avoid cross-reaction of the antiserum with other CNC family members. Antiserum was tested in parallel with preimmune serum to confirm the specificity to human NRF3. In addition, we raised an antiserum specific for a peptide (KLHDLYHDIFSRLLRDDQGRPVNPN) in the carboxy terminus of human NRF3. The peptide was coupled to KLH and used to immunize female New Zealand White rabbits (Pocono Farms). The serum was purified using peptide coupled to Affi-Gel 10 according to the instructions of the manufacturer (BioRad).

6. EMSA experiments

6.1 Oligonucleotide probes

Complimentary oligonucleotides for EMSA probes were obtained from (Sheldon, Montreal, QC). 10 µg of each complimentary oligonucleotide were mixed together in an Oligo buffer (5M NaCl, 0.5M EDTA, 1M Tris pH 8.0). The mixture was then boiled for 10 min and then cooled down slowly to RT. Probes were end-labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase. The stress response element (StRE) DNA binding site oligonucleotide derived from the human HO-1 promoter [126]. A 1200 molar excess of unlabeled oligonucleotide was used for competition. The NF-E2 binding site oligonucleotide derived from the human porphobilinogen deaminase promoter [52]. A 400 molar excess of unlabeled oligonucleotide was used for competition.

6.2 Binding reaction and Specific supershift

For electrophoretic mobility shift assay (EMSA) experiments using the StRE binding site, nuclear extracts were prepared using the NB buffer described above. The reaction was incubated at 4°C for 30 min in the binding reaction containing 18 mM HEPES-KOH (pH 7.9), 80 mM KCL, 2 mM $MgCL_2$, 10 mM DTT, 10% glycerol, poly(dI-dC) 160 µg/ml, 0.2 mg/ml bovine serum albumin and 7K-30K cpm [γ-^{32}P]-ATP labelled probe [126]. For these supershift experiments 3µl of either preimmune or immune serum was added to the reaction mixture as indicated. For EMSA experiments using the NFE2 binding site, nuclear extracts were prepared as described previously [195]. The reaction was incubated at RT for 20 min in the binding reaction containing 20 mM HEPES-KOH (pH 7.9), 60 mM KCL, 60 mM $MgCL_2$, 0.2 mM EDTA, 100 mM DTT, 10% glycerol, poly(dI-dC) 70 µg/ml and 7K-30K cpm [γ-^{32}P]-ATP

labelled probe [196]. For these supershift experiments 1 µl of either preimmune or immune serum was added to the reaction mixture as indicated. Reaction mixtures were analyzed by native 5% polyacrylamide gel electrophoresis and autoradiography.

7. FACS

7.1 Detection of intracellular H_2O_2 levels

Intracellular H_2O_2 is detected when membrane-permeable 2', 7'-dichlorofluorescin diacetate (DCFH-DA) undergoes non-specific cleavage by intracellular esterases, and the resulting DCFH is oxidized to the fluorescent compound, 2', 7'-dichlorofluorescein (DCF) in the presence of H_2O_2 as previously described [197]. JAR cells were seeded at $2x10^5$ cells/ml in six-well plates and were treated the following day with 5 µM As_2O_3 for 6 hours. Cells were then treated with 25 µg/ml DCFH-DA, and incubated at 37°C for 45 min. Positive control groups were treated with 1 mM H_2O_2, and incubated at 37°C for 15 min prior to time of collection (data not shown). The fluorescent intensity of DCF was then measured with a Coulter flow cytometer.

8. Northern Analysis

8.1 RNA Isolation

Total RNA was prepared by using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions.

8.2 Northern Blotting

Northern blotting was performed using standard procedures loading 10 µg of RNA per lane. After subjecting RNA to gel electrophoresis, the gel was transferred overnight using standard techniques. The membrane was then the briefly equilibrated in EQ buffer (50mM PO_4 pH 7.2) at 65°C and pre-hybridized for 1 hour in Church buffer (0.5M PO_4, 1 mM EDTA, 7% SDS) at 65 °C. The denatured labelled probe was than added to the membrane and left to hybridize overnight. Finally, the membrane was washed using WB buffer (50mM PO_4, pH 7.2, 1% SDS) at 65 °C [198] and analyzed using autoradiography.

8.3 cDNA Probes

The random priming labelling kit (Roche) was used to label all cDNA probes. To obtain a NRF3 -specific probe a 950 bp BglII fragment of human NRF3 cDNA was isolated. Human GAPDH cDNA was purchased (Clontech) or recovered by RT-PCR using the following primers hGAPDH-B (5'-CCACAGTTTCCCGGAG-3') and GAPDH-T (5'-GCTGAGTACGTCGTGG-3').

To obtain PTGS2, NRF2, MAFG, MAFF- and MAFK -specific probes for Northern analysis, we performed an EcoRI digestion of the pCRBluntII- COX-2, pCRBluntII- NRF2, pCRBluntII- MAFG404, pCRBluntII- MAFF and pCRBluntII- MAFK plasmids, respectively.

9. Two-hybrid screen

A Matchmaker (Clontech) two-hybrid screen was performed according to the instructions of the manufacturer. Human MAFG cDNA comprising the entire coding region was subcloned into the EcoRI and BamHI sites of the pGBT9 vector coding for the GAL4 DNA-binding domain. The pGBT9-MAFG vector was introduced into HF7c cells. Subsequently, a pGAD10

vector-based placental Matchmaker library (Clontech), containing cDNA sequences fused to the GAL4 activation domain, was transformed into the HF7c (pGBT9-MAFG) cells. The transformation mixture was then plated on Trp^-, Leu^-, His^- selection plates. Twenty-two of 80 colonies showed lacZ activity. The cDNA insert of 11 independent clones was recovered by polymerase chain reaction (PCR) and sequenced. The sequence of 7 clones corresponded to that of NRF2 and one to a cDNA of unknown function. The remaining 3 clones were identical, and contained nucleotides 735 to 2827 of the NRF3 gene fused to the GAL4 activation domain. One of the clones, 2Hyb-33, was further characterized. The 5'end of NRF3 mRNA (clone RT-Pla5pr) was recovered by standard RT-PCR, in the presence of 10% DMSO, using human placental RNA as template. To this end, we designed specific primers, located upstream of the putative start codon using sequence data from human BAC clone (#CTB-119C02) and in the second exon of the NRF3 gene: hNRF3-5'F1 (5'-GTGGCTCCTTCTTCGCTTCT-3') and hNRF3-5'R1 (5'-CAGTGGTCTTTTCTGCCTCC-3').

10. Dual-Luciferase Reporter Assay System

Luciferase activity was determined with a dual luciferase reporter gene kit (DLR) from Promega according to the manufacturer's manual. The cells lysates were analyzed with a Lumat LB9507 luminometer (EG&G Berthold, Wellesley, MA, USA) after automatic injection of the necessary substrate solutions. All samples were measured at least in duplicate. The results for firefly luciferase activity were normalized to Renilla luciferase activity.

11. Quantification and statistical analysis

Quantification of experiments was performed using a phosphoimager (Molecular Dynamics) and Image Quant software (version 5.2) or densitometry using NIH image software (version 1.63). All data are expressed as the mean ± SEM and were analyzed using as stated either one way ANOVA or student T-test or non linear regression analysis. Values of $P<0.05$ (*) or $P<0.01$ (**) were used as criteria for declaring significance. FACS analysis was performed using Cell Quest Pro software.

Chapter III- Results

A. Arsenic-mediated oxidative Stress in the Placenta

Oxidative stress caused by environmental toxicants and proinflammatory cytokines can lead to the pathogenesis of many degenerative and inflammatory diseases, including cancer [112], Alzheimer disease [133], arthritis [134] and gestational disorders [135]. ROS play a dual role in the female reproductive tract, in that they can be both physiologic as well as pathologic. There is growing literature on the pathological effects of oxidative stress in female reproduction and its involvement in causing abortions [139], preeclampsia [140], free radical-induced birth defects [141], and preterm labour [142].

Arsenic, a reproductive and developmental toxicant, is well known for inducing oxidative stress [150, 151]. It is able to cross the placenta and is believed to contribute to spontaneous abortions, birth defects and embryonic lethality [145-147]. However, the molecular mechanisms of arsenic toxicity remain poorly understood. Arsenic can stimulate production of reactive oxygen species (ROS) [199 1999] and alters cellular sulfhydryl levels [200 G.], whereas glutathione depletion enhances arsenic cytotoxicity [201]. Furthermore, it has been well established and documented that NRF2 is a key player in both the constitutive and the inducible expression of ARE-dependent genes in numerous *in vitro* and *in vivo* studies [91, 95, 115-118].

We hypothesized that NRF2 may be involved in antioxidant response to arsenic-mediated stress in placental cells. We selected JAR cells derived from trophoblastic tumors of the placenta, as a model for our studies.

A.1 Upregulation of NRF2 protein levels by different doses of arsenic

We first performed dose-response experiments in the placental JAR cell line. Previous studies had shown treatment of osteoblast cells with arsenic trioxide, an inorganic form of arsenic, induced a marked increase in NRF2 levels in total cell lysates within 4 h, peaking at 12 h and remaining elevated up to 24 h [202]. Hence, we treated JAR cells for 6 hours with different concentrations of arsenic trioxide. We prepared nuclear extracts of JAR cells and analyzed them in immunoblot assays using a NRF2 specific antiserum. We found that nuclear NRF2 protein levels are upregulated by doses of 0.5μM of arsenic trioxide (Fig. 15). Higher concentrations resulted in even stronger inductions of NRF2 protein. We noticed that at doses of 10μM and higher, cells looked unhealthy as evidenced by a rounded appearance resulting in increased numbers of cells that were lifting off the plate, especially at longer time points. We, thus, chose to use 5μM of arsenic trioxide in further experiments, as this dose resulted in a strong induction of NRF2 protein levels with no visible toxic effects, i.e. the cells maintained uniform shape and did not lift off the dish.

A.2 Time course of Arsenic induction

We next analyzed the induction of NRF2 following treatment of the JAR cells with 5 μM arsenic trioxide by preparing nuclear extract at different time points. Immunoblot analysis of nuclear extracts prepared from JAR cells showed upregulation of NRF2 at all the times

Fig.15 Induction of NRF2 by different doses of arsenic.

Immunoblot of nuclear extracts prepared from JAR choriocarcinoma cells exposed to different concentrations of arsenic trioxide (0.5-10μM) or not (control) for 6 hours using antisera specific for NRF2 and Actin, beta (ACTB). 30 μg of protein was loaded per well. Quantification of 3 experiments is shown. Statistically significant differences are indicated by two asterisks ($P<0.01$).

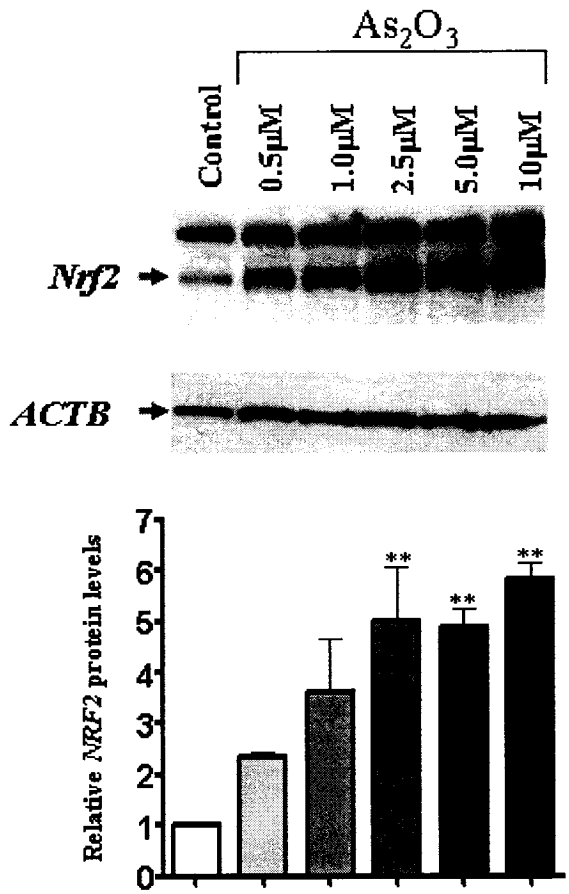
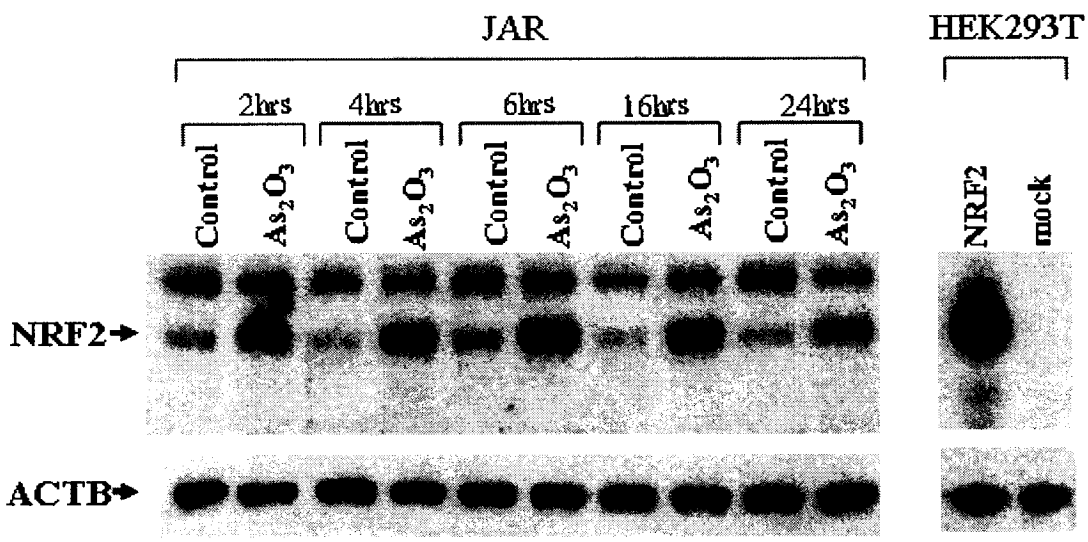

As2O3
Control
0.5μM
1.0μM
2.5μM
5.0μM
10μM
Nrf2
ACTB
Relative NRF2 protein levels
0
1
2
3
4
5
6
7
**
**
**

Fig.16 Time course of arsenic treatment of nuclear NRF2.

Immunoblot of nuclear extracts prepared from JAR choriocarcinoma cells treated or not (control) with 5μM arsenic trioxide for 2 to 24 hours using antisera specific for NRF2. 30 μg of protein was loaded per well. Control extracts from HEK293T cells transfected with a expression vector coding for NRF2 or not (mock) have been analyzed in parallel.


JAR
HEK293T
2hrs
4hrs
6hrs
16hrs
24hrs
Control
As_2O_3
NRF2
mock
NRF2
ACTB

analyzed, from 2 to 24 hours (Fig. 16). The highest induction levels were observed at the 6 hour time point.

A.3 Arsenic treatment results in oxidative stress

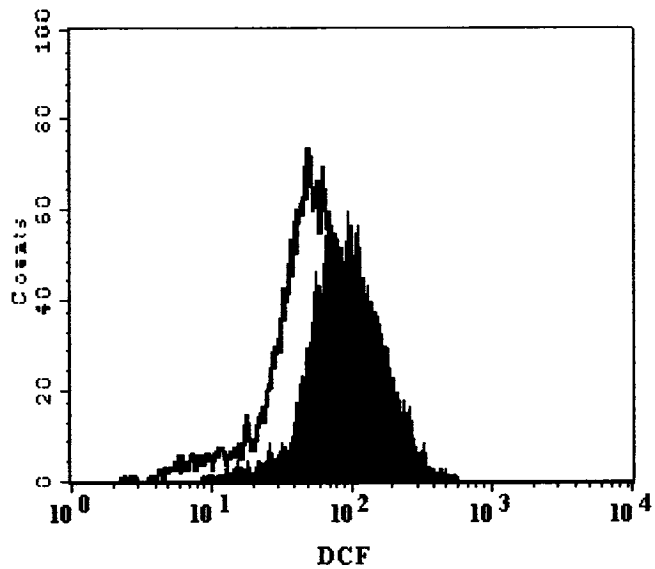
H_2O_2 has been shown to be one of the mediators of NRF2 upregulation due to oxidative stress [203]. To test whether it is involved in our system, JAR cells were treated with 5μM arsenic trioxide for 6 hours, and stained with dichlorofluorescin diacetate (DCFH-DA) for detection of intracellular H_2O_2. Fluorescein-positive cells were detected using FACS analysis. We showed that levels of H_2O_2 are significantly increased in JAR cells (Fig. 17) at 6 hours following exposure to arsenic trioxide.

A.4 Generation of MAFF antiserum

Given that CNC family members including NRF2 require small MAF partners in order to bind to the MARE/ARE binding sites [194], we examined whether the small MAFs where regulated by arsenic treatment. In order to test whether MAFF is induced at the protein level, we needed to generate an antiserum specific for human MAFF. To this end we cloned the full human MafF cDNA into the pGEX-2TK fusion vector (Amersham). The purified GST fusion protein comprising the entire 164 amino acid coding region of human *MAFF* was subsequently used to immunize Rabbits at Pocono Rabbit Farm & Laboratory, Inc. Using immunoblot analysis, the antiserum was shown to specifically recognize MAFF, an 18 kDa protein, in nuclear extracts of HEK293T cells transiently transfected with an expression vector coding for human MAFF (Fig. 18). HEK293T cells were used due to the fact that they express very low

Fig.17 Intracellular H_2O_2 levels in choriocarcinoma cells upon exposure to arsenic.

JAR choriocarcinoma cells were treated with 5μM arsenic trioxide for 6 hours, and stained with DCFH-DA for detection of intracellular H_2O_2. Fluorescein-positive cells were detected through flow cytometry. The histogram shows the plot for the untreated group (unshaded) overlaying that for the treated group (shaded). The histogram is representative of 5 experiments.


Counts
0
20
40
60
80
100
10^0
10^1
10^2
10^3
10^4
DCF

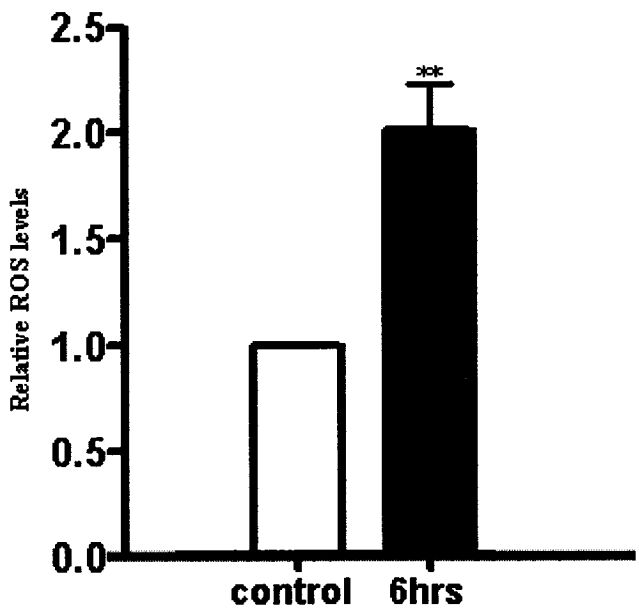
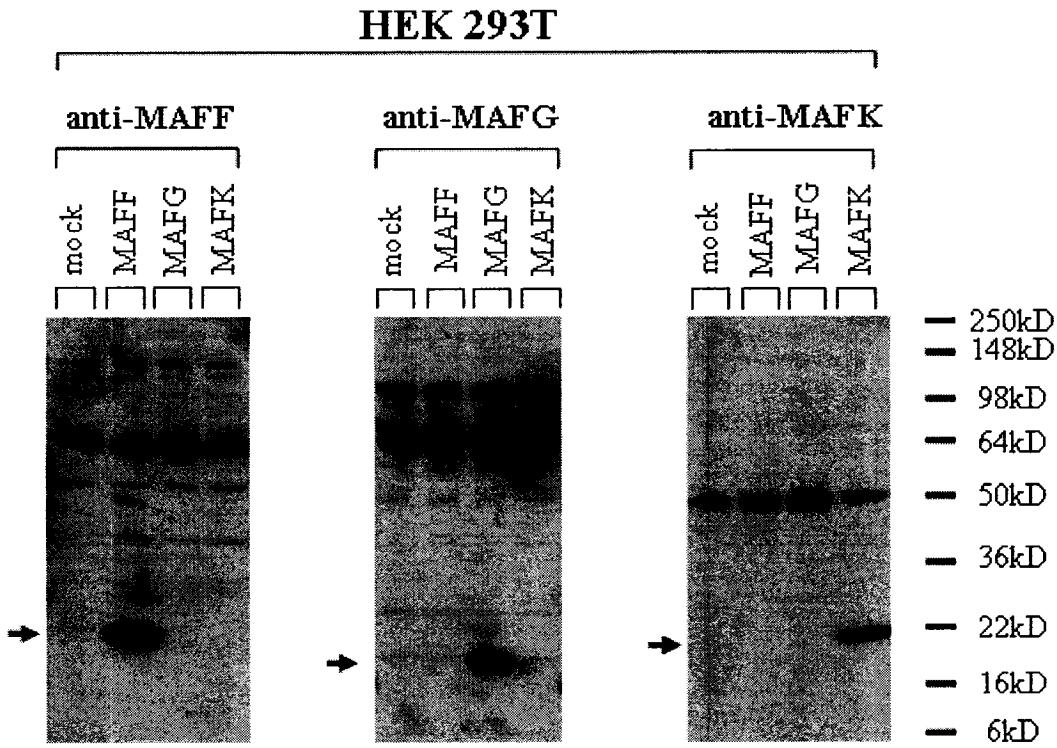

Relative ROS levels
2.5
2.0
1.5
1.0
0.5
0.0
**
control
6hrs

Fig.18: Specificity of MAFF antiserum

Immunoblots of whole cell extracts from HEK293T cells that are not transfected (mock) or transfected with constructs coding either for human MAFF, MAFG or MAFK. The blots were incubated with antisera specific for each of the small MAF. 20 µg of whole cell extract was loaded per well. Small MAFs are indicated by an arrow.


