UNIVERSIDADE DO ALGARVE
DEPARTAMENTO DE CIÊNCIAS BIOMÉDICAS E MEDICINA

Interaction studies of Gla-rich protein with bone morphogenetic proteins

Lúcia Alexandra Rosa dos Santos

Dissertação para a obtenção do grau de
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Trabalho efectuado sob a orientação de:
Prof.ª Doutora Dina Costa Simes
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"Interaction studies of Gla-rich protein (GRP) with bone morphogenetic proteins"

Declaração de autoria de trabalho

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

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(Lúcia Santos)

Agradecimentos

Quero começar por agradecer à professora Dina Simes por me ter aceitado no seu grupo de trabalho, pela sua orientação e pela oportunidade que me deu de expandir os meus conhecimentos.

Quero agradecer à Marta Rafael pela paciência e dedicação que teve comigo. Muito do que aprendi durante este ano devo-o a ti! Obrigada por me ajudares a crescer um pouco mais.

Gostaria também de agradecer à Carla Viegas, pela forma como me recebeu e pela sua ajuda que foi fundamental para a realização deste trabalho.

Um muito obrigado a todos os que fazem e fizeram parte deste grupo. Sofia Cavaco, Rúben Costa, Sofia Santos e Inês Matias Luís obrigado por me terem recebido tão bem, pelos conselhos e pela boa disposição!

Agradeço às minhas colegas de curso, Raquel, Ana Rita e Catarina, pelos momentos de descontração e por ouvirem os meus desabafos. Às minhas amigas, Rita, Verónica, Raquel, Andreia, Jaqueline e Lucianna que me ouviram tantas vezes a falar da minha tese mesmo sem perceberem nada do que dizia! Ao Luís por me ter apoiado sempre e ter tornado tudo muito mais fácil.

Aos meus pais e avós obrigada por acreditarem em mim e apoiarem sempre as minhas escolhas. Espero ter-vos deixado orgulhosos!

Abstract

Cardiovascular disease is one of the main causes of death worldwide. Vascular calcification is a risk factor that strongly contributes to disease progression and to which the vitamin-K dependent family of proteins (VKDPs) appear to play a major role. Gla-rich protein, or GRP, was the last member of the VKDPs to be identified and has been associated with ectopic calcification in tissues such as skin, vasculature and cartilage in cases of dermatomyositis and *pseudoxanthoma elasticum*, chronic kidney disease and osteoarthritis, respectively, suggesting a possible role in the development and/or regulation of pathological calcification. Matrix Gla protein (MGP), another VKDP, is a recognized inhibitor of soft tissue calcification. Although its inhibitory mechanism is still not completely understood, distinct studies reported the binding of MGP to bone morphogenetic proteins (BMPs), known bone formation promoters in both skeletal and vascular tissue, antagonizing its function. However, these mechanisms of action are not enough to explain the numerous reported cases of calcification in humans leading us to hypothesize GRP as one of the missing regulators of calcification in soft tissues. Considering the reported data on MGP-BMP-2 interaction, and since an effect of BMP-2 on GRP expression has been previously demonstrated, we have focused in understanding the importance of GRP in calcification inhibition via interaction with MGP and BMP-2, either as a duplet or as a part of a larger protein complex. To further investigate these possibilities, we have engineered HEK293T cells to overexpress GRP and MGP and used their conditioned media in addition to recombinant BMP-2. Our immunoprecipitation assays demonstrate, for the first time, an interaction between GRP and BMP-2, supporting our hypothesis of GRP acting as a regulator of ectopic calcification via an interaction with BMP-2. Although our novel data indicate that GRP-BMP-2 interaction could be determining to vascular calcification, further functional studies will soon be performed to prove this hypothesis.

Keywords: Vitamin K dependent proteins (VKDPs), Gla-rich protein (GRP), matrix Gla protein (MGP), bone morphogenetic protein-2 (BMP-2), vascular calcification.

Resumo

A doença cardiovascular é uma das principais causas de morte no mundo. A calcificação vascular é um factor de risco que contribui para a sua progressão e para o qual as proteínas dependentes da vitamina K (VKDPs) parecem ter um papel determinante. A proteína rica em Glas (GRP), a última das VKDPs a ser identificada tem sido associada a vários casos de calcificação ectópica na pele, vasculatura e cartilagem, em casos de dermatomiose e *pseudoxanthoma elasticum*, doença crónica do rim e osteoartrite, respectivamente, sugerindo um possível papel na regulação da calcificação patológica. A proteína Gla da matriz (MGP), outra VKDP, é reconhecida como um inibidor da calcificação dos tecidos moles. Embora o seu mecanismo de inibição não seja completamente conhecido, diversos estudos mostram a sua ligação a proteínas morfogenéticas do osso (BMPs), conhecidas por promoverem a formação de osso nos tecidos esquelético e vascular, antagonizando a sua função. Contudo, estas proteínas e os seus mecanismos de acção não são suficientes para explicar os inúmeros casos de calcificação ectópica em humanos levando-nos a propor a GRP como um dos reguladores adicionais envolvidos na inibição da calcificação. Considerando a interacção MGP-BMP-2 e o efeito da BMP-2 na expressão da GRP em condrócitos de ratinho, focámo-nos em investigar a importância da GRP na inibição da calcificação através do estudo da interacção com a MGP e BMP-2, quer como um dupleto ou como parte de um complexo proteico. Para este efeito, sobre-expressámos a GRP e MGP em células HEK293T e o seu meio condicionado, ao qual se adicionou BMP-2 recombinante, foi usado em ensaios de imunoprecipitação. Os nossos resultados demonstram, pela primeira vez, uma interação entre a GRP e BMP-2, apoiando a nossa hipótese de que a GRP actua como um regulador da calcificação ectópica e que esta poderá ocorrer através da interacção com a BMP-2. Neste momento, estamos a desenvolver estudos funcionais que julgamos ser determinantes para provar a nossa hipótese original que foi fortemente reforçada com os resultados deste trabalho.

Palavras-chave: Proteínas dependentes da vitamina K (VKDPs), proteína rica em Glas (GRP), proteína Gla da matriz (MGP), proteína morfogenética do osso-2 (BMP-2), calcificação vascular.

