

André da Soledade Carruna Coelho

CHARACTERIZATION OF THE EFFECT OF CARTILAGE
ACIDIC PROTEIN (CRTAC) ON HUMAN MONOCYTES
THP-1 CELLS



Universidade do Algarve
Faculdade de Ciência e Tecnologia

2016

André da Soledade Carruna Coelho

CHARACTERIZATION OF THE EFFECT OF CARTILAGE
ACIDIC PROTEIN (CRTAC) ON HUMAN MONOCYTES
THP-1 CELLS

Dissertação de Mestrado em Biotecnologia

Trabalho elaborado sob a orientação de:

Professora Doutora Deborah M. Power

Doutora Liliana Anjos



Universidade do Algarve

Faculdade de Ciência e Tecnologia

2016

DECLARAÇÃO DE AUTORIA

Declaro ser o(a) autor(a) deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

A handwritten signature in cursive script, reading "André da Soledade Carruna Coelho", is written on a light-colored rectangular background.

(André da Soledade Carruna Coelho)

COPYRIGHT

A Universidade do Algarve não poderá publicar nem tornar pública esta tese ou os seus conteúdos sem o consentimento das orientadoras e do autor.

ACKNOWLEDGEMENTS

My exceptional thanks go to my thesis supervisors Professora Doutora Deborah M. Power and Doutora Liliana Anjos for their dedication and time spent to help me throughout my academic journey. I appreciate the support given in the laboratory experience and knowledge acquisition, as also the opportunity to work and learn in their team.

A special thanks to Doutora Zélia Velez for all her help during my laboratory experiments and knowledge transmitted.

I'm also thankful to all members of CCMAR for the dedication and time spent to help me, particularly to Doutora Rute Félix and Doutor João Cardoso.

Acknowledgments to Doutor Nuno Rodrigues dos Santos, from Centro de Biomedicina Molecular e Estrutural (CBME), that kindly provide my first THP-1 cell culture for my initial work.

Finally, my acknowledgment and gratitude to my family, especially to my mother for all her love and support, friends, work colleagues and master classmates for their friendship and advice.

This research was funded by the Portuguese National Science Foundation (FCT) (Project: PTDC/MAR/122296/2010) and Operational Competitiveness Programme (COMPETE), Quadro de Referência Estratégico Nacional (QREN) and European Regional Development Fund (ERDF). It was also supported by FCT through the project UID/Multi/04326/2013-plurianual at CCMAR.

ABSTRACT

Cartilage acidic protein 1 (CRTAC1) is an extracellular matrix protein of chondrogenic tissue associated with several diseases, such as multiple sclerosis. *crtac* duplicates emerged in teleost fish as a result of the teleost specific whole genome duplication. The poorly characterized function of CRTAC's was the starting point of the present study. RT-PCR and Western blot analysis demonstrated that the HEK 293 cell line expresses human (h) CRTAC1. *In silico* analysis of the protein using bioinformatic tools indicated that hCRTAC1 and sea bass (*Dicentrarchus labrax*, dl) CRTAC1 and CRTAC2 proteins is most likely located in mitochondria, lysosomes, golgi apparatus and endoplasmic reticulum. Western blot analysis of subcellular fractions of HEK 293 cells indicated that hCRTAC1 is present in the nucleus, endoplasmatic reticulum and mitochondria. Trypan blue exclusion assay with THP-1 cells, indicated that dlCRTAC1 has an inhibitory effect on cell proliferation, did not promote cell death and increased cell adherence with an IC₅₀ of 78.45ng/mL and dlCRTAC2 at 95.50ng/mL after 72 hours of cells incubation. In Hanging drop assays, dlCRTAC1 (30ng/mL) increased the size of THP-1 spheroids and the circularity of the spheroids increased when incubated with dlCRTAC1 (3 and 30ng/mL) and hCRTAC1 (3 and 30ng/mL), for 72 hours. Wound scratch assays with HEK 293 cells, indicated that dlCRTAC1 (0.03ng/mL), dlCRTAC2 (300ng/mL) and hCRTAC1 (0.03 and 0.3ng/mL) inhibited wound healing and that dlCRTAC1 (30ng/mL) and hCRTAC1 (300ng/mL) promoted wound healing, after 18 hours of incubation. In motility assays with macrophage, dlCRTAC1 (300ng/mL) increased cell motility and dlCRTAC2 (30ng/mL) inhibited macrophage motility. These results suggest that CRTAC proteins inhibit THP-1 cells proliferation but promote their adhesion and aggregation and dlCRTAC1 increased and dlCRTAC2 inhibited macrophage motility. Wound healing assays with HEK293 cells revealed that low concentrations of CRTAC inhibited wound healing and higher concentrations promoted wound healing.

Keywords: CRTAC, THP-1, HEK 293, subcellular location, cell adhesion, cell aggregation

RESUMO

A proteína acídica da cartilagem 1 (CRTAC1), também conhecida por proteína expressa por condrócitos 68 kDa (CEP-68), é uma proteína da matriz extracelular da camada condrogénica da cartilagem, utilizada como marcador *in vitro* para diferenciação entre condrócitos e osteoblastos. Esta proteína encontra-se presente noutros locais do corpo humano, existindo dois transcritos variantes: *CRTAC1-A* (presente na cartilagem e pulmões) e *CRTAC1-B* (presente no cérebro, olho, cristalino do olho e na glândula pineal).

Esta proteína tem um peso molecular de 68 kDa e é constituída por 661 aminoácidos, incluindo um péptido de sinal de 27 aminoácidos, localizado na zona N-terminal. É constituída por vários domínios, dos quais: um domínio integrina N-terminal, que contém quatro motivos FG-GAP; seguido de um domínio ASPIC/UnbV; e um domínio EGF (do inglês, *epidermal growth factor*) C-terminal. Encontra-se ainda ao longo da sua sequência proteica um motivo RDG.

Os motivos FG-GAP (FG, motivo conservado fenilalanina-glicina; GAP, motivo conservado glicina-alanina-prolina) são encontrados na zona N-terminal de α e β integrinas, uma família de proteínas que medeia a interação célula-célula e célula-matriz extracelular. Os motivos tripeptídicos RGD (motivo conservado arginina-glicina-ácido aspartático), em comum com os motivos FG-GAP, estão presentes em muitas integrinas e ligantes de integrina. O domínio ASPIC/UnbV encontra-se também associado a interações célula a célula e célula a matriz extracelular. A presença dos motivos FG-GAP 3 e 4, tal como o domínio EGF e a natureza acídica da proteína, são responsáveis pela sua capacidade de se ligar a catiões divalentes como o cálcio.

A CRTAC1 foi inicialmente patenteada como biomarcador de fratura óssea e lesão da cartilagem e, numa segunda patente, como biomarcador e alvo terapêutico de várias doenças, tais como: problemas cardiovasculares, hematológicos, gastrointestinal, urológicos, doenças respiratórias, problemas inflamatórios, distúrbios neurológicos e vários tipos de cancro. A CRTAC1-B é um antagonista endógeno dos receptores Nogo-1, cuja neutralização está associada à regeneração axonal após a lesão, e pacientes que sofrem de esclerose múltipla (uma desordem desmielinizante auto-imune do sistema nervoso central, causada pelo acúmulo de monócitos nesses locais) revelam uma presença incomum desta proteína no líquido cefalorraquidiano. Contudo, ainda não é

conhecida a função destas proteínas nem o seu papel no desenvolvimento destas patologias.

A análise filogenética, com proteínas de diversas espécies, revelou que estas proteínas são ancestrais, uma vez que se encontram presentes desde as cianobactérias até aos vertebrados. No ramo dos vertebrados, surge um cluster correspondente à CRTAC1 (presente em teleósteos e tetrápodes) e outro cluster (presente em teleósteos) que corresponde a uma proteína ortóloga, denominada de CRTAC2, que difere pela ausência do domínio EGF C-terminal. A existência de duas formas de CRTAC em teleósteos é consistente com a hipótese de que o genoma dos peixes teleósteos foi submetido a um evento específico de duplicação total do genoma. As características altamente conservadas e sua manutenção durante os processos evolutivos sugerem que estas proteínas possuem funções vitais que são comuns pelos vários em filós.

A proteína CRTAC1 dos teleósteos possui alta similaridade na sequência de aminoácidos à semelhança das regiões N-terminal e média da CRTAC1 humana (hCRTAC1), indicando que estas podem ter funções semelhantes. A CRTAC1 e CRTAC2 possuem uma distribuição tecidual generalizada de transcritos e proteínas, sendo encontrados no sistema nervoso (glândula pituitária, cérebro e espinal medula), gónadas, fígado, rim, coração, músculo e estruturas calcificadas. Estudos de hibridação *in situ* em pituitárias de *S. auratus*, demonstram uma grande quantidade de RNAm de CRTAC2 em pequenas células de forma irregular com grandes núcleos adjacentes ao tecido neurohipofisários, entre os lóbulos anterior e posterior. A libertação de CRTAC2 a partir da glândula pituitária de *S. auratus* pode sugerir que esta proteína, para além de ser uma proteína de matriz extracelular, pode também ter também funções endócrinas.

A estrutura ancestral e conservada das proteínas CRTAC indica que estas devem ter uma função importante, que ainda não foi caracterizada, sendo este o ponto de partida deste estudo. A análise por RT-PCR e Western blot demonstram que a linha celular HEK 293 expressa a proteína hCRTAC1. A localização subcelular *in silico* da proteína hCRTAC1 e das proteínas de robalo (*Dicentrarchus labrax*, dl) CRTAC1 e CRTAC2 indicam que as CRTACs podem estar presentes nas mitocôndrias, lisossomas, aparelho de Golgi e no retículo endoplasmático. A análise por Western blot de fracções subcelulares de células HEK 293, sugere fortemente que a hCRTAC1 poderá estar presente no núcleo, retículo endoplasmático e nas mitocôndrias.

O teste de exclusão de Trypan Blue com células THP-1, indica que a dlCRTAC1 tem um efeito inibidor da multiplicação celular (IC_{50}) na concentração de 78.45ng/mL e a dlCRTAC2 a 95.50ng/mL, no entanto, não foi observada morte celular, mas sim o aumento da aderência celular. O ensaio de MTS indica que a dlCRTAC1 inibe a proliferação celular. Nos ensaios de hanging drop, a dlCRTAC1 aumenta o tamanho dos agregados de células THP-1 e a circularidade dos agregados aumenta quando incubadas com a dlCRTAC1 e a hCRTAC1.

Os ensaios de wound scratch com células HEK 293, indicam que a dlCRTAC2 inibe a capacidade de cicatrização enquanto que a dlCRTAC1 e a hCRTAC1 promovem esta capacidade. No ensaio de motilidade com macrófagos, a dlCRTAC1 aumenta a motilidade celular e a dlCRTAC2 inibe a motilidade destas células. Estes resultados sugerem que as CRTACs inibem a proliferação de células THP-1, mas promovem a sua capacidades de adesão e agregação; inibem (em baixas concentrações) e promovem (em concentrações mais elevadas) a capacidade de cicatrização de culturas de células HEK 293 em monocamada; e que a dlCRTAC1 promove a motilidade dos macrófagos enquanto que a dlCRTAC2 inibe a motilidade dos mesmos.

Palavras-chave: CRTAC, THP-1, HEK 293, localização subcelular, adesão celular, agregação celular

TABLE OF CONTENTS

ABBREVIATIONS.....	IX
LIST OF FIGURES	XII
LIST OF TABLES	XIV
CHAPTER I	1
1. GENERAL INTRODUCTION.....	2
1.1. BIOTECHNOLOGY CONTEXT.....	2
1.2. CARTILAGE ACIDIC PROTEIN (CRTAC) DISCOVERY.....	2
1.2.1. Human CRTAC1 genomic structure.....	3
1.2.2. Human CRTAC1 protein domains.....	4
1.3. CRTAC EVOLUTION.....	5
1.3.1. Teleost CRTAC1 and CRTAC2.....	7
1.4. CRTAC FUNCTIONS AND FUNCTIONAL DOMAINS.....	8
1.5. CRTAC AND HUMAN DISEASES.....	11
1.5.1. Immune system and autoimmunity.....	12
1.5.2. Human monocytes.....	13
1.5.2.1. THP-1 cell line.....	15
1.5.3. Integrin-like proteins and the immune system.....	16
2. OBJECTIVES.....	18
CHAPTER II	20
1. INTRODUCTION.....	22
2. MATERIAL AND METHODS.....	25
2.1. BIOLOGICAL MATERIALS.....	25
2.1.1. Cell lines.....	25
2.1.2. Recombinant human and fish CRTAC proteins.....	25
2.1.3. Antibodies.....	25
2.2. METHODS.....	26
2.2.1. Cell culture maintenance.....	26
2.2.2. Detection of the human <i>CRTAC1</i> transcript and protein in THP-1 monocytes, Macrophage and HEK 293 cells.....	26
2.2.2.1. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.....	26
2.2.2.2. Protein extraction and western blot analysis.....	27

2.2.3. Subcellular location of human CRTAC1.....	29
2.2.3.1. Prediction of subcellular location.....	29
2.2.3.2. Western blot subcellular location analysis.....	29
2.2.4. Cell culture assays.....	29
2.2.4.1. Cell viability assay.....	30
2.2.4.2. Flow cytometry.....	30
2.2.4.3 Cellular cycle assay.....	31
2.2.4.4. Hanging drop assay.....	31
2.2.4.5. Wound healing assay.....	32
2.2.4.6. Cyclic AMP assay.....	32
2.2.5. Cell transfection and CRTACs stable cell line.....	33
2.2.5.1. Mammalian CRTAC's expression vector constructs.....	33
2.2.5.2. HEK 293 cell transfection.....	34
2.2.6. Data Treatment.....	35
3. RESULTS.....	36
3.1. DETECTION OF HUMAN <i>CRTAC1</i> TRANSCRIPTS AND PROTEIN IN CELL CULTURES.....	36
3.2. SUBCELLULAR LOCATION OF HUMAN CRTAC1.....	38
3.3. CELL CULTURE ASSAYS.....	41
3.3.1. Teleost CRTAC's effect on THP-1 cell viability.....	41
3.3.2. Effect of CRTAC's on THP-1 monocytic cell line differentiation.....	43
3.3.3. CRTAC's effect on THP-1 monocytes cell cycle.....	44
3.3.4. CRTAC's potentiates THP-1 monocytes cell aggregation.....	45
3.3.5. Effects of CRTAC's in the wound healing process and cell motility.....	47
3.3.6. CRTAC's effect on THP-1 cAMP production.....	49
4. DISCUSSION AND CONCLUSION.....	52
5. FUTURE PERSPECTIVES.....	59
REFERENCES.....	60
APPENDICES.....	68

ABBREVIATIONS

A

ASPIC1 Acidic secreted protein in cartilage-1

C

cAMP Cyclic AMP

CD Clusters of Differentiation

cDNA Complementary deoxyribonucleic acid

CEP-68 Chondrocyte Expressed Protein 68

CRTAC Cartilage Acidic Protein

D

DMEM Dulbecco's Modified Eagle's Medium

DMSO Dimethyl sulfoxide

DTT Dithiothreitol

E

ECM Extracellular matrix

EDTA Ethylenediaminetetraacetic Acid

EGF Epithelial Growth Factor

EGTA Ethylene Glycol Tetraacetic Acid

EST Expressed Sequence Tag

F

FBS Fetal Bovine Serum

Fc Crystallisable Fragment region

FG- GAP Phenylalanine/Glycine-Glycine/Alanine/Proline

G

GPI Glycosylphosphatidylinositol

H

HEK 293 Human Embryonic Kidney 293 cells

HepG2 Human liver hepatocellular carcinoma cells

HUVEC-2 Human Umbilical Vein Endothelial Cells

I

IBMX	3-Isobutyl-1-methylxanthine
ICAM1	Intercellular adhesion molecule 1

K

kDa	Kilodalton
-----	------------

L

LB	Laemmli sample loading buffer
LPS	Lipopolysaccharides
LFA-1	Function-associated antigen-1

M

Ma cells	Macrophages derived from THP-1 cell cultures
Mac-1	Macrophage-1 antigen
MALT	Mucosa-Associated Lymphoid Tissue
mRNA	Messenger ribonucleic acid
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), inner salt
M-CSF	Macrophage colony-stimulating factor

N

NgR1	Nogo receptor-1
NO	Nitric oxide

P

PBS	Phosphate Buffered Saline
PenStrep	Penicillin and Streptomycin
PMA	Phorbol-12-myristate-13-acetate

R

Raw 264.7	Abelson murine leukemia virus-induced tumor cells
RGD	Tripeptide Arginyl-glycyl-aspartic acid
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute

RT-PCR	Reverse transcriptase-polymerase chain reaction
S	
SDS	Sodium Dodecyl Sulfate
SP	Signal peptide
T	
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline - Tween 20
THP-1	Human acute monocytic leukemia cell line
TRL	Toll-Like Receptor
U	
UTR	Untranslated region
V	
VCBS	Vibrio, Colwellia, Bradyrhizobium, and Shewanella domain

LIST OF FIGURES

Figure 1. Scheme of the genomic structure and splice variants of the human <i>CRTAC1</i> gene.....	3
Figure 2. Schematic representation of the main structural domains of Human CRTAC1 protein.....	4
Figure 3. Phylogenetic tree of CRTAC1/CRTAC2 protein family.....	6
Figure 4. Phase contrast microscopy of THP-1 and macrophage-like cell line cultures.....	15
Figure 5. Expression of human <i>CRTAC1-A</i> transcripts in cDNA from HEK 293, THP-1 and Ma cells.....	36
Figure 6. Western blot revealing hCRTAC1 expression in HEK 293 cell lines.....	37
Figure 7. Predicted subcellular location of human and teleost CRTAC proteins.....	40
Figure 8. Western blot revealing subcellular location of hCRTAC1 in HEK 293 cells.....	40
Figure 9. Effect of CRTAC's on human monocytes THP-1 cell viability using a MTS assay.....	42
Figure 10. Effect of dlCTRAC1 and 2 on human monocyte (THP-1) cell viability using a Trypan blue exclusion test.....	43
Figure 11. Analysis by flow cytometry of surface expression of the CD11b macrophage marker in cells exposed or depleted of human and teleost CRTAC's proteins.....	44
Figure 12. Effect of CRTAC's on THP-1 cell cycle.....	45
Figure 13. Effect of CRTAC's on THP-1 monocyte aggregate total area (μm^2) and circularity by hanging drop assay.....	46
Figure 14. Formation of small dark areas in THP-1 cell spheroids in a hanging drop.....	47

Figure 15. The effects of CRTAC's during the wound healing process Using a HEK 293 cell assay.....	48
Figure 16. Effects of CRTAC's on macrophage (Ma) derived THP-1 cell motility.....	49
Figure 17. Effects of CRTAC's on cAMP production by THP-1 cells.....	50

LIST OF TABLES

Table 1. Sequence of primers designed to amplify human and <i>D. labrax</i> CRTAC genes for cloning into the pcDNA TM 3.1 expression vector.....	34
Table 2. Predicted subcellular localization of human and teleost CRTAC's.....	39

CHAPTER I

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

The thesis is organized in the format of a scientific paper as foreseen in the regulamentos de Mestrados da Universidade do Algarve. However, initially a detailed overview is given about CRTAC and the objectives of the thesis project are presented before moving to chapter II. The second chapter is organized in the format of a scientific publication and has a short introduction, methods, the original results and a discussion. The MSc thesis work was carried out in part-term since the author has a full time job in Faro Hospital (Centro Hospitalar do Algarve, EPE).

1.1. PLACING THE THESIS IN THE CONTEXT OF BIOTECHNOLOGY

Biotechnology is an area of research that explores and investigates organisms and biological processes with the perspective of identifying products and tools useful in sub-fields such as agriculture (green biotechnology), industry (white biotechnology), medicine (red biotechnology) and marine (blue biotechnology) areas. Globally, the diversity of marine plants and animals is largely unstudied and they represent a vast reserve of resources that may lead to the discovery of new bioactive compounds, materials and unique processes. The present study is firmly based in blue biotechnology as it is focused on two novel marine derived proteins with potential biomedical application and compares them with the human orthologous protein, which is associated with several pathologies but has an unknown function.

1.2. CARTILAGE ACIDIC PROTEIN (CRTAC) DISCOVERY

The cartilage acidic protein 1 gene (*CRTAC1*) was first identified in humans by Steck *et al.*, in 2001, and was designated a novel gene marker for cultured chondrocytes and was originally named as chondrocyte expressed protein 68kDa [CEP-68, also known as acidic secreted protein in cartilage-1 (ASPIC1)]. This gene encodes a glycosylated extracellular matrix protein (ECM) of articular deep zone cartilage and is also found in tissues other than the skeleton and transcript variants occur also in brain, lung, eye, lens, pineal and parathyroid gland (Benz *et al.*, 2002; Rabinowitz *et al.*, 2005; Turner *et al.*, 2007; Steck *et al.*, 2007; Nakajima *et al.*, 2010). The tissue distribution of *cartac1* and the protein structure suggests it may have a multitude of functions most of which still remain to be characterized.

1.2.1 Human *CRTAC1* genomic structure

The human (h) *CRTAC1* gene was identified in chromosome 10 (Steck *et al.*, 2007) which contains one disease-susceptibility locus linked to Alzheimer disease (Grupe *et al.*, 2006). The gene is encoded in a genomic region of 164 kb, in the genomic contig NT_030059, and is located at the cytogenetic q22 band of the long arm of the chromosome (Steck *et al.*, 2007).

The protein is encoded by a predicted open reading frame of 1959 nucleotides. The gene contains 15 exons and 15 introns (Figure 1.A) all of which possess consensus 5' and 3' splice sites and a polyadenylation signal is found 463 nucleotides after the stop codon. The first form of *CRTAC1* identified, splices in exon 15A and was called *CRTAC1-A*, and is 16 amino acids longer than the splice variant *CRTAC1-B* (Figure 1.B). The variant *CRTAC1-B* results from splicing in of exon 15B and the resulting gene transcript encodes a protein with a modified C-terminus, nonetheless the epithelial growth factor (EGF)-Ca²⁺ binding domain (encoded by exon 14 and described later in this work) remains unchanged (Steck *et al.*, 2007) (Figure 1.B).

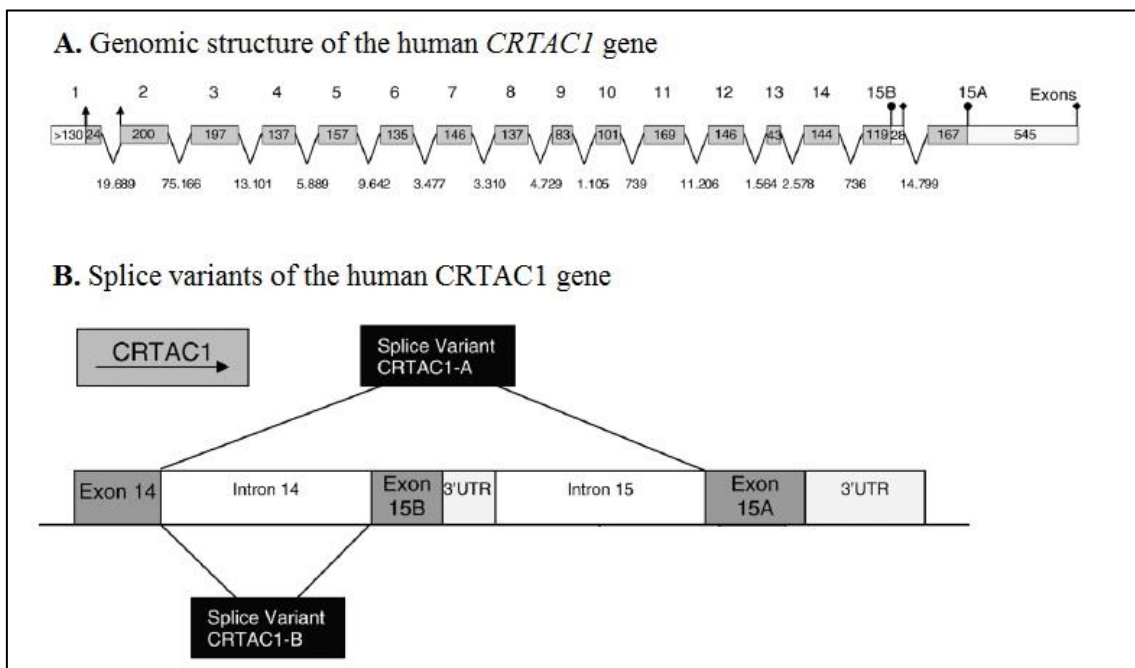


Figure 1. Scheme of the genomic structure and splice variants of the human *CRTAC1* gene (Extracted from Steck *et al.*, 2007). **A.** The Genomic structure of the human *CRTAC1* gene. The gene has 15 exons and 15 introns. The boxes correspond to exons and the exon length is indicated in each exon and the 5' and 3'-untranslated region (UTR) is represented by the light grey boxes. Numbers below the gene represent intron length (lengths in base pairs; ▲ indicate alternative start codons, ● stop codons and ◆ the polyadenylation site). **B.** Splice variants of the human *CRTAC1* that results from alternative splicing of exons of the 3' region of the gene, which gives rise to proteins with a different C-terminus. In variant

CRTAC1-A splicing occurs with exon 15A and the intervening sequences are lost, while *CRTAC1*-B splices in exon 15B (Steck *et al.*, 2007).