HEK 293T
anti-MAFF
anti-MAFG
anti-MAFK
mock
MAFF
MAFG
MAFK
250kD
148kD
98kD
64kD
50kD
36kD
22kD
16kD
6kD

levels of the small MAFs. Interestingly, even though the entire cDNA was used to generate the MAFF antiserum, it did not cross-react with the homologous small MAF transcription factors MAFG and MAFK in immunoblot assays (Fig. 18).

A.5 Protein Analysis of the Small MAFs

We monitored the expression of the dimerization partners of NRF2, the small MAF proteins MAFF, MAFG and MAFK using immunoblot analysis of nuclear extracts prepared from JAR cells treated with 5μM arsenic trioxide for 2 to 24 hours. We found no induction for the small MAF factors MAFG and MAFK, whereas expression of MAFF was slightly upregulated, in particular at 2, 4, 6 and 16 hours following arsenic treatment (Fig. 19A & 19B & 19C).

A.6 Increase of NRF2/small MAF DNA binding activity

We then proceeded to examine whether the induction of NRF2 and the small MAFs translated into increased DNA binding. We used EMSAs to analyze the DNA binding activities in JAR cells. In view of the fact that proteins in this assay remain in their native form we re-assessed the specificities of antisera used for this study. To this end, we transiently transfected HEK293, which express low levels of endogenous small MAFs, with expression constructs coding for NRF2 and either one of the small MAF members, MAFF, MAFG or MAFK. We subsequently collected the nuclear extracts and performed the EMSA. Cotransfection of NRF2 and either of the small MAF constructs resulted in a complex binding to a stress responsive element (StRE) recognition site, derived from the human HO-1 promoter [126] (Fig. 20). DNA binding was specific, as addition of an unlabeled oligonucleotide corresponding to the recognition site abolished the interaction. The NRF2 antiserum disrupted the NRF2/small MAF heterodimer,

Fig.19 Time course of arsenic treatment of nuclear small MAF levels.

Immunoblot of nuclear extracts prepared from JAR choriocarcinoma cells treated or not (control) with 5μM arsenic trioxide for 2 to 24 hours using antisera specific for **(A)** MAFF, **(B)** MAFG, **(C)** MAFK and *ACTB*. 30 μg of protein was loaded per well.

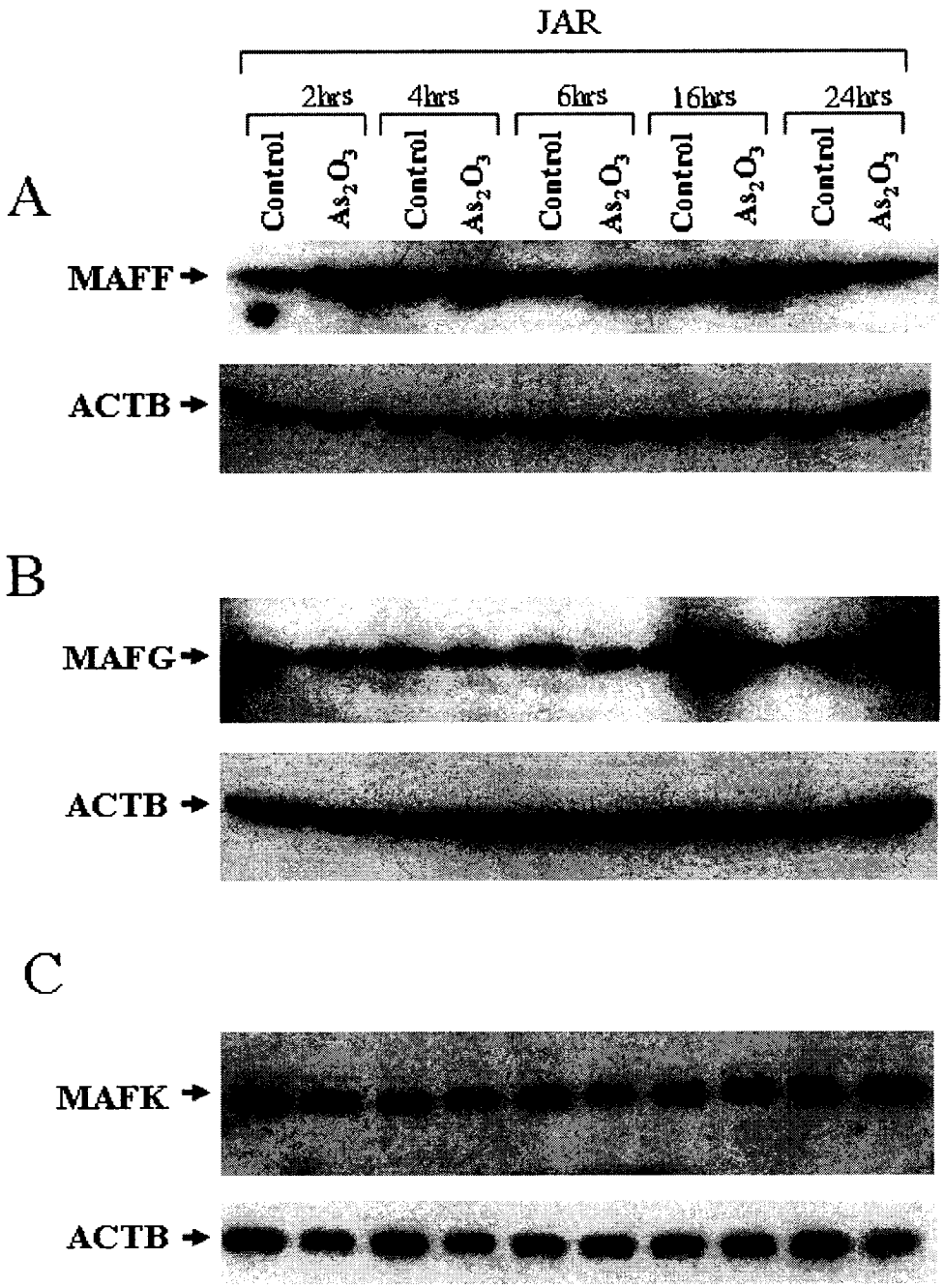
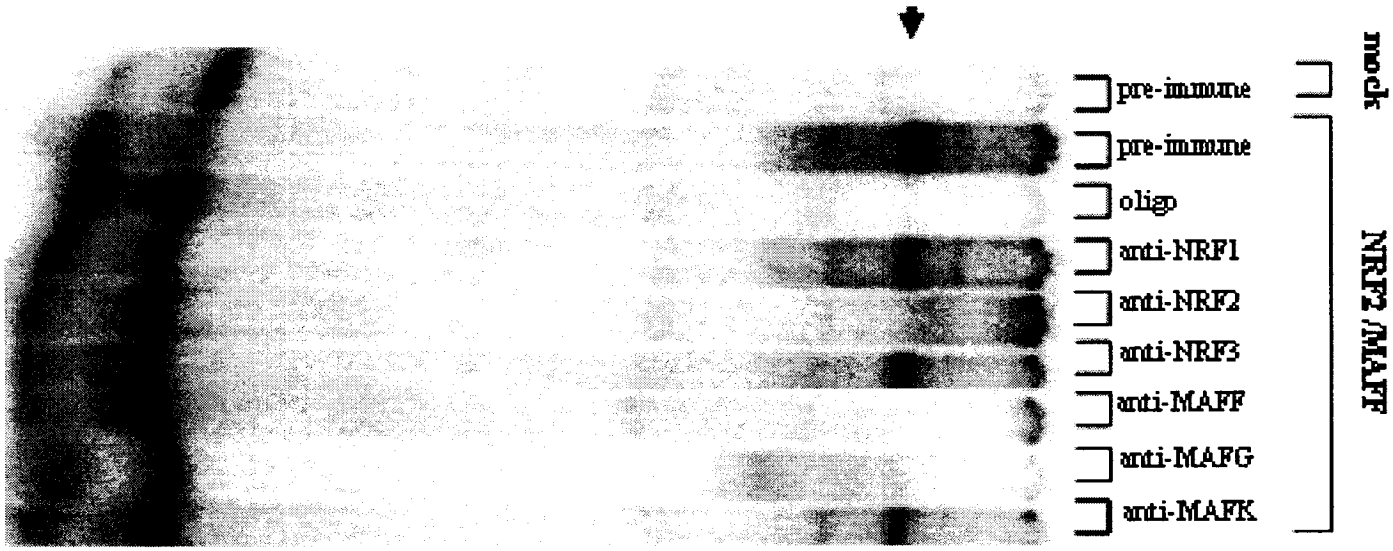
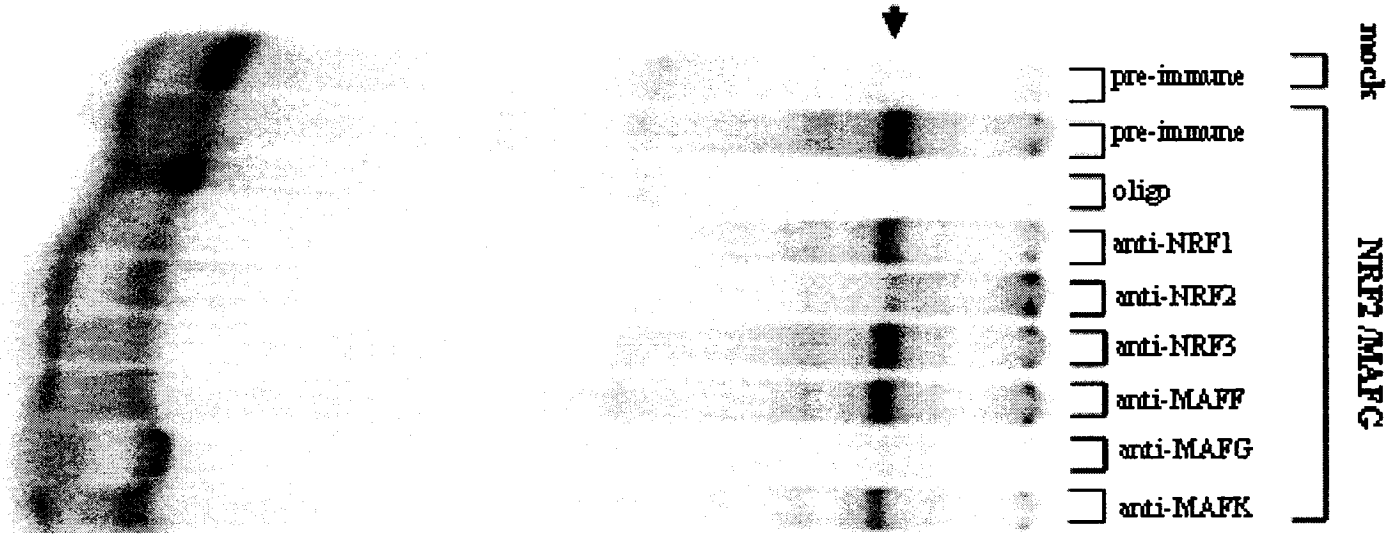
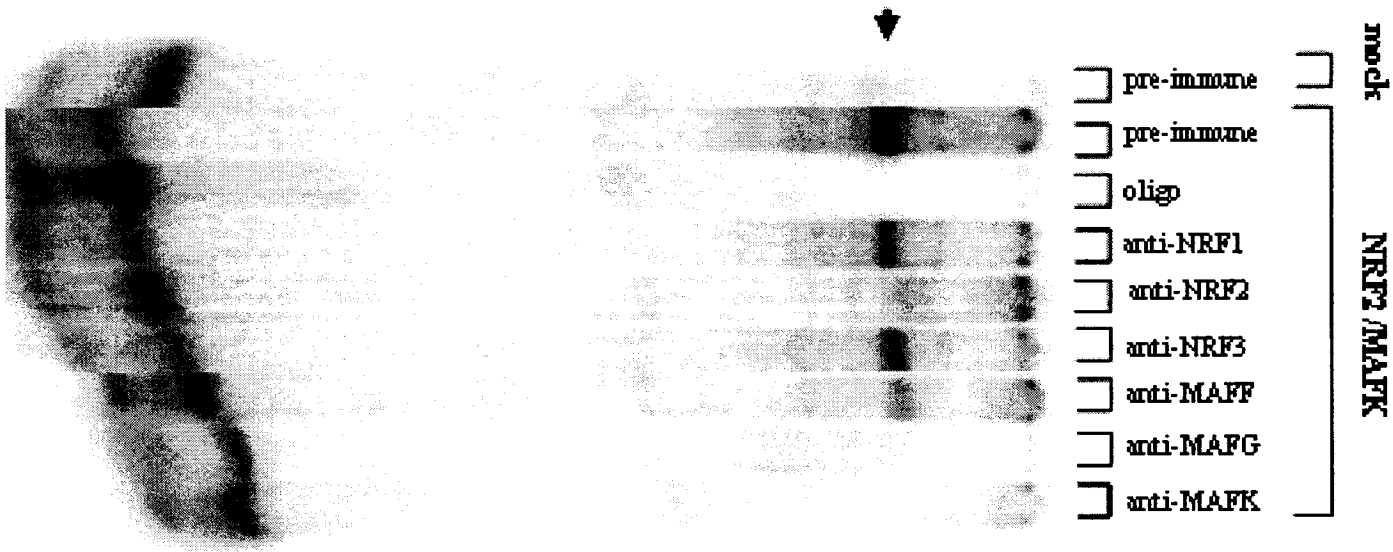

JAR
2hrs
4hrs
6hrs
16hrs
24hrs
Control
As_2O_3
A
MAFF
ACTB
B
MAFG
ACTB
C
MAFK
ACTB

Fig.20 Specificities of NRF2 and small MAF antisera.

EMSA analysis of nuclear extracts for HEK293T cells transfected or not (mock) with expression vectors coding for NRF2 and either MAFF **(left panel)**, MAFG **(center panel)** or MAFK **(right panel)**. A stress responsive element (StRE) recognition site derived from the HO-1 promoter has been used as a probe. Non-labeled competitor StRE oligonucleotide (oligo), preimmune serum and NRF1, NRF2, NRF3, MAFF, MAFG and MAFK specific antisera were added to the reaction mix as indicated. Arrows indicate the position of the different NRF2/small MAF heterodimers.


mock
pre-immune
pre-immune
oligo
anti-NRF1
anti-NRF2
anti-NRF3
anti-MAFF
anti-MAFG
anti-MAFK
NRF2/MAFF


mock
pre-immune
pre-immune
oligo
anti-NRF1
anti-NRF2
anti-NRF3
anti-MAFF
anti-MAFG
anti-MAFK
NRF2/MAFG


mock
pre-immune
pre-immune
oligo
anti-NRF1
anti-NRF2
anti-NRF3
anti-MAFF
anti-MAFG
anti-MAFK
NRF2/MAFK

whereas NRF1 or NRF3 specific antisera did not appear to recognize the complex. As expected, the MAFG specific antiserum supershifted the complex containing MAFG, but also cross reacted with DNA binding complexes comprising MAFF or MAFK. In contrast, both the MAFF and MAFK antisera seem to be highly specific and recognize only complexes that comprise either of these small MAF factors.

Next, we examined the endogenous DNA binding complexes in JAR cells that had been exposed to 5μM arsenic trioxide for 6 hours. Uninduced cells showed only a minor signal, although this basic DNA binding activity appeared to be specific, as competition with non-labeled oligonucleotides abolished this interaction (Fig. 21, left panel). Exposure to arsenic trioxide resulted in the induction of a prominent DNA binding complex (Fig. 21, right panel). This DNA binding activity comprised NRF2, as addition of the NRF2 antiserum clearly disrupted the complex. In contrast, antisera specific for the highly homologous NRF1 and Nrf3 proteins did not appear to have an effect. Addition of the MAFG antiserum that cross reacts with all three small MAF also completely abolished the DNA binding complex, whereas in the presence of the specific MAFF and MAFK antisera, the complex did not fully disappear. Our results suggest that the arsenic-induced complex in JAR cells consists of NRF2/small MAF heterodimers (Fig. 21), in agreement with the fact that all three small MAF proteins are expressed in these cells (Fig. 19).

A.7 Arsenic mediated increase of HO-1 expression

The StRE is found in several copies in the enhancer region of the HO1 gene. As further evidence for the involvement of oxidative stress, we examined the expression of the enzyme

Fig.21 Induction of NRF2/small Maf heterodimers by arsenic in choriocarcinoma cells.

EMSA analysis of nuclear extracts for JAR choriocarcinoma cells not treated (left panel) or treated (right panel) with 5μM arsenic trioxide for 6 hours. A stress responsive element (StRE) recognition site derived from the HO1 promoter has been used as a probe. Non-labeled competitor StRE oligonucleotide (oligo), preimmune serum and NRF1, NRF2, Nrf3, MAFF, MAFG and MAFK specific antisera were added to the reaction mix as indicated. The arrow indicates the position of the endogenous NRF2/small MAF heterodimers.

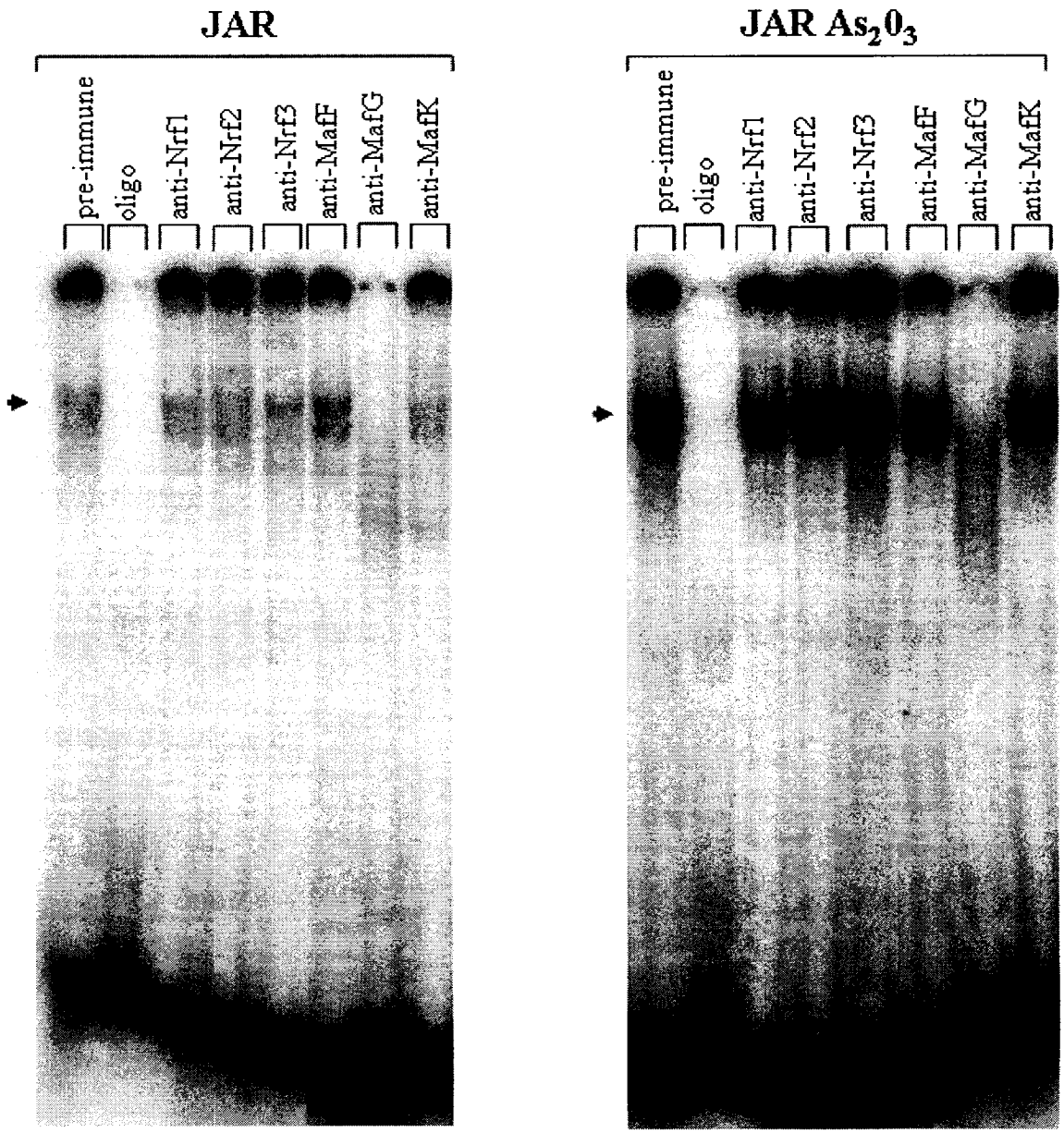
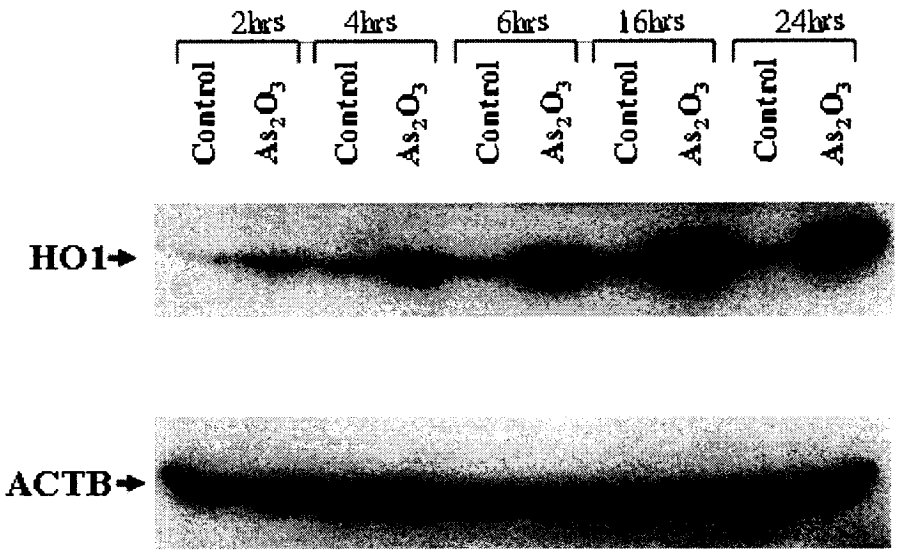

JAR
pre-immune
oligo
anti-Nrf1
anti-Nrf2
anti-Nrf3
anti-MafF
anti-MafG
anti-MafK
JAR As_2O_3
pre-immune
oligo
anti-Nrf1
anti-Nrf2
anti-Nrf3
anti-MafF
anti-MafG
anti-MafK

Fig.22 Arsenic induced upregulation of HO1 expression.