Resumo alargado

Uma dieta pobre em micronutrientes é um problema que afecta não só países pobres como também os países mais desenvolvidos, conduzindo ao aparecimento de diversas doenças tais como anomalias ósseas, calcificação arterial ou doença cardiovascular, osteoartrite, doença crónica do rim (CKD) e cancro.

A vitamina K é um micronutriente cuja principal função é actuar como um co-factor para a γ-glutamil carboxilase (GGCX), enzima responsável pela conversão dos ácidos glutâmicos (Glu) em ácidos glutâmicos γ-carboxilados (Gla). Estes resíduos apresentam elevada afinidade para o cálcio, essencial para as diversas funções biológicas da família de proteínas dependentes da vitamina K (VKDP). Durante a reacção de γ-glutamil carboxilação, a forma reduzida da vitamina K (KH2) é convertida na sua forma oxidada (KO) que, por sua vez, pode ser reciclada pela enzima epóxido reductase da vitamina K (VKOR), promovendo o início de um novo ciclo. No entanto, esta enzima pode ser inibida, no fígado, por antagonistas da vitamina K (VKA), como é exemplo a varfarina, utilizada de forma generalizada como anticoagulante no tratamento de problemas tromboembólicos arteriais ou venosos.

As VKDPs são uma família de proteínas constituída por 16 membros, dos quais os mais conhecidos e melhor caracterizados são os factores de coagulação, cuja função é essencial à sobrevivência. No entanto, existem outros membros desta família, como são exemplo a osteocalcina (OC), proteína Gla da matriz (MGP) e a proteína rica em Glas (GRP), descritas como tendo um papel na remodelação óssea e prevenção da calcificação vascular e uma associação a diversas doenças relacionadas com a calcificação anormal de tecidos.

A MGP, isolada pela primeira vez a partir do osso bovino, é reconhecida pela sua capacidade de inibir a calcificação de tecidos moles. A atribuição desta função à MGP tornou-se clara após estudos em ratinhos *knock out* (KO) para este gene. A calcificação massiva das artérias observada nestes animais é acompanhada por uma transição fenotípica das células vasculares do músculo liso (VSMCs) para células do tipo osteo-condrocítico. O mesmo fenótipo é também observado em ratinhos tratados com varfarina, apontando assim para a importância da γ-carboxilação da MGP na inibição

da calcificação vascular. Embora, o mecanismo pelo qual a MGP exerce a sua função ainda não seja completamente conhecido, são apontadas duas principais hipóteses: i) ligação da proteína a iões ou cristais de cálcio que se encontram em excesso nos tecidos moles levando à sua libertação para a circulação; ii) ligação da MGP a proteínas conhecidas pelo seu papel na osteo/condrogénese, como as proteínas morfogenéticas do osso (BMPs), antagonizando a função das mesmas. Na verdade, vários estudos realizados confirmam a interacção entre a MGP e as BMPs 2, 4 e 7.

A MGP tem sido associada a patologias humanas como são exemplo as *pseudoxanthoma elasticum* (PXE) e esclerose sistémica (Ssc), caracterizadas pela mineralização das fibras elásticas e calcificação da pele, respectivamente. Outro exemplo é a síndrome de Keutel, uma doença autossómica recessiva caracterizada por mutações no gene da MGP. Esta síndrome é caracterizada por uma calcificação anormal da cartilagem, estenose periférica pulmonar e hipoplasia facial leve mas, ao contrário do que acontece em ratinhos *KO*, os doentes vivem até à idade adulta e não têm calcificação arterial severa o que sugere o possível envolvimento de outros reguladores da calcificação neste processo.

A GRP, inicialmente purificada da cartilagem calcificada do esturjão (*Acipenser nacarii*), foi a última das VKDPs a ser identificada. A principal característica estrutural desta proteína é o seu elevado número de resíduos Gla encontrados na sua forma matura (15 em humanos) que lhe confere a capacidade de ligação ao cálcio. A GRP tem sido associada com a calcificação ectópica, tendo já sido demonstrado em humano calcificação da pele, tecidos vascular e cartilagíneo em casos de dermatomyositis, *pseudoxanthoma elasticum* (PXE), doença crónica do rim, cancro e osteoartrite.

A calcificação vascular é uma doença que afecta a população desde há mais de 5 milhares de anos, que por si só é um factor determinante e independente de risco para a morte por doença cardiovascular. Existem dois tipos de calcificação vascular dependendo da sua localização: i) calcificação da camada íntima dos vasos ou calcificação aterosclerótica e ii) calcificação da camada média dos vasos ou esclerose de Monckeberg. A primeira é relacionada com factores de risco como hipertensão, inflamação ou dislipidémia, enquanto a segunda está muito mais associada com o envelhecimento, CKD e *diabetes mellitus.*

Durante muito tempo a calcificação vascular foi considerada uma consequência inevitável do envelhecimento, mas actualmente é considerada um processo activo que implica diferentes mecanismos moleculares de inibição onde as VKDPs podem desempenhar um papel fundamental.

Os cristais de cálcio e fosfato são abundantes na circulação, podendo a sua deposição ser evitada devido à presença de inibidores específicos, como a MGP. Para esta proteína, foi demonstrado que o impedimento da deposição destes cristais depende da presença de resíduos Gla na sua estrutura. Quando o fornecimento de vitamina K é inadequado, ou quando são administrados antagonistas da vitamina K, a proteína MGP torna-se inactiva conduzindo a casos de calcificação ectópica. No entanto, existem indicações de que esta proteína não actua de forma independente: a presença da MGP num complexo com a fetuína-A e minerais de cálcio e fosfato foi descrito por diversos estudos, que sugerem um papel na prevenção da calcificação vascular através do impedimento da deposição de minerais, libertando-os para a circulação.