An additional splice variant of the human *CRTAC1* gene from keratoconus cornea was also reported (Rabinowitz *et al.* 2005), that results from alternatively spliced exons at the 5' region of the gene (exons 1a, 2a and 2b), and the transcript encodes a protein with a longer N-terminal region that has a different amino acid sequence and includes several cysteine residues that are lacking in other variants, although the function of this protein has not been studied so far.

Northern blot analysis of human tissue samples revealed that only *CRTAC1*-A is expressed in cartilage and lung whereas *CRTAC1*-B is expressed in the brain, eye, lens and pineal gland (Steck *et al.*, 2001; Rabinowitz *et al.*, 2005; Turner *et al.*, 2007). Both transcripts exist in brain however *crtac1*-B is the predominant form (Steck *et al.*, 2007).

1.2.2 Human CRTAC1 protein domains

Human CRTAC1-A has a molecular weight of 68 kDa and is composed of 661 amino acids including a 27 amino acid signal peptide at the N-terminus. Several domains and motifs were initially identified and described in the human protein sequence (Steck *et al.*, 2007): FG-GAP conserved motifs (phenylalanine/glycine-glycine/alanine/proline; amino acids 46-88, 105-147, 283-333 and 395-437) are located in the N-terminal integrin α chain like domain, ASPIC/UnbV domain (amino acid 458-527) and EGF-like (Epithelial Growth Factor-like) calcium binding domain (amino acid 559-605) are located at the C-terminus (Figure 2). A RGD motif (arginyl-glycyl-aspartic acid; amino acid 271-273) is also found in the protein sequence.

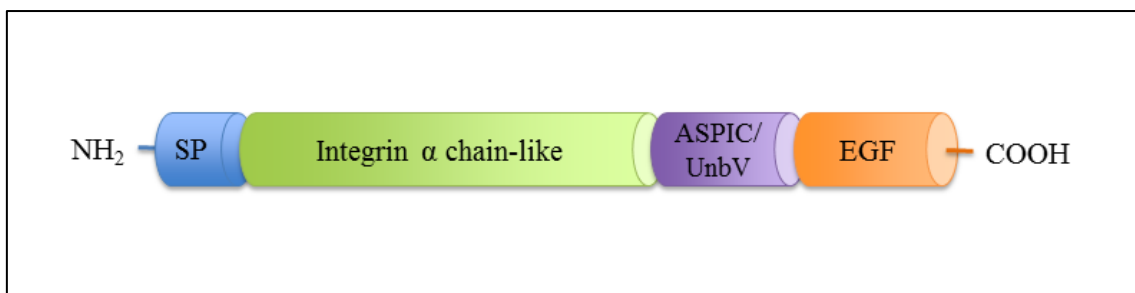


Figure 2. Schematic representation of the main structural domains of human CRTAC1 protein. The protein possesses a signal peptide (SP) in the N-terminus followed by an integrin α chain-like, ASPIC/UnbV (ASPIC) and EFG-like Ca^{2+} binding (EFG) domains in the C-terminus (based on Steck *et al.*, 2007).

The FG-GAP repeats are commonly found in the N-terminal region of α and β integrins, a known protein family that mediates cell-cell and cell-matrix interactions. RGD tripeptide motifs, in common with the FG-GAP domains, are also present in many integrins and integrin ligands, e.g. plasma fibrinogen interaction with platelets (promotes platelet aggregation) and monocytes (Loftus *et al.*, 1994; Springer, 1997; Salsmann *et al.*, 2005). The domains FG-GAP 3 and 4, EGF-like Ca^{2+} domain and the acidic nature of the protein are responsible for its ability to bind divalent cations like calcium (Steck *et al.*, 2007). The ASPIC/UnbV domain is present in several paralogous proteins bacteria such as *Rhodospirellula baltica*, and is also associated with several eukaryote integrin-like proteins (Marchler-Bauer *et al.*, 2015).

1.3. CRTAC EVOLUTION

Initially, database searches for the complete coding sequence of the mouse *crtac1* gene, identified orthologs of *crtac1* in several vertebrates (mouse, rat, chicken and fugu) but not in invertebrates (Steck *et al.*, 2007).

Later, Redruello *et al.* (2010) identified a *crtac1*-like transcript, in the pituitary gland of *Sparus auratus* (teleost) that lacked the C-terminal EGF-like Ca^{2+} -binding domain and called it *crtac2*. Extended phylogenetic analysis, generated with full-length CRTAC proteins of several species, revealed that the identified CRTAC from prokaryotes and eukaryotes clustered into three major clades that included cyanobacteria (*Gloeobacter*), moss (bryophyte) and metazoan (being present in the three kingdoms: prokaryotes, plants and animal) sequences. In the metazoan clade two main branches occur in the vertebrates, one cluster corresponds to teleost and tetrapod CRTAC1 proteins and the other to teleost CRTAC2 (Figure 3) (Redruello *et al.*, 2010). Removal of the EGF-like domain from the CRTAC1 sequences before phylogenetic analysis failed to modify the tree topology and CRTAC1 sequences clustered separately from CRTAC2. The co-existence of *crtac* duplicates in teleosts is consistent with the hypothesis that teleost genomes underwent a specific whole genome duplication event (Jaillon *et al.*, 2004; Steck *et al.*, 2007; Redruello *et al.*, 2010).

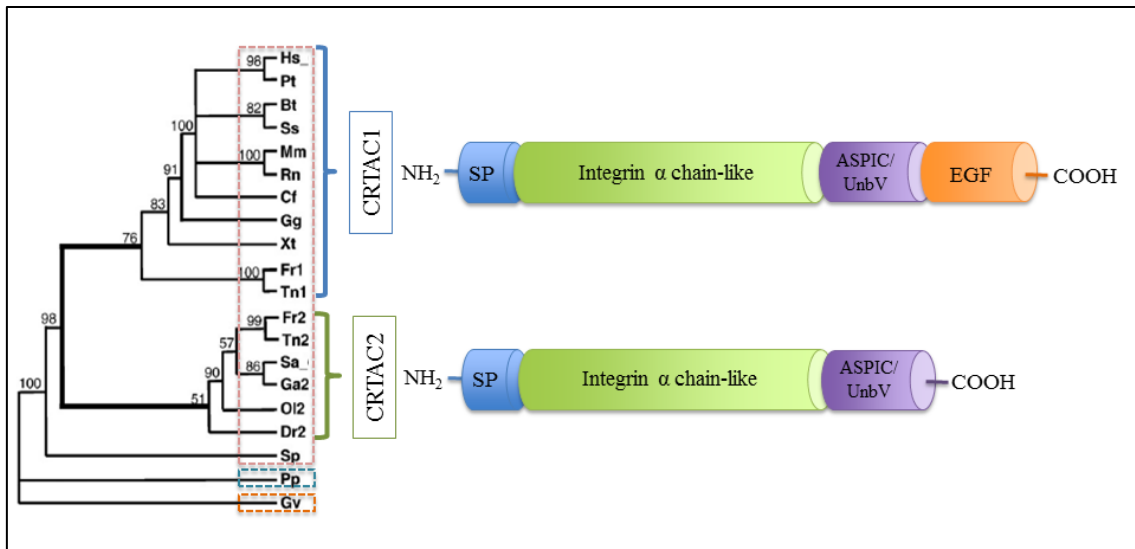


Figure 3. Phylogenetic tree of CRTAC1/CRTAC2 protein family (Adapted from Redruello *et al.*, 2010). Neighbor-joining distance tree of full-length protein sequences (bootstrap values of 100 replicates are indicated). Three major clades separate cyanobacteria (Gv; orange square), bryophyte (Pp; blue square), and metazoan (pink square) sequences. Within the metazoan clade, the sea urchin (Sp) sequence is separated from the vertebrate cluster. In vertebrates, one cluster corresponds to teleost and tetrapod CRTAC1 proteins and the other to teleost CRTAC2 proteins. This indicates the existence of two subfamilies, CRTAC1-like and CRTAC2-like, which differ in the presence or not of a C-terminus EGF-like Ca^{2+} binding domain (EGF), respectively. Hs - *Homo sapiens*, Pt - *Pan troglodites*, Bt - *Bos taurus*, Ss - *Sus scrofa*, Mm - *Mus musculus*, Rn - *Rattus norvegicus*, Cf - *Canis familiaris*, Gg - *Gallus gallus*, Xt - *Xenopus tropicalis*, Fr - *Fugu rubripes*, Tn - *Tetraodon nigroviridis*, Sa - *Sparus auratus*, Ga - *Gasterosteus aculeatus*, Ol - *Oryzias latipes*, Dr - *Danio rerio*, Sp - *Strongylocentrotus purpuratus*, Pp - *Physcomitrella patens*, Gv - *Gloeobacter violaceus* (Redruello *et al.*, 2010).

CRTAC is an ancient family of proteins present from prokaryotes (cyanobacteria) to advanced vertebrates (e.g. human) with highly conserved characteristics and their maintenance during evolution suggests they possess fundamental functions that are common across phyla. During evolution two *crtac* duplicates emerged (*crtac1* and *crtac2*) and teleost fish are the only organisms in which both duplicates co-exists which makes them an interesting model in which to study CRTAC proteins and their evolution. The conservation of genes during evolution can provide important insights into molecular function and may be linked to common actions in metazoans such as cell cycle maintenance or cell-cell interaction (Ohno, 1993; Redruello *et al.*, 2010).

1.3.1. Teleost CRTAC1 and CRTAC2

As mentioned before, the *crtac2* transcript was first isolated from the teleost (*S. auratus*) pituitary gland (Redruello *et al.*, 2010) and database searches also revealed in teleosts an ortholog gene of human *crtac1* and teleost *crtac1* and *crtac2* are considered to be paralogs genes.

In teleosts, the *crtac2* gene and protein is the most studied form. Sea bream (*Sparus auratus*) (sb) CRTAC2 cDNA is approximately 1977 bp and has an open reading frame of 1644 bp (start codon at position 79 and stop codon at position 1720). The protein has a predicted molecular weight of 58.9 kDa including a signal peptide of 19 amino acids at the N-terminus (Redruello *et al.*, 2010), suggesting that it is targeted to the secretory pathway.

Teleosts CRTAC1 and 2 share high amino acid sequence similarity with the N-terminal and mid-region of human CRTAC1 indicating that they may have overlapping functions. CRTAC2 is shorter than CRTAC1 as it lacks the C-terminal EGF-like Ca²⁺ binding domain crucial for protein-protein interactions. Both proteins contain an N-terminal conserved integrin α chain-like domain and an ASPIC/UnbV domain which has been implicated in integrin inside-out cell signaling mechanisms and cell matrix interactions. *In silico* analysis detected seven FG-GAP repeats distributed along the N-terminal integrin α chain-like domain (Redruello *et al.*, 2010) for all CRTAC protein family members, including the human, which contrasts with what has previously been reported (Steck *et al.*, 2007).

Redruello *et al.*, 2010, through *in silico* homology modelling demonstrated that sbCRTAC2 fits the seven-bladed β -propeller structure previously solved for the N-terminal integrin- α and harbours five Ca²⁺ binding β hairpin loops, described as crucial for molecular recognition mechanisms. Moreover it was recently demonstrated that sbCRTAC2 is a high affinity Ca²⁺ binding protein (Anjos *et al.*, 2013). An additional discriminative characteristic between CRTAC1/CRTAC2 is the presence in teleost CRTAC2 of a C-terminal CaaX-box prenylation site, involved in the anchoring of proteins to the plasma membrane (Gao *et al.*, 2009).

In common with human CRTAC, teleost CRTAC1/CRTAC2 also have a widespread tissue distribution and transcripts and proteins were found in nervous tissues (pituitary gland, brain, spinal cord), gonads, liver, kidney, heart, muscle and calcified

structures (Redruello *et al.*, 2010; Anjos *et al.*, 2013). In situ hybridization studies in *S. auratus* pituitary demonstrated a large quantity of CRTAC2 mRNA in small irregular shaped cells with large nuclei adjacent to the neurohypophysial tissue, between the anterior and posterior lobes. The fact that CRTAC2 is released from the *S. auratus* pituitary gland may suggest that this protein in addition to being an extracellular matrix protein may also have endocrine functions.

1.4. CRTAC FUNCTIONS AND FUNCTIONAL DOMAINS

The mechanism of action of the human CRTAC1 and teleost CRTAC1/2 proteins still remains elusive and few functional studies exist. hCRTAC1-A in chondrocyte cultures is secreted with other ECM proteins and interacts with them enhancing cellular adhesion and contributes to the physical characteristics of cartilage (Steck *et al.*, 2006; Huang *et al.*, 2008). In common with human CRTAC1 the teleost CRTAC2 protein promotes skin epithelial cell outgrowth *in vitro* (Anjos *et al.*, 2013), suggesting it may share functional homology and be involved in cell adhesion/proliferation. hCRTAC1B is the dominant transcript in brain (Steck *et al.*, 2007) and recently it was reported as an antagonist of Nogo receptor-1 which is involved in lateral olfactory tract development *in vivo* (Sato *et al.*, 2011) and *in vitro* (Kurihara *et al.*, 2012) suggesting a role in axon formation and migration.

The structure of the teleost CRTAC2 protein has been characterised (Anjos *et al.*, 2013) with the aim of providing insight into protein function. sbCRTAC2 is a high affinity calcium-binding protein, a crucial ion for mechanisms of molecular recognition. Moreover, it has been hypothesized that the Ca²⁺ binding motifs in human CRTAC1-A may have a functional role in calcifying articular cartilage in humans (Steck *et al.*, 2007). In addition, sbCRTAC2 protein is hyperthermostable and retains its native tertiary structure after heating and also form large aggregates *in vivo* and *in vitro* (Anjos *et al.*, 2013) similar to those found in amyloid disease.

When a structure of a protein is predicted evidence for its function may be predicted based upon homology between the protein domains identified and those present in other protein with known function and homologous protein structures have been used to find grooves or surface cavities that may indicate an active site (Teichmann *et al.*, 2001). As mentioned before, CRTAC proteins contain several predicted conserved domains and motifs that are also present in other proteins whose

functions are identified and studied. This type of information can help to predict or generate hypothesis about the role of CRTAC in biological systems:

- **Signal peptides**, similar to that in CRTAC proteins, generally controls the route that a specific protein must take, targeting proteins for insertion in cell membranes or to the secretory pathway in eukaryotes. Generally, a signal peptide is located at the N-terminus of the protein and is positively charged and is followed by a hydrophobic region and a neutral but polar C-region, and is cleaved off while the protein is translocated (Nielsen *et al.*, 1997; Singh *et al.*, 2013).
- **Integrin domain**, also present in CRTAC proteins, are known as vital for cell-cell and cell-matrix adhesion, cell growth, migration, differentiation and in inflammatory processes. This specific domain interacts with other ECM proteins, such as fibronectin, vitronectin and laminin, (Mao & Schwarzbauer, 2005; Zhou, 2007; Nelson *et al.*, 2008; Chouhan *et al.*, 2011). Vertebrate integrins are heterodimeric adhesive receptors and hold at least 18 different integrin α -subunits and eight β -subunits. The N-terminal domain in the α - and β -integrin subunits (the β -propeller structure), provide a ligand-binding site for several ligands. Half of the α -subunits contain an additional domain (a von Willebrand factor type-A), inserted between the second and the third blades of the N-terminal 7-bladed β -propeller, that binds to their ligands via a metal ion dependent site, such as the divalent cations Mg^{2+} or Ca^{2+} . Also, the β -propeller domain contains RGD and FG-GAP motifs in the N-terminal portions of the α - and β -subunits, and is the binding site of Mg^{2+} or Ca^{2+} that are responsible for the attachment of integrin-mediated cell adhesion (Springer, 1997; Shattil *et al.*, 2010; Chouhan *et al.*, 2011).
- **EGF-like Ca^{2+} binding** domains play a role in extracellular activities such as cell adhesion and receptor-ligand interactions, however they are also found in the blood coagulation factors VII, IX, X and protein C and S, where this domain mediates interactions between the various components and most likely has an adhesive function (Downing *et al.*, 1996; Wouters *et al.*, 2004). Structurally it is defined as a small domain of 30 to 40 amino acids, stabilized by three disulfide bonds, forming two β -sheets referred to as the major N-terminal and minor C-

terminal sheets (Wouters *et al.*, 2004). The EGF domain exist as tandem repeats in several proteins and modifications in the amino acids sequence leads to changes in their Ca^{2+} binding capacity and, by consequence, alterations in the stability of the N-Terminal domain of the proteins. These modifications have been identified in patients with Marfan syndrome, hemophilia B and protein S deficiency (Downing *et al.*, 1996).

- **The ASPIC/UnbV domain**, also present in CRTAC1 proteins, discovered in *Rhodopirellula baltica*, is associated with several eukaryotic integrin-like proteins. Although the exact function of this domain remains unknown, the fact that it is associated with integrin-like proteins is related to cell-cell and cell-matrix interaction activity (Studholme *et al.*, 2004; Steck *et al.*, 2007; Wada, 2013; Marchler-Bauer *et al.*, 2015).
- **VCBS**, present in CRTACs was recently identified in databases. This domain is found in large proteins from some pathogenic bacteria, such as *Vibrio*, *Colwellia*, *Bradyrhizobium* and *Shewanella* (VCBS), and suggests a possible role in the pathogen adhesion to host cells. It appears in multiple copies of about 100 amino acid residues (Yousef & Espinosa-Urgel, 2007; Zhu *et al.*, 2014).
- **CaaX-box prenylation site** for CaaX prenyltransferases, only found in teleost CRTAC2 at Cys⁵⁴⁴ (Redruello *et al.*, 2010), are involved in the anchoring of proteins to the plasma membrane and nuclear or mitochondrial membrane as well as in protein to protein interactions (Gao *et al.*, 2009). Prenyltransferases are known to affect cell growth and morphology, cellular cycle and induce apoptosis in cancer cells. Deficiencies in prenylation are associated with several diseases, such as Hermansky-Pudlak syndrome, and inhibition of prenyltransferases causes tumor regression and have beneficial effects in diabetic retinopathy and macular degeneration (Maurer-Stroh *et al.*, 2003).

Knowledge about CRTAC protein domain assembly, the proteins widespread tissue distribution and its conservation in genomes from bacteria to human are clues to the important role of these proteins in an organism. The conserved structural properties of CRTAC during evolution suggests their involvement in cell-cell and cell-matrix interactions and that could be a target of potential interest for biomedicine.

1.5. CRTAC AND HUMAN DISEASES

Even though CRTAC functions are not well established it has been linked to several human pathologies, however the mechanisms behind its role in disease are so far unknown. Nonetheless two patents have been proposed for hCRTAC, i) as a biomarker and ii) as a potential therapeutic target in several pathologies:

- hCRTAC1 protein was first patented as a blood biomarker of bone fracture and cartilage injury, since CRTAC1 peripheral blood levels appear to be significantly elevated in healthy individuals with these kinds of injuries (Grgurevic *et al.*, 2007; Grgurevic *et al.*, 2008). Moreover proteomic analyses of synovial fluid from patients with osteoarthritic knee also showed up-regulation of CRTAC1 protein (Ritter *et al.*, 2013).
- Additional studies also demonstrated the regulation by high Ca^{2+} medium of hCRTAC1 gene expression in parathyroid tissues ex-plants from patients with primary hyperparathyroidism (Nakajima *et al.*, 2010).
- Proteins with a β -propeller fold like CRTAC frequently play key biological roles and have been directly or indirectly associated with several human diseases (Chen *et al.*, 2011) such as, human retinal degeneration, Kallmann syndrome or amyloid diseases, such as Alzheimers or atherosclerosis (Shin *et al.*, 2008). The propensity to form amyloid has been correlated with the high tendency of teleost recombinant CRTAC2 to form large protein aggregates (Anjos *et al.*, 2013).
- CRTAC1-B protein is an endogenous antagonist of Nogo receptor-1 (NgR1, a receptor of myelin-derived axon growth inhibitors) that is present in neurons and neutralization of these kinds of receptors improve axonal regeneration after injury. The ASPIC/UnbV and EGF-like domains alone or together are responsible for the antagonistic action of the CRTAC1-B (Kurihara *et al.*, 2012).
- Proteomic analysis of cerebrospinal fluid from patients suffering from Multiple Sclerosis (autoimmune demyelinating disorder of the central nervous system) revealed the unusual presence of CRTAC1-B protein in the fluid (Hammack *et al.*, 2004; Takahashi *et al.*, 2015).
- A second patent proposed CRTAC1 as a biomarker and therapeutic target of several diseases of the human body system such as cardiovascular, haematological, gastrointestinal and liver, urological and respiratory diseases,

and several types of cancer, inflammation and neurological disorders without further specification (Golz *et al.*, 2008).

In summary, hCRTAC has been associated with cartilage and related diseases, multiple sclerosis, bone fracture, neurofibromatosis type 1-associated glomus tumors (Brems *et al.*, 2009) and Alzheimer disease and it has been patented as a plasma biomarker and potential therapeutic target for diseases of all human body systems including the immune system.

1.5.1. Immune system and autoimmunity

The human immune system consists of isolated cells and organized tissues, spread throughout the entire organism and has as a central function, defence of the organisms from invading microorganisms, toxins and strange molecules. The organized tissues are the lymphoid tissues such as the thymus, spleen lymph nodes and the mucosa-associated lymphoid tissue (MALT), where the main cell types are the B- and T-lymphocytes. Isolated cells such as granulocytes (neutrophils, eosinophils and basophiles), phagocytic mononuclear cells (monocytes) and also lymphocytes are found circulating in the blood stream and lymph and macrophages and dendritic cells are predominantly found in the connective tissue (Junqueira & Carneiro, 2004).

The immune system is responsible for the two main components of the immune response: cellular immunity (innate immunity), where cells recognise and destroy pathogens and strange molecules, and the humoral immunity (acquired immunity), where the antibodies produced by lymphocyte B cells neutralize pathogens and toxic substances, helping in the cellular immune response (Junqueira & Carneiro, 2004). Macrophages cells are well known for their capacity to recognise pathogens and neutralize substances bound to antibodies (opsonise), as they have membrane receptors for the crystallisable fragment (Fc) region, leading to the phagocytosis of these abnormal bodies and playing an important role in the immune response (Aderem & Underhill, 1999).

Autoimmunity occurs when the immune system responds against self-molecules as a result of a failure of immunologic tolerance. Several autoimmune diseases can occur, such as rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis, and affect 3% of the North American and European populations (Smith &

Germolec, 1999). Multiple sclerosis, a chronic inflammation leading to the demyelination of axons, is associated with macrophages infiltrates, axonal damage and neurodegeneration (Vogel *et al.*, 2013; Rawji & Yong, 2013), as well as the unusual presence of CRTAC1-B protein in the cerebrospinal fluid (Hammack *et al.*, 2004; Takahashi *et al.*, 2015). Another autoimmune disease associated with macrophages is Macrophage Activation Syndrome in which inappropriately activated macrophages causes cytopenia, organ dysfunction, and coagulopathy problems (Deane *et al.*, 2010).

1.5.2. Human monocytes

Monocytes and macrophages belong to the innate immune system, and play a key role in the initiation and resolution of inflammatory states. Monocytes and macrophages are responsible for the recognition and phagocytosis of foreign pathogens (such bacteria, viruses and fungi) and for the production of pro-inflammatory chemokines and cytokines, anti-inflammatory cytokines (when the inflammatory/infection state is under control) and reactive oxygen species (ROS). These cells also play an important role in tissue remodelling in ontogenesis, orchestrations of metabolic functions, wound healing and cancer progression (Parihar *et al.*, 2010; Sica & Mantovani, 2012; Chanput *et al.*, 2013).

The precursors of monocytes are located in the bone marrow. They derive from myeloid cluster forming granulocyte-monocyte colonies. These cells differentiate into monoblasts (a controversial matter in the scientific community since their identification in bone marrow is almost impossible) and then into promonocytes (morphological identifiable cells). Promonocyte cells (20µm diameter cells) reside in the bone marrow for approximately 6 days and then become mature monocytes (15-18µm diameter cells) migrating to the blood stream (Auwerx, 1991; Junqueira & Carneiro, 2004; Lewis *et al.*, 2006). The mature monocytes, in the absence of an inflammatory state, migrate randomly into different tissues of the human body such as the liver (Kupffer cells), bone (Osteoclasts), brain (Microglial cells), lung, intestinal wall, spleen, lymph nodes and connective tissue (Auwerx, 1991). This migratory activity has a negative impact when some virus (such as HIV, HCV and Dengue Virus) infect these cells, allowing the virus to spread throughout the body (Ziegler-Heitbrock, 2014). Accumulation of monocytes in certain areas can also aggravate the state of several diseases such as multiple

sclerosis, atherosclerosis, arthritis and lupus nephritis (Parihar *et al.*, 2010; Sica & Mantovani, 2012).