Immunoblot analysis of cytoplasmic extracts prepared from JAR choriocarcinoma cells treated or not (control) with 5μM arsenic trioxide for various lengths of time using antisera specific for HO1 and ACTB. 30 μg of protein was loaded per well.


2hrs
4hrs
6hrs
16hrs
24hrs
Control
As_2O_3
Control
As_2O_3
Control
As_2O_3
Control
As_2O_3
Control
As_2O_3
HO1
ACTB

HO1, a target of NRF2/small MAF DNA binding complexes [126, 204]. We showed expression of the HO1 protein is significantly induced by arsenic trioxide in JAR cells (Fig. 22).

Chapter IV- Results

A. Regulation of *MAFF* and *NRF3* in the Placental Cells and Villi

Another major source of oxidative stress in the female reproductive system occurs at 15 to 16 weeks of pregnancy during the time of fetal trophoblast invasion of the uterus. This is when the placenta is being linked to the maternal decidua by anchoring chorionic villi. During normal placentation, trophoblast cells that originate from the outer layer of cells surrounding the blastocyst inner cell mass differentiate into cytotrophoblasts. These cytotrophoblasts invade the maternal decidua and adjacent spiral arteries thus forming the chorionic villi.

In view of the fact that *NRF3* and the small *MAFF* are highly expressed in the placenta [70, 194] (Appendix A-Fig. 1), we further explored the possible role of these transcription factors in the placenta. We hypothesized that investigating *NRF3* and *MAFF* expression in the different cell types found in the placenta and at different stages during the gestation period would provide some evidence on their role in the placenta.

A.1 Human NRF3 is primarily expressed in Placental Cytotrophoblasts

First, placental chorionic villi from different stages of gestation were obtained. Briefly, placentas were subjected to a series of enzymatic digests, which detached cytotrophoblast progenitors from the stromal cores of the chorionic villi. Once detached, the cells were purified and total RNA isolated. We subsequently used northern blot analysis to detect *NRF3* and *MAFF* mRNA levels (Fig. 23A & 24A). Two human *NRF3* transcripts, a major and a minor mRNA species of 2.9 kb and 4.4 kb, respectively, were detected, whereas the *MAFF* transcript was detected at 2.6 kb. Interestingly, it was revealed that both *NRF3* and *MAFF* were

Fig.23 Expression pattern of human *NRF3* transcripts in placental cells.

Northern analysis of total RNA prepared from placental and choriocarcinoma cells. **A**, placental chorionic villi at different gestation stages (12 weeks to term). Quantification of *NRF3* transcript levels normalized to *GAPDH* mRNA levels was done by phosphoimager analysis. **B**, placental fibroblasts, cytotrophoblast cell islands (ISL) and cytotrophoblast progenitors. **C**, choriocarcinoma cell lines BeWo and JAR. 10 μg of RNA was analyzed per lane. Human *NRF3* and *GAPDH* transcripts are indicated.

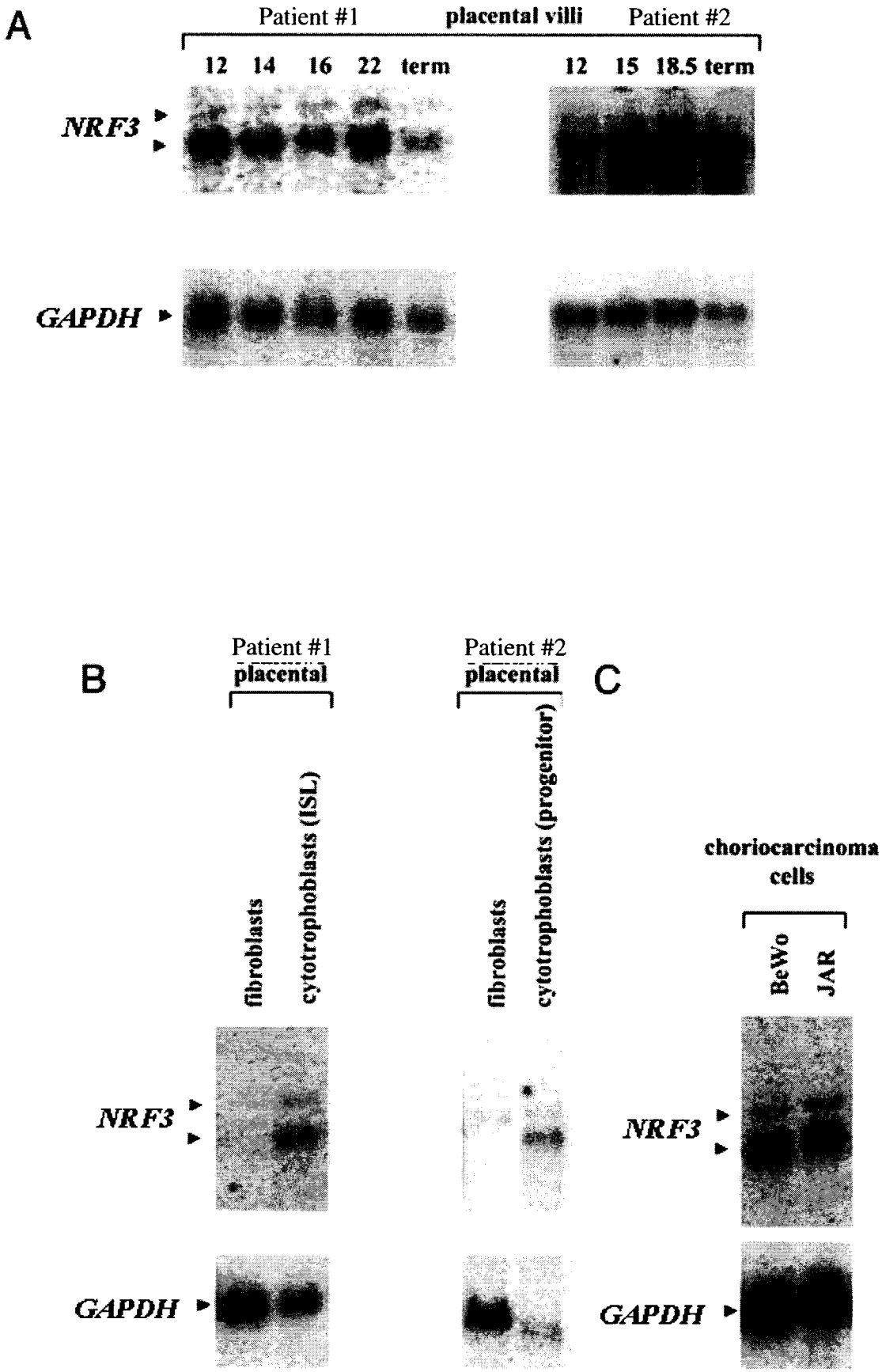
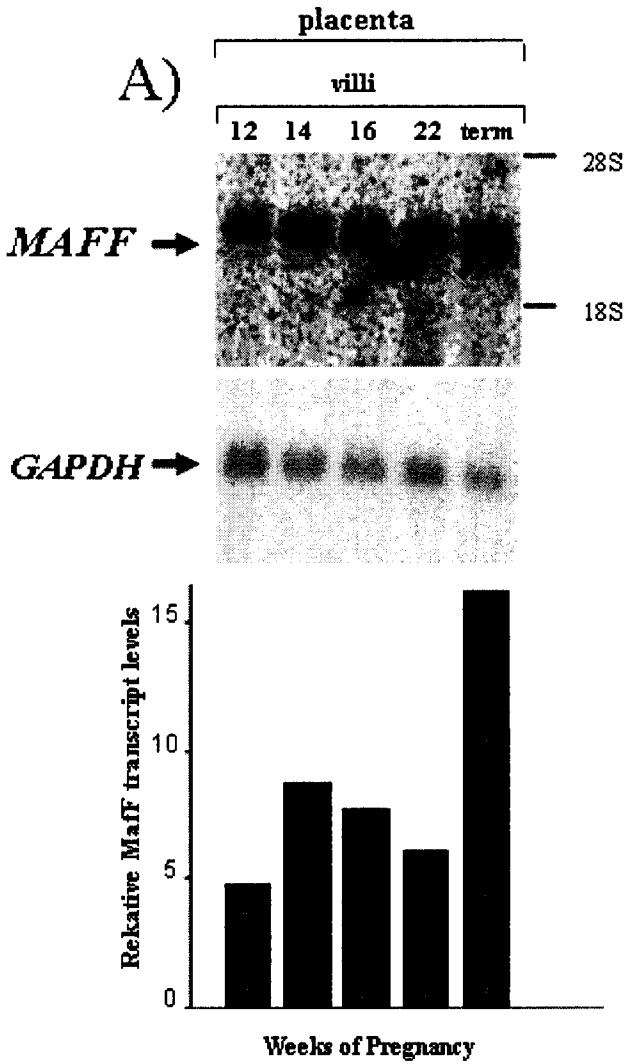
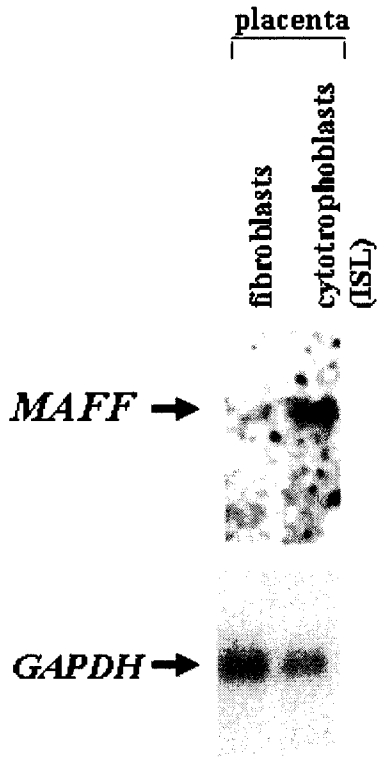

A
Patient #1
placental villi
Patient #2
12 14 16 22 term
12 15 18.5 term
NRF3
GAPDH
B
Patient #1
placental
Patient #2
placental
fibroblasts
cytotrophoblasts (ISL)
fibroblasts
cytotrophoblasts (progenitor)
C
choriocarcinoma cells
BeWo
JAR
NRF3
GAPDH

Fig.24 Expression pattern of human *MAFF* transcripts in placental cells.

Northern analysis of total RNA prepared from placental and choriocarcinoma cells. **A,** placental chorionic villi at different gestation stages (12 weeks to term). Quantification of *MAFF* transcript levels normalized to *GAPDH* mRNA levels was done by phosphoimager analysis. **B**, placental fibroblasts, cytotrophoblast cell islands (ISL) and cytotrophoblast progenitors. Human *MAFF* and *GAPDH* transcripts are indicated.


placenta
A)
villi
12 14 16 22 term
28S
18S
MAFF
GAPDH
Rekative MafF transcript levels
15
10
5
0
Weeks of Pregnancy


placenta
B)
fibroblasts
cytotrophoblasts (ISL)
MAFF
GAPDH

iexpressed throughout gestation, from at least week 12 to term (Fig. 23A & 24A). Second, the expression patterns in specific cell populations of chorionic villi were investigated. Two of the predominant cells types in placental villi, fibroblasts and cytotrophoblasts, were isolated. Fibroblasts make up the stromal compartment while cytotrophoblasts line the placenta and mediate all interactions between fetal and maternal tissues. We isolated cytotrophoblast progenitor cells from second trimester placentas as well as cytotrophoblasts located in cell islands from early gestation placentas, which represent a population differentiating into invasive cells. *NRF3* and *MAFF* transcripts were highly expressed in cytotrophoblast progenitors and those derived from trophoblast islands (ISL), while expression in placental fibroblasts was either weak or undetectable (Fig. 23B & 24B). Finally, we found that the human choriocarcinoma cell lines BeWo and JAR [205, 206], derived from trophoblastic tumors of the placenta, also strongly express *NRF3* transcripts (Fig. 23C). These results taken together show that, in the placenta, cytotrophoblasts are the primary source of *NRF3* and *MAFF* mRNA, suggesting that this transcription factor may function in directing the differentiation of these cells, which is critical to formation of the fetal-maternal interface during normal placentation.

A.2 Expression of human NRF3 is stimulated by TNF

There is increasing evidence that preeclampsia arises from poor placentation due to improper trophoblast invasion [158]. Proinflammatory cytokines are a chief source of ROS during this process. In fact, TNF levels have been shown to be higher in the circulation of women with preeclampsia [165]. This led us to investigate whether NRF3 expression is modulated by TNF in JAR cells. We found a reproducible induction of NRF3 transcripts in JAR cells at 4, 8 and

16 hours following induction (Fig. 25A). Importantly, the increased human NRF3 RNA levels correlated with increased protein levels (2.3 fold) in TNF-treated JAR cells (Fig. 25B), which suggests that NRF3 may participate in late TNF mediated signaling in placental cells.

Only a small amount of information is available on NRF3, the most recently identified member of the CNC family [85]. Hence, we embarked on further characterization of the dimerization, binding and transactivation potential of NRF3.

A.3 NRF3 and MAFG heterodimerize and bind to NF-E2/MARE sites

Isolation of NRF3 using a two-hybrid strategy [207] strongly suggested that NRF3 is able to interact with MAFG. To this end, electrophoretic mobility shift assays (EMSA) were performed to assess DNA binding activity and protein-protein interactions of NRF3. HEK293T cells were transiently transfected with expression constructs coding for human MAFG and a truncated form of NRF3 (525 amino acids), corresponding to the clone isolated in the 2-hybrid assay. We also used a cDNA corresponding to a truncated intermediate size version (607 amino acids) as early experiments resulted only in low level expression of the full-length (694 amino acid) clone. Thus, in subsequent transfection studies we used a higher amount of the expression vector coding for full-length NRF3 (694 amino acids). Co-transfection of expression vectors coding for MAFG and for either of the different NRF3 versions resulted in a strong DNA-binding activity to the NF-E2/MARE-type recognition site, whereas in mock-transfected cells the complex was not present (Fig. 26A). DNA-binding was specific as addition of excess unlabeled oligonucleotide corresponding to the recognition site abolished the interaction. To characterize NRF3 protein complexes biochemically, a specific antiserum

Fig.25 Induction of *NRF3* transcript and protein levels by TNF

A, Northern analysis of total RNA prepared from JAR choriocarcinoma cells that were treated, or not, with 100 ng/ml of TNF for 4, 8 or 16 hours. 10 μg of RNA was analyzed per lane. Human *NRF3* and *GAPDH* transcripts are indicated. Quantification of *NRF3* transcript levels normalized to *GAPDH* mRNA levels was done by phosphoimager analysis of two independent experiments.

B, Immunoblot analysis of JAR choriocarcinoma cell extract treated with 100ng/ml TNF for 16 hours. 30 μg of total proteins were subjected to immunoblotting using purified anti-peptide antiserum raised against NRF3. Quantification of NRF3 protein levels normalized to GAPDH protein levels was performed using NIH image software analysis of three independent experiments. Significant statistical difference is indicated by an asterisk ($P<0.01$).

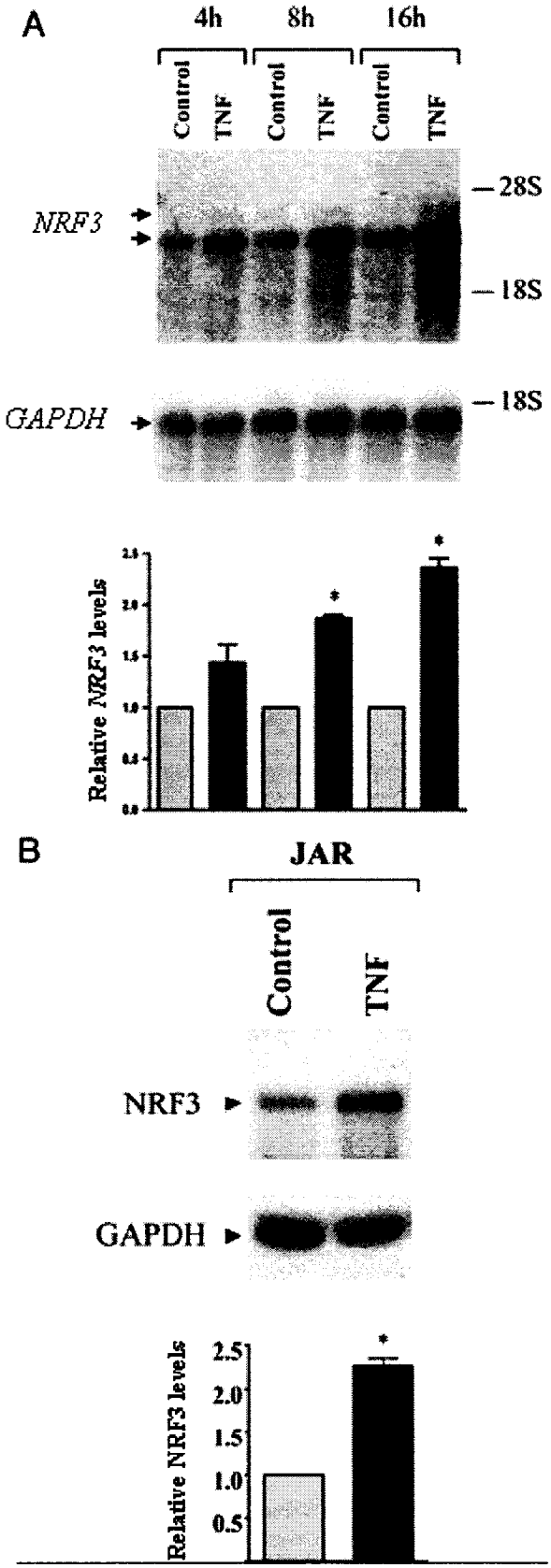
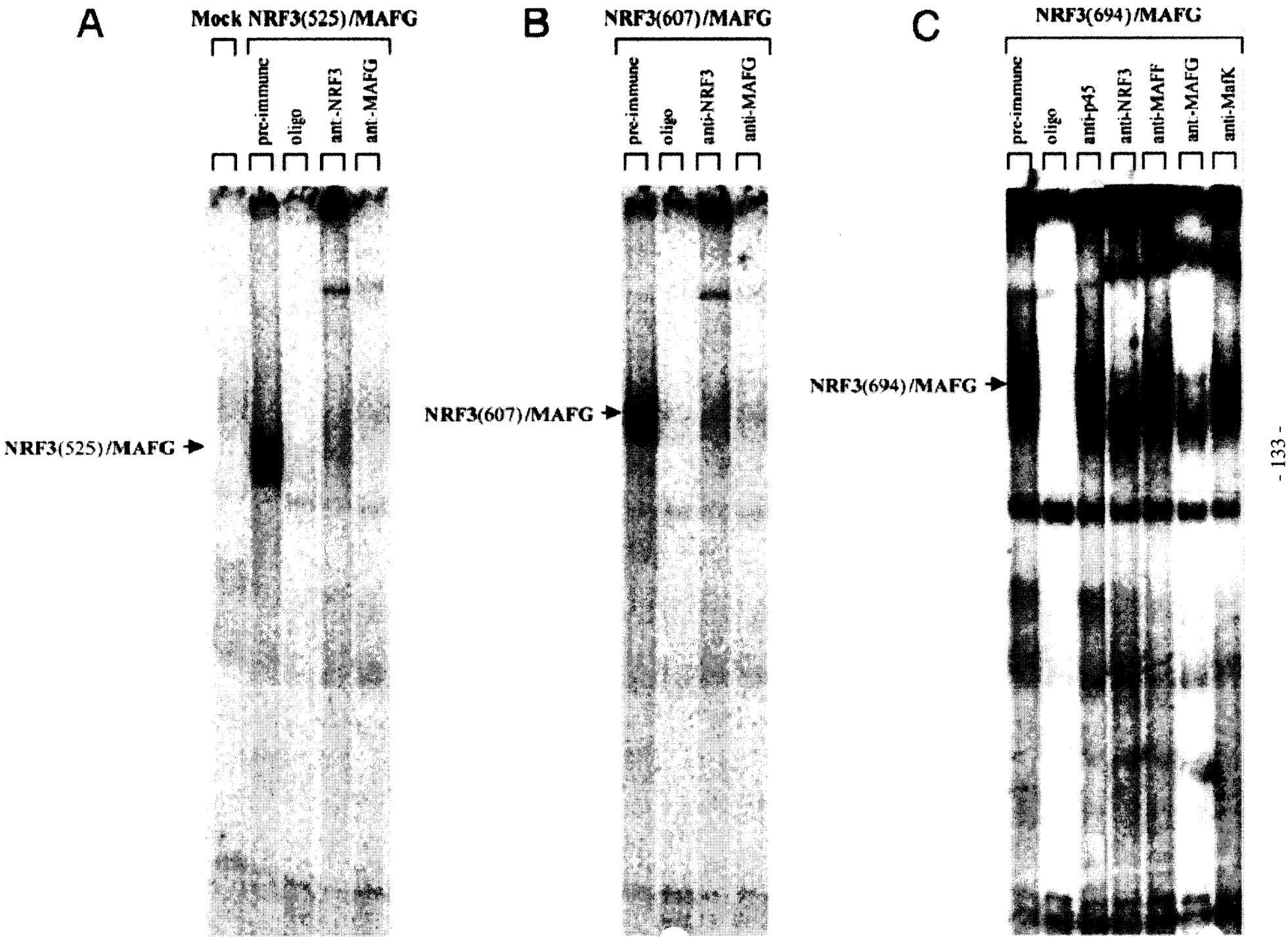

A
4h
8h
16h
Control
TNF
Control
TNF
Control
TNF
NRF3
28S
18S
GAPDH
18S
Relative NRF3 levels
B
JAR
Control
TNF
NRF3
GAPDH
Relative NRF3 levels
2.5
2.0
1.5
1.0
0.5

Fig.26 Heterodimerization of human NRF3 with MAFG.