Como acontece durante a osteogénese e condrogénese, as VSMCs libertam vesículas da matriz (MVs) que servem como local para a deposição de cristais de cálcio e fosfato promovendo a calcificação da matriz extracelular. Em condições normais, estas MVs possuem inibidores da calcificação, como a fetuina-A e MGP, que impedem este processo de deposição de cristais. Contudo, quando a quantidade de cálcio é elevada, como descrito em patologias associadas à calcificação vascular, estas MVs estão aptas a calcificar. Nos locais de calcificação vascular, as VSMCs podem sofrer uma alteração de fenótipo para células do tipo osteocondrocítico. Esta transição pode ser despoletada, por exemplo, pela BMP-2 que promove o aumento da expressão de factores osteogénicos essenciais para a transdiferenciação celular, tais como o *Core-binding factor subunit alpha-1/Runt-related transcription factor 2* (Cbfa/Runx2) e *SRY-related HMG box transcription factor 9* (Sox9). Como mencionado anteriormente, a MGP pode prevenir esta transição através da sua interacção com a BMP-2, impedindo a sua ligação aos receptores, bloqueando assim a transcrição normalmente promovida pela BMP-2.

Embora os estudos feitos indiquem a MGP como um inibidor da calcificação vascular, quer através da sua presença num complexo com a fetuína-A e minerais de cálcio e fosfato, quer através da sua ligação às BMPs, estes mecanismos não são suficientes para explicar o fenótipo associado a casos em que esta proteína está ausente. A presença de outros possíveis inibidores, sugerida recorrentemente na literatura, aliada aos conhecimentos que temos hoje levou-nos a propor a hipótese da GRP desempenhar esse papel. Deste modo, e considerando uma possível relevância da GRP no mecanismo de inibição da calcificação vascular, assim como o facto de tratamentos com BMP-2 em condrócitos de ratinho resultarem numa diminuição da transcrição da GRP, investigámos a possibilidade de regulação via interacção com a BMP-2. Assim, para estudar uma possível interacção entre GRP e BMP-2, começámos por sobre-expressar a GRP humana em células HEK293T tratadas com vitamina K. O mesmo desenho experimental foi utilizado para a produção de MGP humana, com o objectivo de ser usada como controlo positivo da interacção da BMP-2. Os resultados obtidos mostram a presença de GRP no meio condicionado ao contrário do que acontece com a MGP que fica maioritariamente retida no interior da célula e/ou na matriz extracelular e portanto pouco disponível no meio condicionado. Neste caso, é necessário proceder a optimizações do procedimento. Aos meios condicionados obtidos das HEK293T sobre-expressando GRP e MGP foi adicionada BMP-2 recombinante e para testar a interacção entre as proteínas usaram-se técnicas de immunoprecipitação. Os resultados indicam uma ligação entre a GRP e BMP-2, reforçando o potencial papel da GRP na regulação da calcificação ectópica, através dum mecanismo semelhante ao utilizado pela MGP. Estudos funcionais com vista a esclarecer estas questões de forma mais aprofundada estão neste momento a ser desenvolvidos.

Dado o envolvimento da GRP em diversas patologias onde a calcificação ectópica representa um risco determinante para a sua progressão, conhecer o seu mecanismo de acção será essencial para estabelecer esta proteína como um potencial alvo para o diagnóstico e até tratamento e/ou prevenção destas doenças. A co-localização destas três proteínas – GRP, MGP e BMP-2 – e a comparação entre tecidos patológicos e

saudáveis trará com certeza novas perspectivas para o mecanismo de acção da GRP e ajudará na avaliação do seu potencial como biomarcador.

Abbreviations

α-SMA	Alpha smooth muscle actin
aa	Aminoacid
ALK1	Activin-like kinase receptor 1
ALP	Alkaline phosphatase
BAEC	Bovine aortic endothelial cells
BMPs	Bone morphogenetic proteins
bp	Base pair
BSA	Bovine serum albumin
Cbfa1	Core-binding factor subunit alpha-1
CBB	Coomassie brilliant blue
cDNA	Complementary deoxyribonucleic acid
CDS	Complete coding sequence
cGRP	Carboxylated Gla-rich protein
CKD	Chronic kidney disease
C-SMAD	Common mediator SMAD
DMEM	Dulbecco's modified eagle medium
DNA	Desoxyrebonucleic acid
E.Coli	Escherichia coli
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
ER	Endoplasmatic reticulum
FBS	Fetal bovine serum
FMC	Fetuin-mineral complex
Gas6	Growth arrest-specific 6
GGCX	γ-glutamyl carboxylase
Gla	γ-carboxylated glutamic acid
Glu	Glutamic acid

GRP	Gla-rich protein
HA	Hydroxyapatite
HEK	Human embryonic kidney cells
HDL	High density lipoprotein
IP	Immunoprecipitation
I-SMAD	Inhibitory SMAD
KH2	Vitamin K hydroquinone
KO	Vitamin K epoxide
LDL	Low density lipoprotein
MGP	Matrix Gla protein
MK	Menaquinone
mRNA	Messenger ribonucleic acid
MVs	Matrix vesicles
MMLV-RT	Moloney-murine virus reverse transcriptase
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
OA	Osteoarthritis
OC	Osteocalcin
O/N	Overnight
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline tween20
Pro	Proline
PXE	*Pseudoxanthoma elasticum*
qPCR	Quantitative polymerase chain reaction
rBMP	Recombinant BMP
RNA	Ribonucleic acid
TAM	TYRO3, AXL, and MER receptors
tRFP	Total red fluorescent protein
R-SMAD	Restricted SMAD

RT-PCR	Reverse transcriptase polymerase chain reaction
RT	Room temperature
RUNX2	Runt-related transcription factor 2
SDS	Sodium dodecyl sulphate
Ssc	Systemic sclerosis
SMC	Smooth muscle cell
SMADs	SMA and MAD from homologous proteins in *C. elegans* and *D. melanogaster*
SOX9	SRY-related HMG box transcription factor 9
TGFα	Transforming growth factor-alfa
TGFβ	Transforming growth factor-beta
tRFP	Total red fluorescent protein
ucGRP	Undercarboxylated Gla-rich protein
UCMA	Upper zone of growth plate and cartilage matrix associated
ucMGP	Undercarboxylated matrix Gla protein
VEGF	Vascular endothelium growth factor
VKA	Vitamin K antagonist
VKDP	Vitamin K dependent protein
VKOR	Vitamin K epoxide reductase
VSMC	Vascular smooth muscle cell

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1. Introduction

1.1. The relevance of a micronutrient: Vitamin K

The intake of micronutrients lower than recommended is widespread, not only in poor but also in developed countries, due to unbalanced diets rich in calories while deficient in micronutrients [1]. Micronutrients insufficiency leads to their unbalanced usage: short-term survival functions have priority over to those that are less essential [1]. Consequently, persistent deficiency of micronutrients may cause age-related diseases, such as bone abnormalities, osteoarthritis, chronic kidney disease (CKD), cancer and cardiovascular disease [1].