Monocytes can be divided in three subpopulation sets according to expression of certain cell surface proteins called clusters of differentiation (CD). Approximately 90 to 95% of these cells are called “classical monocytes” since they are strongly positive for the CD14 protein (Lipopolysaccharide binding protein receptor) and CD64 protein but negative for the expression of the proteins CD16 (CD14^{hi}CD16⁻, CD64⁺). Non-classical monocytes account for 5-10% of the cells and express CD14^{low}CD16^{hi}, CD64⁻ and a third category, called “intermediary monocytes” are CD14^{hi}CD16⁺, CD64⁺ positive (Parihar *et al.*, 2010).

The differentiation of monocytes into macrophages depends of the response of these cells to environmental signals, and result in distinct populations of functional macrophages. Activation stages include the Classical M1 activation and the alternative M2 activation of macrophage. The M1 macrophage phenotype is stimulated in response to Toll-Like Receptor (TRL) ligands (such as bacterial lipopolysaccharide, LPS) and Interferon- γ and the M2 macrophages phenotype is stimulated in response to Interleukin 4 and Interleukin 13 (Parihar *et al.*, 2010; Sica & Mantovani, 2012; Martinez & Gordon, 2014). M1 macrophages have a high expression level of pro-inflammatory cytokines and produce oxygen and nitrogen intermediates, a strong antimicrobial and antitumor activity and are also responsible for the response/activation of T helper-1 cells and the presentation of antigens to B cell. M2 macrophages are involved in parasite phagocytosis, promotion of tissue remodelling, immunoregulatory functions, response/activation of T helper-2 cells and down-regulation of pro-inflammatory cytokines (Parihar *et al.*, 2010; Sica & Mantovani, 2012; Martinez & Gordon, 2014).

Although monocytes and macrophages are phenotypically different (macrophages are huge cells with amoeboid movement, compared with the round monocytes), other changes also occur e.g. the decrease of expression of CD14 and increased levels of CD11b in macrophages cells (Parihar *et al.*, 2010). CD11b is one protein subunit that forms the heterodimeric surface receptor integrin CD11b/CD18 (also known as $\alpha_M\beta_2$ or macrophage-1 antigen, Mac-1), a pattern recognition receptor for LPS and functional aggregated beta-amyloid proteins (Orchekowski *et al.*, 2000; Zhou *et al.* 2013).

1.5.2.1. THP-1 cell line

THP-1 cell line (Figure 4.A) culture is used in the present study and was established by Tsuchiya *et al.* (1980) from the peripheral blood of a 1 year old infant male patient with an acute monocytic leukemia. This cell line has highly similar morphology and differentiation properties with the primary monocytes and macrophages produced by humans (Adati *et al.*, 2009; Qin, 2012;

Several chemical substances, bacterial molecules and stimulating factors can be used to differentiate THP-1 monocyte cells into macrophages-like cells (Figure 4.B), in a similar way to the peripheral blood monocytes (Qin, 2012; Chanput *et al.*, 2013). After exposure to these substances, THP-1 cells start to suffer clear morphological changes since they start to adhere to culture plates, and have a flat amoeboid-like shape (Tsuchiya *et al.*, 1982; Qin, 2012). The most commonly substances used to induce THP-1 cells differentiation are phorbol-12-myristate-13-acetate (PMA), 1α 25-dihydroxyvitamin D₃ [1α 25(OH)₂D₃], LPS from *Escherichia coli* and Macrophage colony-stimulating factor (M-CSF) (Daigneault *et al.*, 2010; Qin, 2012; Chanput *et al.*, 2013).

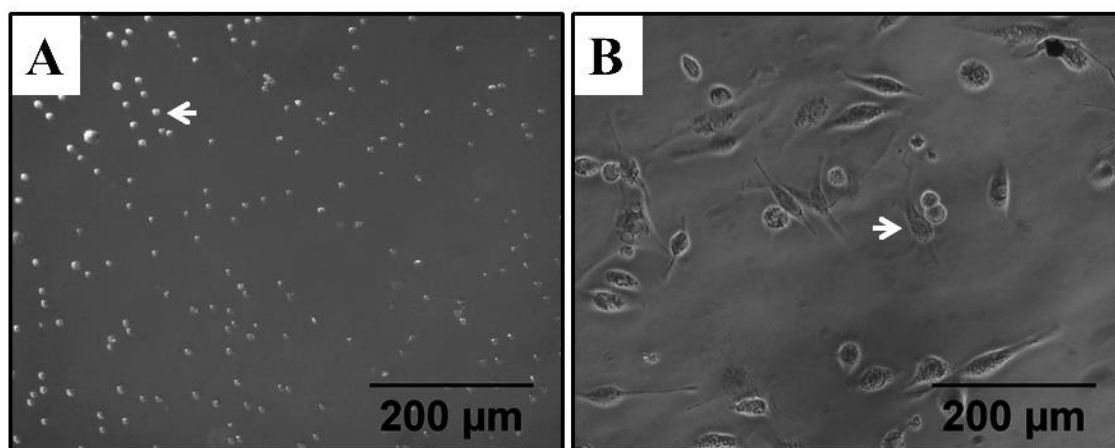


Figure 4. Phase contrast microscopy of THP-1 and macrophage-like cell line cultures. A. Monocytic THP-1 cell (white arrow) suspension culture in RPMI 1640 medium supplied with 20% FBS and 1% PenStrep. **B.** Adherent macrophage-like cells (white arrow) derived from a THP-1 cell culture after 72hours incubation with PMA (100nM). Shown a 20x amplification.

PMA is more effective as differentiation inducer of THP-1 derived macrophages-like cells to macrophages, since it increases cell adherence, phagocytic activity and expression of cell surface marker such as TLR-2, CD36, CD11b and CD18. 1α 25(OH)₂ D₃ provoked differentiation of THP-1 cells in macrophages-like cells is less

effective when classified in relation to the production of TNF- α (Tumor Necrosis Factor alpha) and phagocytic capability. M-CSF plays a different role in the process of differentiation of monocytes, as it differentiates early stem cell-monoblast into monocytes before they are released into peripheral blood (Adati *et al.*, 2009; Daigneault *et al.*, 2010; Qin, 2012; Chanput *et al.*, 2013).

In common with normal human peripheral blood monocytes, THP-1 cells can also be classified as M1 and M2 macrophages-like cells if challenged with LPS or Interleukin 4, respectively. The THP-1 cells stimulated with PMA are classified as being M0 and from this state they can be then polarized into either the M1 or M2 state macrophage-like cells (Chanput *et al.*, 2013).

The use of these cancerous cells is an important scientific research tool for cellular function studies, mechanisms and signalling pathways in response of drug treatments *in vitro*, as a method of selection of future medical/pharmacological compounds. The high growth rate, capability of propagation in culture, absence of virus or toxic products, existence of easy storage protocols and homogeneous genetic background are the most important advantages of the THP-1 cell cultures (Chanput *et al.*, 2013).

1.5.3. Integrin-like proteins and the immune system

Integrin proteins are crucial cell surface preceptors that mediate cell-pathogen, cell-cell and cell-ECM interactions and out-side-in signals across the cell membrane with other self-receptors, particularly important in the immune system. Integrin functions can be modulated by conformational changes that modify the affinity for the ligand, formation of integrin clusters by additional integrin receptors recruitment and association with molecules to form active signalling complexes (Cambi *et al.*, 2000).

Non activated blood leucocytes, such as monocytes, show low levels of integrin activity which increase after stimulation by agonist molecules. In activated macrophages, e.g., the integrin lymphocyte function-associated antigen-1 (LFA-1) form clusters when it binds to the intercellular adhesion molecule 1 (ICAM1) leading to an increase in the adhesion capacity of cells to the endothelium (Cambi *et al.*, 2006; Evans *et al.*, 2009).

Another important feature is the association of integrin proteins and glycosylphosphatidylinositol-linked (GPI) proteins in which the lipid content of the cell membrane (mainly glycosphingolipids and cholesterol) provide scaffolding platforms that mediate specific cell signaling events (Cambi *et al.*, 2000; Ferguson *et al.*, 2009), e.g., CRTAC1-B, an integrin-like protein has been shown to be an endogenous antagonist of NgR1 receptors, a GPI-anchored protein found in neurons and neutralization of this kind of receptor improves axonal regeneration after injury (Sato *et al.*, 2011; Kurihara *et al.*, 2012).

As mentioned before, CRTAC1-B levels increase in the cerebrospinal fluid of patients with multiple sclerosis (Hammack *et al.*, 2004; Takahashi *et al.*, 2015), and it is known that monocytes migrate and accumulate in the brain aggravating the state of this pathology (Parihar *et al.*, 2010; Sica & Mantovani, 2012). In addition, there are evidences of CRTAC1 being expressed by human monocytes (GeneCards database, <http://www.genecards.org>). When all the information about CRTAC is taken together it suggests that the proteins may have an important role in the immune system.

Preliminary unpublished experiments (Anjos L., Velez Z., and Power D. M.) screened for the potential effects of CRTAC on several mammalian cell lines, by exposing them to several different concentrations of sbCRTAC2. The cell lines tested included: Abelson murine leukemia virus-induced tumor (Raw 264.7, macrophage cells), Human liver hepatocellular carcinoma cells (HepG2, hepatocytes), Human Umbilical Vein Endothelial Cells (HUVEC) and THP-1. The effect of sbCRTAC2 on cell viability, motility, adhesion, aggregation and its anti-inflammatory capacity was established and suggested that CRTAC may affect the proliferation, aggregation and viability on the immune cells, macrophage (Raw 264.7) and monocyte (THP-1) cell lines. The results are consistent with some functional role for CRTAC associated with the immune system and in the present thesis the effect of CRTAC on THP-1 monocyte cell line will be explored.

2. OBJECTIVES

Preliminary results (from the host laboratory) suggest that sbCRTAC2 affects cell proliferation in Raw 264.7 macrophage cell line and affect the viability on THP-1 monocyte cells. The general aim of the present thesis was to validate these promising results and characterize the effect of human and teleost CRTACs (through a comparative approach) on THP-1 monocytic and on Human Embryonic Kidney 293 (HEK 293) cell activity (proliferation, aggregation and adhesion) in order to infer CRTACs function. This work makes use of important tools and knowledge already developed in the host laboratory such as the availability of CRTAC's recombinant proteins and their specific antibodies. Several tasks with defined objectives were performed for this purpose:

- a) Establish the intrinsic expression of hCRTAC1-A in the cell lines under study and evaluate the subcellular location of CRTAC proteins through *in silico* analysis using bioinformatic tools coupled to Western blot analysis in subcellular fractions;
- b) Characterization of the effect of human CRTAC1-A and teleost (*D. labrax*) CRTAC1/2 proteins on THP-1 monocytic cell cultures. This task involves the performance of several *in vitro* cell assays in order to evaluate the effect of CRTACs on: cell viability, motility, adhesion/aggregation capacity, modifications in the cell cycle or induction of cell differentiation, anti-inflammatory effects and cell signalling by cAMP detection;
- c) Transfection of HEK 293 cell lines with human and teleost *crtac* genes in order to assess the cellular pathway of the proteins.

The scientific output of this thesis was to increase basic knowledge about the function of CRTAC's (taking a comparative approach by comparing hCRTAC1 and teleost CRTAC1 and CRTAC2 proteins) on cell proliferation and related cellular processes and generate knowledge that may be useful for the development of potential

applications for medical diagnosis and drug discovery and of interest for the biotechnological, medical and pharmaceutical industries.

CHAPTER II

CHARACTERIZATION OF THE EFFECT OF CARTILAGE ACIDIC PROTEIN (CRTAC) ON HUMAN MONOCYTES THP-1 CELLS

**CHARACTERIZATION OF THE EFFECT OF CARTILAGE ACIDIC
PROTEIN (CRTAC) ON HUMAN MONOCYTES THP-1 CELLS**

André Coelho, Liliana Anjos ^a, Zélia Velez and Deborah M. Power ^a

^a*Comparative Endocrinology and Integrative Biology Group (CEIB), CCMAR, Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.*

Corresponding author:

1. INTRODUCTION

Cartilage acidic protein (CRTAC) is an extracellular matrix protein of chondrogenic tissue of unknown function that has been associated with several diseases, including cartilage related disease and multiple sclerosis (Steck *et al.*, 2001; Steck *et al.*, 2007; Hammack *et al.*, 2004; Takahashi *et al.*, 2015). Two splice variants were found in humans (h), *CRTAC1-A* that is expressed in cartilage and lung with residual levels in brain and *CRTAC1-B*, a shorter transcript that is the dominant form in brain and nervous tissue but is also present in the eye, lens and pineal gland (Benz *et al.*, 2002; Rabinowitz *et al.*, 2005; Turner *et al.*, 2007; Steck *et al.*, 2007; Nakajima *et al.*, 2010).

CRTAC is an ancient protein found in prokaryotes through to vertebrates and its overall conservation between species despite their evolutionary distance suggests it has an important and fundamental cellular function, although this still remains to be established. During evolution *crtac* duplicates (*crtac1* and *crtac2*), that are homologues of *CRTAC1* in humans (*hCRTAC1*), have emerged in teleost (t) fish as a result of the teleost specific whole genome duplication (Jaillon *et al.*, 2004). Cartilage Acidic Protein 2 (CRTAC2) was initially isolated from the teleost pituitary gland (Redruello *et al.*, 2010) and is a paralogue of *hCRTAC1*. In common with humans, *tCRTAC*'s are widespread in tissues and transcripts and proteins are found in nervous tissues (brain, pituitary gland and spinal cord), heart, liver, gills, gonads, kidney, white muscle and calcified structures (Redruello *et al.*, 2010; Anjos *et al.*, 2013).

tCRTAC1 and 2 share high amino acid sequence similarity with the N-terminus and mid-region of human *CRTAC1* indicating that they may have overlapping functions. CRTAC-like proteins contain an N-terminal conserved integrin α chain-like Ca^{2+} binding domain, followed by an ASPIC/UnbV domain, both associated with cell-cell and cell-matrix interactions (Studholme *et al.*, 2004; Redruello *et al.*, 2010; Marchler-Bauer *et al.*, 2015). CRTAC2 differs from CRTAC1 as it lacks the C-terminus EGF-like Ca^{2+} binding domain that is crucial for protein-protein interactions (Selander-Sunnerhagen *et al.*, 1992; Rao *et al.*, 1995; Redruello *et al.*, 2010). Structural studies demonstrate that sea bream (sb, an advanced teleost) CRTAC2 is a protein with high propensity to form large aggregates *in vivo* and *in vitro* (Anjos *et al.*, 2013), similar to those found in amyloid diseases such as Alzheimer or atherosclerosis. In addition it is a high affinity calcium-binding protein, a crucial ion for mechanisms of molecular recognition (Anjos *et al.*, 2013). The conserved structural properties of CRTACs

strongly suggest their involvement in cell-cell and cell-matrix interactions and its structural characteristics and importance in human pathologies makes this protein an interesting target for biomedicine.

The mechanism of action of CRTAC and its relationship with human pathologies still remains elusive. Nevertheless hCRTAC has been patented and is proposed as a biomarker and potential therapeutic target for diseases of numerous human systems and pathologies, such as: bone and cartilage, inflammatory diseases, numerous types of cancer and neurological disorders (Grgurevic *et al.*, 2007; Grgurevic *et al.*, 2008; Golz *et al.*, 2008). Recent studies indicate that CRTAC1-B is an endogenous antagonist of Nogo receptor-1, present in neurons, and neutralization of these receptors improves axonal regeneration after injury (Sato *et al.*, 2011; Kurihara *et al.*, 2012). For example, patients suffering from multiple sclerosis, an autoimmune demyelinating disorder of the central nervous system associated with chronic inflammation caused by macrophages infiltration, have increased CRTAC1-B protein concentrations in their cerebrospinal fluid (Hammack *et al.*, 2004; Vogel *et al.*, 2013; Rawji & Yong, 2013; Takahashi *et al.*, 2015). In multiple sclerosis, and several other diseases such as atherosclerosis, arthritis and lupus nephritis accumulation of monocytes in certain regions of the tissue are associated with aggravation of the disease state and its progression (Parihar *et al.*, 2010; Sica & Mantovani, 2012).

Monocytes have low levels of integrin activity that increases after stimulation by agonist molecules that activate these cells. Integrin-like proteins are crucial cell surface receptors that mediate cell-pathogen/-cell/-ECM interactions and out-side-in signals across the cell membrane with other self-receptors, showing that these proteins have an important role in the immune system (Cambi *et al.* 2000; Cambi *et al.* 2006). CRTAC is a non-integrin protein that harbours an integrin α chain-like domain and additionally there are evidences that CRTAC1 is expressed in human monocytes and it also circulates in serum (GeneCards database, <http://www.genecards.org>). All these facts together suggest that this protein may have a function related to the immune system.

Preliminary experiments (realized in the host laboratory, Anjos L., Velez Z., and Power D. M.) screened for potential effects of sbCRTAC2 using several mammalian cell lines (exposed or depleted of recombinant sbCRTAC2) suggested that sbCRTAC2 affects cell proliferation in a rat macrophage cell line (Raw 264.7) and affect the viability on THP-1 monocytic cells. Therefore, we propose characterize the effect of

CRTAC proteins in the THP-1 monocytic cell line as an attempt to increase the knowledge about the functionality of these proteins and their potential relation with the immune system and pathologies.

2. MATERIAL AND METHODS

2.1. BIOLOGICAL MATERIALS

2.1.1. Cell lines

The human monocyte THP-1 cell line was purchased from the German Collection of Microorganisms and Cell Culture (DSMZ, Germany) and the Human Embryonic Kidney 293 (HEK 293) cell line was purchased from the American Tissue Culture Collection (ATCC, USA).

2.1.2. Recombinant human and fish CRTAC proteins

Human CRTAC1-A and *Dicentrarchus labrax* (dl) CRTAC1 and CRTAC2 mature proteins were produced without fusion tags by recombinant technology at the host laboratory [Comparative Endocrinology and Integrative Biology Group (CEIB), CCMAR - Laboratório Associado, University of Algarve, Portugal] and were kindly provided by Dr. L. Anjos. The recombinant proteins were solubilized in 1x Phosphate buffer saline (PBS - 137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄ and 2mM KH₂PO₄) at a concentration of 70µg/mL and stored at -20°C.

2.1.3. Antibodies

Two primary antibodies were used to identify hCRTAC protein: a commercial goat anti-hCRTAC1 IgG (SantaCruz Biotechnology, USA) and a polyclonal rabbit anti-sbCRTAC2 serum produced against *S. aurata* CRTAC2 recombinant protein as previously described (Anjos *et al.*, 2013). The secondary antibodies used were commercially obtained and were rabbit anti-goat IgG conjugated to Horseradish Peroxidase (HRP) (Sigma-Aldrich, USA) and the biotinylated donkey anti-rabbit IgG (GE Healthcare, Sweden) followed by streptavidin conjugated to HRP (GE Healthcare, Sweden). A commercial mouse anti-human CD11b conjugated to Allophycocyanin (APC) (BioLegend Inc., USA) was purchased and used in the flow cytometry assays.

2.2. METHODS

2.2.1. Cell culture maintenance

THP-1 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640 medium; Sigma-Aldrich, USA) supplemented with 20% inactivated fetal bovine serum (FBS; Sigma-Aldrich, USA) and 1% antibiotic PenStrep solution (containing 100U/ml Penicillin and 10mg/ml Streptomycin; Sigma-Aldrich, USA). Macrophages derived from THP-1 cell cultures (Ma cells) were obtained by incubating THP-1 cells (1×10^5 cells/mL) with 100nM of PMA (Sigma-Aldrich, USA) diluted in 100% Dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA), for 48 hours.

HEK 293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, USA) supplemented with 10% FBS and 1% of antibiotic PenStrep solution.

Cell cultures were grown in a humidified incubator at 37°C, 95% air atmosphere and 5% CO₂ and all cell preparations were performed at room temperature, using aseptic techniques in a laminar flow cabinet.

2.2.2. Detection of the human *CRTAC1* transcript and protein in THP-1, Ma and HEK 293 cells

The expression of *hCRTAC1-A* transcript or hCRTAC1 protein in the cell lines selected for analysis was investigated by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analysis. Additionally and in parallel a bioinformatics search was also carried out in GeneCards (<http://www.genecards.org/>) and the Human Protein Atlas (<http://www.proteinatlas.org/>) database.

2.2.2.1. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA from THP-1, HEK 293 and Ma cells (1.5×10^7 cells/mL) was extracted using the E.Z.N.A.[®] Total RNA Kit I (Omega Bio-tek Inc., USA) according to the manufacturer instructions. RNA (500ng) was treated with 1U DNase I (Ambion[®], Thermo Fisher Scientific Inc., USA) for 30 min at 37°C, according to the manufacturer instructions. DNase treated RNA was denatured at 65°C for 5 min, cooled on ice for 5min and used for cDNA synthesis using the RevertAid Reverse Transcriptase kit (Thermo Fisher Scientific Inc., USA) in a 20µl reaction volume containing (100pmol)

random hexamer primers, 1mM dNTPs, 200U of RevertAid Reverse Transcriptase and 20U RiboLock RNase Inhibitor. cDNA was synthesized for 10 min at 25°C followed by 60 min at 42°C and 70°C for 10 min. The quality and quantity of the cDNA were assessed by PCR amplification of ribosomal subunit 18S rRNA using the following cycle: 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, followed by 72°C for 5 min.

Two sets of primers were designed using Primer Premier v5 (Premier Biosoft, Sigma-Aldrich, USA) to amplify the mature (438_forward 5' - TCC CAG CGG GCT GAA CCC ATG TTC - 3'; 2340_reverse 5' - CTA GCA GCT GGG CTC GCA GCT CTC C - 3') and truncated (1882_forward 5' - ATG GCA AGA TGG TGA GCC GGA ACG T - 3'; 2112_reverse 5' - ACC TGT AGC TTC CAT AGG TG - 3') forms of *hertac1-a*. The thermocycle consisted of: 95°C for 5 min followed by 35 cycles of 30 s at 95°C, 30 s at 60°C or 2 min at 60°C (for the truncated form) and 45 s at 72°C, followed by a final cycle of 5 min at 72°C. The resulting cDNA fragments were amplified in a 15µL reaction volume containing 0.3U DreamTaq DNA polymerase (5U/µl, Thermo Fisher Scientific Inc., USA), 1.5mM MgCl₂ (Thermo Scientific Inc., USA), 0.2mM dNTP's (GE Healthcare, Spain) and 0.25µM of each gene specific primer pairs. Amplified PCR products were separated by gel electrophoresis (1% agarose/1×Tris Acetate-EDTA gel and 1µg/mL ethidium bromide) and were sequenced to confirm the gene identity. All primers were obtained from Sigma-Aldrich, USA.

2.2.2. Protein extraction and western blot analysis

For hCRTAC1 protein detection by western blot, total cell protein extracts were prepared from HEK 293 cells. Cells (1.5x10⁷ cells/mL) were collected from culture flasks, washed with 1x PBS, divided into two samples and lysed separately in two different protein extraction buffers (1:10 ratio) in order to optimize the best extraction conditions: 1) RIPA Modified Buffer (150mM NaCl, 50mM Tris HCl pH=7.4, 1mM EDTA, 1% Triton X-100, 1% Deoxycholic acid, 0.1% SDS and 1x Protease Mix Inhibitor) and 2) Denaturing Buffer (8M Urea, 50mM Tris pH=8, 100mM NaCl, 100mM EDTA, 1% Triton X-100, 10mM DTT and 1x Protease Mix Inhibitor (Sigma-Aldrich, USA). Lysed cells were incubated for 1h on ice (stirring every 10min), sonicated for 1 min, and centrifuged at 12000rpm for 10 min at 4°C. Soluble protein was collected and quantified using a Quick Start™ Bradford Protein Assay (Bio-Rad, USA) following the manufactures instructions.

Equal amounts of cell protein extracts (4.5 μ g) were diluted in 2x Laemmli sample loading buffer (2x LB; 0.1% Bromophenol blue, 10% SDS, 20% Glycerol, 125mM Tris HCl pH=6.8 and 0.2M DTT) and denatured at 100°C for 5 min. Protein samples were fractionated by SDS-PAGE electrophoresis onto a 10% polyacrylamide gel (4% of stacking and 10% of resolving gel; at 70V constant for 7 min and 120V constant for 2 hours) using a Mini-PROTEAN[®] Tetra System (Bio-Rad, Hercules, USA) and the gels were stained with Coomassie blue R solution [0.1% (w/v) Coomassie blue R250, 10% Acetic acid, 40% Metanol; 60 min at room temperature] and destained (10% Methanol, 10% acid acetic) to check the quality of the protein extracts or were used for blotting. PageRuler Prestained Protein Ladder (Fermentas, USA) was run on all gels.