NFE2 DNA binding site oligonucleotide derived from the human PBGD promoter was used as a probe in an EMSA to detect NRF3 binding activity in nuclear extracts from HEK293T cells transfected with constructs coding for MAFG and either truncated NRF3 versions (**A**, 525; **B**, 607 amino acids) or full-length NRF3 (**C**, 694 amino acids). Non-labeled competitor human PBDG promoter oligonucleotide, pre-immune serum (PI) and/or p45-, NRF3-, MAFG-, MAFF-, MAFK-specific antisera were added to the reaction mix as indicated. Arrows indicate the position of the different NRF3/MAFG heterodimers.


A
Mock
NRF3(525)/MAFG
pre-immune
oligo
ant-NRF3
ant-MAFG
NRF3(525)/MAFG
B
NRF3(607)/MAFG
pre-immune
oligo
anti-NRF3
anti-MAFG
NRF3(607)/MAFG
C
NRF3(694)/MAFG
pre-immune
oligo
anti-p45
anti-NRF3
anti-MAFF
ant-MAFG
anti-MafK
NRF3(694)/MAFG

was generated using a GST-NRF3 fusion protein comprising solely the center portion of NRF3 to avoid cross-reaction with other CNC proteins. Indeed, the antiserum supershifted the NRF3/MAFG heterodimers (Fig. 26A & 26B & 26C), and did not cross-react with other CNC proteins such as p45, NRF1 and NRF2 (Fig. 27B & 27A & 20). The formerly described MAFG antiserum also disrupted formation of the DNA/protein complex, unique to HEK293T cells expressing NRF3 and MAFG proteins, confirming that this complex contained both factors (Fig. 26). In contrast, antisera raised against the bZIP transcription factors p45, MAFF and MAFK had no effect (Fig. 26). It was also confirmed that NRF3 (525 amino acids) can dimerize with MAFF. However, attempts to demonstrate full length NRF3 heterodimerzing with MAFF were unsuccessful. This can be attributed to not using the ideal MARE binding site for NRF3(694)/MAFF dimers. In summary, these results show that the full-length human NRF3 transcription factor can form heterodimers with small MAF proteins resulting in complexes that recognize NF-E2/MARE-type DNA-binding motifs.

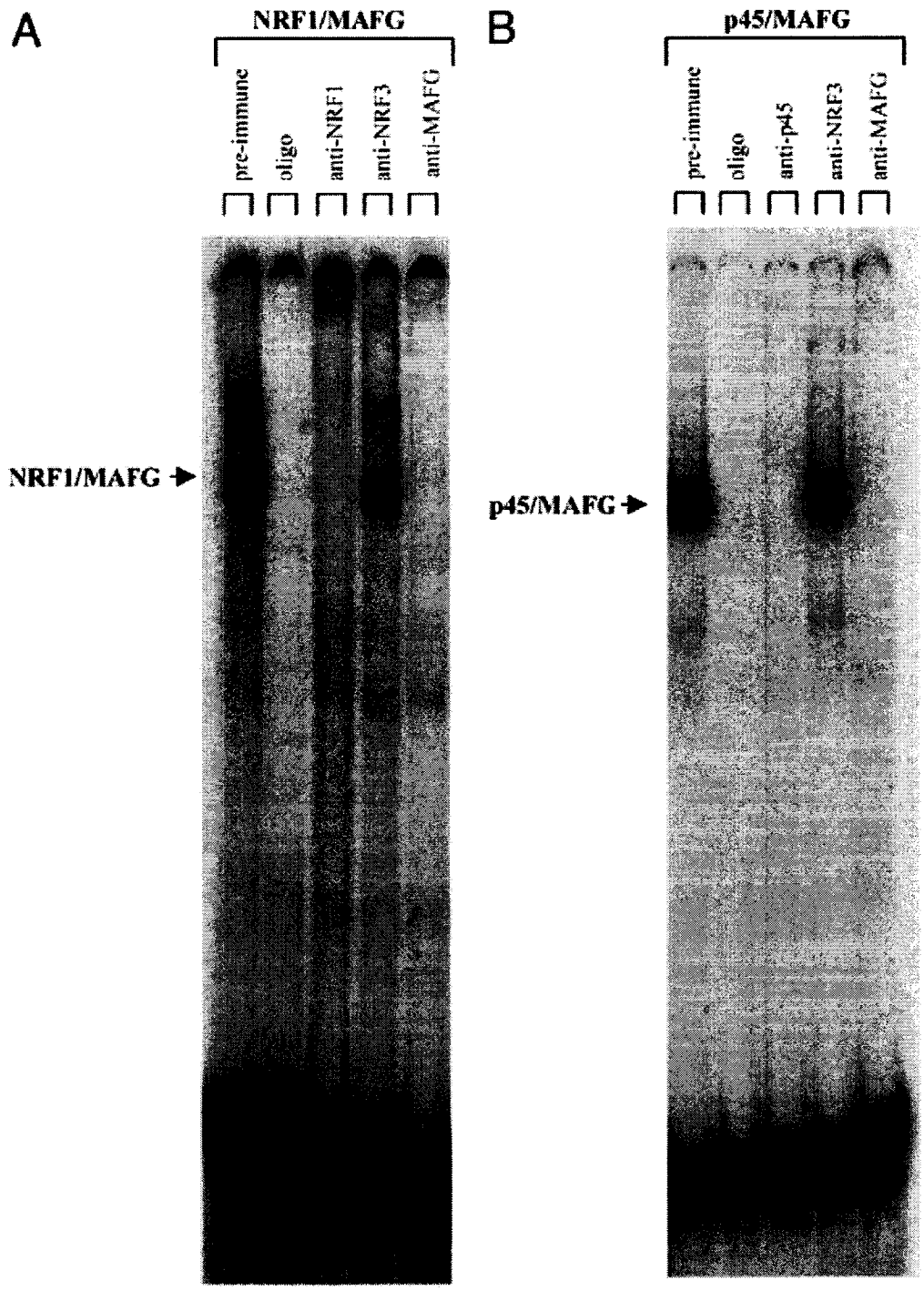
A.4 Analysis of endogenous human NRF3 protein expression

As the anti-GST-NRF3 fusion protein antiserum, used in EMSA experiments, only weakly recognized full-length NRF3 protein in an immunoblot, we raised an antiserum specific for a peptide (KLHDLYHDIFSRLLRDDQGRPVNPN) in the carboxyl-terminus of human NRF3. The peptide was coupled to KLH and used to immunize female New Zealand White rabbits (Pocono Farms). This serum was further purified using affinity chromatography and was subsequently used to analyze the expression of endogenous NRF3 at the protein level in the BeWo and JAR cell lines, as well as in transfected HEK293T cells. Whole cell extracts from HEK293T cells transfected with constructs coding for the 525 amino acid, 607 amino acid and

full-length (694 amino acids) versions of NRF3 were prepared and run on an SDS polyacrylamide gel. Immunoblot analysis revealed the presence of the corresponding protein

Fig.27 Specificity of NRF3 antiserum.

NFE2 DNA binding site oligonucleotide derived from the human PBGD promoter was used as a probe in an EMSA to detect binding activity in nuclear extracts from HEK293T cells transfected with constructs coding for MAFG and either **A**, NRF1 or **B**, p45. Non-labelled competitor human PBDG promoter oligonucleotide, pre-immune serum (PI) and/or p45, NRF1-, NRF2-, NRF3-, MAFG-specific antisera were added to the reaction mix as indicated. Arrows indicate the position of the different CNC protein/MAFG heterodimers.


A
NRF1/MAFG
pre-immune
oligo
anti-NRF1
anti-NRF3
anti-MAFG
NRF1/MAFG
B
p45/MAFG
pre-immune
oligo
anti-p45
anti-NRF3
anti-MAFG
p45/MAFG

versions, whereas in mock-transfected cells no NRF3 was detectable (Fig. 28B). In addition, whole cell extracts from JAR as well as BeWo cells, were analyzed and confirmed that the endogenous protein displayed the same apparent molecular weight (~120 kDa) as full length NRF3 (694 amino acids) from transfected HEK293T cells (Fig. 28). The use of preimmune serum yielded multiple bands, but the appearance of NRF3 protein is evident only in the presence of NRF3-specific antiserum (Fig. 28A & 28B). Specificity of the antiserum was further established by competition with the peptide antigen in an immunoblot assay (Fig. 28C). It was noticed that in western blot experiments, full-length NRF3 gave rise to shorter versions, migrating at a lower molecular weight. Thus, it was speculated that the faster migrating species may be degradation products of the full-length version. The shorter products observed in transfected HEK293T cells are not seen in the BeWo and JAR cell lines that express endogenous NRF3, suggesting cell-specific modifications may be needed to yield a stable full-length NRF3 protein.

A.5 Human NRF3 comprises a potent transactivation domain

To determine whether human NRF3 can act as a transcriptional activator we performed transfection experiments in HEK293T cells using Gal4 DNA binding domain-NRF3 fusion protein constructs jointly with a Gal4 DNA binding site-luciferase reporter vector (Fig. 29). Full-length NRF3 is able to activate approximately 6 fold when compared to HEK293T cells transfected with Gal4 DNA binding domain (Gal4DBD) vector (pSG424) alone (Fig. 29). A chimeric protein (Gal4DBD-NRF3$_{1\text{-}536}$) without CNC and bZIP domains showed increased transactivation potential and stimulated the reporter gene by 18 fold. A more drastic increase was observed when the first 88 amino acids of the NRF3 amino terminus were removed.

Fig.28 Expression of endogenous NRF3 protein.

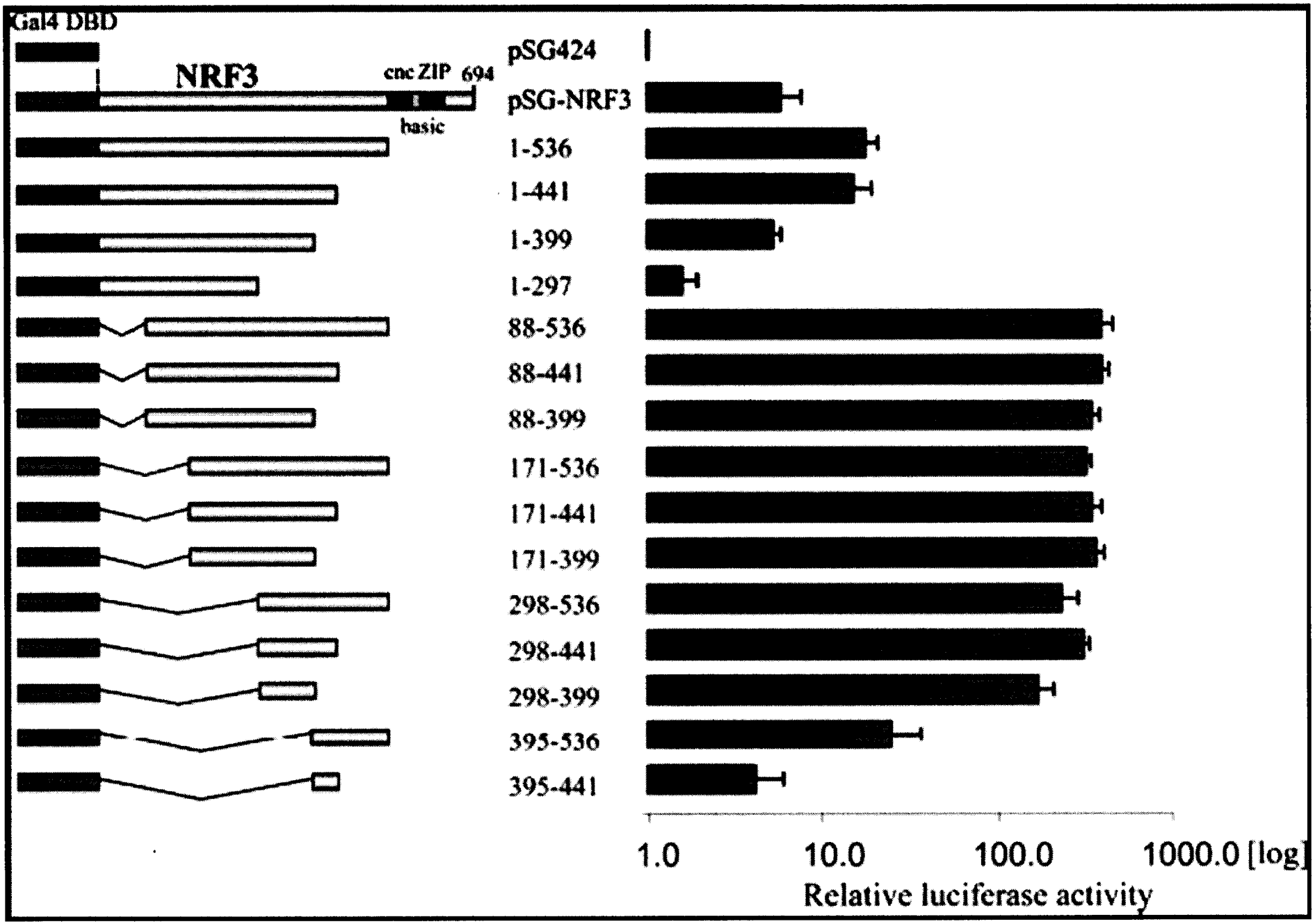
Immunoblot analysis of total protein extracted from HEK293T cells transfected or not (mock) with expression plasmids coding for shorter versions of NRF3 (525 and 607 amino acids), full-length NRF3 (694 amino acids), or from the choriocarcinoma cell lines BeWo and JAR. HEK293T (7 µg), BeWo (30 µg), and JAR (30 µg) whole-cell extracts were subjected to immunoblot analysis. **A**, Preimmune control serum. **B**, Purified antipeptide NRF3-specific antiserum. To reveal the presence of the endogenous NRF3, the lanes corresponding to the BeWo and JAR cell extracts of the same blot have been exposed 4 times longer. **C**, Purified antipeptide NRF3-specific antiserum in the presence of 50 µM peptide antigen. To reveal possible nonspecific bands, this blot was exposed 10 times longer than the blot A and the lanes corresponding to HEK293T cell extracts of blot B. *Arrows* indicate the position of the shorter NRF3 versions (525 and 607 amino acids) and full-length NRF3 (694 amino acids).

A
pre-immune
mock
NRF3 (525)
NRF3 (607)
NRF3 (694)
HEK 293T
BeWo
JAR
B
anti-NRF3
mock
NRF3 (525)
NRF3 (607)
NRF3 (694)
HEK 293T
BeWo
JAR
NRF3(694)
NRF3(607)
NRF3(525)
NRF3
C
anti-NRF3
& peptide
mock
NRF3 (525)
NRF3 (607)
NRF3 (694)
HEK 293T
BeWo
JAR
kDa
191
97
64
51
39

Chimeric proteins, having in common at least amino acids 298 to 399 of NRF3, were extremely potent activators and induced transcription approximately 200- to 400- fold. A series of deletion mutants comprising different regions of the center portion of NRF3 were used to map the transactivation domain. We observed a drastic decrease in the transactivation potential of chimeric protein Gal4DBD-NRF3$_{395\text{-}536}$ when compared to the Gal4DBD-NRF3$_{298\text{-}399}$ (Fig. 29). In summary, our results reveal the presence of a potent transactivation domain in the center portion (amino acids 298-399) of NRF3, suggesting that this protein can function as a potent activator of gene transcription.

Fig.29 Mapping of the NRF3 transactivation domain.

Gal4 DNA binding domain-NRF3 fusion constructs and the control plasmid (pSG424) comprising the Gal4 DNA binding domain (DBD) alone that were analyzed in transactivation studies. HEK293T cells were transfected with the $(Gal4)_5$-TK/luc reporter plasmid and various Gal4-NRF3 chimeric expression plasmids. Firefly luciferase activity was analyzed 48h post-transfection. Relative luciferase activity was measured as fold activation relative to the basal level of the reporter gene in the presence of pSG424 vector after normalization to cotransfected renilla luciferase activity (pRL-TK vector). The results shown are from at least three independent experiments each done in triplicate, with variability shown by the error bars.


Gal4 DBD
NRF3
cnc ZIP
694
basic
pSG424
pSG-NRF3
1-536
1-441
1-399
1-297
88-536
88-441
88-399
171-536
171-441
171-399
298-536
298-441
298-399
395-536
395-441
1.0
10.0
100.0
1000.0 [log]
Relative luciferase activity

Chapter V- Results

A. Regulation of MAFF in Myometrium

Our data so far had suggested an important role for the CNC members, NRF2 and NRF3, and MAFF in the placenta, an essential transient organ of the female reproductive system. The myometrium is another vital organ in this system, because during labor it becomes active and develops high frequency, high-amplitude contractions that are an essential part of the labor process. The involvement of proinflammatory cytokines in the myometrium has been extensively investigated in the context of both normal and abnormal pregnancy and delivery. In fact, not only has it been shown that a systemic fetal proinflammatory cytokine response (IL1B, IL6 and TNF) is followed by the onset of spontaneous preterm parturition in patients [208], but it has also been shown that IL1R1/TNFR double knock-out mice have significantly lower rates of preterm delivery [184].

The mechanism by which inflammation may lead to labor is postulated to involve release of proinflammatory cytokines, which in turn stimulate prostaglandin synthesis by gestational tissues via the activation of prostaglandin-endoperoxide synthase 2 (PTGS2, previously known as COX2), thereby causing uterine contractions [184].

The human MAFF protein has previously been identified in a 1-hybrid assay as a factor binding to the US-2 motif in the human oxytocin receptor (OTR) gene [69]. This, along with the fact that MAFF transcripts are highly expressed in term myometrium, compared to the absence of the transcript in early gestation (14 weeks) and non-pregnant human myometrial

tissue [69], led our hypothesis that MAFF expression may be modulated by proinflammatory cytokines in the myometrium.

1.1 Preparation of primary myometrial cells

Primary myometrial cells were derived from a myometrial tissue dissected from a patient after a caesarian delivery. The tissue was rinsed, cut into smaller pieces and enzymatically digested. After terminating the enzymatic reaction, the tissue was filtered through a sterile gauze. Finally, the eluate was spun and the pellet of primary myometrial cells was washed and plated into 6 well plates.

1.2 Induction of *MAFF* by Cytokines in Primary Myometrial Cells.

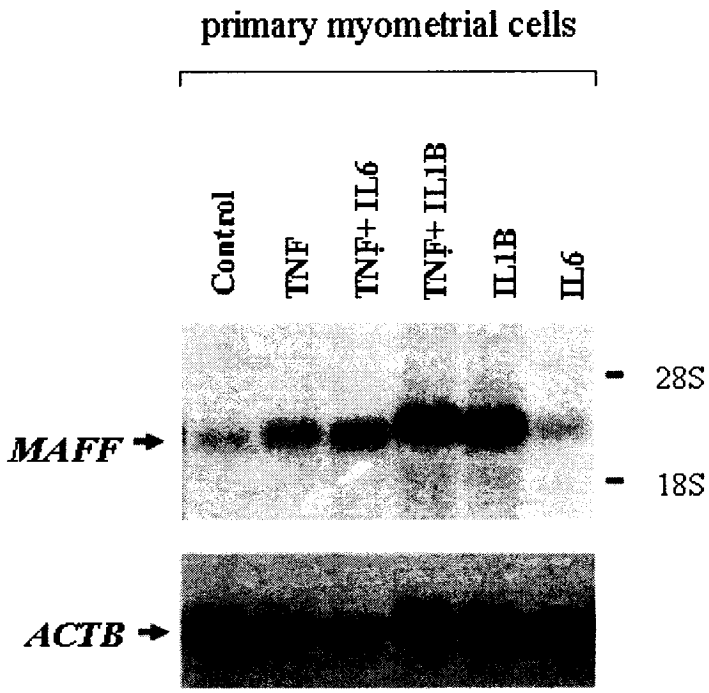
Initial experiments sought to test whether MAFF is involved in uterine gene regulation. Primary myometrial cells were cultured and once they reached 80% confluency were induced for 3 hours with IL1B (100ng/ml), IL6 (100ng/ml) and TNF (100ng/ml) or combinations of these cytokines. The RNA was then isolated and subjected to northern analysis. Interestingly, *MAFF* mRNA levels were induced by IL1B and TNF, but not by IL6 (Fig. 30).

1.3 Immortalized Pregnant Human Myometrial Cells (PHM1-31)

To minimize the need for primary myometrial tissue obtained from patients undergoing cesarian section, myometrial PHM1-31 cells were acquired and used in subsequent studies. These cells were derived from primary myometrial cells of pregnant women undergoing cesarian sections but who were not in labor. Immortalization was attained through overexpression of the human papilloma virus E6/E7 proteins [186]. The appearance of

Fig.30 Small *MAF* expression in the primary myometrial cells.