Vitamin K is a micronutrient which major function is to act as a cofactor for the γ-glutamyl carboxylase (GGCX), an essential enzyme for the activation of vitamin K-dependent proteins (VKDPs) biological function **(Fig. 1.1)** [2,3]. VKDPs, initially described when studying coagulation cascade, namely coagulation factors II, VII, IX and X, are critical for short-term survival as shown by mice knockouts [1,2,4,5]. Beyond coagulation factors, there are other VKDPs, such as matrix Gla protein (MGP) and osteocalcin (OC), that although less critical for survival, when vitamin K supply is inadequate have long-term consequences and may lead to age-associated conditions [1].

Discovered by the Danish scientist Henrik Dam in 1930s, vitamin K belongs to a family of lipid-soluble vitamins that includes a common 2-methyl-1,4-napthoquinone ring [2,3,7]. Depending on the structure of the substituted ring derivative, it can be classified in two biological active forms, vitamin K1 (or phylloquinone) and vitamin K2 (or menaquinone, MK) that can be obtained from diet [8]. While vitamin K1, the major form obtained by diet, is found in high concentrations in green leafy vegetables, vitamin K2 is found in fermented food such as cheese and Japanese natto, but can also be produced by intestinal bacteria in distal colon [3,5,6]. There is also a third form, the synthetic vitamin K3 (or menadione), that needs to be converted into vitamin K2 to exhibit vitamin K activity [1,2,5].

Dietary vitamin K is emulsified by bile salts to form micelles which are taken up by intestinal cells and incorporated into chylomicrons entering the blood stream and to

be transported to different target tissues [4]. In spite of both forms being absorbed by small intestine, vitamin K1 is incorporated in lipoproteins enriched in triacylglicerol while vitamin K2 is transported by low and high-density lipoproteins (LDL and HDL, respectively) [10]. Different tissue distribution is directly related to distinct transport mechanisms: whereas the vitamin K1 is mostly found in liver, vitamin K2 is distributed systemically through extra-hepatic tissues, and can actually be more active than vitamin K1 in these tissues [1,9].

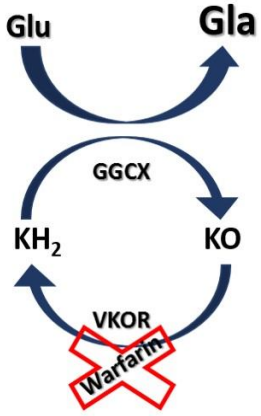

Figure 1.1 - The vitamin K cycle. Gamma-glutamyl carboxylase (GGCX) converts glutamate (Glu) into γ-carboxylated glutamate (Gla) residues through conversion of reduced vitamin K (KH2) to vitamin K epoxide (KO). In order to be reused, KO is recycled by vitamin K epoxide reductase (VKOR) which can be inhibited by warfarin. Adapted from Willems *et al* (2014)[8].

1.2. VKDPs and the importance of vitamin K recycling

Vitamin K dependent proteins become functional after being γ-carboxylated by the γ-glutamyl carboxylase (GGCX) enzyme that is localized in the endoplasmatic reticulum (ER) membrane [11]. This enzyme is responsible for the conversion of glutamic acid (Glu) into γ-carboxylated (Gla) residues which exhibit high affinity for calcium ions and calcium crystals, a property being intimately related to VKDPs specific functions [11]. The reduced form of vitamin K (KH2) is used by the GGCX as a co-factor for γ-glutamyl carboxylation [3]. During this reaction, KH2 is converted into vitamin K epoxide (KO) that can be recycled by the vitamin K epoxide reductase (VKOR) enzyme promoting the beginning of new cycle **(Fig. 1.1)** [12]. This recycling enzyme can be inhibited in liver by vitamin K antagonists (VKA), such as 4-OH coumarin analogs (e.g. warfarin) widely used in anticoagulant therapies for the treatment of arterial and venous thromboembolic

disorders **(Fig.1.1)** [3,14,15]. In these cases, and when vitamin K concentrations are increased, an alternative pathway occurring in liver, independent of coumarins, can be used to provide the γ-carboxylation cofactor. This pathway uses NAD(P)H dehydrogenases (DT-diaphorases) to reduce vitamin K [13].

Vitamin K dependent proteins constitute a family of 16 described members – Table 1 [9]. The most well-known VKDPs are coagulation factors such as prothrombin (factor II), factors VII, IX and X, and anticoagulation factors such as proteins C, S and Z, that are synthesized mostly in liver with the exception of protein S [10,14,15].

Table 1 – List of described VKDPs [1, 9]

	Protein	Tissue of γ-carboxylation	Function
Coagulation	Factor II (prothrombin)	Liver, limited extra-hepatic	Coagulation, regulation of inflammation
	Factor VII		Coagulation
	Factor IX	Liver	
	Factor X		
Anticoagulation	Protein C	Liver	Coagulation and anti-inflammatory regulation
	Protein S	Liver and endothelial cells	Coagulation, anti-inflammatory, phagocytosis and apoptosis
	Protein Z	Liver	Degradation of factor Xa
Bone/vascular related	Osteocalcin	Primarily osteoblasts	Bone homeostasis
	Matrix Gla protein	Bone, cartilage, vascular tissues and macrophages	Inhibitor of vascular calcification
	Gla-rich Protein	Most soft tissues, cartilage	Regulator of mineralisation
	Growth arrest specific gene 6 (Gas6)	Vascular smooth muscle cells (VSMCs) and endothelial cells	TYRO3, AXL, and MER receptors (TAM) activating ligand
Other VKDPs	Transforming growth factor (TGF-α) inducible protein	Most soft tissues	Cell proliferation
	Proline-rich Gla protein		Unknown
	Transmembrane Gla protein		
	Periostin	Bone marrow, mesenchymal stromal cells, cardiomyocytes	Collagen assembly and homeostasis

Other members of this family of proteins were identified and shown to be synthesized in extra-hepatic tissues, with widespread physiologic activities such as regulation of bone turnover, prevention of vascular calcification and apoptosis [10,14]. Osteocalcin, MGP, GRP and growth arrest specific gene 6 protein (Gas6) can be included in this group of proteins, exhibiting highly specific physiologic functions – Table 1 [10,14].