SDS-PAGE resolved proteins were blotted onto a 0.2 μ m nitrocellulose membrane (Bio-Rad, Hercules, USA) for 2h30 at 300mA and 4°C in a vertical tank transfer system (TE22 Mighty Small Tank Transfer System, Hoefer Pharmacia Biotech, USA) and the success of transfer was confirmed by Ponceau S dye [0.1% (w/v) Ponceau S; 5% acetic acid] staining for 5 min followed with a wash step with distilled water. The membrane was blocked with 3% BSA/1x TBST (20mM Tris HCl pH=7.4, 150mM NaCl and 0.1% Tween-20) for 60 min at room temperature. hCRTAC1 expression was immuno-labeled using the goat anti-hCRTAC1 IgG (1/2.5K) as primary antibody and by incubation overnight at 4°C, followed by incubation with the secondary antibody rabbit anti-goat-HRP (tested for 1/80K, 1/100K and 1/120K), for 1hour at room temperature. All the antibodies were diluted in sterile 1x TBS (20mM Tris HCl pH=7.4 and 150mM NaCl) and all the washing steps in between each antibody incubation were carried out in 1x TBST during 3x 10 min with agitation, except that two additional washing steps of 5 min with 1x TBS were added in the last incubation. As a positive reaction control, recombinant purified hCRTAC1 protein (1 μ g per well) was also resolved. An additional primary antisera [rabbit anti-sbCRTAC2 (1/300K)] was also tested once it is known that cross react with the fish dlCRTAC1 protein and the procedure followed was as in Anjos *et al.*, 2013. Reaction controls were antisera preabsorbed with antigen [10 μ L of purified hCRTAC1 (0.71 μ g/ μ L) in 5mL of anti-hCRTAC1 diluted 1/2.5K in 1x TBS], pre-immune serum and omission of primary antisera.

Membranes were developed by chemiluminiscence with ECL™ Prime Western Blotting detection reagent (Amersham GE Healthcare, Uppsala, Sweden) according to manufactures guidelines and images were captured in ImageQuant LAS 500 (GE healthcare, UK) system until the best signal was achieved.

2.2.3. Subcellular location of human CRTAC1

The subcellular location of human CRTAC1 was assessed using *in silico* sequence analysis through bioinformatics tools and western blot analysis under protein extracts of subcellular fractions.

2.2.3.1. Prediction of subcellular location

The prediction of subcellular localization was carried out using the amino acid sequences of hCRTAC1-A [AC: NP_060528.3 (NCBI); Q9NQ79 (UniProtKB/Swiss-Prot) in Annex II], dlCRTAC1 and dlCRTAC2 (not published, provided by Dra. Anjos L.) in several software's: PSORT II (<http://psort.hgc.jp/form2.html>; Nakai & Horton, 1999), WoLF PSORT (http://www.genscript.com/psort/wolf_psort.html; Horton *et al.*, 2007), TargetP v1.1 (<http://www.cbs.dtu.dk/services/TargetP/>; Emanuelsson *et al.*, 2000) and Predotar v1.03 (<https://urgi.versailles.inra.fr/predotar/predotar.html>; Small *et al.*, 2004). The cutoff value of the predictions was automatically set (default option) and none threshold value was considered (all results were considered).

2.2.3.2. Western blot subcellular location analysis

Western blot was performed using 10µg HEK 293 protein extracts after subcellular fractionation [nuclear (including nuclear membrane), membranes (plasma membrane, endoplasmic reticulum and golgi apparatus), mitochondrial and citoplasmatic fractions) using centrifugation method as described by Huang *et al.* (2012). The Western blot protocol used was as described above (point 2.2.1.2.), the primary antisera was rabbit anti-sbCRTAC2 serum (1/300K) and the secondary antisera donkey anti-rabbit IgG biotinylated (1/80K) antibodies.

2.2.4. Cell culture assays

Cell culture assays were performed with three replicates (n=3 replicates) for each independent experiment (for n=3 or 6 replicated independent experiments) and cells were exposed to the recombinant proteins (hCRTAC1-A, dlCRTAC1 and dlCRTAC2

mature tag-free proteins) at 0.03ng/mL, 0.3ng/mL, 3ng/mL, 30ng/mL and 300ng/mL. Other protein concentrations used are indicated as appropriate.

Incubation of cells in the absence of protein was used as a negative control in order to present a dose-response correlation and to evaluate the behavior (inhibitory or stimulatory effect) between treated and non-treated cells. Different time points of protein/cells incubation were used according to the assays executed.

2.2.4.1. Cell viability assay

MTS proliferation assay and a Trypan blue exclusion test were performed to measure cell viability. MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), inner salt] cell proliferation assays were also performed using the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (Promega, USA) according to manufactures instructions. Briefly, 100µL of THP-1 cells were seeded into 96-well plates at a concentration of 1.5×10^5 cells/mL in RPMI 1640 containing 20% FBS and 1% PenStrep. Cells were incubated in the presence and absence (negative control) of different concentrations of hCRTAC1, dICRTAC1 or dICRTAC2 for 72 hours before analysis. MTS reagent was added directly to the cells and the multi-well plates were incubated at 37°C for 60 min in a humidified 5% CO₂ atmosphere protected from light and the absorbance at 490nm recorded using a Synergy[™] 4 microplate reader and Gen5 Data Analysis Software (BioTeck Instruments Inc., USA).

In the Trypan blue exclusion test, 100µL of THP-1 cells at a density of 10^4 cell/mL were seeded into 96-well plates (SARSTEDT, USA) in RPMI 1640 medium supplemented with 10% FBS and 1% PenStrep. THP-1 cells were exposed to hCRTAC1, dICRTAC1 or dICRTAC2 at 0.01, 0.1, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250 and 500ng/mL for 24 hours, 48 hours and 72 hours. Cell viability was assessed after 24, 48 and 72 hours exposure to protein by counting the number of viable cells using a Neubauer Chamber (Hausser Scientific, USA) according to manufactures instruction after staining with 0.4% trypan blue solution (diluted 1/100, Gibco[®], USA).

2.2.4.2. Flow cytometry

THP-1 monocyte differentiation into macrophage-like cells assays were performed as described by Phillip *et al.* (2005) using THP-1 cells cultured in RPMI

1640 containing 20% FBS and 1% PenStrep. THP-1 cells (1×10^6) were seeded into T25 Flask (SARSTEDT, USA) and grown for 48 hours. THP-1 cells were exposed to 30, 100 and 500ng/mL of hCRTAC1, dlCRTAC1 or dlCRTAC2 and differentiation into macrophages was assessed. Non treated THP-1 cell were used as negative control and Ma cells were used as a positive control. Exposed cells were centrifuged (1000rpm for 10 min) and washed twice with ice-cold staining buffer (1x PBS and 3% FBS) and the cell pellet was surface-stained with $0.2 \mu\text{g}/\mu\text{L}$ of mouse IgG anti-human CD11b conjugated to Allophycocyanin (APC) (BioLegend Inc., USA) by incubation for 60 min at 4°C . Following incubation, the cell pellet was washed twice and resuspended in 1mL of ice-cold staining buffer. Cells were analyzed using the BD FACSCalibur™ flow cytometer and BD CellQuest Pro version 6 software (Becton Dickinson, USA).

2.2.4.3. Cell cycle assay

Cell cycle assays were performed using 1×10^6 THP-1 cells cultured in RPMI 1640 containing 20% FBS/1% PenStrep and seeded into T25 Flask and allowed to grow for 48 hours. Non treated cells were used as a negative control and Ma cells were used as a positive control and the effect of exposure of THP-1 cells to hCRTAC1, dlCRTAC1 or dlCRTAC2 (30, 100 and 500ng/mL) was determined. The cells were centrifuged (1000rpm for 10 min), washed once with ice-cold 1x PBS/3% FBS and fixed with ice-cold 100% absolute ethanol for 60 min at -20°C . The cells were centrifuged again, washed twice with ice-cold 1x PBS/3% FBS and centrifuged. The resulting cell pellet was incubated for 15 min at 37°C with $100 \mu\text{L}$ RNase A ($100 \mu\text{g}/\text{mL}$; Sigma-Aldrich, USA) followed by incubation with 1mL of Propidium iodide ($20 \mu\text{g}/\text{mL}$; Sigma-Aldrich, USA) at room temperature for 20 min. Samples were analyzed using a BD FACSCalibur™ flow cytometer and BD CellQuest Pro version 6 software (Becton Dickinson, USA).

2.2.4.4. Hanging drop assay

Hanging drop assays were performed as described by Kumar *et al.* (2014) using THP-1 cells at a concentration of 1×10^5 cells/mL in RPMI 1640 containing 20% FBS and 1% PenStrep. Droplets ($30 \mu\text{L}$) containing 3000 cells/drop and protein (hCRTAC1, dlCRTAC1 or dlCRTAC2) were pipetted onto the upper side of the lid of 100 x 20 mm diameter tissue culture dishes (SARSTEDT, USA). The lid was inverted and placed on top of the culture dish filled with 5mL of sterile 1x PBS and incubated in a humidified

5% CO₂ atmosphere for 72 hours allowing cells to form aggregates at the base of the drop. Hanging droplets were photographed using an Olympus SZX7 stereo microscope (Olympus America Inc., USA) and the area, circularity and number of cell aggregates was quantified using ImageJ 1.48v software (National Institutes of Health, USA).

2.2.4.5. Wound healing assay

Wound healing assays were performed using HEK 293 cells (5×10^5 cells/mL) in DMEM medium containing 10% FBS and 1% PenStrep and with Ma cells (3.5×10^5 cells/mL) in RPMI 1640 medium containing 20% FBS and 1% PenStrep. Before seeding, a line was drawn at the underside of the culture wells with a marker pen and served to mark the area to be analyzed. Cells were seeded into 6-well plates (SARSTEDT, USA) and grown to confluence. 1x PBS replaced the medium and three parallel scratch wounds were made perpendicular to the marker lines with a blue P1000 pipette tip (approximately 400 μ m width wound). Culture medium containing several concentrations of hCRTAC1, dICRTAC1 or dICRTAC2 was added to the plates after removal of the 1x PBS. Images of the areas flanking the intersections of the wound and the marker line were taken at time zero and after 18 hours of incubation at 37°C. Images were collected using an Olympus SZX2 stereo microscope (Olympus America Inc., USA) and the distance between the borders of the wound were measured using ImageJ 1.48v software (National Institutes of Health, USA). The time for termination of the assay was selected after preliminary small scale assays to establish the ontogeny of wound closure.

2.2.4.6. Cyclic AMP assay

The ability of hCRTAC1, dICRTAC1 and dICRTAC2 proteins to stimulate intracellular cyclic adenosine monophosphate (cAMP) production in THP-1 cells was assessed using cAMP dynamic 2 kit (Cisbio Bioassays, France) as described by Cardoso *et al.* (2015), following the manufactures instructions. Approximately 10.000 cells/well were assayed and incubations were performed in white 384 well small VolumeTM HiBase Polystyrene microplates (Greiner Bio-One, Germany). Prior to the assay, THP-1 cells were resuspended in 1x PBS/1mM 3-isobutyl-1-methylxantine (IBMX; Sigma-Aldrich, USA) and incubated for 5 min at 37°C in a humidified 5% CO₂ atmosphere. Several concentrations of hCRTAC1, dICRTAC1 or dICRTAC2, diluted in 1x PBS/1mM IBMX, were added to the cells for 30 min at 37°C. As a final point, anti-

cAMP-Cryptate and cAMP-d2 were added and the multi-well plates were incubated for 2 hours at 4°C. Forskolin (10µM, Sigma-Aldrich, USA) was used as positive control to assess cellular responsiveness of the cAMP pathway. Two additional assay controls were also performed according to the manufacturers recommendations: **a)** a negative background control (absence of cAMP-d2) and **b)** a non-stimulated cell control (no proteins added). cAMP production was monitored using a Synergy™ 4 microplate reader (BioTek, USA) and Gen5 Data Analysis Software (BioTek, USA). The data obtained was normalized using the absorbance ratio 665nm/620nm and expressed in 1/Delta F (%), following the manufactures recommendations.

2.2.5. Cell transfection and CRTACs stable cell line

2.2.5.1. Mammalian CRTAC's expression vector constructs

pGEM-T easy vector (Promega, USA) encoding the full-length of human *CRTAC1-A* (clone acquired from GeneCopoeia™, USA) and *D. labrax crtac1* and *crtac2* (cloned in the host laboratory) were provided by Dr. L. Anjos and were used as templates for PCR amplification products and subcloning into pcDNA™ 3.1 (Directional TOPO® Expression Kit mammalian expression vector, Invitrogen, USA; pcDNA™ 3.1 map vector in Appendix III). Specific primers flanking the proteins mature region and incorporating enzyme restriction sites (5' *Not* I and 3' *Hind* III) for each *crtac* were designed using Primer Premier v5 software (Premier Biosoft, Sigma-Aldrich, USA) (Table 1). The thermocycle consisted of: 95°C for 5 min followed by 35 cycles of 30 s at 95°C, 30 s at 63.5°C (human *CRTAC1-A*) or 59.6°C (*D. labrax crtac1*) or 60°C (*D. labrax crtac2*), 72°C for 2 min followed by a final cycle of 10 min at 72°C. All PCR reactions were amplified in a 15µL reaction volume containing 0.3U DreamTaq DNA polymerase (5U/µl, Thermo Fisher Scientific Inc., USA), 1.5 mM MgCl₂ (Thermo Scientific Inc., USA), 0.2 mM dNTP's (GE Healthcare, Spain) and 0.25 µM of each gene specific primer pairs. Amplified PCR products were separated by gel electrophoresis (1% agarose/1×Tris Acetate-EDTA gel and 1µg/mL ethidium bromide) and products of the correct size were purified from gel band using an illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Spain) and sequenced to confirm the gene identity.

Table 1. Primers sequences to amplify human and *D. labrax* CRTAC's genes to clone into the pcDNATM3.1 vector.

Gene	Primer Name	Primer sequence ¹
Human <i>CRTAC1-A</i>	LAfw-hCRTAC1pcDNA	Fw 5' - GCG AAGCTT ATGGCTCCGAGCGCTG - 3'
	LArev-hCRTAC1pCDNA	Rv 5' - CGC GCGGCCG CCTAGCAGCTGGGCT - 3'
<i>D. labrax crtac1</i>	LAfw-dlCRTAC1pcDNA	Fw 5' - GCG AAGCTT ATGTTGTTGTGGCTGG - 3'
	LArev-dlCRTAC1pCDNA	Rv 5' - CGC GCGGCCG CCTACAGTAGTCCAG - 3'
<i>D. labrax crtac2</i>	LAfw-dlCRTAC2pcDNA	Fw 5' - GCG AAGCTT ATGTGGGCTTCAGGTC - 3'
	LArev-dlCRTAC2pCDNA	Rv 5' - CGC GCGGCCG CTCACAGACCTGCAC - 3'

The blue color in the primer sequences represent the restriction site for *Hind* III and green color the restriction site for *Not* I. ¹ Sequences of forward (Fw) and reverse (Rv) primers (5' to 3' sense) for *crtac* genes.

The amplified products and the pcDNATM3.1 (5µg) were digested separately using (0.5µL or 1 µL, respectively) FastDigest *Not* I (Fermentas, Thermo Scientific Inc., USA) and FastDigest *Hind* III (Fermentas, Thermo Scientific Inc., USA), in a reaction volume of 20µL for 1 hour at 37°C. Digested and purified pcDNATM3.1 was treated with 0.003U of Calf Intestinal Alkaline Phosphatase (CIAP; 0.01U/µL, Promega, USA) in a reaction volume of 20µL for 30 min at 37°C. Lastly, digested gene products were cloned in treated pcDNATM3.1 vector using 3.5U of T4 DNA Ligase, (5U/µL, Fermentas, Thermo Scientific Inc., USA) in a reaction volume of 10µL for 3 hour at room temperature. All these enzymatic reactions were executed according to manufactures instructions.

The ligation products were used to transform XL1–Blue *E. coli* strain (Stratagene, Agilent Technologies Inc., USA) and a colony PCR was performed to select the positive clones for the recombinant vector. After positive clone selection the recombinant plasmids were extracted using the GeneJetTM plasmid Miniprep kit (Fermentas, Thermo Scientific Inc., USA), according to manufactures instructions and were sequenced to confirm if the coding regions of CRTAC was in frame with the vector promoter pCMV.

2.2.5.2. HEK 293 cell transfection

Stable transfection of CRTAC's/pcDNATM3.1 was performed using FuGENE[®] 6 Transfection Reagent (Promega, USA). Each recombinant CRTAC's/pcDNATM3.1 mammalian expression vector (5µg) was digested with 100U *Bst*BI (10U/µL; Fermentas, Thermo Fisher Scientific Inc., USA) for 60 min at 37°C, followed by 20 min

at 80°C, in order to linearize the recombinant expression vectors for cell nuclear DNA integration. On the day prior to transfection, HEK 293 cells (2×10^5 cell/mL seeded in 6-well tissue culture plates) were transfected with 2.5µg of linearized expression vectors and FuGENE[®] 6 reagent (ratio 1:6). Cells were incubated for 24 hours at 37°C with DMEM medium containing 10% FBS and 1% PenStrep, and then maintained in DMEM medium containing 10% FBS, 1% PenStrep and supplemented with 800µg/mL of Geneticin (G418; GibcoBRL, USA) for at least 4 weeks for permanent transfected cell selection. Cell recovery was monitored daily by constant changes of antibiotic selective medium until no cell death was observed.

The success of transfection of HEK 293 cells and CRTAC's protein expression will be performed by PCR and western blot, but it was not possible to complete this work in the timescale of the thesis. This work will be finalized in a subsequent study.

2.2.6. Data Treatment

The percentage (%) of cell viability in the Trypan blue exclusion test was determined using the following equation:

$$\% \text{ Cell Viability} = [1 - (\text{Number of blue cells} / \text{Number of total Cells})] \times 100$$

The IC₅₀, defined as the CRTAC concentration necessary to cause a 50% decrease in the initial number of cells, was calculated by plotting the percentage of cell viability in each condition against the concentration of human and teleost CRTAC used in the assay.

The percentage (%) of healing and motility capability in the wound healing assay was determined using the following equation:

$$\% \text{ Healing or Motility} = 100 - \left[\left(\text{distance between borders}_{18h} \text{ (mm)} / \text{distance between borders}_{0h} \text{ (mm)} \right) \times 100 \right]$$

All data were expressed as the mean ± standard error of the mean (SEM). Statistical differences between experimental groups were determined using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. All statistical analyses were performed using GraphPad Prism 6 version 6.01 (GraphPad Software, Inc., USA). Differences were considered to be significant at P < 0.05.

3. RESULTS

3.1. DETECTION OF HUMAN *CRTAC1* TRANSCRIPTS AND PROTEIN IN CELL CULTURES

Initially, the intrinsic expression of *hCRTAC1*-A transcript or hCRTAC1 protein in THP-1, Ma and HEK 293 cells was investigated by RT-PCR and western blot.

The *hCRTAC1*-A transcript was amplified in cDNA of HEK 293 cells (Figure 5. A and B) with a molecular weight as expected for both, the mature and the truncated transcript (of ≈ 1900 bp and ≈ 230 bp respectively). The identity of the RT-PCR products was positively confirmed by sequencing (results not shown). No RT-PCR products were amplified for *hCRTAC1*-A in THP-1 monocytes or after they were induced to differentiate into macrophages cells nor in the reaction control (absence of cDNA).

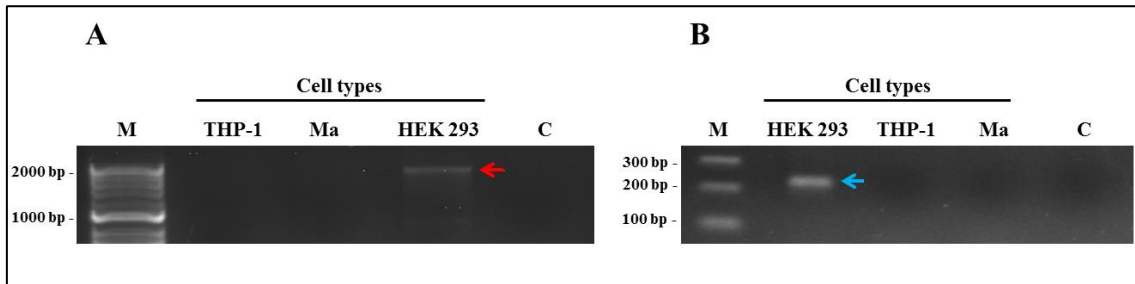


Figure 5. Expression of human *CRTAC1*-A transcripts in cDNA from HEK 293, THP-1 and Ma cells. RT-PCR product amplification using primer pairs flanking the mature *hCRTAC1*-A transcript (A) and a truncated form (B). In A and B the arrows highlight the amplicons obtained with a molecular weight of ≈ 2000 bp (red arrow) and 200bp (blue arrow), close to the expected (of ≈ 1902 bp and ≈ 230 bp). M – is the molecular weight marker and the molecular sizes in base pairs (bp) are indicated on the left hand side. Control samples: (C) – PCR reaction lacking cDNA, reaction control.

Two different protein extraction buffers (RIPA modified buffer and a denaturing buffer) were tested for protein extraction from HEK 293 cells in order to optimize the best hCRTAC1 extraction conditions. SDS-PAGE analysis showed no relevant differences between the two extraction methods (results not shown), so all the total protein extracts were performed using the denaturing buffer, to take into account the propensity of CRTAC to aggregate *in vivo* and *in vitro* (Anjos *et al.*, 2013) and also the increased availability of the sequential epitope regions for antibodies recognition.

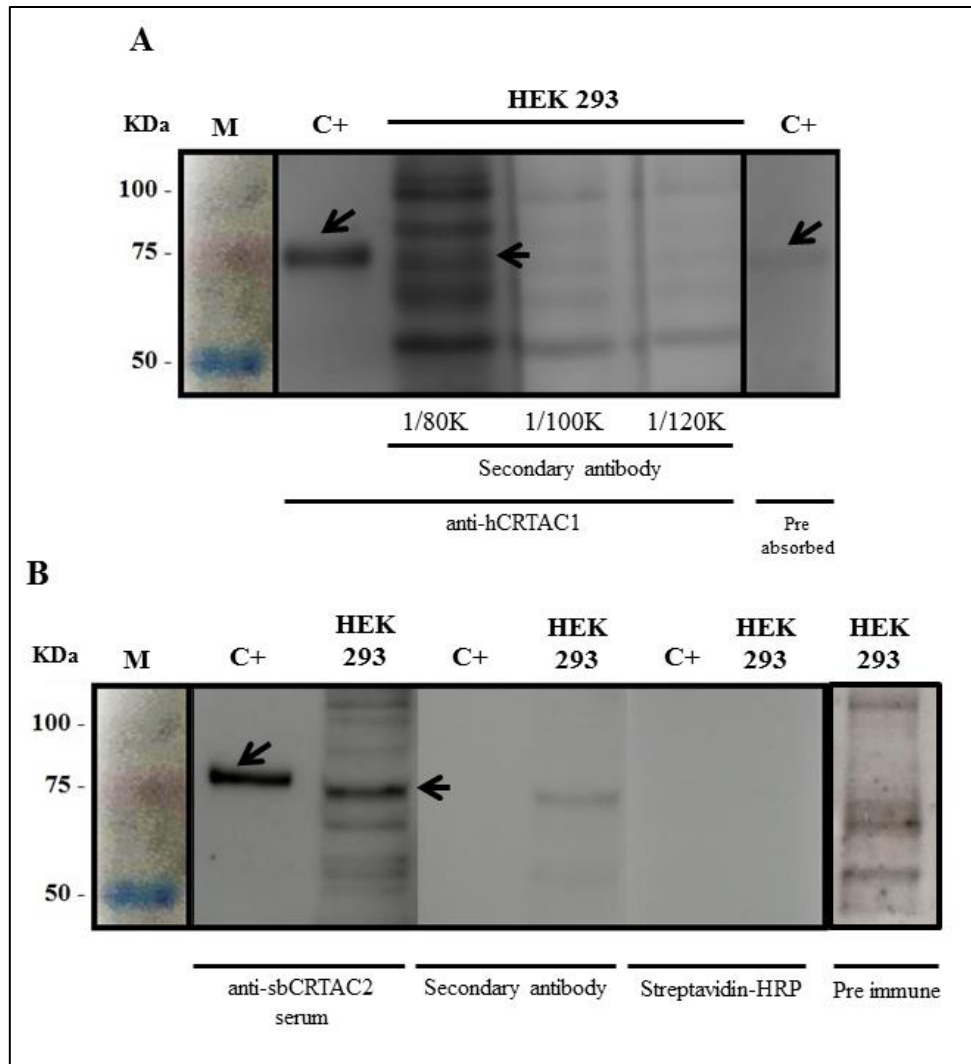


Figure 6. Western blot revealing hCRTAC1 expression in HEK 293 cell lines. 2 μ g of recombinant hCRTAC1-A (positive control, C⁺) and 4.5 μ g of total HEK 293 cell protein extracted (in denaturing buffer) were fractionated by SDS-PAGE (10% polyacrylamide) under reducing conditions and blotted onto a nitrocellulose membrane. In panel (A) the primary antisera was the goat anti-hCRTAC1 IgG (diluted 1/2500) or pre absorbed antisera (1/2.5K, saturated goat anti-hCRTAC1 with 7.1 μ g recombinant hCRTAC1-A for specificity control) followed by the secondary antibody rabbit anti-goat-HRP (1/80K, 1/100K and 1/120K). In panel (B) the primary antisera was the rabbit anti-sbCRTAC2 antisera (1/300K, which cross react with the hCRTAC1-A) and as secondary antibody was the donkey anti-rabbit IgG biotinylated (1/35K) followed by Streptavidin-HRP (1/50K). Antibody specificity controls were incubation with the secondary antibody and streptavidin and only streptavidin. The exposure time for image capture was 2min (A) and 5min (B) respectively. Molecular weight markers (M, in kDa, Precision Plus ProteinTM Standards Dual Color; Bio-Rad Laboratories, USA) are indicated and immunoreactive hCRTAC1 bands are highlighted with black arrows.