Northern analysis of total RNA from primary myometrial cells induced for 3 hours with IL1B (10ng/ml), IL6 (10ng/ml) and TNF(10ng/ml) or combinations of these cytokines. 10μg of total RNA per lane was loaded. *MAFF* and *ACTB* transcripts are indicated.


primary myometrial cells
Control
TNF
TNF+ IL6
TNF+ IL1B
IL1B
IL6
MAFF
28S
18S
ACTB

PHM1-31 cells was determined by phase contrast light microscopy (Fig. 31). The morphology of the cells is similar to that of regular proliferating smooth muscle cells. They appear long, spindle shaped with a central nucleus and a sheet-like growth pattern at confluency.

1.4 Induction of *MAFF* by Cytokines in PHM1-31 Myometrial Cells.

MAFF mRNA expression in PHM1-31 cells treated with proinflammatory cytokines was examined (Fig. 32). A significant upregulation of *MAFF* mRNA levels by TNF, and an even stronger induction by IL1B was found. Similar to the primary myometrial cells, treatment with IL6 did not result in a significant change of *MAFF* mRNA levels (Fig. 32A). Combination of TNF and IL1B did not increase *MAFF* mRNA levels further than IL1B treatment alone (Fig. 32A). These results suggest two things; that PHM1-31 cells, although immortalized, have conserved their myometrial phenotype with respect to cytokine responses and that perhaps the mechanism of action of IL1B and TNF is different from that of IL6. What was even more fascinating in subsequent experiments was that in contrast to *MAFF*, the transcript level of the highly homologous *MAFG* and *MAFK* genes were not induced by IL1B or TNF. Small MAF protein homology has made it difficult to assign specific roles *in vitro* and *in vivo* for each of the factors. These results suggest that stimulation by these cytokines is specific for the *MAFF* gene (Fig. 32B & 32C).

1.5 Regulation of *MAFF* expression at the transcriptional level

We started by determining the dosage and time response of IL1B in order to detect and quantify the lowest concentration and earliest time by which to achieve the maximal response. Linear regression is often used for building a purely empirical model between stimulus and

Fig.31 PHM1-31 cells.
Phase contrast image of the immortalized pregnant human myometrial cell line PHM31 in culture at passage 19. Magnification is 200X.

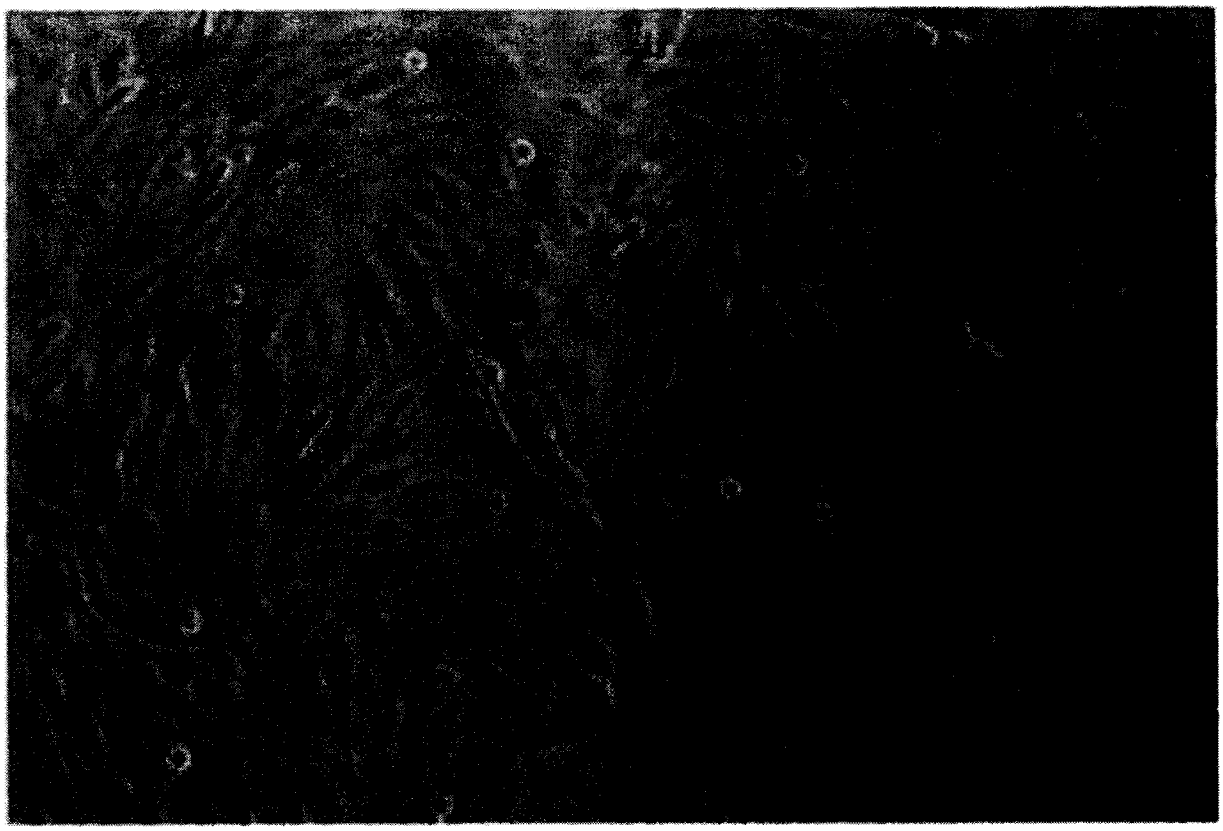
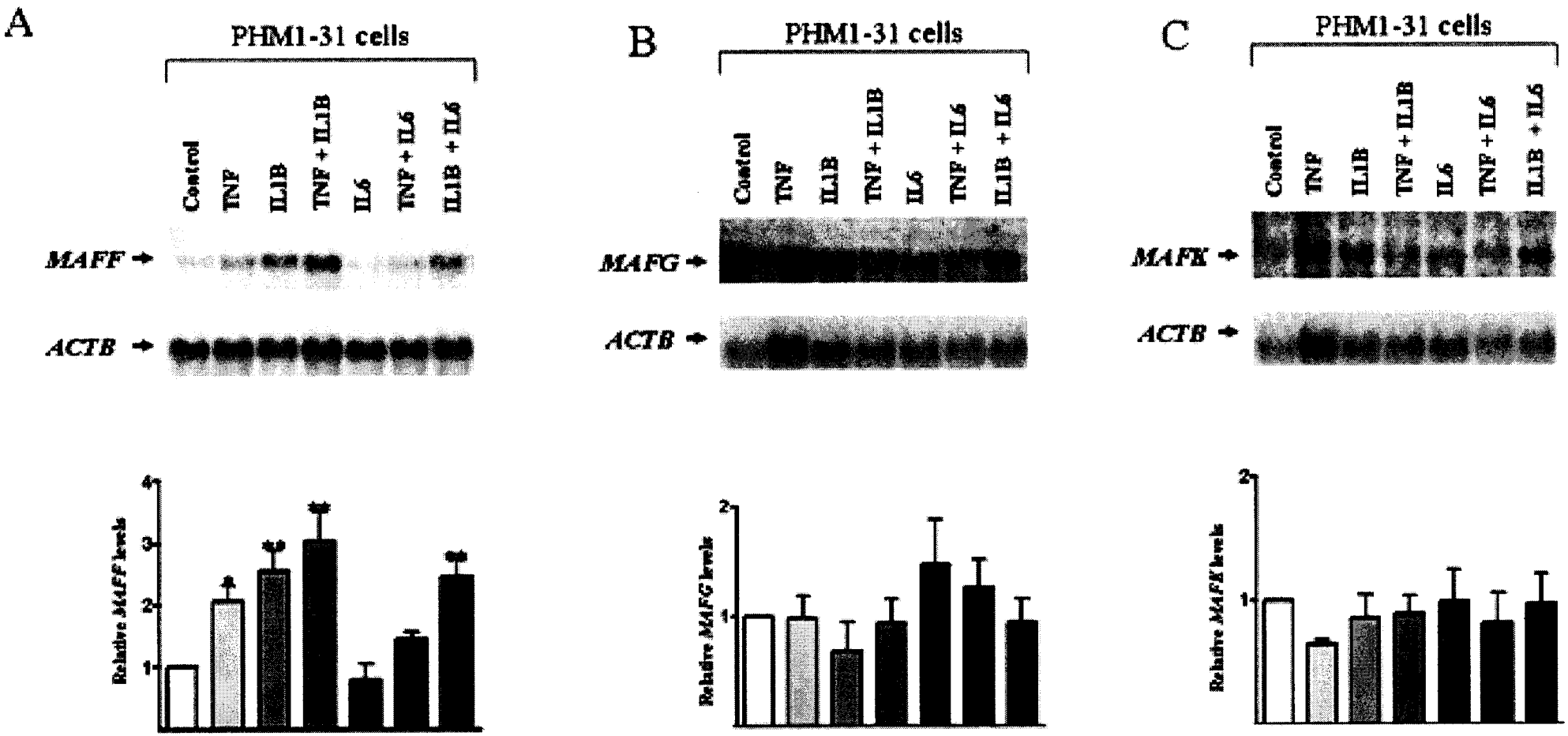


Fig.32 Small *MAF* expression in the PHM1-31 myometrial cell line.

Northern analysis of total RNA from PHM1-31 cells induced for 3 hours with IL1B (10ng/ml), IL6 (10ng/ml) and TNF(10ng/ml) or combinations of these cytokines. 10µg of total RNA per lane was loaded. *MAFF* **(A)**, *MAFG* **(B)** and *MAFK* **(C)** and *ACTB* transcripts are indicated. Quantification of at least 3 independent experiments is shown. Statistically significant differences are indicated by one ($P<0.05$) or two asterisks ($P<0.01$).


A
PHM1-31 cells
Control
TNF
IL1B
TNF + IL1B
IL6
TNF + IL6
IL1B + IL6
MAFF
ACTB
Relative MAFF levels
B
PHM1-31 cells
Control
TNF
IL1B
TNF + IL1B
IL6
TNF + IL6
IL1B + IL6
MAFG
ACTB
Relative MAFG levels
C
PHM1-31 cells
Control
TNF
IL1B
TNF + IL1B
IL6
TNF + IL6
IL1B + IL6
MAFK
ACTB
Relative MAFK levels

response. However, since the receptors provide a physical limiting factor for the binding of the IL1B, we used nonlinear regression to describe the relationship between the transcript levels of *MAFF* and the concentration of IL1B.

We induced PHM1-31 cells using IL1B between 0.005 ng/ml and 100ng/ml and revealed that 0.005 ng/ml of IL1B upregulated *MAFF* gene expression, with maximal induction being reached with 0.5 ng/ml of IL1B (Fig. 33A). Time course experiments were carried out between 15 minutes to 24 hours. These experiments showed induction of *MAFF* gene transcription by 30 minutes reaching maximal levels by 1 hour. Furthermore, elevated *MAFF* transcript levels were sustained over a 24 hour period (Fig. 33B & 33C).

In order to test whether regulation of the MAFF transcript is due to novel RNA synthesis, we preincubated the PHM1-31 cells with actinomycin D. Actinomycin D intercalates between GC base pairs of the DNA strands and thus, inhibits transcription without interfering with translation or DNA replication [209]. We found that actinomycin D inhibited induction of *MAFF* mRNA by IL1B (Fig. 34) suggesting regulation at the transcriptional level.

1.6 Analysis of MAFF protein levels in myometrial cells.

MAFF protein expression in whole cell extracts, nuclear and cytoplasmic extracts prepared from PHM1-31 cells was also analyzed (Fig. 35A). In accordance with its function as a transcription factor, we provide the first evidence confirming the presence of MAFF in the nucleus. We also corroborated the presence of MAFG and MAFK in the nucleus (Fig. 35B & 35C) which had been previously reported [31, 210]. Finally, we examined whether the upregulation of *MAFF* transcripts also leads to an increase in MAFF protein levels. Analysis of nuclear extracts of cytokine induced PHM1-31 cells showed that both TNF and IL1B induce

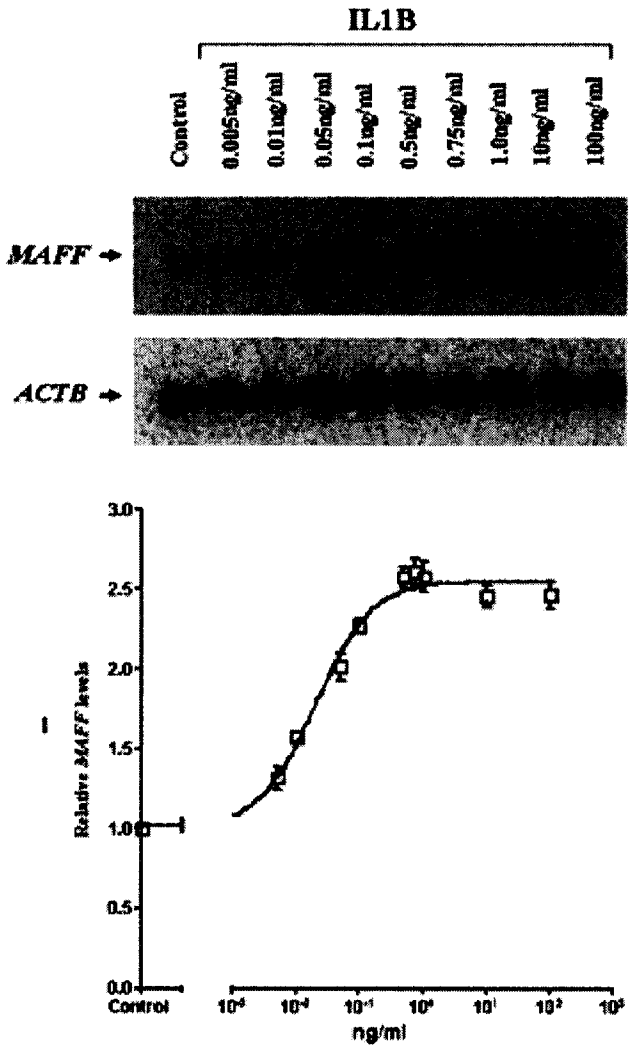
Fig.33 Dose response and time course of *MAFF* induction by cytokines.

(A) Dose response of *MAFF* transcript levels to IL1B (0.005 –100ng/ml) in PHM1-31 cells. 10µg of total RNA per lane was loaded. *MAFF* and *ACTB* (control) transcripts are indicated. Quantification of 4 independent experiments is shown using non linear regression analysis.

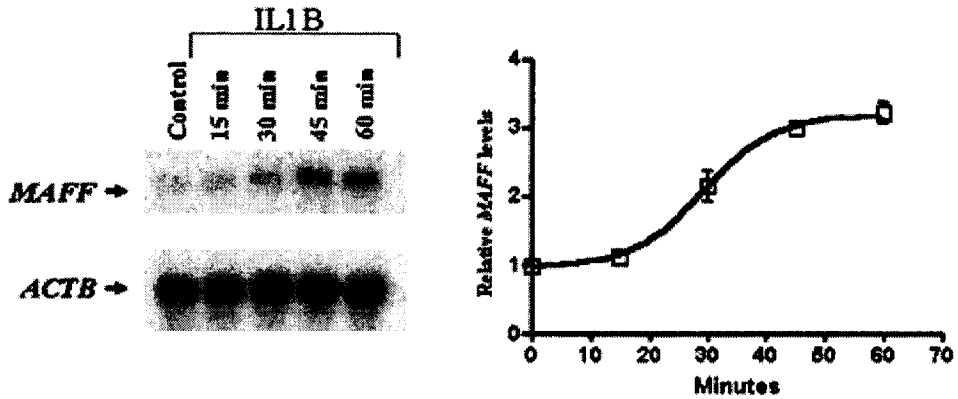
(B) Short time course analysis of *MAFF* mRNA induction by IL1B (1ng/ml) in PHM1-31 cells. 10µg of total RNA per lane was loaded. *MAFF* and *ACTB* (control) transcripts are indicated. Quantification of 4 independent experiments is shown using non linear regression analysis.

(C) Extended time course analysis of *MAFF* mRNA induction by IL1B (100ng/ml) in PHM1-31 cells. 10µg of total RNA per lane was loaded. *MAFF* and *ACTB* (control) transcripts are indicated. Figure is representative of 3 independent experiments.

A


IL1B
Control
0.005ng/ml
0.01ng/ml
0.05ng/ml
0.1ng/ml
0.5ng/ml
0.75ng/ml
1.0ng/ml
10ng/ml
100ng/ml
MAFF
ACTB
Relative MAFF levels
Control
ng/ml

B


IL1B
Control
15 min
30 min
45 min
60 min
MAFF
ACTB
Relative MAFF levels
Minutes

C

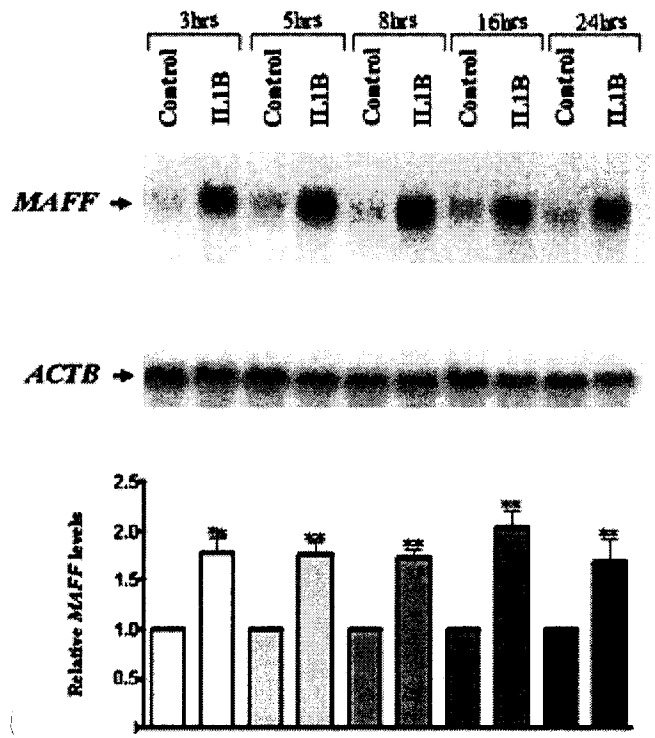

3hrs
5hrs
8hrs
16hrs
24hrs
Control
IL1B
MAFF
ACTB
Relative MAFF levels

Fig.34 Inhibition of *MAFF* induction by actinomycin D.

MAFF transcript levels in control and IL1B (1ng/ml) induced PHM1-31 cells in the presence or absence of actinomycin D. The presence of DMSO, the vehicle for actinomycin D is indicated. 3 independent experiments were performed with similar results.

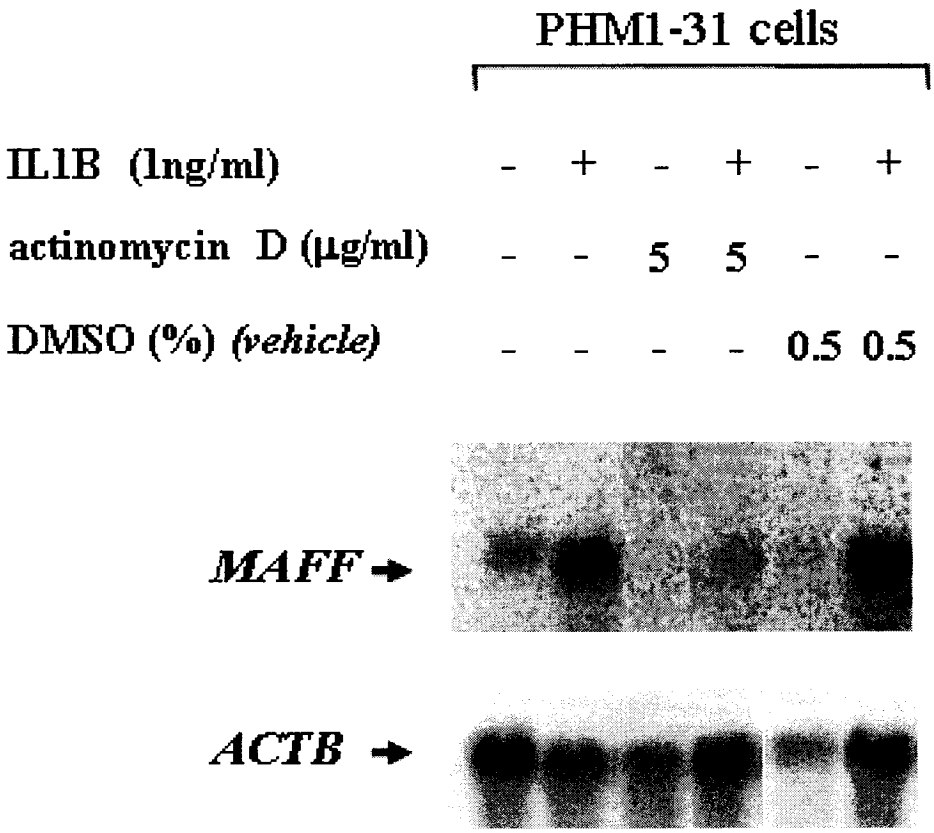
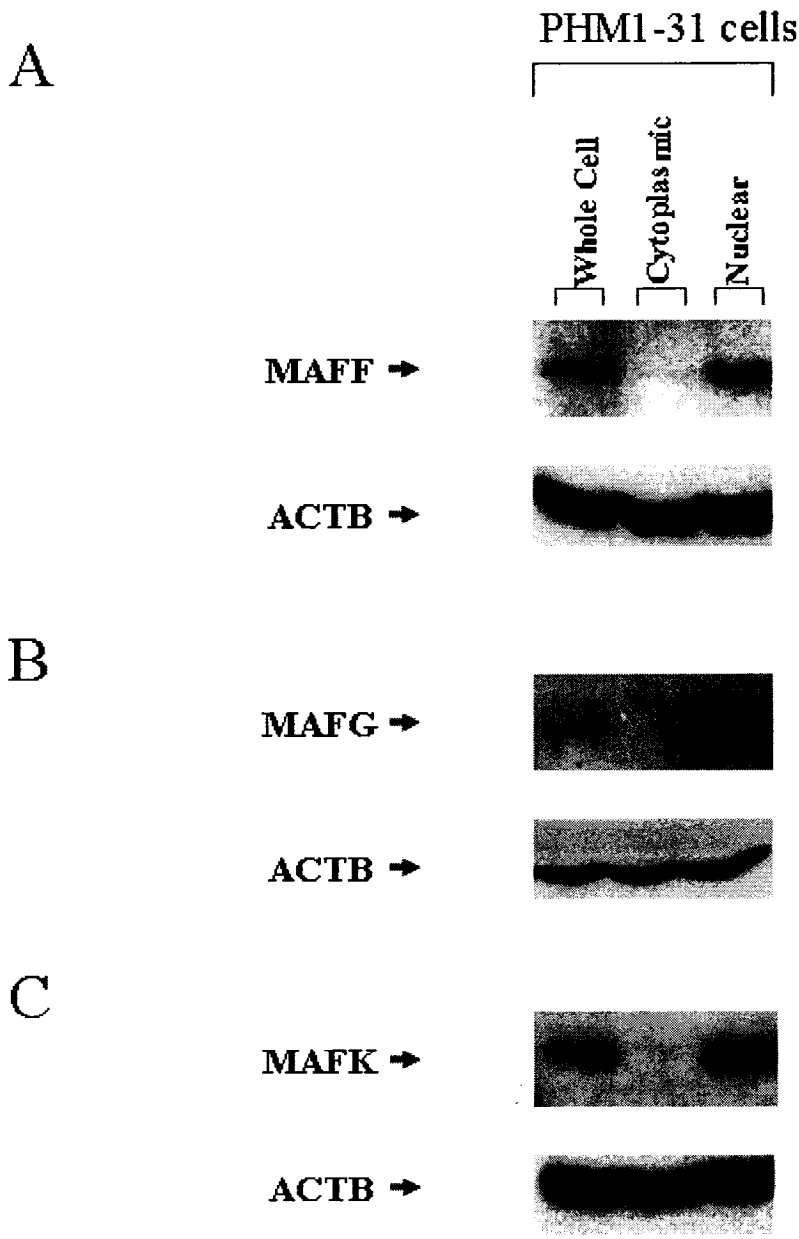

PHM1-31 cells
IL1B (1ng/ml) - + - + - +
actinomycin D (μg/ml) - - 5 5 - -
DMSO (%) (vehicle) - - - - 0.5 0.5
MAFF
ACTB

Fig.35 Cellular localization of small MAF protein in myometrial cells.