VKDPs exhibit a general structural homology: a signal peptide, a propeptide and a mature protein that contains the Gla domain **(Fig 1.2A)** [13,16]. The signal peptide is required for protein translocation into the ER where VKDPs bind to carboxylase [16]. The propeptide contains the GGCX recognition site being shown to play an important role in the γ-carboxylation of these proteins [13,16,17]. After carboxylation, all known VKDPs are released from the ER and directed to the Golgi, where the propeptide is further processed by the endopeptidase furin (with the exception of MGP**, Fig. 1.2B)** [13].

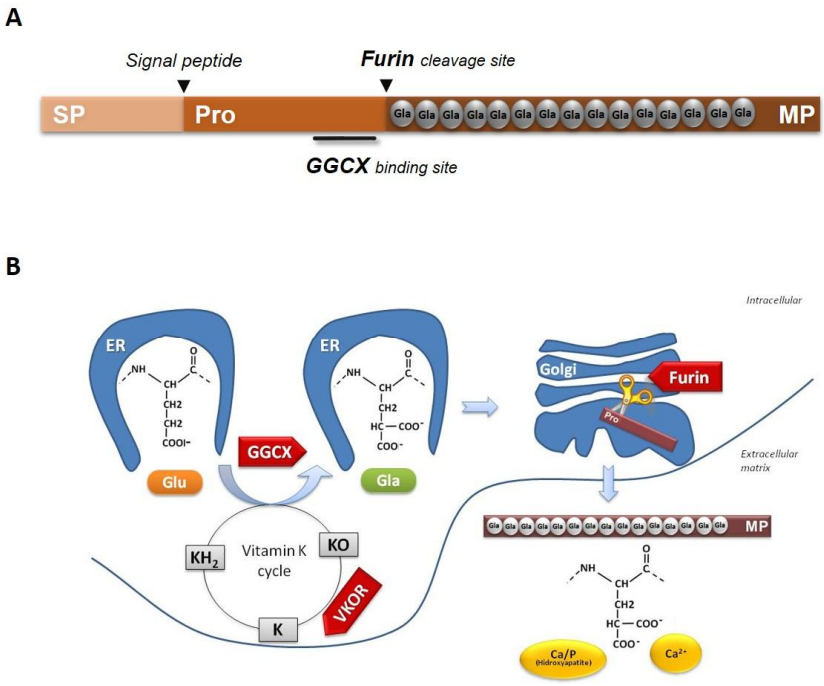

Figure 1.2 - Vitamin K dependent proteins (VKDPs) general structure and processing. A) General structure of a VKDP. VKDPs are composed by a signal peptide (SP), a propeptide (Pro) that contains the GGCX recognition site and a mature protein (MP) including the Gla domain. **B**) VKDPs cellular processing: SP is required for protein translocation into the endoplasmatic reticulum (ER) where VKDPs bind to GGCX and γ-carboxylation occurs. After carboxylation, VKDPs are released into the Golgi, where the Pro is further processed by furin. Mature VKDPs (MP) are then released to the extracellular matrix where they can exert their specific function. Adapted from Bristol et al (1996) [18]

1.3. Matrix Gla protein and Gla-rich protein: complementary partners (in health and disease)?

1.3.1. Matrix Gla protein

Matrix Gla protein was identified for the first time in 1983 in bovine bone by Price and co-workers [19]. It was the second Gla protein to be isolated from bone and demonstrated to be strongly associated with collagenous bone matrix [19]. Despite its high accumulation in extracellular matrix (ECM) of bone, this protein is also synthetized in other tissues such as cartilage, lung, kidney, heart, arterial vessel wall and in calcified atherosclerotic lesions [19–22]. MGP was also identified in rat, shark, chicken, xenopus and human, either as protein or as mRNA [23–26].

1.3.1.1. Protein and gene structural characterization

MGP is a small secreted VKDP that can undergo two types of post-translational modifications, γ-carboxylation and phosphorylation [27]. The human mature protein consists of 89-aa, with a molecular weight of 14 kDa; five out of the nine Glu residues can be γ-carboxylated while three of its five serine residues can be phosphorylated [27]. The human MGP gene is located on chromosome 12 (p13.1 - p12.3) and consists of four exons and three large introns with a total length of 3.9 kb **(Fig. 1.3A)** [27].

Matrix Gla protein share common features with other VKDPs, such as a signal peptide, a mature protein containing the Gla domain, a AXXF motif and a RXXR furin-like cleavage site **(Fig. 1.3B)** [16]. In MGP the GGCX recognition site, although included in its propeptide, is not cleaved after the γ-carboxylation reaction and therefore is integrated in the mature protein, a feature that is unique among VKDPs [2,27]. Additionally, MGP has a motif (Ser-X-Glu) recognized for serine phosphorylation [27].

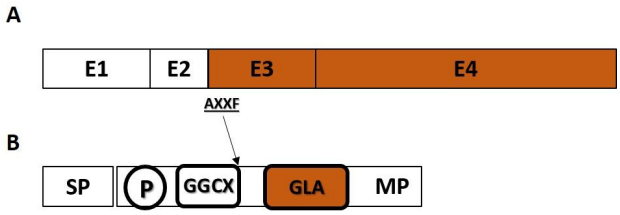

Figure 1.3 - Structural organization of MGP at gene and protein levels. A) Gene structure of MGP. Boxes represent coding exons and orange boxes represent exons coding for the Gla domain. **B**) Illustration of MGP protein structure: SP, signal peptide; MP, mature protein; P, serine phosphorylation; GGCX, γ-glutamyl carboxylase recognition site; AXXF, proteolytic cleavage site in MGP; Gla, gla domain. Adapted from Viegas *et al* (2008) [28].