Western blot analysis validated the RT-PCR results and a single immunoreactive band with the expected molecular weight (Mw) of 68.6 kDa was identified in HEK 293 cell protein extracts (Figure 6. A and B). The commercial anti-hCRTAC1 antibody did not result in conclusive results due to cross-reaction (Figure 2. A) with other factors in HEK 293 cell extracts and it has not been possible to resolve this problem so far.

However, the anti-sbCRTAC2 antibody available in house and already characterised (Anjos *et al.*, 2013) that detects sea bream and sea bass dICRTAC1 was also tested with HEK 293 cell protein extracts (Figure 2. B). An immunoreactive protein band was observed with a Mw close to that of recombinant hCRTAC1-A (68.6 kDa) in HEK 293 cell protein extracts (Figure 2. B) and this signal was ablated in the western blot validation controls.

3.2. SUBCELLULAR LOCATION OF HUMAN CRTAC1

The subcellular localization of hCRTAC1-A and teleost dICRTACs (CRTAC1 and 2) was established through *in silico* sequence analysis using several software's as a tactic for comparison and collection of as many data as possible (Table 2). Additionally, the subcellular localization of hCRTAC1 was also established by western blot analysis.

Predicted sub cellular localization analysis (Table 2) indicated with a high probability that hCRTAC1-A, dICRTAC1 and dICRTAC2 are extracellular proteins [PSORT II (<http://psort.hgc.jp/form2.html>): 66.7-77.8% in 100%; WoLF PSORT (http://www.genscript.com/psort/wolf_psort.html): 19-23k-NN in 32k-NN] and follow the secretory pathway [TargetP (<http://www.cbs.dtu.dk/services/TargetP/>): 0.888-0.946 in 1], which is consistent with the presence of a signal peptide. The sub cellular localization of CRTAC that was less probable was localization in vacuoles (PSORT II: 22.2%) and for hCRTAC1-A localization in mitochondria (PSORT II: 11.1%). Predictions from WoLF PSORT show that all the proteins may be present (with a lower score) in lysosomes (6-9k-NN in 32k-NN) and that dICRTAC1 may be also present in peroxisomes and in the golgi apparatus (3k-NN in 32k-NN).

Predotar v1.03 software (<https://urgi.versailles.inra.fr/predotar/predotar.html>; a prediction tool for identification of putative mitochondrial and endoplasmic reticulum N-terminal targeting sequences) predicts that all proteins possess an endoplasmic reticulum targeting sequence (0.99-0.92 in 1) and that piscine dICRTAC2 may also be present in mitochondria.

Table 2. Predicted subcellular location of human and teleost CRTAC's. The table content shows the predicted results for the subcellular location of human and *D. labrax* CRTAC proteins using available bioinformatics tools.

Species	Protein name	Accession number	Subcellular location prediction results in 4 software's			
			PSORT II http://psort.hgc.jp/form2.html	WoLF PSORT http://www.genscript.com/wolf-psort.html	TargetP v1.1 http://www.cbs.dtu.dk/services/TargetP/	Predotar v1.03 https://urgi.versailles.inra.fr/predotar/predotar.html
<i>Homo sapiens</i> (Human)	hCRTAC1-A	NP_060528.3 (NCBI) Q9NQ79 (UniProtKB/Swiss-Prot)	Extracellular (66.7%) Vacuolar (22.2%) Mitochondrial (11.1%)	Extracellular (23k-NN) Lysosome (8k-NN)	Secretory pathway (0.946)	Endoplasmic reticulum (0.99)
<i>Dicentrarchus labrax</i> (European seabass)	dICRTAC1	Na	Extracellular (77.8%) Vacuolar (22.2%)	Extracellular (19k-NN) Lysosome (6k-NN) Peroxisome (3 k-NN) Golgi (3 k-NN)	Secretory pathway (0.888)	Endoplasmic reticulum (0.92)
	dICRTAC2	Na	Extracellular (77.8%) Vacuolar (22.2%)	Extracellular (20k-NN) Lysosome (9k-NN)	Secretory pathway (0.924)	Endoplasmic reticulum (0.99) Mitochondrial (0.11)

Na – accession number not available. k-NN – k-Nearest Neighbors (algorithm in which a query is classified by a majority of votes assigned to the most common class of nearest neighbors; 32k-NN is the maximum value provided and represents the proteins with similar PSort scores as the query).

A search in the GeneCards[®] Human Gene Database (<http://www.genecards.org/cgi-bin/carddisp.pl?gene=CRTAC1>) supported our results and revealed that hCRTAC1-A is secreted to the extracellular space and also introduced the possibility of the protein being located at the cell plasma membrane (Figure 7).

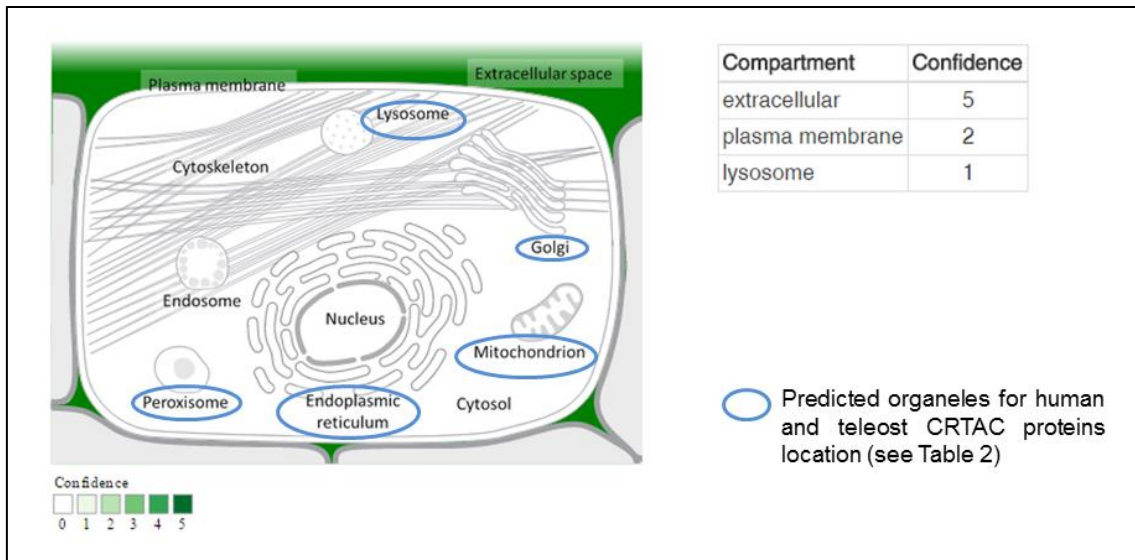


Figure 7. Predicted subcellular location of human and teleost CRTAC proteins. Predicted subcellular location for human CRTAC1, with the respective grade of confidence (as indicated by green colours). Blue rings indicate the predicted organelles where human CRTAC1-A and teleost CRTAC proteins may be present (see Table2). Image extracted from GeneCards® Human Gene Database (<http://www.genecards.org/cgi-bin/carddisp.pl?gene=CRTAC1&keywords=crtac1#localization>) and based in data obtained from UniProtKB/Swiss-Prot and Ensembl databases.

In summary, all the predictions about CRTAC sub-cellular localization indicate it is most likely associated with the secretory pathway (extracellular proteins) and may also co-localize in the endoplasmic reticulum, vacuoles, mitochondria and lysosomes.

Analysis of the sub-cellular location of hCRTAC1 by Western blot using subcellular fractions of extracted HEK 293 cells (Figure 8) confirmed the predicted subcellular localization and was used to infer potential protein functions.

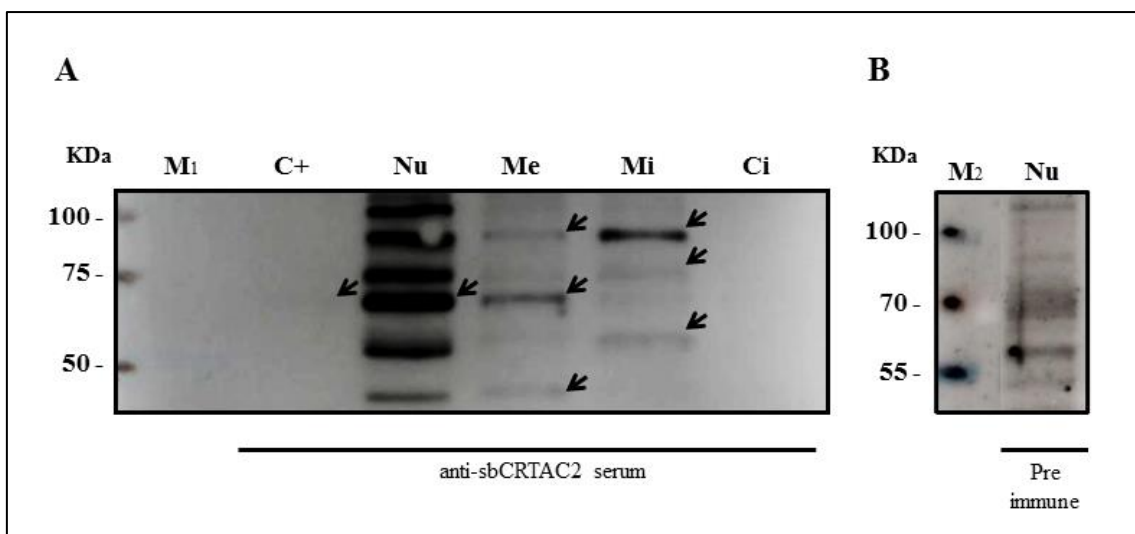


Figure 8. Western blot revealing subcellular location of hCRTAC1 in HEK 293 cells. 2µg of recombinant hCRTAC1-A (positive control, C⁺) and 10µg of each cellular fraction of HEK 293 cell

protein extracts were fractionated by SDS-PAGE (10% polyacrylamide) under reducing conditions and blotted onto a nitrocellulose membrane. **(A)** The immunodetection was carried out using the primary antisera rabbit anti-sbCRTAC2 antisera (1/300K, which cross react with the hCRTAC1-A) and the secondary antibody was the donkey anti-rabbit IgG biotinylated (1/35K) followed by Streptavidin-HRP (1/50K). **(B)** Antibody specificity controls were incubation with the pre immune serum (1/300K). The exposure time for image capture was 3min. Molecular weight markers [M_1 , in kDa, Precision Plus Protein™ Standards Dual Color (Bio-Rad Laboratories, USA); M_2 , in kDa, PageRuler™ Plus Prestained Protein Ladder (Thermo Fisher Scientific Inc., USA)] are indicated and immunoreactive hCRTAC1 bands are highlighted with black arrows in each cellular fraction. Nu – Nuclear fraction, Mi – Mitochondrial fraction, Me – Membrane fractions (includes endoplasmatic reticulum and golgi apparatus), Ci – Citoplasmatic fraction.

hCRTAC1 was highly abundant in the nuclear fraction (Figure 8). Several immunoreactive proteins of between 100-55 kDa were detected, including one with the predicted Mw, 68.6 kDa, of hCRTAC. Additional immunoreactive protein bands were also observed in the membrane fraction (including the endoplasmatic reticulum and golgi apparatus \approx 90, 70, 40 kDa) and mitochondrial (\approx 90, 75, 55 kDa) fraction. No protein bands were observed in the citoplasmatic fraction.

Combining the overall results of the predicted *in silico* and verified sub-cellular location strongly suggests that the majority of hCRTAC1 trafficked via the extracellular pathway and lesser amounts are also present in the nucleus, endoplasmatic reticulum and mitochondria. Teleost CRTAC proteins subcellular results *in silico* indicate that the proteins also follow a secretory pathway and may also be present in the endoplasmatic reticulum, mitochondria, vacuolar organelles and lysosomes.

3.3. CELL CULTURE ASSAYS

3.3.1. Teleost CRTAC effect on THP-1 cell viability

The effect of hCRTAC1 and fish dlCRTAC1 and dlCRTAC2 on THP-1 cell viability was evaluated by MTS proliferative and Trypan blue exclusion assays. MTS assays (in which the quantity of formazan compound formed is directly proportional to the number of proliferative cells in culture) revealed that dlCRTAC1 at the concentration of 30ng/mL ($p < 0.05$) to 300ng/mL ($p < 0.001$) decreased the proliferative activity of THP-1 cells (Figure 9. A) relative to the control (non-treated cells). No significant cell viability effects were observed for THP-1 cells treated with dlCRTAC2 or with hCRTAC1-A (Figure 9. B and C) in the range of concentrations tested.

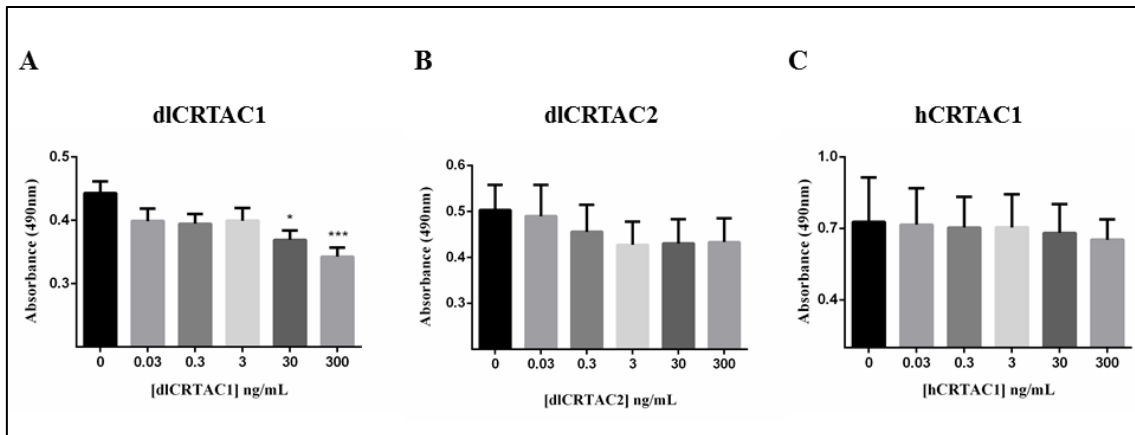


Figure 9. Effect of CRTAC's on human monocytes THP-1 cell viability using a MTS assay. Cells (1.5×10^5 cells/mL) were incubated for 72 hours in medium with or without proteins at 37°C and the concentration of formazan product was measured by absorbance at 490nm. **A.** Measurements of THP-1 cells treated with dlCRTAC1 (**A**), dlCRTAC2 (**B**) and hCRTAC1-A (**C**) at concentrations of 0.03, 0.3, 3, 30 and 300ng/mL (Bars represents mean \pm SEM; tested for n = 6 independent experiments). * and *** indicates treatments with statistical significance for $p < 0.05$ and $p < 0.001$ (One-way ANOVA).

The trypan blue exclusion assay, used to determine the percentage of viable cells, based on the principle that viable cells possess an intact cell membrane that excludes this dye, indicated that the treatment of the cells with the hCRTAC1-A did not influence the cell viability, irrespective of the concentration used and the incubation times tested (data not shown). In contrast, dlCRTAC1 and 2 affected THP-1 cell viability in a dose dependent manner (Figure 10). dlCRTAC1 caused a dose response inhibitory effect with an IC_{50} of 78.45ng/mL and dlCRTAC2 had an IC_{50} of 95.50ng/mL (Figure 10. A) after 72 hours of cells incubation. This means that at this concentration the number of viable cells decreased to half the number of the cell present in the control (non-treated cells). The same effect was also observed in cells incubated for 48 hours (data not shown).

Despite the decrease in cell number over the duration of the culture (assessed with a Neubauer Chamber using cells stained with trypan blue dye), no cell death (data not shown) was observed for dlCRTAC1 and dlCRTAC2 treated cells. However, observations by microscopy indicated that THP-1 cells started to adhere to the culture plates when dlCRTAC1 (e.g. 500ng/mL) was present in the culture medium (Figure 10. B I and II) and this presumably explained the apparently reduced cell number.

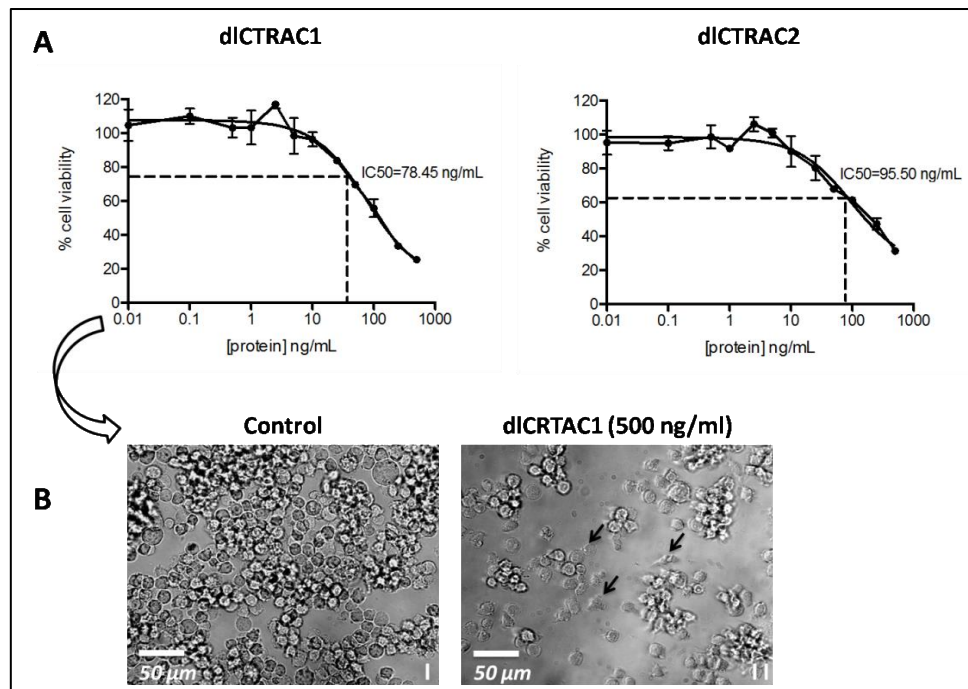


Figure 10. Effect of dlCTRAC1 and 2 on human monocyte (THP-1) cell viability using a Trypan blue exclusion test. Cells (10^4 cells/mL) were incubated for 72 hours in medium with or without CRTAC at 37°C. Panel (A), plotting the % of cell viability against several concentration of dlCTRAC's (0.01, 0.1, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250 and 500 ng/mL). Cell viability was assessed with a Neubauer Chamber using cells stained with Trypan blue dye. The IC_{50} values obtained are the concentrations required to reduce cell number to a point half way between the top (0.01 ng/mL treated cells) and bottom (500 ng/mL treated cells) plateaus of the curve ($n = 6$ independent experiments and $p < 0.05$). Panel (B), representative microscopic images of THP-1 control cells (I) and cells incubated with 500 ng/mL dlCTRAC1 (II). Black arrows indicate that THP-1 cells start to adhere to the culture plate (images captured using an inverted microscope, x400 amplification).

The decrease in cell proliferation determined using the MTS assay (result of the continuous proliferation of the control cells against the lower rate of cells division of the treated cells with the higher protein concentrations) is consistent with the results of the Trypan blue exclusion assay, where THP-1 cells seem to become adhesive. Based on the results, we hypothesize that dlCTRAC's could have some effect on THP-1 cell differentiation.

3.3.2. Effect of CRTAC's on THP-1 monocyte cell line differentiation

Since the cell viability assays suggested that THP-1 cells became adherent when treated with CRTACs, suggesting a change in differentiation state occurred (e.g. monocyte to macrophage). A flow cytometry assay using a macrophage marker (CD11b) was performed to investigate the effect of CRTAC on THP-1 monocytic cell differentiation into macrophage-like cells.

The results using the anti-CD11b antibody (macrophage cells marker) indicated that neither human nor fish CRTAC stimulate THP-1 monocyte differentiation into macrophages at the concentrations and incubation times tested. No significant differences were observed between control (non-stimulated cells, with $12.22 \pm 0.88\%$ of cell that were differentiated into macrophages) and CRTAC's exposed cells (using IC_{50} equivalent protein concentrations, 100ng/mL) in the number of macrophages, which was of 11.80 ± 1.63 ; 11.69 ± 1.47 ; $11.71 \pm 1.76\%$ (Figure 11). The macrophage inducer PMA was used as the positive control (Ma cells) and a significant increase $90.46 \pm 0.83\%$ in macrophage-like cells (Figure 11) was registered relative to the control, untreated cells. Similar results were obtained for the concentrations (30 and 500ng/mL) of CRTAC proteins tested (data not show).

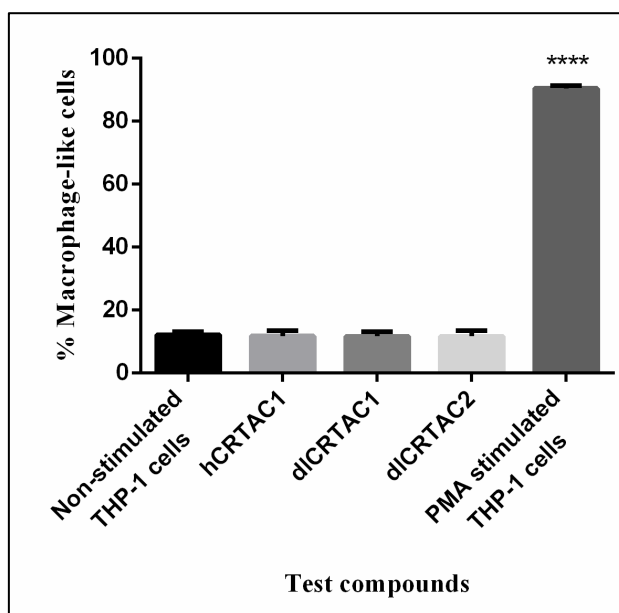


Figure 11. Analysis by flow cytometry of surface expression of the CD11b macrophage marker in cells exposed or depleted of human and teleost CRTAC's proteins. Percentage of macrophage-like cells was determined in non-treated THP-1 cells (basal control), THP-1 cells induced Ma differentiation (PMA induced monocytes differentiation into Ma cells, positive control) and in THP-1 cells treated with (100ng/mL) of human and teleost CRTAC proteins (Bars represents mean \pm SEM; tested for $n = 3$ independent experiments). **** indicates treatment with statistical significance for $p < 0.0001$ (One-way ANOVA).

3.3.3. CRTAC's effect on the THP-1 monocyte cell cycle

To determine whether the inhibitory effect of CRTAC on THP-1 cell viability involved cell cycle changes, we examined cell cycle phase distribution of treated cells by flow cytometry, namely G0/G1 (G0, cells stop dividing; G1, cells prepared for DNA synthesis), S (DNA synthesis) and G2 (cells that are prepared for mitosis or/and

continues to growth) phase (Cooper, 2000). A propidium iodide staining assay revealed that THP-1 monocyte cells treated with human or teleost CRTACs (100ng/mL) caused no significant change in the cell cycle compared to the non-treated THP-1 monocytes (basal control) (Figure 12). In the basal control 53.21 ± 0.32% of cells were in G0/G1, 28.37 ± 1.06% in S phase and 18.85 ± 1.35% in the G2 phase and similar values were obtained for cells exposed to human and teleost CRTACs (Figure 12). Ma cells (positive control or assay control) were also evaluated and 65.78 ± 1.32% of cells were in the G0/G1 phase, 9.02 ± 0.49% in S phase and 24.74 ± 0.69% in G2 phase (data not show). This is consistent with the known behavior of Ma cells that stop dividing and start to increase in size. Human and teleost CRTAC's had no effect on the THP-1 monocytes cell cycle at the concentration and incubation times tested. For the other two concentrations of CRTAC protein tested the results were similar to the presented results (data not show).