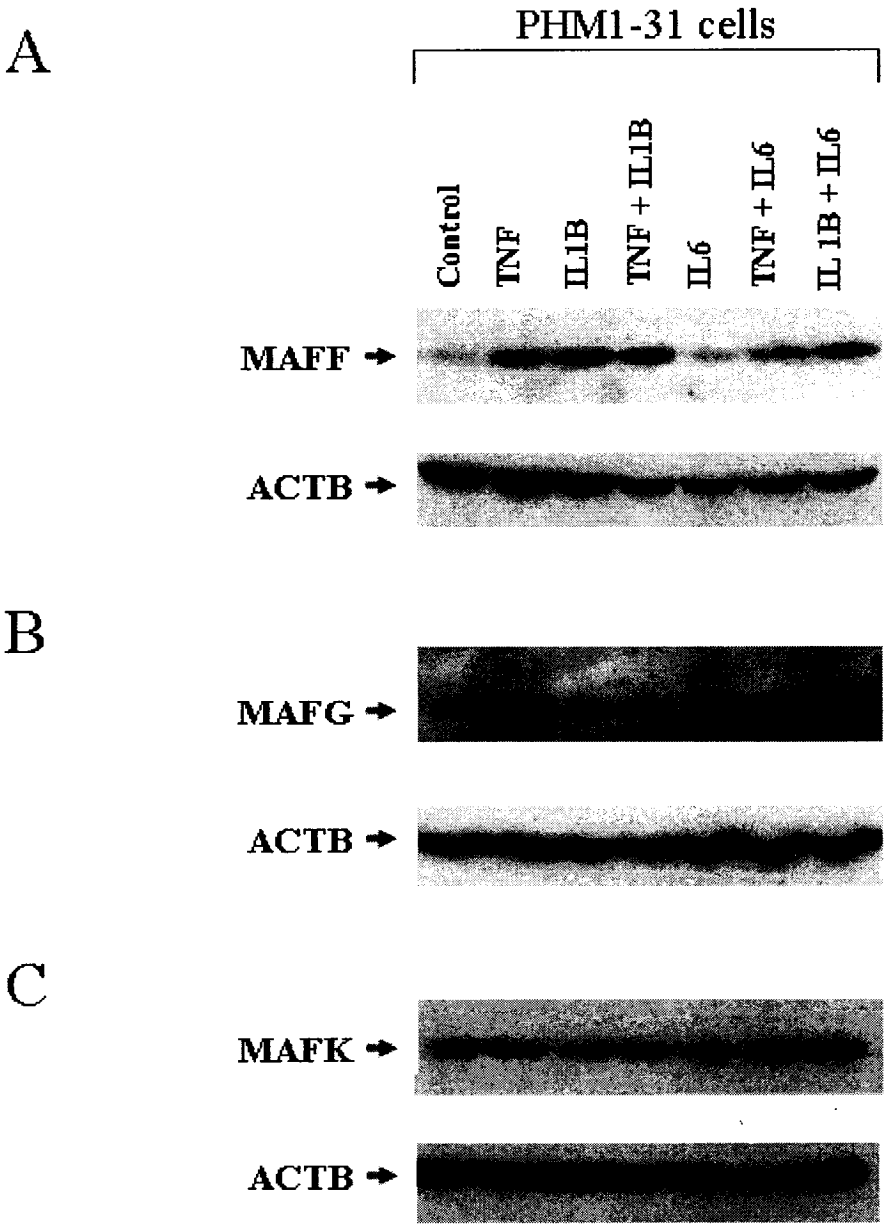
Immunoblot of whole cell, cytoplasmic or nuclear extracts prepared from PHM1-31 cells incubated with antisera specific for MAFF (**A**), MAFG (**B**) or MAFK (**C**) and ACTB. 20-30 μg of protein was loaded per well.


A
PHM1-31 cells
Whole Cell
Cytoplasmic
Nuclear
MAFF
ACTB
B
MAFG
ACTB
C
MAFK
ACTB

MAFF protein expression (Fig. 36A). In contrast, expression of MAFG and MAFK protein was not affected by cytokine treatment (Fig. 36B & 36C). Hence, cytokine regulation of MAFF mRNA and protein is coordinate.

Fig.36 Small MAF protein levels following cytokine treatment.

Immunoblot of nuclear extracts from PHM1-31 cells induced with cytokines for 3 hours. The blots were incubated with antisera specific for either MAFF (**A**), MAFG (**B**) or MAFK (**C**) and ACTB. 20-30 μg of protein was loaded per well.


A
PHM1-31 cells
Control
TNF
IL1B
TNF + IL1B
IL6
TNF + IL6
IL 1B + IL6
MAFF
ACTB
B
MAFG
ACTB
C
MAFK
ACTB

Chapter VII

DISCUSSION

Oxidative stress plays a significant role in the pathogenesis of a number of disorders such as preterm labor [135] and preeclampsia [133]. Oxidative stress may be defined as an imbalance between cellular production of ROS and antioxidant defence mechanisms. ROS are constantly produced as a result of metabolic reactions in living systems. The antioxidant defence pathway involves the upregulation of phase II enzymes which protect against the cell against the damaging effects of ROS. The expression of these enzymes, including HO and NQO1, is dependent on the activity of the ARE present in their promoters [113, 114]. NRF2 has emerged as an indispensable factor in coordinating the induction of these cytoprotective genes through binding to ARE. However, in order to achieve its transactivation potential NRF2 requires dimerization with one of the members of the small MAF or JUN/FOS family [91, 95, 115-118]. We investigated the role that the CNC and small MAF family members play in the antioxidant response and maintaining a healthy redox balance in placental and myometrial cells.

1. Modulation of arsenic-mediated oxidative stress in the placenta

We used arsenic, a known teratogen and developmental toxicant in many animal models [150], which is able to cross the placenta [145-147], to induce oxidative stress in JAR cells (derived from trophoblastic tumors of the placenta). We established a link between arsenic exposure and the rapid induction of nuclear NRF2 in this placental cell line [211]. It appears that the expression of the small MAFF factor is also slightly induced by arsenic, whereas the levels of the two other small MAFs, MAFG and MAFK, are not modulated. This is of interest as the

small MAF proteins are highly homologous, and functional redundancy has made it difficult to assign specific roles *in vitro* and *in vivo* for each of these factors [15, 50]. In contrast, studies performed in HeLa cells showed that *MAFG* mRNA levels are induced by arsenic [129]. Upon closer examination of these results, we also noticed the induction of a *MAF* homologous mRNA species by arsenic, with a transcript size corresponding to that of the *MAFF* gene [129]. Hence, it appears that regulation of the small MAFs by arsenic is cell line dependant.

We confirmed that arsenic causes oxidative stress in our JAR choriocarcinoma cell model, as shown by an increase in intracellular H_2O_2 levels (Fig. 5). In addition, we found the expression of the HO1 protein, the rate-limiting enzyme for heme degradation and a major stress responsive protein is significantly induced by arsenic in JAR cells (Fig. 6). Our results are in agreement with earlier reports showing upregulation of HO1 in the presence of arsenic in osteoblasts and macrophages [202, 212]. Furthermore, using EMSAs, we were able to show that arsenic treatment causes an increase in endogenous NRF2/small MAF heterodimer binding to the stress responsive element (StRE) recognition site (Fig. 4). The StRE is derived from the human HO1 promoter [126] and is structurally and functionally similar to the ARE [213]. Cross reactivity of the antiserum raised against MAFG, MAFF and MAFK prevents us from drawing specific conclusions on the nature of the small MAF forming the heterodimer with NRF2, but the slight upregulation of MAFF protein levels suggests that MAFF may be an important partner of NRF2 in the formed complex. Nevertheless, this does not exclude the possibility that all three members might be heterodimerizing with NRF2 in response to arsenic treatment.

Another laboratory has recently provided further evidence for the involvement of NRF2/small MAF heterodimers in arsenic-mediated oxidative stress in mouse hepa1c1c7 cell line [214]. Using ChIP analysis they demonstrated that arsenic treatment leads to increased binding of NRF2/small MAF heterodimers to the ARE enhancer of NQO1 [214]. They postulate that the small MAFs in these complexes are primarily MAFK and MAFG. However, we suggest that this still does not exclude MAFF as a player in the response to arsenic mediated oxidative stress because the small MAF antiserum (Santa Cruz) used in this study recognizes all three small MAFs.

Our results thus identify NRF2 as an important stress regulator in placental cells and confirm earlier data showing that NRF2 is involved in the response to arsenic insult in other cell types such as peritoneal macrophages, keratinocytes and osteoblasts [202, 203, 212].

Great strides have been made in unraveling the mechanism of NRF2 regulation. It has become clear that the BTB protein KEAP1 acts as a cytoplasmic repressor of NRF2 activity [113, 215, 216]. KEAP1, in association with CUL3 and the RING box protein 1 (RBX1), appears to form an E3 ubiquitin ligase complex that mediates the rapid degradation of NRF2 via the proteosome [124, 217] (Appendix A-Fig. 2). It is known that arsenic perturbs the natural oxidation and reduction balance through a variety of mechanisms that are involved in redox reactions with endogenous oxidants and cellular antioxidant systems [218]. This is of interest, as the redox capacities of many proteins reside in the sulfhydryl groups on cysteines. As proposed earlier [219], the accumulation of nuclear NRF2 may at least in part be mediated by modification of the sulfhydryl groups on cysteines in KEAP1 by arsenic. Hence, KEAP1

appears to serve as a core component in the regulation of NRF2, providing at least three functions: as a scaffold to anchor NRF2 with the cytoskeleton filaments in the cytoplasm, as a CUL3 substrate adaptor to bring NRF2 into the CUL3-dependent E3 complex for ubiquitination of NRF2, and as a sensor to interact with oxidative/electrophilic stimuli for induction of target genes.

In addition, it has been suggested by several groups that activation of NRF2 is also regulated by phosphorylation of this transcription factor [216]. ERK mitogen activated protein kinase (MAPK) activation has been shown to be required for NRF2 nuclear localization during pyrrolidine dithiocarbamate induction of glutamate cysteine ligase gene expression in HepG2 cells [220]. Induction of ERK MAPKs by arsenic has been observed previously in the human HaCat keratinocytes and JB6 mouse epidermal cell lines [221, 222]. Intriguingly, recent studies in our laboratory found that ERK MAPKs are activated in the presence of arsenic in our choriocarcinoma cell model.

Hence, we can propose that the ubiquity in the environment and the pleiotropy of toxicity of arsenic have thus provided selective pressure for evolution of various strategies to defend against oxidative stress mediated by toxic environmental contaminants.

2. Proinflammatory cytokines in the myometrium.

Proinflammatory cytokine-driven inflammation was initially recognized as a simple allergic reaction. Currently, inflammation is being considered to underline pathophysiology of a much broader spectrum of diseases than previously expected. Recent evidence suggests that

proinflammatory cytokines may play an important role in the events that lead to preeclampsia and preterm labor, causing morbidity and mortality of the fetus and serious health complications for the mother [166, 167].

One of the mechanisms by which infection and/or inflammation is believed to lead to labor is involves the infection-induced release of proinflammatory cytokines, which in turn stimulate prostaglandin synthesis via the induction of PTGS2 activity in gestational tissue, thereby causing uterine contractions [184]. On the other hand, the mechanism in which inflammation leads to preeclampsia is believed to be attributed to incomplete trophoblast invasion [223, 224]. This impaired placentation leads to placental hypoxia and reperfusion injury due to ischemia and the resultant oxidative stress triggers the release of proinflammatory cytokines and prostaglandins, which results in endothelial cell dysfunction [225].

These mechanisms are supported by several lines of evidence, for instance, elevated levels of IL1B are observed in the amniotic fluid only late in pregnancy, whereas with infection, the levels of IL1B, IL6, TNF and IL8 rise even further, and this increase is linked to the early onset of labor [167, 183]. Furthermore, the circulating levels of TNF and IL1B have been reported to be much more robust in preeclamptic women [226].

We set out to examine the involvement of some of the CNC and small MAF family members in proinflammatory cytokines mediated stress in the gestational organs, the placenta and the myometrium. We found an intriguing new link between proinflammatory cytokine signalling and MAF transcription factor gene regulation in uterine smooth muscle cells of the

myometirum. In our studies, we examined the small MAF expression in the myometrial cell line, PHM1-31. We found that PHM1-31 cells mimic primary myometrial cells with respect to morphology and upregulation of *MAFF* in response to cytokines [227, 228] and hence provide a valuable model to study myometrial gene expression and function. Most importantly, our novel data showed that *MAFF* mRNA is rapidly induced by IL1B and TNF in primary myometrial and PHM1-31 cells. The induction of MAFF also occurs at the protein level in the PHM1-31 cell line. It is of interest to note that IL6 does not modulate MAFF transcript and protein levels, especially when this is considered with the findings from another laboratory which postulate that IL6 acts downstream of IL1B and TNF in the inflammatory response of mice [229].

The two most prominent transcription factors responsible for the effects of TNF and IL1B are NFκB and AP1 [230]. Activation of NFκB depends on the signal-induced phosphorylation of IκB, which initiates the conjugation of the inhibitor to ubiquitin and its subsequent degradation by the proteosome. The liberated transcription factor NF-kB can then translocate to the nucleus and activate gene expression [231]. On the other hand, activation of the AP1 factors is regulated by induction of the c-JUN and c-FOS genes and by phosphorylation of both of its components; both levels of regulation are under the control of distinct mitogen activated protein kinase (MAPK) cascades [232].

Promoter analysis of the human *MAFF* gene revealed the presence of two relevant transcription factor binding sites (Appendix A-Fig. 3). An NFκB site, a well known downstream target for

IL1B [233] and TNF signalling [234] and a MARE binding site containing a core AP1 site. This suggests that MAFF may be regulated by both NFκB and AP1.

Experiments targeting MAPK and NFκB inhibition (Appendix B-6) were also carried out, however results were inconsistent. For further functional analysis, a Bacterial Artificial Chromosome (BAC) containing the promoter region of the MAFF gene was obtained and used to generate different promoter constructs (Appendix B-5) for future luciferase assays. Testing these constructs with different combination of proinflammatory cytokines would help determine the important regions within the MAFF promoter that are required for IL1B and TNF signal transduction.

ChIP assays would also be a great tool to verify the composition of the DNA binding sites found in the *MAFF* promoter. Furthermore, using MAFF dominant negative and wild type MAFF overexpression plasmids generated by our lab (Appendix B-2) should provide even more clues on the exact role the CNC and small MAFs may play in the response to proinflammatory cytokines mediated oxidative stress.

3. Proinflammatory cytokines in the Placenta.

In this respect, it is of interest to note that just like MAFF, we found that NRF3 mRNA and protein expression is also upregulated by the proinflammatory cytokine TNF in placental cells. The actions of TNF in pregnancy are not well understood, but there is evidence that it may play an important role in placental biology. For example, both placental cytotrophoblasts and choriocarcinoma cell lines express members of the TNF receptor superfamily suggesting an

autocrine role for this cytokine [235]. Furthermore, circulating levels of TNF are elevated in preeclampsia [161], which could contribute to this syndrome that is characterized by maternal high blood pressure and proteinurea. Increased TNF levels have also been observed in pathological placental tissue derived from intrauterine growth restricted (IUGR) pregnancies [164, 236].

Speculation on the role NRF3 may play in the placenta arose after viewing the expression pattern of NRF3. In contrast to the widely expressed NRF1 and NRF2 genes, NRF3 transcripts are primarily present in the placenta (Fig. 6), although they are expressed at lower levels in a series of other tissues [85]. This data hinted at a possible function for NRF3 in the placental TNF signalling cascade and led us to further explore the possible role of the NRF3 transcription factor in the placenta.

We found *NRF3* mRNA is expressed in placental chorionic villi throughout gestation, from at least week 12 to term [207]. *NRF3* mRNA is thus present during the period when fetal cells penetrate maternal tissue and develop connections with the maternal vasculature, a process termed endovascular invasion, that takes place during the first half of pregnancy [237].

As placental chorionic villi are made up of multiple cell types including cytotrophoblasts, syncytiotrophophoblasts, fibroblasts and macrophages among others, we performed additional analyses in specific cell types. We found that high *NRF3* transcript levels are present in cytotrophoblast progenitor cells and in cytotrophoblasts from cell islands, representing cells that are differentiating down the invasive pathway. In contrast, the stromal component,

placental fibroblasts, do not or only minimally express *NRF3* transcripts [207]. Interestingly, we also observed a similar pattern of *MAFF* expression to that of *NRF3* (Fig. 25). Additionally, we were the first to show that MAFF heterodimerizes with NRF3 and binds to NFE2/MARE binding sites (Fig. 26C), allowing us to speculate that NRF3/MAFF heterodimers may be involved in essential cytotrophoblast functions or possibly in the molecular mechanisms that specify important cell fate decisions that include acquisition of tumor cell-like invasiveness.

Besides NRF3, other transcription factors also belonging to the AP1 family are expressed in specific placental celltypes, for example cytotrophoblasts switch from expressing c-JUN to JUND as they differentiate and invade the uterine wall [156]. JUNB deficient mice die of placental abnormalities during embryogenesis in part due to defects in the spatial distribution of trophoblast giant cells, which correspond to the invasive cytotrophoblasts in the human placenta [238]. As it has been shown that NRF2 can interact with JUN proteins [239], it is probable that NRF3 may interact with other members of the AP1 family in the placenta to regulate specific target genes.

What are the potential target genes of transcription factors in placental cells? Like all tissues, placental development involves cell proliferation, differentiation and apoptosis [240]. We hypothesize that genes that play important roles in placental function, such as placental hormones like placental lactogen, may represent possible NRF3 targets [240]. In addition, matrix metalloproteinases (MMPs) such as MMP9 that are essential for the degradation of extracellular matrix and thus for tissue invasion, are highly regulated in the placenta [156]. In this context, it is interesting to note that both, placental lactogen and MMP9 can be regulated

by AP1 transcription factors as indicated by the presence of typical AP1 regulatory sequences in their promoter regions [241, 242]. As CNC/small MAF heterodimers can bind to a subset of AP1 like recognition sites, these genes may be potential targets for NRF3.

Our simplified hypothetical model (Appendix A-Fig.4) depicts the CNC family members, NRF2 and NRF3, and MAFF playing a central role in maintaining the redox balance in myometrial and placental cells. In the placenta, MAFF heterodimerizes with NRF2 in order to upregulate phase II detoxifying enzymes such as NQO1 and HO1. In the myometrium MAFF may heterodimerize with one of these CNC members and prevents ROS from reaching dangerous levels and thus allowing myometrial muscles to remain in quiescent state until the fetus fully matures and is ready to be delivered.

On the other hand, our hypothetical model (Appendix A-fig. 4) illustrates that in conditions where exogenous sources of ROS or inflammation, such as environmental arsenic contamination or bacterial LPS infection, lead to increased amounts of ROS generation tipping of the balance that the CNC/MAFF heterodimers struggle to maintain. Once overpowered the effects manifest into overproduction of PTGS2 and hence early muscle contractions or in the case of placenta poor placentation and hence the clinical symptoms of preeclampsia.