1.3.1.2. MGP, the calcification inhibitor

The function of MGP as an inhibitor of soft tissue calcification just became clear after studies in MGP null-mice [29]. These animals died six to eight weeks after birth due to an extensive calcification that caused rupture of the thoracic and/or abdominal aorta [29]. Moreover, the authors demonstrated a phenotypic transition in vascular smooth muscle cells (VSMCs) to chondrocyte-like cells [27,28]. A phenotype similar to MGP null-mice was obtained after treatments with warfarin, with rats developing massive calcification of their cartilage, arteries and heart valves [29,30], demonstrating that the capacity of MGP to inhibit calcification is dependent of its γ-carboxylation status [31].

The precise molecular mechanism by which MGP exerts its function is not yet completely understood but has been proposed to be through binding of calcium ions or calcium crystals present in excess in soft tissues and clearance into circulation [32]. Supporting this hypothesis is the fact that MGP mRNA is expressed in many tissues while the protein usually accumulates in sites of ectopic calcification and circulates in plasma [32]. To confirm these facts Roy and Nishimoto showed that MGP binds to increasing concentrations of hydroxyapatite (HA), further suggesting a binding site for HA in MGP. Moreover, Hackeng and colleagues demonstrated a Ca^{2+} binding to MGP and consequently a possible conformational change in protein's structure [26,32]. It was also suggested that MGP could antagonize proteins known to have a role in chondrogenesis and bone formation, such as bone morphogenetic proteins (BMPs), an issue that will be further discussed in this section [34].

Because MGP is poorly soluble in its mature secreted form, it was though that this protein circulates either in an aggregated form or bound to higher molecular weight chaperones [32]. Price and co-workers were the firsts to identify MGP as part of a protein-mineral complex composed by hydroxyapatite, fetuin-A and other proteins in serum of rats treated with bisphosphonate etidronate, an inhibitor of bone mineralization [35]. Nishimoto and colleagues, showed that MGP binds to vitronectin which is a multifunctional plasma and ECM protein with a role in cell adhesion, complement activation, coagulation and fibrinolysis [36]. This interaction was proved to occur *in vitro* by colocalization and immunolocalization studies and may be a mechanism to anchor MGP to the ECM that could modify its activity [36]. This group has also identified a binding site for vitronectin in a region flanking MGP *C-terminus* [36].

1.3.1.3. MGP-related disorders

Matrix Gla protein has been associated to several human disorders, such as *pseudoxanthoma elasticum* (PXE) and systemic sclerosis (Ssc) characterized by elastic fibres mineralization and skin calcification, respectively [36,37]. Two different studies investigating MGP protein accumulation, both in dermis sections from PXE and skin biopsies from Ssc patients, respectively, have shown that MGP is more abundant in calcified tissues of these patients when compared to healthy controls [36,37]. MGP has also been associated in cases of vascular calcification. For example, a study demonstrates, by immunohistochemistry, that undercarboxylated MGP (ucMGP) is strongly associated with intimal and medial calcification [39].

The Keutel syndrome, a human autosomal recessive disorder is characterized by mutations on MGP gene predicting the production of a non-functional protein [40]. This syndrome is characterized by abnormal cartilage calcification, peripheral pulmonary stenosis and mild facial hypoplasia [40]. Unlike the mouse model, Keutel patients survive until adulthood and lack severe arterial calcification suggesting the involvement of alternative calcification regulators [40].

1.3.2. Gla-rich protein

Gla-rich protein (GRP), also known as upper zone of growth plate and cartilage matrix associated protein (UCMA[41,42]), firstly isolated from the calcified cartilage of Adriatic sturgeon (*Acipenser nacarii*), was the latest VKDP to be discovered [28]. This protein is highly conserved amongst vertebrates and exhibits two paralogs, GRP1 and GRP2, in teleost fishes, whose existence might be explained due to a genome duplication event [28,43].

1.3.2.1. Protein and gene structural characterization

GRP is a small secreted protein, negatively charged although highly insoluble at neutral pH [28]. The protein exhibits a VKDP general protein structure, as previously mentioned in section 1.1.1 **(Fig. 1.4A)** [28]. Sturgeon mature GRP, purified from calcified cartilage, was shown to be a 74-aa long protein, exhibiting a molecular weight of 10.2 kDa where all its Glu residues were experimentally shown to be γ-carboxylated [28]. Its gene structure is also highly conserved amongst species, comprising four introns and five exons [28]. Exons 3, 4 and 5, all containing putative Gla residues, code for the mature protein but exon 4, by exhibting the larger number of Gla residues, is considered to be the functional core of the protein [28].

Although its highly conserved gene structure, GRP alternative transcription appears to be species-specific **(Fig. 1.4B)**. In murine embryo chondrocytes, during development, four distinct transcripts of GRP gene were reported, as well as in the two zebrafish paralogs [40,41]. In both species, GRP-F1 is the longest and most abundant transcript; the other three, GRP-F2, GRP-F3 and GRP-F4, lack exon 2, exon 4 or both, respectively [44]. Overexpression of mouse GRP isoforms, in HeLa cells, evidenced that GRP-F1 and GRP-F3 are secreted proteins that were localized, and detected by immunofluorescence, in the Golgi apparatus [44]. On the contrary, GRP-F2 and GRP-F4 isoforms are not secreted, once they lost exon 2 they lack the signal peptide, and aggregate in a structure similar to the aggresome [44]. Since exon 4 comprises most of the Gla residues, these transcripts give rise to two isoforms missing 60% of the putative Gla residues most possibly affecting protein function [44].

Recently, Rafael and colleagues identified in human osteoarthritis (OA) cartilage two novel alternatively spliced variants of human GRP gene, GRP-F5 and GRP-F6, and a new alternative transcript in cartilage from wild-type murine femurs, GRP-F7 [45]. The two novel human transcripts, both GRP-F5 and GRP-F6, lack exon 3 leading to the loss of furin-like cleavage site for propeptide processing and GGCX recognition site [45]. GRP-F6 also lacks exon 2 leading to the translation of a protein with shortening signal peptide length [45]. Using an overexpression system in HEK293T cells, it was demonstrated that the new human protein isoforms, GRP-F5 and GRP-F6 are both secreted but GRP-F6 is secreted later than GRP-F5 since this transcript have a shorter signal peptide, pointing to a lower secretion potential and efficiency during secretory process [45].