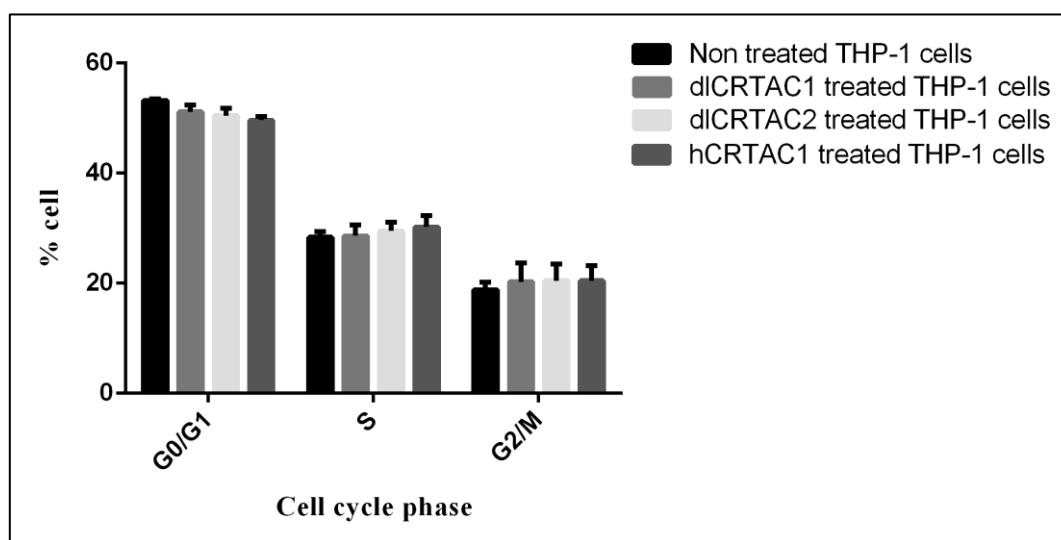


Figure 12. Effect of CRTAC's on THP-1 cell cycle. Percentage of macrophage-like cells was determined in non-treated THP-1 cells (basal control) and in THP-1 cells treated with (100ng/mL) of human and teleost CRTAC proteins. Bars represents mean ± SEM; tested for n = 3 independent experiments.

3.3.4. CRTAC potentiates THP-1 monocytes cell aggregation

Hanging drop assays were used to investigate the effect of CRTACs on cell aggregation (Foty, 2011). During this experiment the total area (μm^2) of the spheroids/cell aggregates formed by THP-1 cells and their circularity was measured and compared between treatment conditions. Only dlCRTAC1 potentiated an increase in

THP-1 spheroid area at the concentrations of 0.03, 3 and 30ng/mL (for $p < 0.05$, 0.01 and 0.001, respectively) in comparison to the control (non-treated THP-1 cells) and no significant effect was observed for hCRTAC1-A or dICRTAC2 (Figure 13. panel I-A, B and C).

Circularity measurements of THP-1 cell spheroids revealed that all CRTACs significantly potentiated the formation of circular cell aggregates in relation to the control (non-treated THP-1 cells) (Figure 13. panel II-D, E and F), as the cell aggregates became more cohesive. hCRTAC1-A and dICRTAC1 (Figure 13. panel II-D and E) had the most pronounced effect at concentrations of 0.03-30 and 0.3-30ng/mL ($p < 0.01$ -0.001 and 0.01-0.0001) respectively. dICRTAC2 had a lesser effect (Figure 13. panel II-F) on the formation of circular cell aggregates, and the effect was only significantly different at 0.3 and 3ng/mL ($p < 0.05$).

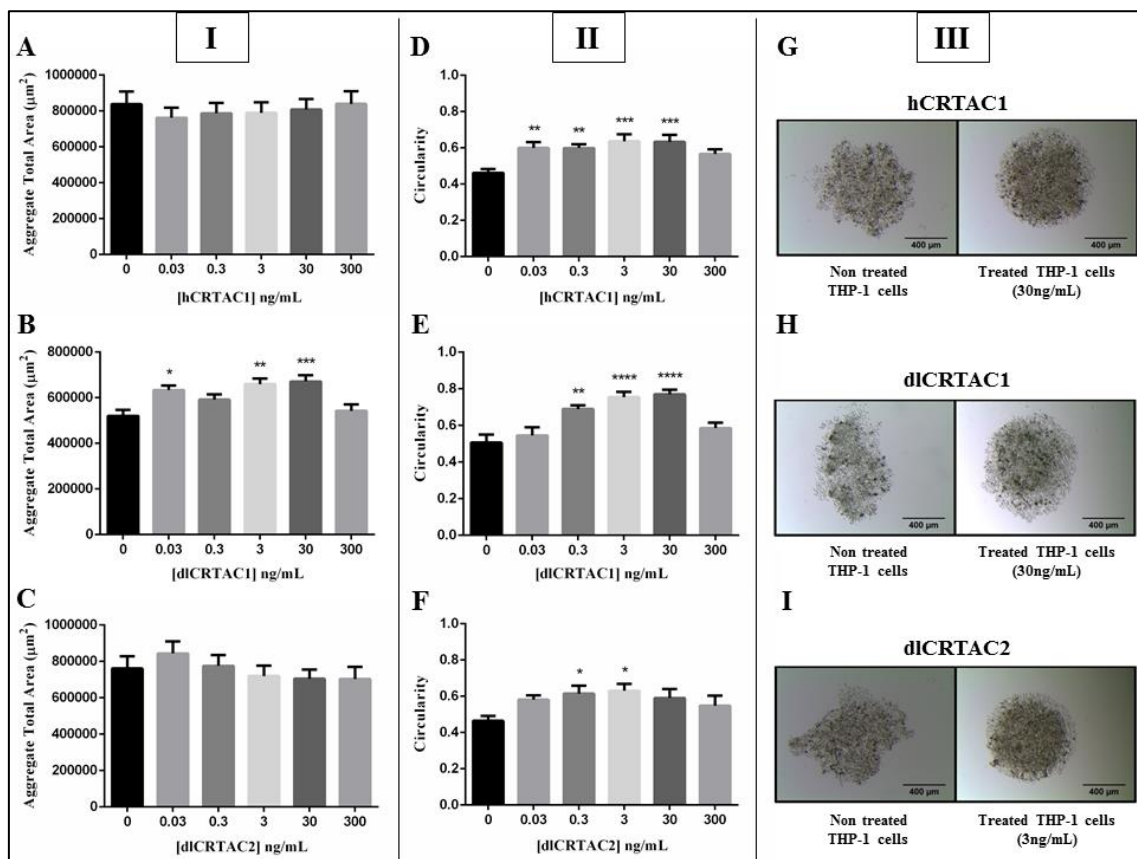


Figure 13. Effect of CRTAC's on THP-1 monocyte aggregate total area (μm^2) and circularity by hanging drop assay. Droplets ($30\mu\text{L}$) containing ≈ 3000 THP-1 cells/drop and CRTAC proteins (at 0.03; 0.3; 0.3; 3; 30 and 300ng/mL) were incubated for 72 hours for cellular aggregate formation (Bars represents mean \pm SEM; tested for $n = 6$ independent experiments). In panels: (I), Aggregate/spheroid total area measurements (μm^2) plotted against hCRTAC1 (A), dICRTAC1 (B) and dICRTAC2 (C) concentrations; (II), Circularity measurements plotted against hCRTAC1 (D), dICRTAC1 (E) and dICRTAC2 (F) concentrations; (III), Microscopic images of THP-1 multicellular spheroids formed by the hanging drop method and harvested 72h after hCRTAC1-A (G), dICRTAC1 (H) and dICRTAC2 (I) incubation or not

(control), scale bars - 400 μ m. *, **, *** and **** indicates significant differences for $p < 0.05$, 0.01, 0.001 and 0.0001 (One-way ANOVA).

The hanging drop method enabled successful formation of compact THP-1 cell spheroids under the effect of CRTAC proteins. CRTACs circularity effects on THP-1 cell aggregates is clear in figure 13 panel III-G, H and I, in which the cells incubated with protein exhibited a cohesive circular aggregate form and compact spheroids relative to control cell aggregates that were more disordered.

An additional observation was the formation of dark areas inside the THP-1 cell spheroids (Figure 14) when supplemented with dlCRTAC1 and mainly at the concentration of 30ng/mL. This effect was not observed in the control cells or in cells incubated with hCRTAC1 or dlCRTAC2. Similar structures have been designated melanomacrophage centers and are normally found in teleost lympho-haematopoietic tissues and liver (Reddy, 2012). These structures have also been described in humans suffering from melanomalytic glaucoma (McMenamin & Lee, 1986). Additional experiments will be necessary to investigate the formation of these structures and their relationship with the CRTAC's.

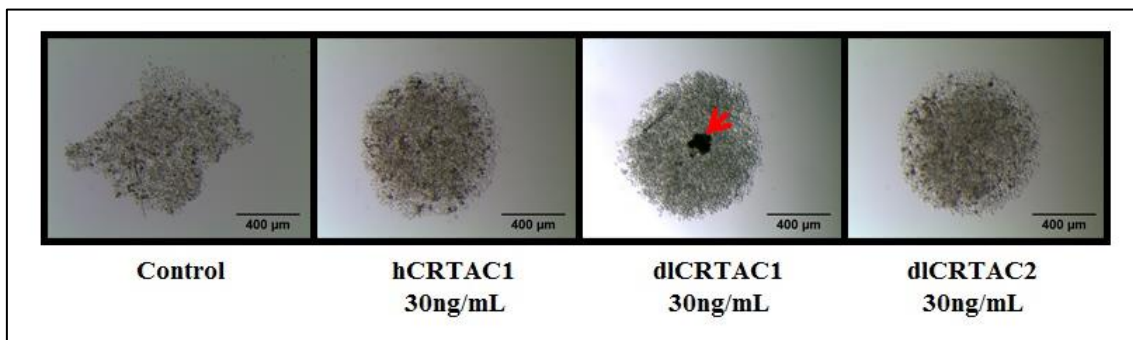


Figure 14. Formation of small dark areas in THP-1 cell spheroids in a hanging drop. Representative microscopic images of THP-1 multicellular spheroids formed by the hanging drop method and harvested 72 hours after incubation or not (control) with hCRTAC1-A, dlCRTAC1 and dlCRTAC2 at 30ng/mL. Images captured using an oscilloscope and a digital camera (Scale bars - 400 μ m). Note the dark area indicated by the red arrow.

3.3.5. Effects of CRTAC's in the wound healing process and cell motility

The effect of human and piscine CRTAC's on the healing process was investigated in HEK293 cells and on the motility of macrophage derived THP-1 monocytic cells, since THP-1 cells are normally non-adherent and unable to build an ECM.

Wound scratch or wound healing assays were performed using confluent monolayers of HEK 293 cells and gave a measure of CRTAC's effectiveness in the healing process. dlCRTAC1 inhibited the healing process at a concentration of 0.03ng/mL ($p < 0.001$) and promoted the healing process at a concentrations of 30ng/mL ($p < 0.05$) (Figure 15. A) when compared with the untreated control cells (HEK293 cells in the absence of protein). dlCRTAC2 inhibited the healing process at concentrations of 3, 30 and 300ng/mL ($p < 0.05$ and 0.01, respectively) in comparison with the control cells (Figure 15. B). hCRTAC1-A, in common with dlCRTAC1, inhibited the healing process at concentration of 0.03 and 0.3ng/mL ($p < 0.01$) and stimulated the healing process at concentration of 300ng/mL ($p < 0.05$) (Figure 15. C) in comparison with the control cells.

CRTAC's wound healing effects on HEK 293 cell monolayers is clearly illustrated in figure 15 (panel II), in which cells incubated with protein exhibited the inhibitory effect of dlCRTAC1, dlCRTAC2 and hCRTAC1-A (panel II - D, F and G, respectively) and an enhancer effect for dlCRTAC1 and hCRTAC1-A at high concentrations (panel II - E and H, respectively).

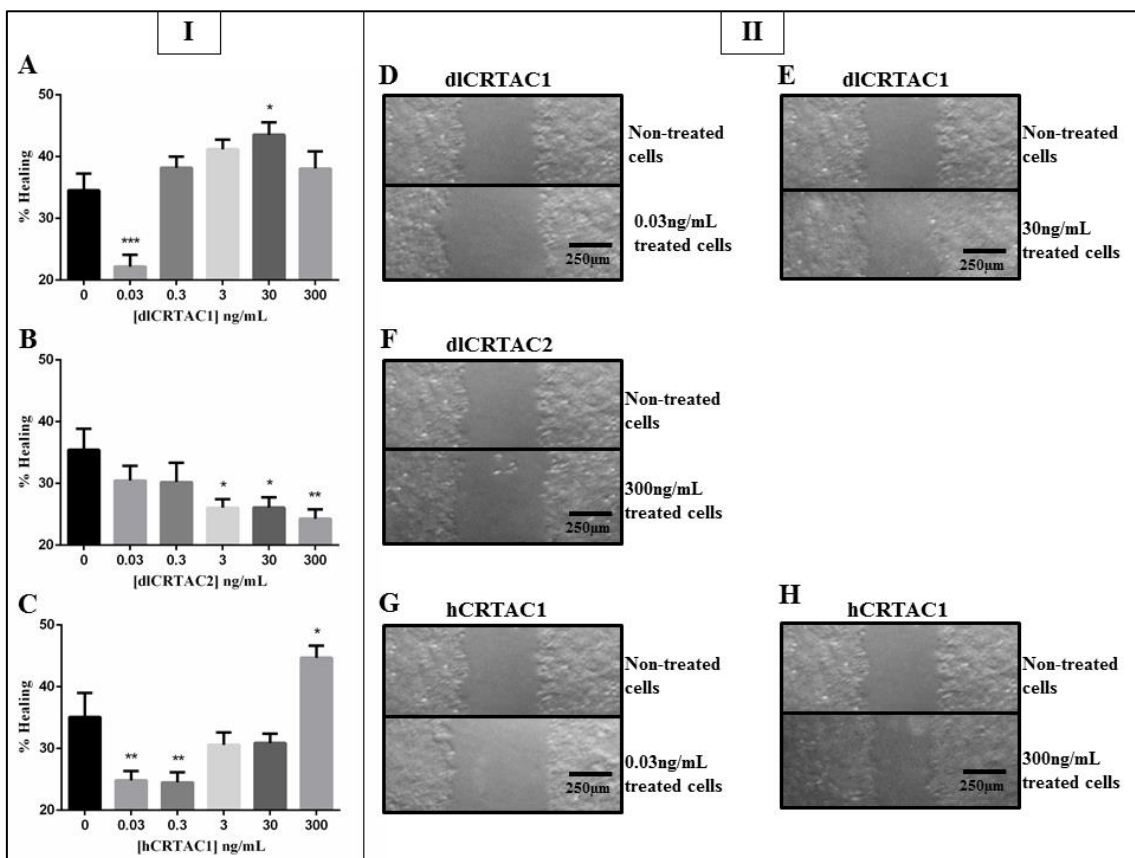


Figure 15. The effects of CRTAC's during the wound healing process using a HEK 293 cell assay. Confluent HEK 293 cell cultures were scratch wounds ($\approx 400\mu\text{m}$ width wound) and incubated with

CRTAC proteins (at 0.03; 0.3; 3; 30 and 300ng/mL) and images of the areas flanking the intersections of the wound were taken and measured at 0 hours and after 18 hours of incubation at 37°C (Bars represents mean \pm SEM; tested for n = 4 independent experiments). In panels: (I), healing capacity (%) plotted against dICRTAC1 (A), dICRTAC2 (B) and hCRTAC1 (C) concentrations after 18 hours of incubation; and (II), representative images of the wound healing process comparing to the control (non-treated cell) and cells exposed to dICRTAC1 (D and E), dICRTAC2 (F) and hCRTAC1-A (G and H) after 18 hours of incubation. *, ** and *** indicates treatments with statistical significance for p < 0.05, 0.01 and 0.001, respectively (one-way ANOVA). Images captured using an oscilloscope and a digital camera (scale bar 250 μ m).

Motility assays with macrophage derived THP-1 monocyte cells (Figure 16) were used to quantify the capability of Ma cells to move and occupy free space in a wound, by measuring the distance between the two borders of a wound. dICRTAC1, at the concentration 300ng/mL (p < 0.05) increased the ability of Ma cells to migrate into the wound area (Figure 16. A). In contrast, piscine dICRTAC2 (Figure 16. B) was effective in decreasing the motility of Ma cells and hCRTAC1-A did not have an effect on the motility of Ma cells (Figure 16. C).

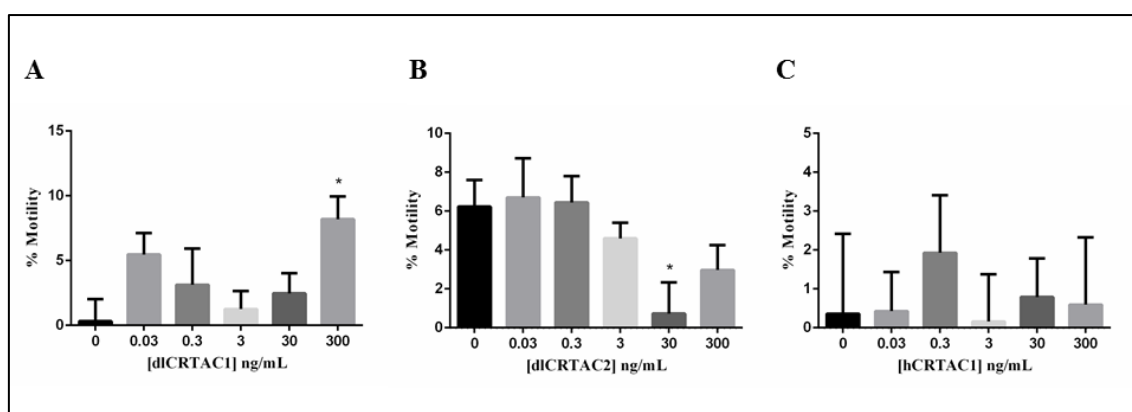


Figure 16. Effects of CRTAC's on macrophage (Ma) derived THP-1 cell motility. Confluent Ma cell cultures were wounded (\approx 400 μ m width wound) and incubated with dICRTAC1 (A), dICRTAC2 (B) and hCRTAC1 (C) proteins (at 0.03, 0.3, 3, 30 and 300ng/mL). Images of the areas flanking the intersections of the wound were taken and measured at 0 hours and after 18 hours of incubation at 37°C for % of motility calculation (Bars represents mean \pm SEM; tested for n = 3 independent experiments). * indicates treatments with statistical significance for p < 0.05 (one-way ANOVA).

3.3.6. CRTAC's effect on THP-1 cAMP production

Cyclic AMP (cAMP) is one of the most important secondary messenger signaling pathways involving activation of cell surface membrane receptors to intracellular responses. cAMP is synthesized by adenylate cyclase, which is associated with the release of the α -subunit of G-binding proteins (Lodish *et al.* 2000; Tuteja, 2009). The cAMP dynamic 2 kit, used in this work, measures the agonist (G α s) effect

and the range of activation of cAMP production should be a Delta F value between non-stimulated cells and cells stimulated to their maximum capacity (e.g. forskolin). Since the value of Delta F is inversely proportional to the production of cAMP, the data are expressed in 1/Delta F (%) to facilitate interpretation of the results.

This assay was complimentary to the viability and hanging drop assays since it is indicative of differentiation into macrophage-like cells as is the formation of melanomacrophage-like centers in CRTAC stimulated THP-1 cell spheroids. Comparison between the non-stimulated THP-1 cells (negative control, [CRTAC] = 0ng/mL) and cells stimulated with 10 μ M of forskolin (Fsk) revealed that the production of cAMP increased almost 5-fold (from $0.00551 \pm 1.73 \times 10^{-6}\%$ in non-stimulated THP-1 cells to $0.02720 \pm 9.27 \times 10^{-5}\%$ in cells stimulated with Fsk; data not show). This result indicates that THP-1 cells respond to Fsk and that this activation pathway can be used as a positive control.

dlCRTAC1 protein, at a concentrations of 0.03 to 30ng/mL have an agonist effect, since the value of 1/Delta F (%) increased compared with non-stimulated cells ($0.0071 \pm 1.5 \times 10^{-5}\%$ and $0.0061 \pm 1.8 \times 10^{-5}\%$ respectively, $p < 0.0001$), although the increase in cAMP production was only 1.25 and 1.11-fold relative to Fsk (Figure 17. A). hCRTAC1-A and dlCRTAC2 proteins had a similar effect at all the protein concentrations tested (Figure 17. B and C) with exception of dlCRTAC2 at 300ng/mL that failed to stimulate cAMP production ($0.00557 \pm 1.15 \times 10^{-5}\%$) relative to the non-stimulated cells (Figure 17. B). In general, treatments with CRTAC at 0.03ng/mL had the highest stimulation effect and hCRTAC1 protein had the highest effect ($0.00815 \pm 5.25 \times 10^{-5}\%$; $p < 0.0001$). This value represents an increase of 1.5-fold of cAMP (Figure 17. C).

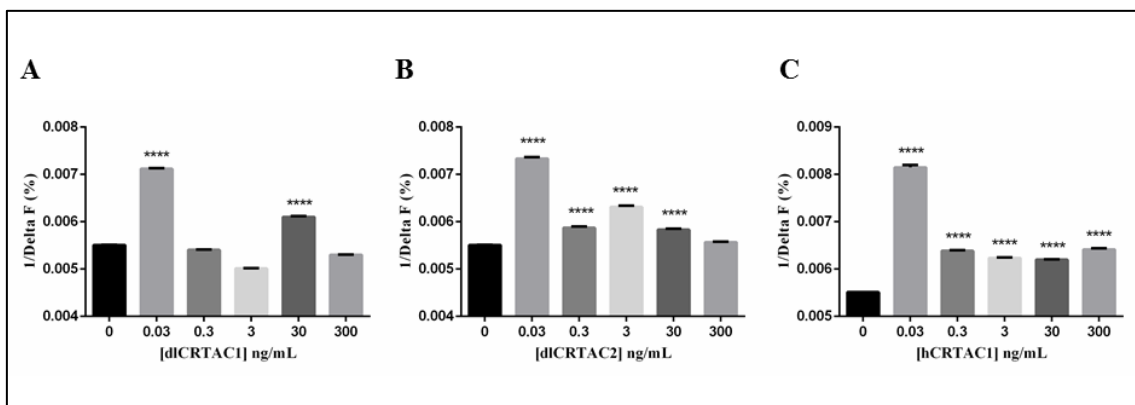


Figure 17. Effects of CRTAC's on cAMP production by THP-1 cells. Cells (10.000cells/well) were incubated for 30min in 1x PBS/IBMX with or without (non-stimulated cells, negative control) proteins at

37°C and the concentration of cAMP was measured by absorbance at 665nm/620nm. THP-1 cells treated with dlCRTAC1 (**A**), dlCRTAC2 (**B**) and hCRTAC1-A (**C**) at concentrations of 0.03, 0.3, 3, 30 and 300ng/mL (Bars represents mean \pm SEM; tested for n = 3 independent assays). **** indicates treatments with statistical significance for $p < 0.0001$ (One-way ANOVA).

4. DISCUSSION AND CONCLUSION

CRTAC1 proteins are an ancient family of proteins with unknown function, present from prokaryotes (e.g. cyanobacteria) to advanced vertebrates (e.g. human), that are patented as blood biomarkers for bone fracture and cartilage injury and also as a therapeutic target (Grgurevic *et al.*, 2008; Golz *et al.*, 2008; Redruello *et al.*, 2010). The highly conserved structural properties during evolution strongly suggests their involvement in cell-cell and cell-matrix interactions and in human pathologies making these proteins an interesting study object and a target for biomedicine applications.

At the start of the study, the intrinsic expression of *hCRTAC1-A* transcripts or hCRTAC1-A protein was investigated by RT-PCR and western blot, respectively, in THP-1, Ma and HEK 293 cells and the results consists of the first real evidence of the expression of *hCRTAC1-A* transcript and hCRTAC1-A protein by HEK 293 cell line, since no other records were found in the available databases.

Previous research indicates that human CRTAC1 is an ECM protein and is efficiently secreted by cultured human chondrocytes *in vitro* (Steck *et al.*, 2007). The *in silico* analysis of the subcellular location of human and teleost CRTAC proteins, performed in this work, support previous results and indicate, with a high probability, that in common with hCRTAC1 the teleost CRTAC's are EC proteins that follows the cellular secretory pathway (which is consistent with the existence of a signal peptide in the N-terminal region of this proteins) and this is also supported by their predicted presence in the endoplasmic reticulum.