In conclusion, we provide here the first evidence that NRF2/small MAF complexes may also play a role in the cellular stress response in placental cells. This is important as arsenic appears to cause developmental toxicity leading to spontaneous abortions, birth defects and embryonic lethality [145-147]. We also suggest a possible role for NRF3 and MAFF in the control of

transcriptional events in placental and myometrial cells in response to proinflammatory cytokines. This is important because of the possible role that proinflammatory cytokines may play in preterm and normal term labor [167].

Three new experimental approaches have recently emerged that will prove to be powerful tools that should facilitate the study of the molecular signal transduction pathways involved in premature labor and preeclampsia, respectively. The first approach established by Zingg et al. consists of layering non-transformed human myometrial cell lines on a collagen lattice and then assessing the surface area of the lattices before and after treatments. This system allows for the direct assessment of muscle contractions. Furthermore, it would be interesting to investigate effects of transfecting the MAFF dominant negative and the wild type MAFF overexpression plasmids and observing the effects that they may have on myometrial contractions. We can speculate that whereas MAFF dominant negative might lead to muscle contractions, the overexpression of MAFF would be concentration dependant.

The second approach, involves the activation of soluble fms –like tyrosine kinase 1 (sFLT1). sFlt1, a splice variant of the vascular endothelial growth factor (VEGF) receptor Flt1 lacks the transmembrane and cytoplasmic domains, and acts as a potent VEGF antagonist [243]. There is circumstantial evidence that antagonism of VEGF may have a role in hypertension, proteinuria and angiogenesis [244, 245].

Another interesting model to use is the BPH/5, which is an inbred mouse model of preeclampsia with borderline hypertension before pregnancy [246]. BPH/5 mice develop

hypertension, proteinuria, and endothelial dysfunction during late gestation. The mice, like humans, are more likely to have impaired placental development and their fetuses are smaller, further making them a good model for the research.

It would be intriguing to examine the regulation of NRF3 and MAFF factors in these mice during different stage of gestation. Furthermore, one can study the involvement of these factors after using an oral treatment of an antioxidant such as Tempol, which has been shown to prevent the onset of preeclampsia in pregnant mice (Hoffmann et al., 2006 unpublished).

Therefore, using these systems to test the involvement of the CNC and small MAF members, might help in the quest to understand the cause, as well as to test preventative and therapeutic strategies in the management of premature labor and preeclampsia.

The biological mechanisms by which ROS exerts their harmful activities are still not well understood at this time. To completely assess the potential their adverse health risks in various exposure situations, it is important not only to understand their mechanism(s) of actions but also the mechanisms in which the body responds to its toxic effects. This research should eventually lead to a better understanding of the molecular mechanisms governing CNC/small MAF factors-mediated regulation of gene expression and help to uncover their possible functions in gestational disorders.

Chapter VII

Contributions to Original Knowledge

The results presented in this thesis have shed a new light on the role of the CNC family members, NRF2 and NRF3 in the placenta. These studies are the first to link the small MAF family member MAFF to proinflammatory cytokine signalling which plays a major role in oxidative stress which contributes to many human diseases. The candidate's major contributions to original knowledge are summarized below:

1. The candidate was the first to identify endogenous NRF2/small MAF heterodimers DNA binding in response to arsenic in placental cells.

2. The candidate was the first to show full length NRF3/MAFG heterodimers bind the NFE2/MARE binding site (Fig. 26C) and is also the first show that MAFF heterodimerizes with p45, NRF1, NRF2 and $NRF3_{525}$ and that these heterodimers bind to NFE2/MARE binding sites (Appendix A-Fig. 5). He also demonstrated the presence of the CNC members' transcripts (*NRF1*, *NRF2* and *NRF3*) in the PHM1-31 cell line (Appendix A-Fig. 6).

3. The candidate provides the first evidence that CNC/small Maf complexes may also play a role in the cellular stress response in placental cells. Furthermore, his results are in agreement with earlier reports showing upregulation of HO1 in the presence of arsenic. He is the first to reproduce this in placental cells.

4. The candidate demonstrated that proinflammatory cytokines regulate MAFF, and that this regulation occurs at the transcriptional level in uterine smooth muscle cells.

5. The candidate was the first to generate specific human MAFF antisera and to confirm the presence of MAFF protein in the nucleus.

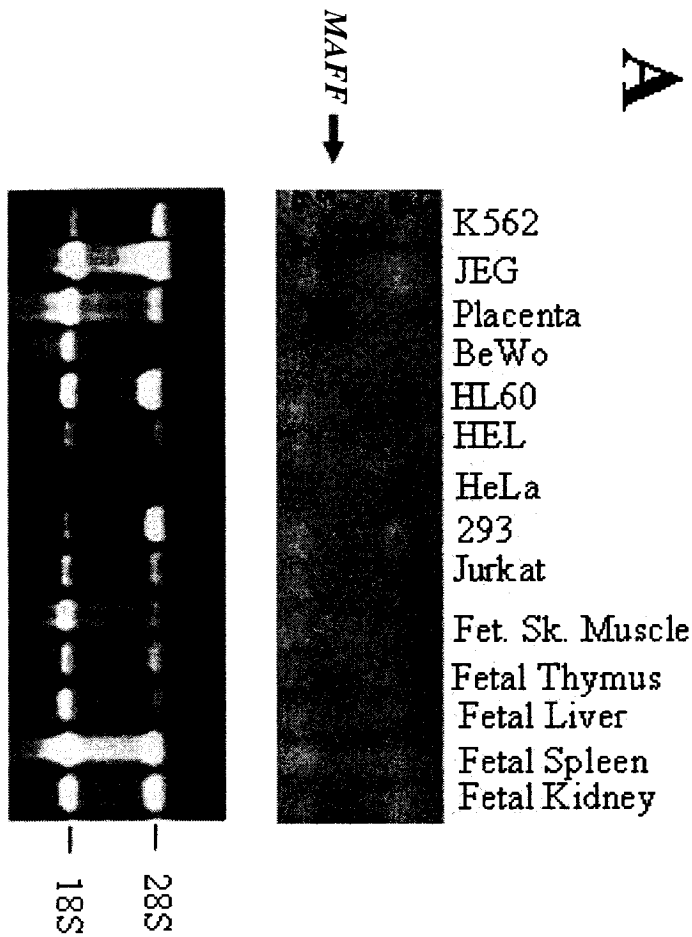
6. Finally, the candidate also aided in determining that globin gene activation by heme is independent of the putative HRMs in the p45 subunit of the NFE2 transcription factor by showing that the mutated versions are expressed and can still recognize a NFE2 DNA binding element.

Chapter VIII (Appendices)

Appendix A: Results

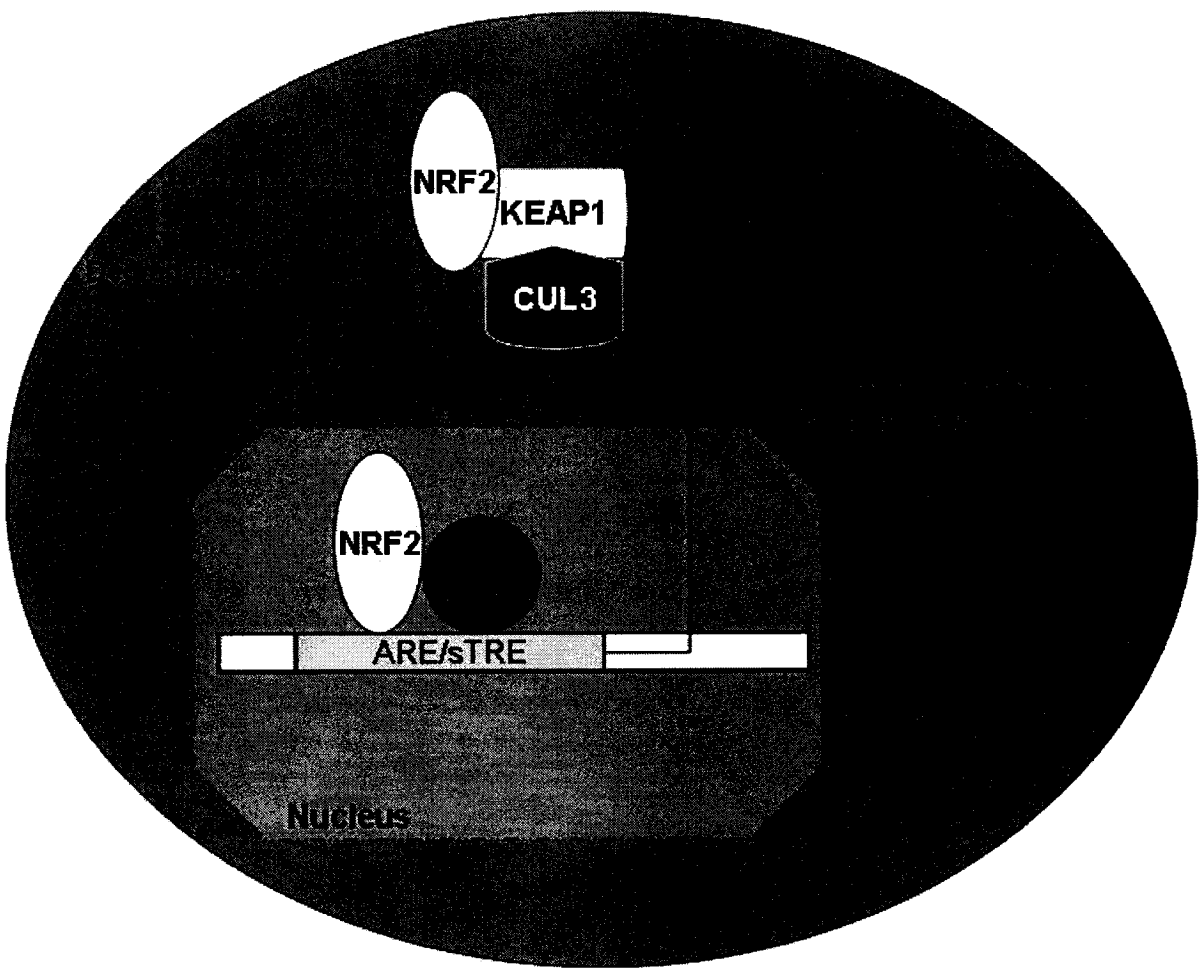
Appendix A-Fig. 1 *MAFF* gene expression

MAFF transcript levels in various human tissues and cell lines.


A
MAFF
K562
JEG
Placenta
BeWo
HL60
HEL
HeLa
293
Jurkat
Fet. Sk. Muscle
Fetal Thymus
Fetal Liver
Fetal Spleen
Fetal Kidney
18S
28S

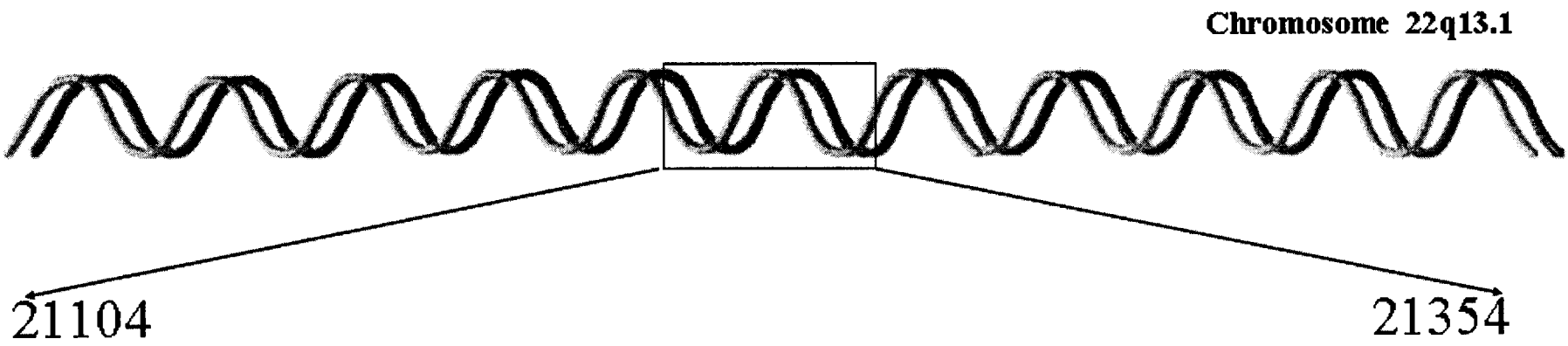
Appendix A-Fig. 2 NRF2 Regulation Model

Involvement of NRF2 in ARE-mediated transcription obviates the involvement of the small MAF partners in order to bind to the ARE sequence. NRF2/small MAF heterodimers are involved in the regulation of detoxifying enzyme genes and in the response to oxidative stress.


NRF2
KEAP1
CUL3
NRF2
ARE/sTRE
Nucleus

Appendix A-Fig. 3 *MAFF* Promoter Analysis

Nucleotide Sequence of the human MAFF promoter region. Location of potential response elements in the 5'- flanking resion are marked. (*) Asterix indicates the transcriptional start site.



agagggccaggaccactacctgtaaaatgagccaatgacacctacaccccGG

GGCTCTgcgaggtgtcacgtgaacgcacccagcgagccctggaaccag
NF-kB

gcagagaatcgcggcagccccgggggcggggcttcaggggcggggtcata

cgggggcggggccgagggaccaagcggccgctcctggccggagggcggcc

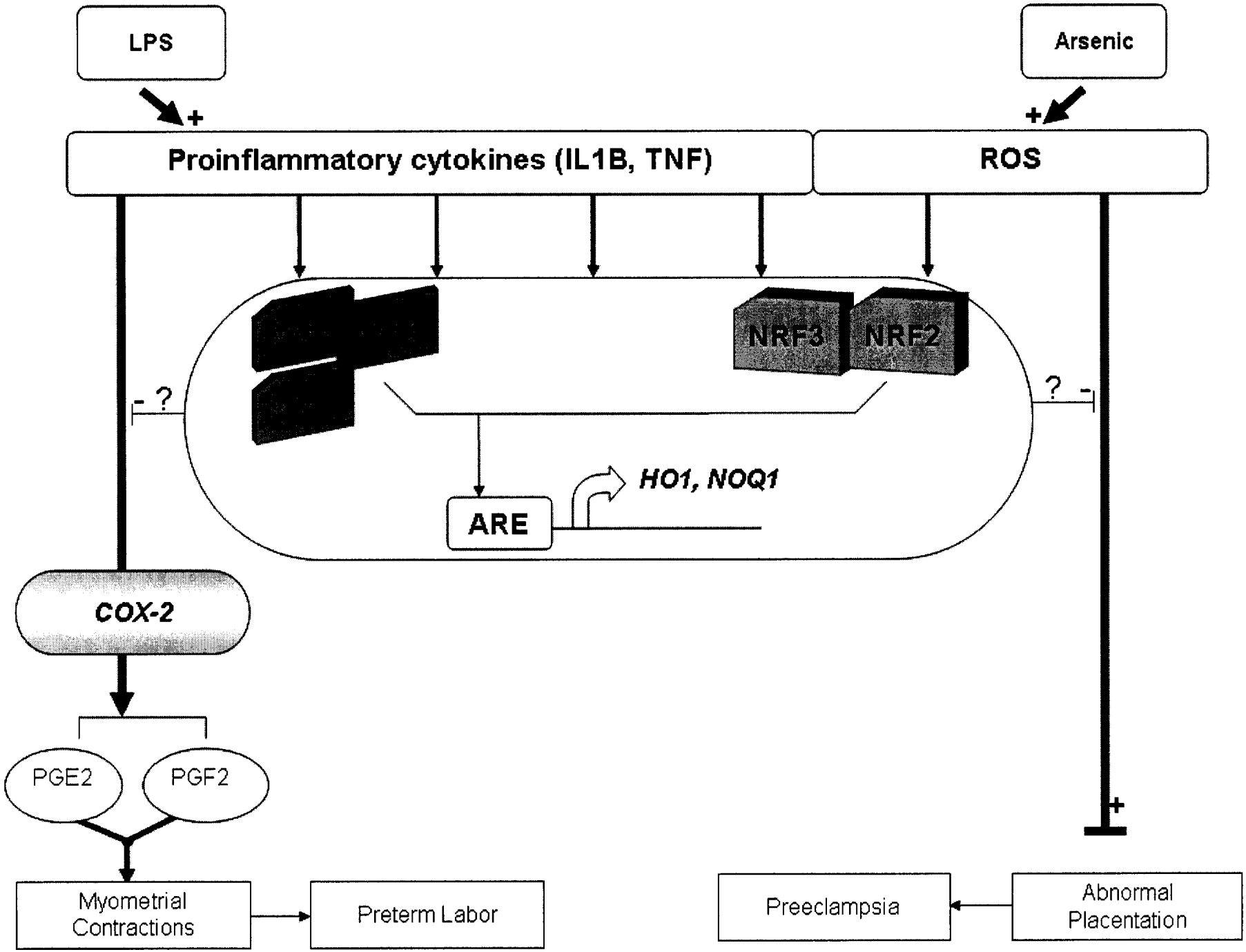
gggtcggtGACGTCaccgcatgactgggtTTTTATgaatgaaagga
MARE TATA Box

aggaatcctgtgagtgagtaattccgggaagctcg*ccttacaactccgcgc

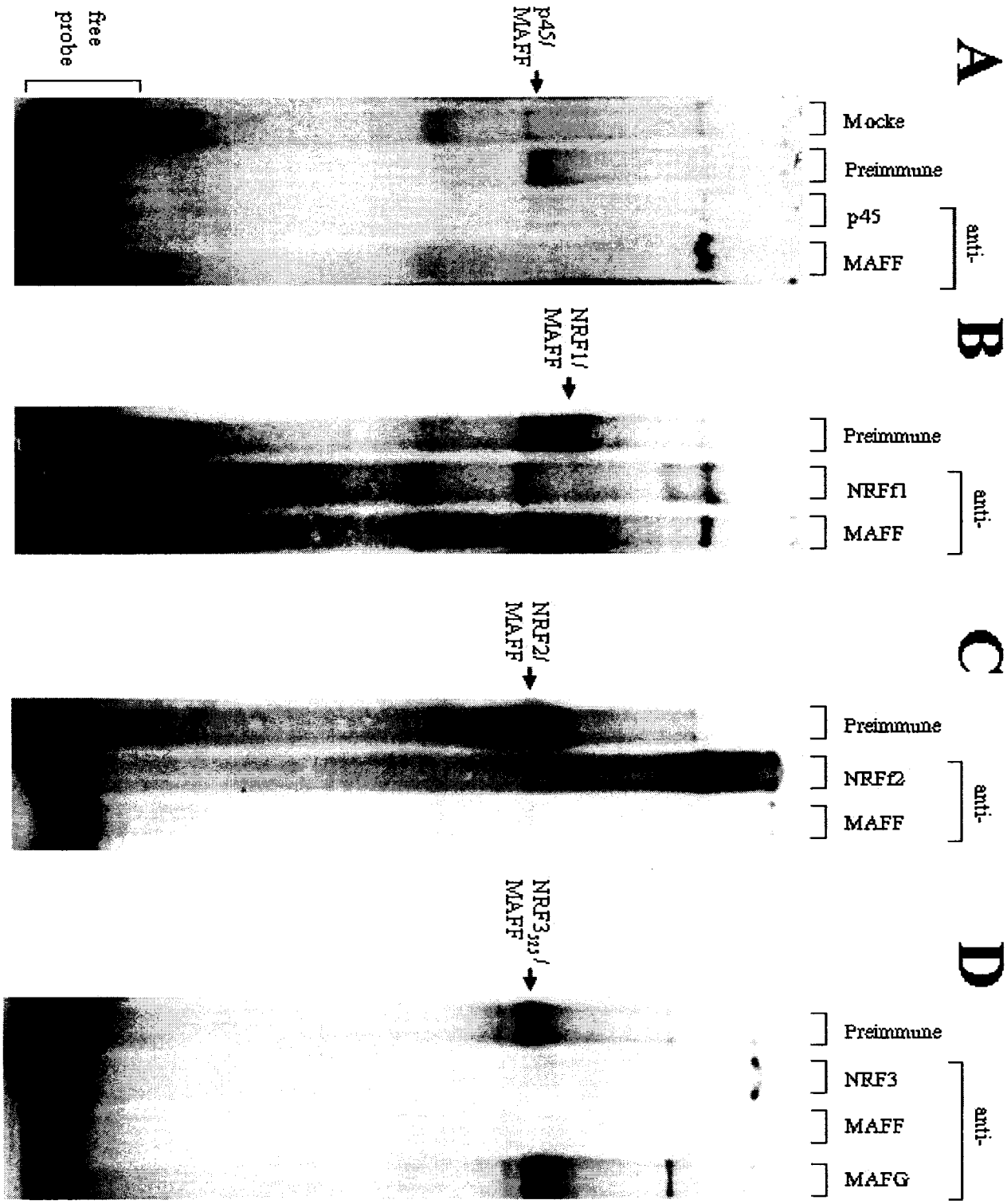
Appendix A-Fig. 4 Hypothetical Model for CNC/Small MAFs network in the Myometrium and the Placenta.

The CNC family members, NRF2 and NRF3, and MAFF playing a central role in maintaining the redox balance in myometrial and placental cells. In the placenta, MAFF heterodimerizes with NRF2 in order to upregulate phase II detoxifying enzymes such as NQO1 and HO1. In the myometrium MAFF may heterodimerize with one of these CNC members and prevents ROS from reaching dangerous levels and thus allowing myometrial muscles to remain in quiescent state until the fetus fully matures and is ready to be delivered.

On the other hand, when exogenous sources e.g. arsenic contamination or bacterial LPS infection lead to an increased amounts of ROS generation they tip the redox balance. The effects manifest into overproduction of PTGS2 and hence early muscle contractions or in the case of placenta poor placentation and hence the clinical symptoms of preeclampsia.