In terms of tissue distribution, at the transcriptional level, human GRP-F1 alternative spice variant is ubiquitously expressed in foetal and adult tissues whereas GRP-F5 and F6 are mostly present in foetal tissues [45]. These data suggest that the biological function of splice variants should be further evaluated, specially throughout development [45].

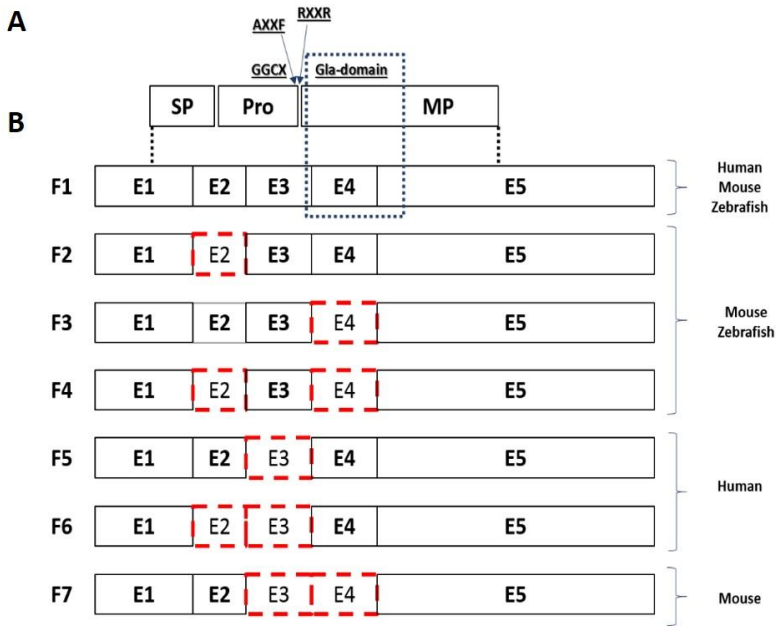

Figure 1.4 - GRP protein structure and splice variants. A) Protein structure of GRP. SP, signal peptide; Pro, propeptide; GGCX, γ-glutamyl carboxylase recognition site; AXXF, proteolytic cleavage site; RXXR, furin-like cleavage site; MP, mature protein. **B)** GRP-splice variants. Boxes represents coding exons and red boxes represents absent exons in each variant. Adapted from Rafael et al (2014)[45].

1.3.2.2. *Novel protein*: old function?

The main characteristic of GRP is its high number of Gla residues found in its mature protein form (15 putative Gla residues in humans) conferring it the highest Gla residues/protein size ratio [46]. This feature has been conserved over 450 million years of evolution, with an impressive conservation degree in number and position of Glu residues as well as in its binding site for GGCX [28]. This high density of Gla residues indicates a high calcium binding capacity for GRP, a function that has been extensively shown for other VKDPs [46]. Nevertheless, the Gla capacity to bind calcium can confer different functions to distinct proteins, whether it is related to the coagulation cascade or involved in physiological or pathological mineralization of tissues, such as in case of vascular calcification [14].

Gla-rich protein was found to be associated with ectopic calcification in human skin, vascular and cartilaginous tissues in cases of dermatomyositis, PXE, CKD, cancer and OA [46,47]. Although experimental data demonstrating GRP degree of carboxylation in humans is still not available, the use of conformational antibodies, specific to carboxylated GRP (cGRP) and undercarboxylated GRP (ucGRP) protein forms, respectively, indicates a different pattern of association of these two forms to healthy or pathological conditions [47]. Two studies demonstrated a preferential association of ucGRP with pathological situations, in cases of osteoarthritis and skin and breast cancers, while cGRP is more prevalent in corresponding healthy tissues [45,47]. It was also demonstrated the colocalization of cGRP and ucGRP at sites of microcalcifications, suggesting that both forms have affinity to bind calcium, while the colocalization of both antibodies indicates an incomplete γ-carboxylation in healthy conditions [47].

Different studies indicate that the role of GRP remains to be further clarified. Although at first GRP expression cannot be confirmed in no other tissues than in mice cartilage, later it was shown that GRP is expressed in rat and human skeletal tissue and that is accumulated in soft tissues, such as skin and the vascular system [43,45,46]. Eitzinger and colleagues show that GRP-deficient mice have a normal development where no skeletal abnormalities or calcification of cartilage and bone is identified, indicating that this protein is not essential for normal cartilage development and

endochondral ossification [48]. Nevertheless, the authors point to a possible phenotype related to calcification and bone turnover during pathological conditions and/or later in development [48]. In contrast, in zebrafish, the knockdown of GRP1 leads to severe growth retardation and abnormal skeletal development and a similar phenotype occurs when γ-carboxylation is inhibited by warfarin [49]. These results show that GRP1 and the γ-carboxylation of its Glu residues are crucial for zebrafish skeletal development [49]. These contradictory data may be partially explained by the finding that GRP1, in zebrafish, is earlier expressed than its murine orthologue [48].

1.4. Bone Morphogenetic Proteins

Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor-beta (TGF-β) superfamily of proteins [50]. These molecules were discovered by Dr. Marshal Urist in the 1960s and since then twenty BMP family members have been identified [50].

BMPs are synthesized as large precursors consisting of a i) signal peptide, which directs the protein to the secretory pathway; ii) a prodomain, that mediates proper folding and iii) the mature peptide [50,51,52]. After signal peptide cleavage, prodomain undergoes glycosylation and dimerization followed by proteolytical cleavage of the mature protein that becomes active [50]. Active BMPs are composed by 50-100 amino acids among which seven cysteines, where six are used to form intramolecular disulphide bonds while the seventh forms a covalent disulphide bond with another monomer and is secreted as a dimer [51].

Although primarily known for their capacity to induce ectopic bone formation, BMPs have a role in a variety of cell functions [50]. These proteins are well known to act in mesenchymal stem cells differentiation and consequently leading to bone and cartilage formation, but are also involved in other diverse processes such as embyogenesis, organogenesis and tooth morphogenesis, cell proliferation and differentiation, apoptosis, chemotaxis, glucose homeostasis and modulation of iron homeostasis [51,52].