Although CRTAC's are mainly EC proteins our results also indicated that they may be present in other cellular organelles such as vacuoles [organelles enriched with hydrolytic enzymes similar to those found in lysosomes (Wada, 2013)], lysosomes and mitochondria in the case of hCRTAC1 and dLCRTAC2 and peroxisomes and the golgi apparatus in the case of dLCRTAC1, which suggests that they can co-accumulate other potential functions. Western blot analysis of subcellular fractions of HEK 293 cells for hCRTAC1-A confirmed their presence in membranes (including the endoplasmic reticulum and golgi apparatus), in mitochondrial fractions and in the nuclear fraction (several protein forms of differing Mw, including one with the expected Mw of 68.6 kDa). These results are consistent with the data obtained by the previous software predictions and the GeneCards database (<http://www.genecards.org/cgi-bin/carddisp.pl?gene=CRTAC1&keywords=CRTAC1#lo>

calization). In addition, the presence of CRTAC proteins in organelles such as the golgi apparatus and the endoplasmic reticulum is consistent with the fact that these organelles are responsible for protein transport through the secretory pathway, synthesis, folding and posttranslational modifications (Bravo *et al.*, 2013; Han *et al.*, 2013). In fact human CRTAC1-A has been described as an O-glycosylated extracellular protein (Steck *et al.*, 2007).

Cell viability was assessed in this work using an MTS proliferation assay and Trypan blue exclusion test. The two techniques were used since soluble formazan assays, such as MTS assay, although easy to perform and good for screening are less sensitive and are susceptible to inaccuracies in the absorbance readings when compared with the very time consuming counting of viable cells (Trypan blue assay) (Huang *et al.*, 2004; Wang *et al.*, 2010). The MTS proliferation assay showed that only dlCRTAC1 at 300ng/mL ($p < 0.0001$) decreased cell proliferation, while the Trypan blue exclusion test revealed that only teleost CRTAC proteins decreased the number of THP-1 cells after 72 hours of incubation and that cell viability was dose dependent, and gave an $IC_{50} = 78,45\text{ng/mL}$ and $95,50\text{ng/mL}$ for dlCRTAC1 and CRTAC2, respectively. Only teleost CRTAC's affected THP1 cell viability. In such conditions no cell death was observed but an increase in cell adherence to the bottom of the culture plates was observed by visual inspection. The results of both assays indicate that THP-1 cells seem to become adhesive in the presence of CRTAC's, which is consistent with cell differentiation into macrophages. This may be due to the fact that the teleost proteins, although similar to the human protein sequence, because of their slightly different amino acid sequence and fish origin may trigger an immune response by the monocytes leading to their differentiation.

Since THP-1 cells becomes adherent when differentiated into macrophage-like cells, a differentiation assay using anti-CD11b macrophage marker was performed to access if the CRTAC proteins induced THP-1 cell differentiation into macrophages. During the procedure, the culture flasks were scraped (and observed into the microscope) to ensure that adherent cells did not stayed attached to the bottom and all cell were harvested in order to assess the THP-1 cell differentiation capability of CRTAC proteins. However, no differences were observed between non-stimulated cells and those treated with proteins in the incubation times and concentrations used, meaning that CRTAC proteins do not stimulate monocytes differentiation into

macrophages under these culture conditions. Nonetheless, integrins and EGF-like domains (which are both present in CRTACs) are known to be involved in cell-cell and cell-ECM interactions (Downing *et al.*, 1996; Wouters *et al.*, 2004; Chouhan *et al.*, 2011) and we hypothesize that these domains may somehow contribute to increase the adherence of that THP-1 cells to the culture plates in the presence of CRTAC's. Thereby, an accurate study of THP-1 cell adhesion capacity should be performed to confirm this effect of teleost CRTAC.

Cell cycle analysis was performed in order to explain the adherence of the THP-1 cell to the bottom of the culture plates, in the viability assays. In this assay, the non-treated THP-1 cells (control) had a similar cell cycle status to previously reported results (Ding *et al.*, 2007; Hsieh *et al.*, 2013). Thus, the experiments (n = 3 independent experiments) were performed 1 week apart from each other, and revealed that the cell culture conditions were normal and the cell cycle did not change. Thereby, it was concluded that human and teleost CRTAC proteins, at the concentrations of 30, 100 and 500ng/mL for 48 hours incubation, did not alter the normal cellular cycle.

Hanging drop assays demonstrated that dlCRTAC1 (30ng/mL, p < 0.001) was the only studied protein that was able to increase the size of THP-1 spheroids and that dlCRTAC1 and hCRTAC1 were able to form more circular spheroids. dlCRTAC2 had only a small influence in THP-1 spheroid circularity at the concentrations of 0.3 and 3ng/mL (p < 0.05). Several studies indicate that, in patients suffering from stage II colorectal carcinoma, the ability of tumors to metastasize is related with circular-type carcinoma shape and the bulk of cell dissemination (Jimi *et al.*, 2008; Coghlin & Murray, 2010). Besides, carcinoma EGF ligand-receptor interaction are known to recruit bone marrow-derived mesenchymal stem cells into the stroma of developing tumors, sustaining cancer progression and promoting neo-angiogenesis and tumor cell migration (Luca *et al.*, 2011). These data are consistent with the fact that both hCRTAC1 and dlCRTAC1 harbour an EGF-like Ca²⁺-binding and integrin domains, associated with cell-cell and cell-ECM interactions (Downing *et al.*, 1996; Wouters *et al.*, 2004; Huttenlocher & Horwitz, 2011). Moreover, hCRTAC1-A has been indicated as a possible biomarker for several types of cancer and the available data, e.g. show that in the colon tumor the relative expression of hCRTAC1 is higher than that in normal tissue (Golz *et al.*, 2008), and this cancer has the capacity to metastasize and has a circular-type shaped (Jimi *et al.*, 2008; Coghlin & Murray, 2010).

Another interesting feature, observed in the hanging drop spheroid formation, was the presence of dark aggregates similar to those found in teleost fish reticulo-endothelial supporting matrix of haemopoietic tissues (spleen and in the kidney interstitium). In teleosts, these dark aggregates are macrophage aggregates (known as Melano-macrophage centres) that appear after exposure to chronic environmental stress and consist of macrophage cells closely packed and enlarged after active phagocytosis of cell debris and melanin pigments (Agius & Roberts, 2003). In humans, the existence of similar structures has been described in patients suffering from melanolytic glaucoma (McMenamin & Lee, 1986). This finding deserves to be better explored, since these melano-macrophage-like centres were only found in the THP-1 cells incubated with dlCRTAC1 when exposed to higher concentrations of the protein (mainly at 30 and 300ng/mL; data not show), and this may indicate changes in the phagocytic capacity of THP-1 cells after exposure to dlCRTAC1 protein.

In the present study the wound healing assays were performed using two types of cells: HEK 293 and Ma cells. HEK 293 cells were used to monitor the capability of CRTAC to promote healing since these cells are adherent and grow as a monolayer epidermis-like surface. On the other hand, Ma cells are adherent cells (obtained by PMA differentiation induction of suspension cultures of THP-1 monocyte cells) but don't behave as a monolayer epidermis-like surface, being used for motility assays due to the capacity of macrophages for amoeboid movements. Tissue engineering uses living cells and/or components of the ECM in the development of implants or devices that may lead to tissue repair (Goessler *et al.*, 2005). CRTAC's are ECM proteins and their structural domains (integrin α chain-like, ASPIC/Unvb and EGF-like domains) strongly suggest that it may play a key role in tissue regeneration. HEK 293 wound healing assay was performed as an approach to evaluate the role of CRTAC proteins in wound healing and tissue regeneration capacity. Our results reveal that hCRTAC1-A seems to inhibit wound repair, at concentrations of 0.03 and 0.3ng/mL ($p < 0.01$), and promote repair at 300ng/mL ($p < 0.05$). dlCRTAC1 had a similar effect to that of hCRTAC1-A, since it inhibited healing at 0.03ng/mL ($p < 0,001$) and promoted wound regeneration at 30ng/mL ($p < 0,05$). However, dlCRTAC2 had an inhibitory effect on wound regeneration at concentrations of 3, 30 ($p < 0.05$) and 300ng/mL ($p < 0.001$). Human and teleost CRTAC1 affect the healing capacity in both senses (inhibitory and promoter) and this is dependent on the protein concentrations. In contrast, dlCRTAC2 is

an inhibitor of healing capacity and this action may be related to the absence of the C-terminal EGF-like Ca^{2+} binding domain, a crucial domain known to be involved in cell adhesion (Downing *et al.*, 1996).

Cell migration is a key process in the defense mechanisms of organisms against pathogens and it involves a set of surface receptors that contribute directionality to movement. Integrin proteins enable the recruitment of macrophage to inflamed areas in response to extracellular stimuli as also mediates adhesion signaling via integrin receptors (Abshire *et al.*, 2011). Here we investigated the effect of CRTAC's, an integrin like protein, on Ma cell motility. Our results shown that only dlCRTAC1 at a concentration of 300ng/mL ($p < 0.05$) potentiate the capability of Ma cells to move into free areas, while dlCRTAC2 appears to inhibit that ability (at the concentration of 30ng/mL, $p < 0.05$) and hCRTAC1-A had no effect on the motility of Ma cells. Teleost CRTAC1 and 2 affect the motility of Ma cells but have opposing effects which suggests these proteins have different functions in the immune system.

Since hCRTAC1-A and dlCRTAC1 are homologous proteins a similar effect might be expected, nevertheless their composition in amino acids is different and these differences could be the reason for their different effects on the motility of Ma cells. An example of this difference is the presence of the RGD peptide in hCRTAC1 (but absent from dlCRTAC1), this is a conserved motif known to be responsible for cell adherence and cell migration. When this RGD motif is used to coat a surface it promotes cell adhesion, however inhibits it when presented to cells in a solubilized form (Ruoslahti, 1996).

dlCRTAC2 had an inhibitory effect on the wound healing process in HEK 293 cells and the same effect was kept in the motility capacity of Ma cells indicating that their role is in the sense of anti-tissue regeneration capacity or in the anti-inflammatory process. As referred above and apparently the main difference between CRTAC1 and 2 is the absence of the C-terminal EGF-like Ca^{2+} domain and the presence of a prenylation site for CaaX prenyltransferases in the latter (Redruello *et al.*, 2010) and these are factors that are known to affect cell growth and morphology in cancer cells (Maurer-Stroh *et al.*, 2003) and could be responsible for the inhibitory effect of CRTAC2 on cell migration/motility.

In the present study we investigated cAMP production by THP-1cells under the effect of human and teleost CRTACs. cAMP is a common secondary messenger that

regulates numerous cellular events such as calcium handling, secretion, ion channel conductance, barrier functioning, metabolic functions, cardiac and smooth muscle contraction, immune activation and control, cell growth and differentiation and apoptosis (Wand *et al.*, 2001; Torgersen *et al.*, 2002; Oldenburger *et al.*, 2012). In general CRTACs did not stimulate cAMP production in the same order of magnitude as Forskolin (the positive control of the assay that leads the maximum stimulation which is almost 5-fold that of non-stimulated cells). Treatments with CRTAC proteins at 0.03ng/mL caused a small but significant increase of cAMP production (about 1.5-fold) ($p < 0.0001$), when compared with the non-stimulated cells. Despite the absence of a dose response signal, the results are consistent in the treatments with human and teleost CRTAC proteins and through the experiments.

Furthermore, some proteins e.g. relaxin has a biphasic cAMP accumulation in THP-1 cells, in which a first response occurs at 1 min after exposure and a second increase of cAMP accumulation is observed after 20 min (Nguyen *et al.*, 2003; Dessauer & Nguyen, 2005). In the present study, a single measure point of cAMP production was performed, after CRTAC exposure, which could be inappropriate to assess cAMP accumulation. Beyond that, cAMP assays are used to evaluate a specific ligand-GPC receptor response, however, in this assay a whole-cell response was considered since no CRTAC receptor has been described or found to our knowledge. The low response from THP-1 cell could be related with events of simultaneous stimulation and inhibition or simply CRTAC proteins use other signaling pathways to interact with THP-1 cells.

In summary, in this thesis we try to clarify previous results and better characterize the effect of human and teleost CRTACs on human monocytes (THP-1) and on Human Embryonic Kidney 293 (HEK 293) cell activity (proliferation/aggregation/adhesion) in order to increase the knowledge about CRTAC protein function. Now we know that HEK 293 cell line expresses *hcrtac1-a* transcripts and hCRTAC1-A protein and we have indications that CRTAC proteins not only follow a secretory pathway as might could be present in other organelles such as the nucleus, mitochondria, lysosome, peroxisome and golgi apparatus. CRTACs affect THP-1 monocytes cell viability, promotes aggregation and spheroids circularization and do not induce cell differentiation into macrophages. Human and teleost CRTAC1 affect the wound healing capacity of HEK 293 cells in both senses (inhibitory and promoter effect

of cell monolayer regeneration) and this is dependent on the protein concentrations, while dlCRTAC2 inhibits the capability of wound healing.

The function of CRTAC's have been partially clarified and the results of the present study that reveal aggregating and proliferative activities consistent with a role for these proteins in cell interaction/proliferation/migration. In this way CRTACs may be an interesting target molecule for regenerative medicine as well as potential pharmacological tools in inflammatory therapy or metastatic invasion.

5. FUTURE PERSPECTIVES

In order to extend the present study of CRTAC's protein function and its role at the cellular and systemic level, different approaches could be followed:

- 1) Implement other cellular assays using the THP-1 cell line, such as inflammatory and phagocytic capacity, attachment capacity to a protein covered surface using e.g. a biosensor and invasion assays;
- 2) Execute the previous cellular assays with other commercial cell lines, e.g., epithelial lung cells, thyroid and neural cells, since CRTAC proteins appear to be present in these tissues in large quantities;
- 3) And finally, perform the cellular assays with the fractioned/digested proteins, that is to test independently the major protein domains to assess which domain is responsible for each response or function.

REFERENCES

- Abshire, M. Y., Thomas, K. S., Owen, K. A. & Bouton, A. H. (2011). Macrophage motility requires distinct $\alpha 5\beta 1$ /FAK and $\alpha 4\beta 1$ /paxillin signaling events. *J Leukoc Biol.*, 89 (2): 251–257.
- Adati, N., Huang, M-C., Suzuki, T., Suzuki, H. & Kojima, T. (2009). High-resolution analysis of aberrant regions in autosomal chromosomes in human leukemia THP-1 cell line. *BMC Research Notes*, 2: 153.
- Aderem, A. & Underhill, D. M. (1999). Mechanism of phagocytosis in macrophages. *Annu. Rev. Immunol.*, 17: 593–623.
- Agius, C. & Roberts, R. J. (2003). Melano-macrophage centres and their role in fish pathology. *Journal of Fish Diseases*, 26: 499–509.
- Anjos, L., Gomes, A. S., Melo, E. P., Canário, A. V. & Power, D. M. (2013). Cartilage Acidic Protein 2 a hyperthermostable, high affinity calcium-binding protein. *Biochem. Biophys. Acta*, 1834: 642-650.
- Auwerx, J. (1991). The human leukemia cell line, THP-1 : A multifaceted model for the study of monocyte-macrophage differentiation. *Experientia*, 47: 22-31.
- Benz, K., Breit, S., Lukoschek, M., Mau, H. & Richter, W. (2002). Molecular analysis of expansion, differentiation, and growth factor treatment of human chondrocytes identifies differentiation markers and growth-related genes. *Biochem. Biophys. Res. Commun.*, 293: 284-292.
- Bravo, R., Parra, V., Gatica, D., Rodriguez, A. E., Torrealba, N., *et al.* (2013). Endoplasmic Reticulum and the Unfolded Protein Response: Dynamics and Metabolic Integration. *Int Rev Cell Mol Biol*, 301: 215-290.
- Brems, H., Park, C., Maertens, O., Pemov, A., Messiaen, L. *et al* (2009). Glomus tumors in neurofibromatosis type 1: genetic, functional, and clinical evidence of a novel association. *Cancer Res.*, 69 (18): 7393–7401.
- Cambi, A, van Helden, S. F. G. & Figdor, C. G. (2000). Roles for Integrins and Associated Proteins in the Haematopoietic System. In: *Madame Curie Bioscience Database* [Internet]. Austin: Landes Bioscience.
- Cambi, A., Joosten, B., Koopman, M., Lange, F., Beeren, I., Torensma, R. *et al.* (2006). Organization of the Integrin LFA-1 in Nanoclusters Regulates Its Activity. *Molecular Biology of the Cell*, 17: 4270–4281.
- Cardoso, J. C. R., Felix, R. C., Martins, R. S. T., Trindade, M., Fonseca, V. G., Fuentes, R. & Power, D. M. (2015). PACAP system evolution and its role in melanophore function in teleost fish skin. *Molecular and Cellular Endocrinology*, 411: 130-145.
- Chanput, W., Mes, J. J., Savelkoul, H. F. & Wichers, H. J. (2013). Characterization of polarized THP-1 macrophages and polarizing ability of LPS and food compounds. *Food Funct.*, 4 (2): 266-276.

- Chen, C. K. M., Chan, N-L. & Wang, A. H. J. (2011). The many blades of the beta-propeller proteins: conserved but versatile. *Trends Biochem Sci*, 36 (10): 553-561.
- Chouhan, B., Denesyuk, A., Heino, J., Johnson, M. S. & Denessiouk, K. (2011). Conservation of the Human Integrin-Type Beta-Propeller Domain in Bacteria. *PLoS ONE*, 6 (10): e25069.
- Coghlin, C. & Murray, G. I. (2010). Current and emerging concepts in tumour metastasis. *J Pathol.*, 222 (1): 1-15.
- Cooper, G. M. (2000). *The Cell: A Molecular Approach*. (2nd Ed.). Sunderland (MA): Sinauer Associates, The Eukaryotic Cell Cycle. Available from: <http://www.ncbi.nlm.nih.gov/books/NBK9876/>.
- Daigneault, M., Preston, J. A., Marriott, H. M., Whyte, M. K. & Dockrell, D., H. (2010) The Identification of Markers of Macrophage Differentiation in PMA-Stimulated THP-1 Cells and Monocyte-Derived Macrophages. *PLoS ONE*, 5 (1): e8668. doi:10.1371/journal.pone.0008668.
- De Luca, A., Gallo, M., Aldinucci, D., Ribatti D, Lamura, L., *et al.* (2011). Role of the EGFR ligand/receptor system in the secretion of angiogenic factors in mesenchymal stem cells. *J Cell Physiol.*, 226 (8): 2131-2138.
- Deane, S., Selmi, C., Teuber, S. S. & Gershwin, M. E. (2010). Macrophage Activation Syndrome in Autoimmune Disease. *Int Arch Allergy Immunol*, 153: 109–120.
- Dessauer, C. W. & Nguyen, B. T. (2005). Relaxin Stimulates Multiple Signaling Pathways: Activation of cAMP, PI3K, and PKC ζ in THP-1 Cells. *Ann N Y Acad Sci.*, 1041: 272-279.
- Ding, Q., Jin, T., Wang, Z. & Chen, Y. (2007). Catalase potentiates retinoic acid-induced THP-1 monocyte differentiation into macrophage through inhibition of peroxisome proliferator-activated receptor γ . *Journal of Leukocyte Biology*, 81: 1568-1576.
- Downing, A. K., Knott, V., Werner, J. M., Cardy, C. M., Campbell, I. D. & Handford, P. A. (1996). Solution Structure of a Pair of Calcium-Binding Epidermal Growth Factor-like Domains: Implications for the Marfan Syndrome and Other Genetic Disorders. *Cell*, 85: 597–605.
- Emanuelsson, O., Nielsen, H., Brunak, S. & von Heijne, G. (2000). Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.*, 300: 1005-1016.
- Evans, R., Patzak, I., Svensson, L., De Filippo, K., Jones, K., McDowall, A. *et al.* (2009). Integrins in immunity. *Journal of Cell Science*, 122: 215-225.
- Ferguson, M. A. J., Kinoshita, T. & Hart, G. W. (2009). Chapter 11: Glycosylphosphatidylinositol Anchors. In: Varki, A., Cummings, R. D., Esko, J. D. *et al.*, (eds). *Essentials of Glycobiology*. 2nd edition. New York: Cold Spring Harbor Laboratory Press.

- Foty, R. A. (2011). Simple Hanging Drop Cell Culture Protocol for Generation of 3D Spheroids. *J. Vis. Exp.* (51), e2720, doi:10.3791/2720.
- Gao, J., Liao, L. & Yang, G-Y. (2009). CAAX-box protein, prenylation process and carcinogenesis. *Am J Transl Res*, 1(3): 312-325.
- Goessler, U. R., Bugert, P., Bieback, K., Huber, K., Fleischer, L. I., *et al.* (2005). Differential Modulation of Integrin Expression in Chondrocytes during Expansion for Tissue Engineering. *in vivo*, 19: 501-508.
- Golz, S., Summer, H., Geets, A., Brueggemeier, U., Albrecht-Kuepper, B. & Klein, M. (2008). Crta as a biomarker, therapeutic and diagnostic target. Bayer Schering Pharma Aktiengesellschaft, Patent n° WO 2008046511 A1.
- Grgurevic, L., Macek, B. & Vukicevic, S. (2008). Blood biomarkers for bone fracture and cartilage injury. Bayer Schering Pharma Aktiengesellschaft, Patent n° WO2008134054 A1.
- Grgurevic, L., Macek, B., Durdevic, D. & Vukicevic, S. (2007). Detection of bone and cartilage-related proteins in plasma of patients with a bone fracture using liquid chromatography-mass spectrometry. *Int. Orthop.*, 31: 743-751.
- Grupe, A., Li, Y., Rowland, C., Nowotny, P. *et al.* (2006). A Scan of Chromosome 10 Identifies a Novel Locus Showing Strong Association with Late-Onset Alzheimer Disease. *Am. J. Hum. Genet.*, 78: 78–88.
- Hammack, B. N., Fung, K. Y., Hunsucker, S. W., Duncan, M. W., Burgoon, M. P., Owens, G. P. & Gilden, D. H. (2004). Proteomic analysis of multiple sclerosis cerebrospinal fluid. *Mult. Scler.*, 10: 245-260.
- Han, H-M., Bouchet-Marquis, C., Jan Huebinger, J. & Grabenbauer, M. (2013). Golgi apparatus analyzed by cryo-electron microscopy. *Histochem Cell Biol*, 140: 369-381.
- Horton, P., Park, K-J., Obayashi, T., Fujita, N., Harada, H. *et al.* (2007). WoLF PSORT: protein localization predictor. *Nucleic Acids Research*, 35: W585–W587.
- Hsieh, Y-J., Chang, C-J., Wan, C-F., Chen, C-P., Chiu, Y-H., *et al.* (2013). *Euphorbia formosana* Root Extract Induces Apoptosis by Caspase-Dependent Cell Death via Fas and Mitochondrial Pathway in THP-1 Human Leukemic Cells. *Molecules*, 18: 1949-1962.
- Huang, A. H., Yeger-McKeever, M., Stein, A. & Mauck, R. L. (2008). Tensile properties of engineered cartilage formed from chondrocyte- and MSC-laden hydrogels. *Osteoarthritis Cartilage*, 16 (9): 1074-82.
- Huang, K. T., Chen, Y. H. & Walker, A. M. (2004). Inaccuracies in MTS assays: major distorting effects of medium, serum albumin, and fatty acids. *BioTechniques*, 37: 406-412.
- Huang, Z., Cheng, Y., Chiu, P. M., Cheung, F. M., Nicholls, J. M., Kwong, D. L., Lee, A. W., Zabarovsky, E. R., Stanbridge, E. J., Lung, H. L. & Lung, M. L. (2012). Tumor suppressor Alpha B-crystallin (CRYAB) associates with the cadherin/catenin adherens

junction and impairs NPC progression-associated properties. *Oncogene*, 31 (32): 3709-3720.

Huttenlocher, A. & Horwitz, A. R. (2011). Integrins in Cell Migration. *Cold Spring Harb. Perspect. Biol.*, 3: a005074.

Jaillon, O., Aury, J-M., Brunet, F., Petit, J-L., Stange-Thomann, N., et al. (2004). *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature*, 432 (21): 946-957.

Jimi, S., Hotokezaka, M., Eto, T. A., Hidaka, H., Maehara, N. & Chijiwa, K. (2008). Circular tumor growth: a prognostic factor in stage II colorectal carcinoma. *Hepatogastroenterology*, 55 (84): 926-929.

Junqueira, L. C. & Carneiro, J. (10th Ed.). (2004). *Histologia Básica*. Rio de Janeiro: Guanabara Koogan.

Kumar, A., Fan, D., DiPette, D. J. and Singh, U. S. (2014). Sparstolonin B, a Novel Plant Derived Compound, Arrests Cell Cycle and Induces Apoptosis in N-Myc Amplified and N-Myc Nonamplified Neuroblastoma Cells. *PLoS ONE* 9 (5): e96343.

Kurihara, Y., Arie, Y., Iketani, M., Ito, H., Nishiyama, K., Sato, Y., Nakamura, F., Mizuki, N., Goshima, Y. & Takei, K. (2012). The carboxyl-terminal region of Crtac1B/LOTUS acts as a functional domain in endogenous antagonism to Nogo receptor-1. *Biochem. Biophys. Res. Commun.*, 418: 390-395.