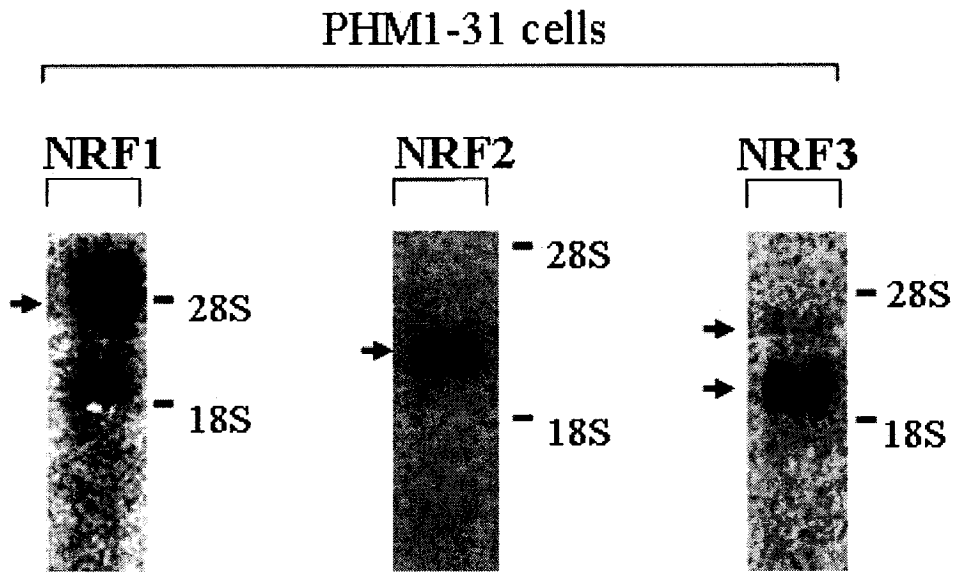

LPS
Arsenic
+
+
Proinflammatory cytokines (IL1B, TNF)
ROS
NRF3
NRF2
? -
- ?
ARE
HO1, NOQ1
COX-2
PGE2
PGF2
+
Myometrial Contractions
Preterm Labor
Preeclampsia
Abnormal Placentation

Appendix A-Fig. 5 Heterodimerization of human CNC family members with MAFF. NFE2 DNA binding site oligonucleotide derived from the human PBGD promoter was used as a probe in an EMSA to detect binding activity in nuclear extracts from HEK293T cells transfected with constructs coding for MAFF and either **A**, p45; **B**, NRF1 **C**, NRF2, **D**, NRF3 (525). Non-labeled competitor human PBDG promoter oligonucleotide, pre-immune serum (PI) and/or p45, NRF1-, NRF2-, NRF3-, MAFF-specific antisera were added to the reaction mix as indicated. Arrows indicate the position of the different CNC protein/MAFF heterodimers.


A
free probe
p45/ MAFF
Mocke
Preimmune
p45
MAFF
anti-
B
NRF1/ MAFF
Preimmune
NRFf1
MAFF
anti-
C
NRF2/ MAFF
Preimmune
NRFf2
MAFF
anti-
D
NRF3ss/ MAFF
Preimmune
NRF3
MAFF
MAFG
anti-

Appendix A-Fig. 6 CNC family members expression in PHM1-31 cells

Northern analysis of total RNA prepared from PHM1-31 cells. *NRF1, NRF2* and *NRF3* transcripts are indicated.


PHM1-31 cells
NRF1
NRF2
NRF3
28S
18S
28S
18S
28S
18S

Appendix A: Results

Members of the MAF and CNC families of bZIP transcription factors play diverse roles in mammalian gene regulation, differentiation, oncogenesis and development [15, 50]. Initial experiments sought to characterize the role that the MAF and CNC families play in erythropoiesis. Erythropoiesis is the process in which new erythrocytes are produced and entails the production of hemoglobin. Hemoglobin synthesis requires the coordinated production of heme and globin [247]. Heme is the prosthetic group that mediates reversible binding of oxygen by hemoglobin. Globin, which is comprised of two α-chains and two β-chains, is the protein that surrounds and protects the heme molecule. In particular, studies on the erythroid specific transactivator NFE2 showed that it promotes the opening of chromatin throughout the β-globin loci [248-250]. Furthermore, it has been reported that even though the binding of RNA polymerase II to the LCR is independent of NFE2, its recruitment to the hyperacetylated β-globin promoter requires NFE2 [251]. We showed that induction of β-globin gene transcription in CB3 cells by hemin (a form of heme) is dependent on the presence of NFE2. CB3 cells have proved to be a useful tool in the study of NF E2 function in erythroid differentiation since they do not express the p45 subunit of NF-E2 due to a Friend leukemia virus insertion in one allele and the loss of the other [252, 253]. Thus, CB3 cells express only minimal levels of α- and β-globin mRNA.

We proceeded to investigate the mechanism of regulation of p45 by heme. Previous studies have shown that the CNC protein BACH1, a homolog of p45 and a repressor of globin gene transcription, is a heme binding protein and is negatively regulated through the presence of heme regulatory motifs (HRM)s [254]. Newer evidence further supports a role for the HRMs in the negative regulation of mitochondrial import of ALAS1, a mitochondrial enzyme that

catalyzes the first step of the heme biosynthetic pathway. These findings show that mutation of the HRMs reverse the inhibition of ALAS1's import into the mitochondria, indicating that the HRMs are essential for the heme-mediated inhibition of ALAS1 transport into the cell [255]. Comparison among different HRM motif-containing proteins previously suggested that only the cysteine-proline (CP) dipeptide is absolutely conserved (Appendix A-Fig. 7A) [256]. This is further supported by the alignment of HRM sequences found in various proteins that have been shown to confer regulation by heme [254, 256-261]. We noted two putative HRMs, both comprising a CP dipeptide, in the amino terminus of p45 that are conserved among different species at amino acids 4/5 and 66/67 (Appendix A-Fig. 7B). We performed mutagenesis of two potential HRMs in p45 and found that the mutated versions (A4, A66 and A4/A66) are expressed (Appendix A-Fig. 8A) and can still recognize an NFE2 DNA binding element (Appendix A-Fig. 8B). In addition, we showed that p45 HRM mutants are able to restore β-globin gene transcription in CB3 cells upon induction by hemin (Appendix A-Fig. 8C). Contrary to our hypothesis, these results suggest that globin gene activation by heme appears to be independent of the putative HRMs in the p45 subunit of the NFE2 transcription factor [262]. Similar findings were reported in the case of heme oxygenase 2 (HO2), an isoform of HO1 which also catalyzes the conversion of heme to biliverdin and CO, and releases of free Fe^{2+} [261]. One may reason that in these cases, HO2 and p45 may function as "heme sensors" in which heme bound by the HO2 or p45 HRMs would be protected from degradation and that when the concentration of heme exceeds the binding capacity of HRMs then excess heme would become available to serve as substrate for catalysis.

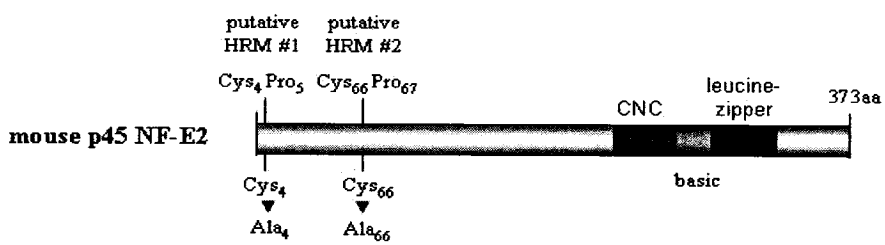
Appendix A-Fig. 7 Putative HRM motifs in p45 NF-E2

(A) Alignment of HRM motifs in proteins that have been reported to confer regulation by heme. This includes the HAP1 and Bach1 transcription factors as well as the enzymes aminolevulinic acid synthase 1, aminolevulinic acid synthase 2, cytochrome c_1 heme lyase and heme oxygenase 2, **(B)** Diagram illustrating the putative HRMs comprising the CP dipeptide in the p45 subunit of the NF-E2 transcription factor. The cysteine to alanine mutations introduced by site-directed mutagenesis are shown. CNC, bZIP domains are indicated. Conservation of the CP dipeptide in the p45 protein of different species is shown.

A

Hon et. al, 2000	
HAP1	KCPINH
(*S. cerevisiae*)	KCPVDH
	KCPVDH
	RCPVDH
	KCPVDH
	RCPIDH
	KCPVYQ
Ogawa, et. al, 2001	
Bach1	LCPKYR
(*Mus musculus*)	QCPAEQ
	ECPWLG
	NCPFIS
	PCPYAC
	DCPLSF
Munakata et. al, 2004	
Aminolevulinic acid synthase 1	RCPFLS
(*Rattus norvegicus*)	NCPKMM
Lathrop et. al, 1993	KCPFLA
Aminolevulinic acid synthase 2	SCPVLS
(*Mus musculus*)	RCPILA
Steiner et. al, 1996	HCPFML
Cytochrome c_1 heme lyase	KCPVDE
(*S. cerevisiae*) *McCoubrey et. al,1997*	
Heme oxygenase 2	KCPFYA
(*Rattus norvegicus*)	NCPFRA

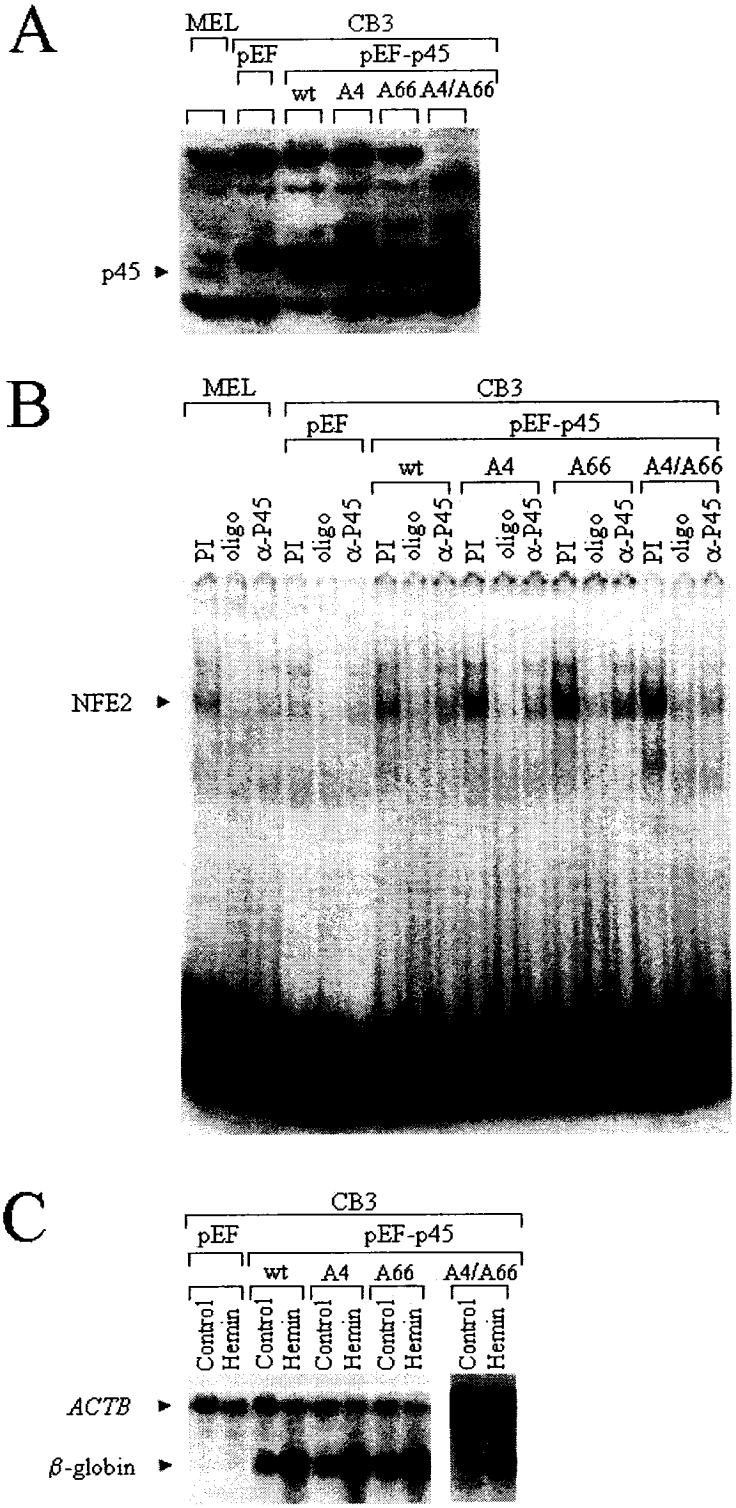
B



p45 NF-E2	putative HRM #1	putative HRM #2
Homo sapiens	.SPCPPQQS..	..TYCPCSIHP..
Pongo pygmaeus	.SPCPPQQS..	..TYCPCSIHP..
Bos taurus	.SPCPPQQS..	..SYCPCSIHS..
Mus musculus	.PPCPPQQN..	..TYCPCSIHP..
Rattus norvegicus	.PPCPPQPN..	..TYCPCSIHP..

Appendix A-Fig.8 Analysis of putative HRMs in p45 NF-E2

(A) Immunoblot analysis of nuclear extracts prepared from MEL cells and CB3 cell clones stably transfected with the pEF1αneo expression vector without insert (pEF) or coding either for p45 wild-type (wt), p45-A4, p45-A66 or p45-A4/A66. For immunoblot analysis a p45 specific antiserum was used [52]. Equal protein amounts (10μg) have been loaded per lane. The p45 protein is indicated by an arrow, **(B)** EMSA analysis of nuclear extracts from MEL cells and CB3 cell clones. An oligonucleotide corresponding to the NF-E2 in the porphobilinogen deaminase promoter has been used as a probe. Non-labeled NFE2 binding site competitor oligonucleotide (oligo), pre-immune (PI) or anti-p45 NF-E2 serum (α-p45) was added to the reaction as indicated, **(C)** Northern blot analysis of total RNA prepared from MEL cells and CB3 cells clones. 10μg of total RNA was loaded per lane. *β-globin* and *ACTB* transcripts are indicated.


A
MEL
CB3
pEF
pEF-p45
wt
A4
A66
A4/A66
p45
B
MEL
CB3
pEF
pEF-p45
wt
A4
A66
A4/A66
PI
oligo
α-P45
NFE2
C
CB3
pEF
pEF-p45
wt
A4
A66
A4/A66
Control
Hemin
ACTB
β-globin

Appendix B: Materials and Methods

The candidate has also generated plasmids and optimised assays that although yielded no publishable results will be essential for future studies.

1. Ribonuclease Protection Assay plasmid Constructs

339BAC: We preformed a PCR using the Bacterial Artificial Chromosome (BAC447C4 MP1) DNA, which was obtained from a source in the United Kingdom. The primers used were 339F1"33951BAC" (5'-CCTTTTGTGTCCCGATCCTA-3') and 339R1" 33613BAC" (5'-CGTAGCCACGGTTTTTGAGT-3'). The fragment was then cloned directly into pCRBluntII.

2. Dominant Negative and Overexpression plasmids.

The dominant-negative MAFF mutant was constructed by overlap extension using PCR. The full length human MAFF fragment was obtained by performing RT-PCR on PHM1-31 total RNA using MAFF-F2 (5'-GGGCACCTTCTGCAAACATGT-3') and MAFF-R2 (5'-GAGGCGGCGCTCAGGCACTTT-3'). The fragment was then cloned into pCRBluntII yielding the pCRBluntII P-31hMAFF plasmid. Mutant oligonucleotide primers used in the PCR were as follows: hMAFFDomNeg F4 (5'-GGCGCCGCACACTCGCCGCGGCTGCTACGCCG-3') and hMAFFDomNeg R1 (5'-GCGTAGCCAGCCGCGGCGAGTGTGC -3'). The resulting PCR fragment was cloned into the pCR-BluntII-TOPO vector (pCRBluntII) (Invitrogen) to yield pCRBluntII hMafFDN.

Subsequently, the MAFF dominant negative fragment was subcloned into pTRE2, pMT2 and pEF1α expression vectors.

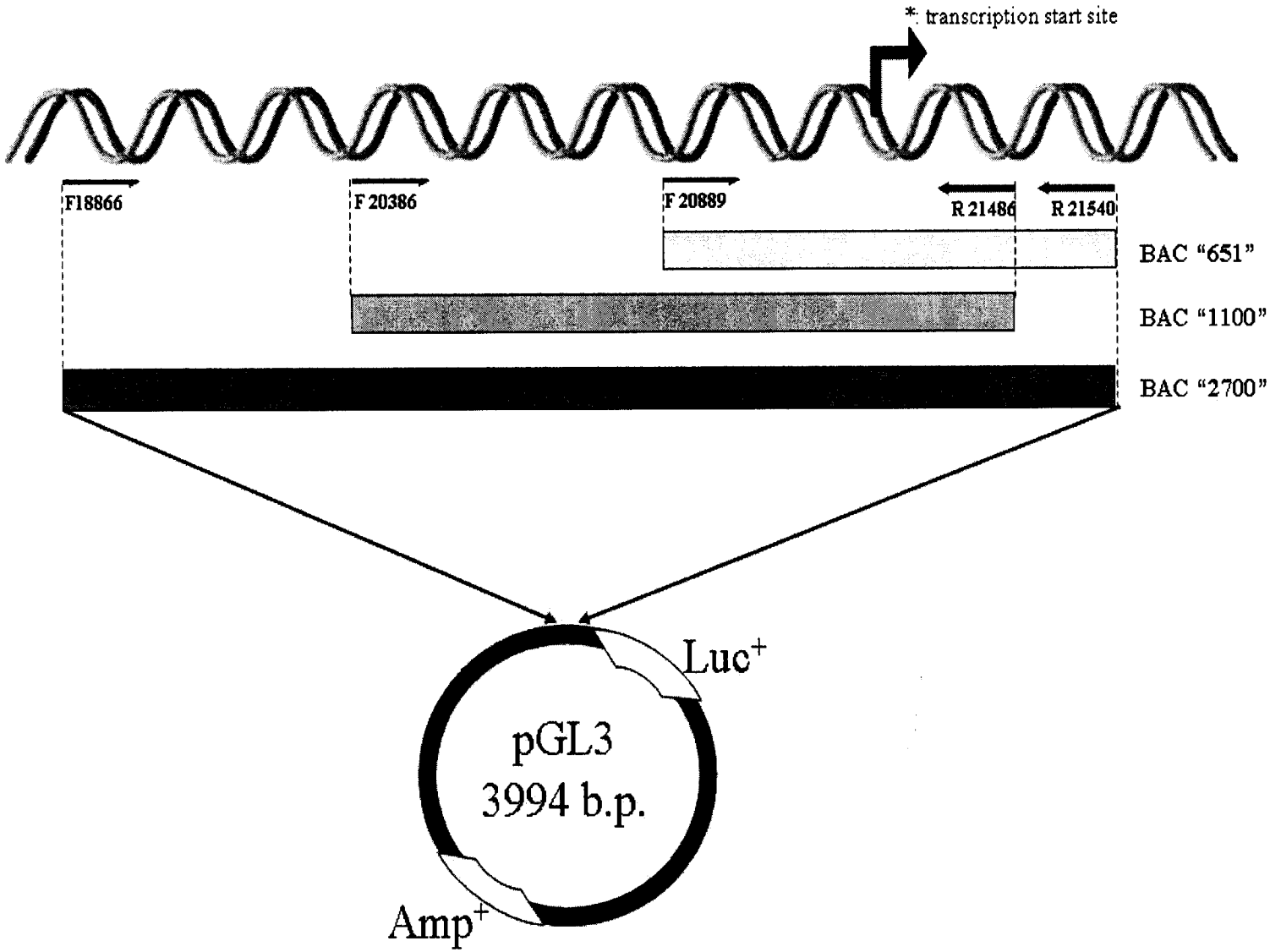
4. OTR Plasmid

cDNA fragments were recovered by RT-PCR using these respective oligonucleotides. Oxytocin Receptor (OXYR): OTR-F2 (5'-GGGTCATGGAGGGCGCGCTCGCA-3') and OTR-R1 (5'-GGGTCACGCCGTGGATGGCTG-3'). Resulting amplicon was cloned into pCR-BluntII-TOPO vector (Invitrogen). Full length cDNA was used as a probe for northern blotting.

5. MAFF Luciferase Reporter Constructs

All cDNA fragments were recovered by PCR on the BAC447C4 MP1 DNA using their respective oligonucleotides.

651BAC: MAFFBAC "21486"R(5'-CTGAGCCGCAGGTCGCTCTGA -3') and MAFFBAC"20889"F(5'-GGGCTGACGATCAGGAGCGGGTT -3'). 1.1BAC: MAFFBAC"21486"R(5'-TGAGCCGCAGGTCGCTCTGA-3') and MafFBAC"20386"F(5'-GACTGCTTTAACCAGGGTGGTCA-3').


*: transcription start site
F18866
F 20386
F 20889
R 21486
R 21540
BAC "651"
BAC "1100"
BAC "2700"
Luc+
pGL3
3994 b.p.
Amp+

6.1 NFκB Inhibition

Calpain inhibitor I (Sigma-Aldrich) was resuspended in DMSO to a concentration of 25μg/ml. It was then added to the cells at a concentration of (20μM, 50μM, 100μM, and 20μM) one hour prior to induction by IL1B.

6.2 MAPK inhibition

One hour prior to adding IL1B, 3μM specific ERK inhibitor U0126 (Promega) or 5μM p38 inhibitor SB202190 (Calbiochem) was added to the cells.

7. Primer Extension Analysis

PKS229 was obtained by subcloning the fragment yielded from the digestion of PCRBI651 with SacI into PKS. PKS229 was then linearized using XhoI and the riboprobe was produced using T7.

Chapter IX

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