Lewis, S. M., Bain, B. J. & Bates, I. (9th Ed.). (2006). *Hematologia Prática de Dacie e Lewis*. Porto Alegre: Artmed.

Lodish, H., Berk, A., Zipursky, S. L., et al. (2000). *Molecular Cell Biology*. 4th edition. New York: W. H. Freeman. Section 20.3, G Protein-Coupled Receptors and Their Effectors. Available from: <http://www.ncbi.nlm.nih.gov/books/NBK21718/>.

Loftus, J. C., Smith, J. W. & Ginsberg, M. H. (1994). Integrin-mediated Cell Adhesion: The Extracellular Face. *J. Biol. Chem.*, 269 (41): 25235-25238.

Mao, Y. & Schwarzbauer, J. E. (2005). Fibronectin fibrillogenesis, a cell-mediated matrix assembly process. *Matrix Biology*, 24: 389-399.

Marchler-Bauer, A., Derbyshire, M. K., Gonzales, N. R., Lu, S. et al. (2015). CDD: NCBI's conserved domain database. *Nucleic Acids Research*, 43 (D): 222-226.

Martinez, F. O. & Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Reports*, 6: 1-13.

Maurer-Stroh, S., Washietl, S. & Eisenhaber, F. (2003). Protein prenyltransferases. *Genome Biology*, 4 (4): 212.

McMenamin, P. G. & Lee, W. R. (1986). Ultrastructural pathology of melanolytic glaucoma. *British Journal of Ophthalmology*, 70: 895-906.

- Nakai, K. and Horton, P. (1999). PSORT: A program for detecting the sorting signals of proteins and predicting their subcellular localization, *Trends Biochem. Sci.*, 24 (1): 34–35.
- Nakajima, K., Okazaki, T., Okamoto, T., Kimura, H., Takano, K. & Sato, K. (2010) Genes up- or down-regulated by high calcium medium in parathyroid tissue explants from patients with primary hyperparathyroidism. *Endocr J.*, 57(2): 153-9.
- Nelson, J., McFerran, N. V., Pivato, G., Chambers, E., Doherty, C. Steele, D. & Timson, D. J. (2008). The 67 kDa laminin receptor: structure, function and role in disease. *Biosci. Rep.*, 28: 33–48.
- Nguyen, B. T., Yang, L., Sanborn, B. M. & Dessauer, C. W. (2003). Phosphoinositide 3-Kinase Activity Is Required for Biphasic Stimulation of Cyclic Adenosine 3',5'-Monophosphate by Relaxin. *Molecular Endocrinology*, 17(6): 1075-1084.
- Nielsen, H., Engelbrecht, J., Brunak, S. & Heijne, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering vol.*, 10 (1): 1–6.
- Ohno, S. (1993). Patterns in genome evolution. *Curr. Opin. Genet. Dev.*, 3: 911–914.
- Oldenburger, A., Maarsingh, H. & Schmidt, M. (2012). Multiple Facets of cAMP Signalling and Physiological Impact: cAMP Compartmentalization in the Lung. *Pharmaceuticals*, 5: 1291-1331.
- Orchekowski, R. P., Plescia, J., Altieri, D. C. & Bajt, M. B. (2000). $\alpha_M\beta_2$ (CD11b/CD18, Mac-1) integrin activation by a unique monoclonal antibody to β_M I domain that is divalent cation-sensitive. *J. Leukoc. Biol.*, 68: 641–649.
- Parihar, A., Eubank, T. D. & Doseff, A. I. (2010). Monocytes and Macrophages Regulate Immunity through Dynamic Networks of Survival and Cell Death. *J Innate Immun*, 2: 204–215.
- Phillips, R. J., Lutz, M. & Premack, B. (2005). Differential signaling mechanisms regulate expression of CC chemokine receptor-2 during monocyte maturation. *Journal of Inflammation*, 2: 14. doi:10.1186/1476-9255-2-14.
- Qin, Z. (2012). The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature. *Atherosclerosis*, 221: 2-11.
- Rabinowitz, Y. S., Dong, L. & Wistow, G. (2005). Gene Expression Profile Studies of Human Keratoconus Cornea for NEIBank: A Novel Cornea-Expressed Gene and the Absence of Transcripts for Aquaporin 5. *IOVS*, 46 (4): 1239-1246.
- Rao, Z., Handford, P., Mayhew, M., Vroni Knott, V., George G. Brownlee, G. G. & Stuart, D. (1995). The structure of a Ca^{2+} -binding epidermal growth factor-like domain: Its role in protein-protein interactions. *Cell*, 82 (1): 131-141.

- Rawji, K. S. & Yong, V. W. (2013). The Benefits and Detriments of Macrophages/Microglia in Models of Multiple Sclerosis. *Clinical and Developmental Immunology*, 2013.
- Reddy, S. J. (2012). Cadmium Effect on Histo-Biomarkers and Melano-Macrophage Centers in Liver and Kidney of *Cyprinus carpio*. *World Journal of Fish and Marine Sciences* 4 (2): 179-184.
- Redruello, B., Louro, B., Anjos, L., Silva, N., Greenwell, R. S., Canário, A. V. M. & Power, D. M. (2010). CRTAC1 homolog proteins are conserved from cyanobacteria to man and secreted by the teleost fish pituitary gland. *Gene*, 456: 1-14.
- Ritter, S. Y., Subbaiah, R., Bebek, G., Crish, J., Scanzello, C. R., Krastins, B., Sarracino, D., Lopez, M. F., Crow, M. K., Aigner, T., Goldring, M. B., Goldring, S. R., Lee, D. M., Gobezie, R. & Aliprantis, A. O. (2013). Proteomic Analysis of Synovial Fluid From the Osteoarthritic Knee. *Arthritis & Rheumatology*, 65 (4): 981-992.
- Ruoslahti, E. (1996). RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol.*, 12: 697-715.
- Salsmann, A., Schaffner-Reckinger, E., Kabile, F., Plançon, S. & Kieffer, N. (2005). A New Functional Role of the Fibrinogen RGD Motif as the Molecular Switch That Selectively Triggers Integrin μ IIb β 3-dependent RhoA Activation during Cell Spreading. *The Journal of Biological Chemistry*, 280 (39): 33610–33619.
- Sato, Y., Iketani, M., Kurihara, Y., Megumi Yamaguchi, M., Yamashita, N. *et al.* (2011). Cartilage Acidic Protein-1B (LOTUS), an Endogenous Nogo Receptor Antagonist for Axon Tract Formation. *Science*, 333 (6043): 769-773.
- Selander-Sunnerhagen, M., Ullner, M., Persson, E., Teleman, O., Stenflo, J. & Drakenberg, T. (1992). How an Epidermal Growth Factor (EGF)-like Domain Binds Calcium. *The Journal of Biological Chemistry*, 267 (27): 19642-19649.
- Shattil, S. J., Kim, C. & Ginsberg, M. H. (2010). The final steps of integrin activation: the end game. *Nat Rev Mol Cell Biol.*, 11 (4): 288–300.
- Shin, T. M., Isas, J. M., Hsieh, C-L., Kaye, R., Glabe, C. G., Langen, R. & Chen, J. (2008). Formation of soluble amyloid oligomers and amyloid fibrils by the multifunctional protein vitronectin. *Mol Neurodegener*, 3 (1): 1-12.
- Sica, A. & Mantovani, A. (2012). Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest*, 122 (3): 787–795.
- Singh, P., Sharma, L., Kulothungan, S. R., Adkar, B. V., Prajapati, R. S., et al. (2013). Effect of Signal Peptide on Stability and Folding of *Escherichia coli* Thioredoxin. *PLoS ONE* 8(5): e63442. doi:10.1371/journal.pone.0063442.
- Small, I., Peeters, N., Legeai, F. & Lurin, C. (2004). Predotar: A tool for rapidly screening proteomes for N-terminal targeting sequences. *Proteomics*, 4 (6): 1581–1590.
- Smith D. A. & Germolec, D. R. (1999). Introduction to Immunology and Autoimmunity. *Environmental Health Perspectives*, 107 (5): 661-665.

- Springer, T. A. (1997). Folding of the N-terminal, ligand-binding region of integrin α -subunits into a β -propeller domain. *Proc. Natl. Acad. Sci.*, 94: 65–72.
- Steck, E., Benz, K., Lorenz, H., Loew, M., Gress, T. & Richter, W. (2001). Chondrocyte expressed protein-68 (CEP-68), a novel human marker gene for cultured chondrocytes. *Biochem. J.*, 353: 169-174.
- Steck, E., Braun, J., Pelttari, K., Kadel, S., Kalbacher, H. & Richter, W. (2007). Chondrocyte secreted CRTAC1: a glycosylated extracellular matrix molecule of human articular cartilage. *Matrix Biol.*, 26: 30-41.
- Studholme, D. J. Fuerst, J. A. & Bateman, A. (2004). Novel protein domains and motifs in the marine planctomycete *Rhodopirellula baltica*. *FEMS Microbiology Letters*, 236: 333–340.
- Takahashi, K.; Kurihara, Y., Suzuki, Y., Goshima, Y., Tanaka, F. & Takei, K. (2015). Association of Cerebrospinal Fluid Levels of Lateral Olfactory Tract Usher Substance (LOTUS) With Disease Activity in Multiple Sclerosis. *JAMA Neurol.*, 72 (2): 176-179.
- Teichmann, S. A., Murzin, A. G. & Chothia, C. (2001). Determination of protein function, evolution and interactions by structural genomics. *Curr. Opin. Struct. Biol.*, 11: 354-363.
- Torgersen, K. M., Vang, T., Abrahamsen, H., Yaqub, S. & Taskén, K. (2002). Molecular mechanisms for protein kinase A-mediated modulation of immune function. *Cellular Signalling*, 14: 1–9.
- Tsuchiya, S., Kobayashi, Y., Goto, Y., Okumura, H., Nakae, S., Konno, T. & Tada, K. (1982). Induction of Maturation in Cultured Human Monocytic Leukemia Cells by a Phorbol Diester. *Cancer Res.*, 42: 1530-1536.
- Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T. & Tada, K. (1980). Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int J Cancer*, 26 (2): 171–6.
- Turner, H. C., Budak, M. T., Murat Akinci, M. A. & Wolosin, J. M. (2007). Comparative Analysis of Human Conjunctival and Corneal Epithelial Gene Expression with Oligonucleotide Microarrays. *Invest. Ophthalmol. Vis. Sci.*, 48 (5): 2050-2061.
- Tuteja, N. (2009). Signaling through G protein coupled receptors. *Plant Signaling & Behavior*, 4 (10): 942-947.
- Valledor, A. F., Borràs, F. E., Cullèll-Young, M. & Celada, A. (2004). Transcription factors that regulate monocyte/macrophage differentiation. *Journal of Leukocyte Biology*, 63: 405-417.
- Vogel, D. Y. S., Vereyken, E. F. F., Glim, J. E., Heijnen, P. D. A. M., Martina Moeton, van der Valk, P., Amor, S., Teunissen, C. E., van Horssen, J. & Dijkstra, C. D. (2013). Macrophages in inflammatory multiple sclerosis lesions have an intermediate activation status. *Journal of Neuroinflammation*, 10 (35).

Wada, H. (2013). Domain Shuffling and the Evolution of Vertebrate Extracellular Matrix. In Keeley, F. W. & Mecham, R. P. (eds.), *Evolution of Extracellular Matrix*. (2013). Berlin: Springer-Verlag.

Wada, Y. (2013). Vacuoles in mammals: A subcellular structure indispensable for early embryogenesis. *Bioarchitecture*, 3 (1): 13-19.

Wand, G., Levine, M., Zweifel, L., Schwindinger, W. & Abel, T. (2001). The cAMP-Protein Kinase A Signal Transduction Pathway Modulates Ethanol Consumption and Sedative Effects of Ethanol. *The Journal of Neuroscience*, 21 (14): 5297–5303.

Wang, P., Henning, S. M. & Heber, D. (2010). Limitations of MTT and MTS-Based Assays for Measurement of Antiproliferative Activity of Green Tea Polyphenols. *PLoS ONE*, 5 (4): e10202.

Wouters, M. A., Rigoutsos, I., Chu, C. K., Feng, L. L., Sparrow, D. B. & Dunwoodie, S. L. (2004). Evolution of distinct EGF domains with specific functions. *Protein Science*, 14: 1091–1103.

Yousef, F & Espinosa-Urgel, M. (2007). In silico analysis of large microbial surface proteins. *Research in Microbiology*, 158: 545-550.

Zhou, A. (2007). Functional structure of the somatomedin B domain of vitronectin. *Protein Science*, 16: 1502–1508.

Zhou, H., Liao, J., Aloor, J., Nie, H., Wilson, B. C., Fessler, M. B. *et al.* (2013). CD11b/CD18 (Mac-1) Is a Novel Surface Receptor for Extracellular Double-stranded RNA to Mediate Cellular Inflammatory Responses. *J Immunol.*, 190 (1): 115–125.

Zhu, W-N., Huang, L-L., Zeng, L-B., Zhuang, X-R., Chun-Yan Chen, C-Y. *et al.* (2014). Isolation and Characterization of Two Novel Plasmids from Pathogenic *Leptospira interrogans* Serogroup Canicola Serovar Canicola Strain Gui44. *PLoS Negl Trop Dis* 8 (8): e3103.

Ziegler-Heitbrock, L. (2014). Monocyte subsets in man and other species. *Cellular Immunology*, 289: 135–139.

APPENDICES

Appendix I. *hcrta1-a* gene and PCR primers location

- Homo sapiens cartilage acidic protein 1 isoform A, mRNA (AC: NM_018058):

```

1 ctggctgccg gctgctgcca ccgcaatccc ggctcctaaa tcagcgcggg gaggcgctcc
61 ctccccacgc ccggctctcc gggctctcgg ggccgcgatt ggccgcgccc gcgcccccca
121 ccccgggccc ccggctccag ctgccgcgcc attggctgcg ggcctccgcc agcctttaca
181 taagaccggg cgcgctcgag tggagttgta taaagcgagc gcgcggcgtc ggggcgggag
241 gctcgaggcc agccccggac cggggctggg agcaagcagg cggcggcgcc ggcggcagag
301 gcggcagcga gcgcccgctt cccacgcccc taggcgccgg ggccgagagc gggaggatgg
361 ctccgagcgc tgaccccggc atgtccagga tgttaccggt cctgctgctg ctctggtttc
421 tgcccatcac tgaggggtcc cagcgggctg aaccatggtt cactgcagtc accaactcag
481 ttctgcctcc tgactatgac agtaatccca cccagctcaa ctatgggtg gacgttactg
541 atgtggacca tgatggggac tttgagatcg tcgtggcggg gtacaatgga cccaacctgg
601 ttctgaagta tgaccgggccc cagaagcggc tgggtgaacat cgcggctgat gagcgcagct
661 caccctacta cgcgctgcgg gaccggcagg ggaacgccat cggggtcaca gcctgcgaca
721 tcgacgggga cggcggggag gagatctact tcctcaacac caataatgcc ttctcggggg
781 tggccacgta caccgacaag ttgttcaagt tccgcaataa ccgggtggaa gacatcctga
841 gcgatgaggt caacgtggcc cgtgggtggt ccagcctctt tgccggacgc tctgtggcct
901 gtgtggacag aaagggctct ggacgctact ctatctacat tgccaattac gcctacggta
961 atgtgggccc tgatgcccctc attgaaatgg accctgaggc cagtgcacct tcccggggca
1021 ttctggcgct cagagatgtg gctgctgagg ctggggtcag caaatataca gggggccgag
1081 gcgtcagcgt gggccccatc ctcagcagca gtgcctcggg tatcttctgc gacaatgaga
1141 atgggcctaa cttccttttc cacaaccggg gcgatggcac ctttgtggac gctgcggcca
1201 gtgctggtgt ggacgacccc caccagcatg ggcgaggtgt cgccctggct gacttcaacc
1261 gtgatggcaa agtggacatc gtctatggca actggaatgg cccccaccgc ctctatctgc
1321 aaatgagcac ccatgggaag gtccgcttcc gggacatcgc ctcacccaag ttctccatgc
1381 cctccccctg ccgcacggtc atcaccgccc actttgaaa tgaccaggag ctggagatct
1441 tcttcaacaa cattgcctac cgcagctcct cagccaaccg cctcttccgc gtcacccgta
1501 gagagcacgg agacccccctc atcgaggagc tcaatcccgg cgacgccttg gagcctgagg
1561 gccggggcac aggggggtgtg gtgaccgact tcgacggaga cgggatgctg gacctcatct
1621 tgtcccatgg agagtccatg gctcagccgc tgtccgtctt ccggggcaat cagggcttca
1681 acaacaactg gctgcgagtg gtgccacgca cccggtttgg ggcctttgcc aggggagcta
1741 aggtcgtgct ctacaccaag aagagtgggg cccacctgag gatcatcgac gggggctcag
1801 gctacctgtg tgagatggag cccgtggcac actttggcct ggggaaggat gaagccagca
1861 gtgtggaggt gacgtggcca gatggcaaga tggtagccg gaacgtggcc agcggggaga
1921 tgaactcagt gctggagatc ctctaccccc gggatgagga cacacttcag gaccagccc
1981 cactggagtg tggccaagga ttctcccagc aggaaaatgg ccattgcatg gacaccaatg
2041 aatgcatcca gttcccattc gtgtgcctc gagacaagcc cgtatgtgtc aaacacctatg
2101 gaagctacag gtgcccggacc aacaagaagt gcagtcgggg ctacgagccc aacgaggatg
2161 gcacagcctg cgtggggact ctcggccagt caccgggccc ccgcccacc acccccaccg
2221 ctgctgctgc cactgccgct gctgctgccg ctgctggagc tgccactgct gcaccggtcc
2281 tcgtagatgg agatctcaat ctggggctcg tggttaaggga gagctgcgag cccagctgct
2341 gagcaggggt gggacatgaa ccagcggatg gagtccagca ggggagtggt aaagtgggct
2401 tgtgctgctg cctagacagt agggatgtaa aggcctggga gctagaccct cccaagccc
2461 atccatgcac attacttagc taacaattag ggagactcgt aaggccaggc cctgtgctgg
2521 gcacatagct gtgatcacag cagacagggt cgctgccctg atggcgctta cattccagtg
2581 ggtctaataa ccatatctta ggacacagat gtgccaggg aggtgggtgc actgcacagg
2641 aagtatgagg acttttagtgt cctgagttca aatcctgatt caggaactca caaagctatg
2701 tgaccttaca ccagtcactt aacttgtagt ccatccatta tcgcatctgc aaaatgggga
2761 ttaagaatag aatcctgggg ttagtgtgga gattagatta aatgtatgta agacacttgg
2821 cacaaaacct ggcacatagt aaaggctcaa taaaaacaag tgcctctcac tgggctttgt
2881 caacacgtga aaaaaaaaaa aaaaaaaaaa aa

```

Highlight = 438_forward 5' - TCC CAG CGG GCT GAA CCC ATG TTC - 3'

Highlight = 2340_reverse 5' - CTA GCA GCT GGG CTC GCA GCT CTC C - 3'

Highlight = 1882_forward 5' - ATG GCA AGA TGG TGA GCC GGA ACG T - 3'

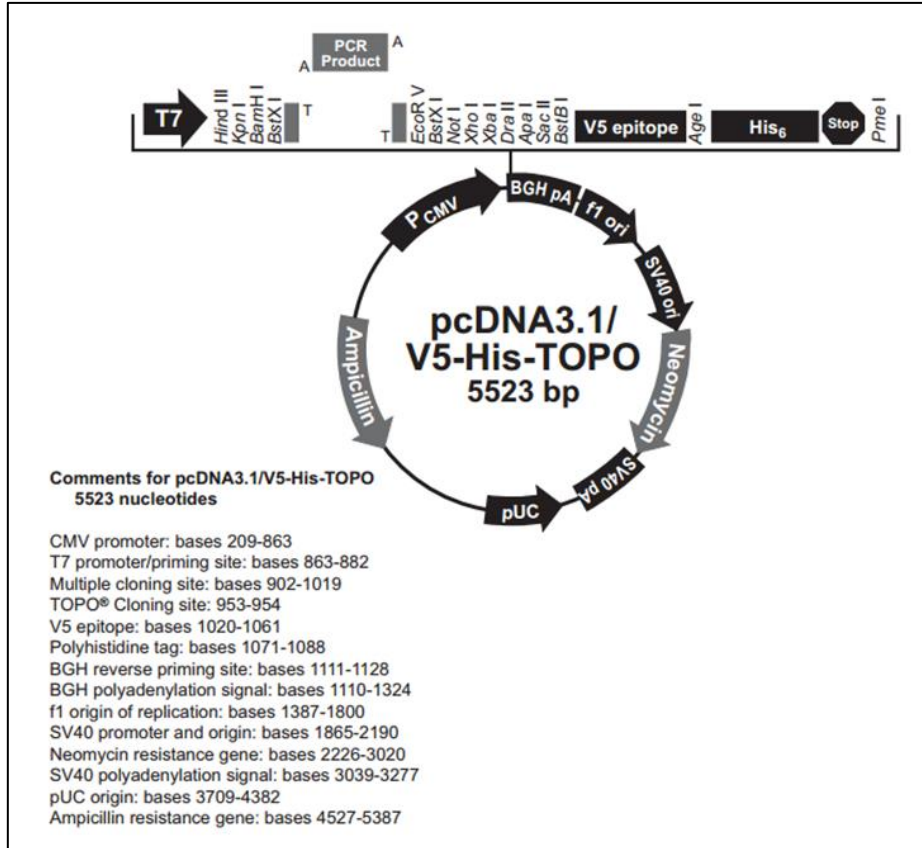
Highlight = 2112_reverse 5' - ACC TGT AGC TTC CAT AGG TG - 3'

Appendix II. Human and teleost CRTACs accession numbers (NCBI and UniProtKB/Swiss-Prot) and protein sequences

Appendix II. Human and teleost CRTAC proteins NCBI and UniProtKB/Swiss-Prot accession numbers, number of amino acids of each proteins and the amino acid sequence.

<i>Homo sapiens</i> cartilage acidic protein 1 isoform A precursor (hCRTAC1-A)	
NCBI accession number: NP_060528.3	UniProtKB/Swiss-Prot accession number: Q9NQ79
Number of amino acids: 661	
Amino acid sequence:	
<p>MAPSADPGMSRMLPFLLLLWFLPITEGSQRAEPMFTA VTN SVLPDPDYDSNPTQLNYGVA VTDVDHHDGDFEIVVAGYNG PNLVLKYDRAQKRLVNI AVDERSSPYALRDRQGNAIGVTACDIDGDGREEIYFLNTNNAFSGVATYTDKLFKFRNRR WEDILSDEVNVARGVASLFAGRSVACVDRKSGGRYSIYIANYAYGNVGPDALIEMDPEASDL SRGILALRDVAAEAGV SKYTGGRGVSVGPILSSASDI FCDNENGPNFLFHNRGDGTFVDAASAGVDDPHQHGRGVALADFN RDGKVDIVYGN WNGPHRLYLQ MSTHGKVRFRDIASPKFSMPSPVRTVITADFDNDQELEIFFN NIAYRSSSANRLFRVIRREHGDFLIE ELNPGDALEPEGRGTGGVVTDFDGDGMLDLILSHGESMAQPLSVFRGNQGFNNWLRVVPRTREFGAFARGAKV VLYTK KSGAHLRIIDGSGYLCEMEPV AHFGLGKDEASSVEVTWPDGKMVSRNVASGEMNSVLEI ILYPRDEDTLQDPAPLECG QGFSQQENGHCM DTNECIQFPFVCPRDKPVCVNTYGSYRCRTNKKCSRGYEPNEDGTACVGT LGQSPGPRPTPTAAA ATAAAAAAAAAGAATAAPV LVDGDLNLGSVVKESCEPSC</p>	
<i>Dicentrarchus labrax</i> cartilage acidic protein 1 (dlCRTAC1)	
NCBI accession number: not available	UniProtKB/Swiss-Prot accession number: not available
Number of amino acids: 634	
Amino acid sequence: not published	
<i>Dicentrarchus labrax</i> cartilage acidic protein 2 (dlCRTAC2)	
NCBI accession number: not available	UniProtKB/Swiss-Prot accession number: not available
Number of amino acids: 547	
Amino acid sequence: not published	

Appendix III. pcDNATM3.1 Directional TOPO[®] Expression Kit mammalian expression vector map and cloning site



Appendix III. pcDNATM3.1 Directional TOPO[®] Expression Kit mammalian expression vector map (Extracted from pcDNATM3.1 Directional TOPO[®] Expression Kit – User Manual Version F 2010; https://tools.thermofisher.com/content/sfs/manuals/pcdna3_1dv5histopo_man.pdf, accessed at 28th August 2015). Schematic representation of the pcDNATM3.1 expression vector (5523bp) map and cloning site, as also the position of promoters, priming sites, alternative cloning sites, resistance genes and tag sequences.