1.4.1. Cellular signalling

BMPs binds to membrane type I and type II serine/threonine kinases receptors that are required for cellular signalling **(Fig. 1.5)** [50]. This binding results in type I receptor phosphorylation promoted by type II receptor which recruits Smads proteins (fusion of names SMA and MAD from homologous proteins in *C. elegans* and *D. melanogaster*, respectively), that are part of the signalling cascade [50]. After phosphorylation of restricted Smads (R-Smads) these proteins recruit common mediator Smads (C-Smads) to form a complex that migrates to the nucleus and promotes transcription of specific target genes [50,53]. The signalling can be regulated at four levels: extracellular, in the membrane, intracellular and in the nucleus [50]. Extracellularly, the presence of antagonists with high affinity to BMPs receptors prevent its binding [50]. Receptors oligomerization can determine the specificity of signalling pathway activation [50]. In the cytoplasm, the signal transmitted can be modulated by inhibitory Smads (I-Smads), which compete with R-Smads for binding type I receptors, or Smurf1 [50,53]. Finally, in the nucleus, the activation of specific target genes and their transcription can be inhibited by co-repressors [50].

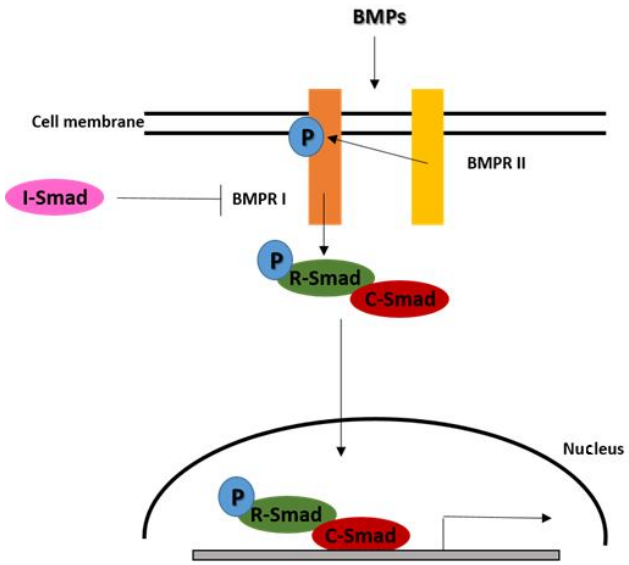

Figure 1.5 - Bone morphogenetic proteins (BMPs) signalling. BMPs binds to type I receptor (BMPR I) that became phosphorylated by type II receptor (BMPR II) which recruits Smads. After phosphorylation of restricted Smads (R-Smads) these proteins recruit common mediator Smads (C-Smads) to form a complex that migrates to the nucleus and promotes transcription of specific target genes. The signalling can be inhibited by inhibitory Smads (I-Smads), which compete with R-Smads to binding type I receptors. Adapted from Yamaguchi et al (2000) [53].

1.4.2. BMP-2

One of the most important known functions of BMP-2 is in bone and cartilage formation [54]. These skeletal tissues are composed by several mesenchymal cell types, such as osteoblasts and chondrocytes, that differentiate in response to Core-binding factor subunit alpha-1/Runt-related transcription factor 2 (Cbfa/Runx2) and SRY-related HMG box transcription factor 9 (Sox9), respectively [53,55]. Regulation of osteoblast differentiation is coordinated by various local factors in a paracrine and/or autocrine manner [53]. It was demonstrated that BMP-2, BMP-4 and BMP-7, are responsible for osteoblast and chondrocytes differentiation [53,54,56]. In particular, BMP-2 was shown to promote the expression of osteoblast phenotypic markers alkaline phosphatase (ALP), collagen type I and OC by increasing the expression of the transcription factor Cbfa/Runx2 [50,53].

1.4.3. BMP's relation with VKDPs

When Urist and colleagues first discovered BMPs, they almost immediately realise the tight relation between BMP and MGP, ie, BMPs and a regulator counterpart. Since then, several studies were performed to prove this interaction and to understand how it occurs *in vivo* [57]. Since these proteins are both synthesized by smooth muscle cells (SMCs), Wallin and co-workers suggested that MGP may neutralize the bone promoting effect of BMP-2 in the vascular wall by forming a complex between the two proteins and, although not proving a regulatory mechanism for MGP, their data confirms the binding between MGP and BMP-2 [34]. Later, the same group, showed that Ca^{2+} and γ-carboxylation of MGP is important for optimal BMP-2 binding to MGP and further demonstrated, by immunofluorescence in glandular tissues, the existence of MGP-BMP-2 *in vivo* [34,58]. Boström and colleagues, by overexpressing MGP in a mesenchymal cell line with the capacity to differentiate in different lineages and by adding recombinant BMP-2 (rBMP-2), showed that MGP modulates the capacity of BMP-2 to induce differentiation [59]. It was suggest that MGP binding to BMP-2 probably prevents its interaction with cell surface receptors [59]. In fact, Zebboudj and co-works

studies indicates that MGP inhibits BMP-2 signalling by preventing receptor binding and Smad1 activation [60].

Once BMP-4 is closely related with BMP-2, Yao and colleagues hypothesized that the inhibitory effect MGP is not limited to BMP-2 but could also include BMP-4 [61]. Immunoprecipitation studies confirmed an interaction between the two proteins [61]. It was also proved that, as in the case of MGP and BMP-2 interaction, calcium ions and Gla residues were important for the binding between MGP and BMP-4, but proline 64 (Pro64), a conserved residue in human MGP sequence, was also critical for binding and inhibition of BMP-4 [62].

It was proposed a model of a regulatory pathway where MGP and BMP-2 and BMP-4 are involved [61,63]. BMP-2 and BMP-4 induce the expression of activin-like kinase receptor 1 (ALK1), a transforming growth factor-β (TGF-β) type I receptor associated with angiogenesis, that when stimulated induces the expression of MGP and vascular endothelial growth factor (VEGF) [61,63]. MGP provides both a positive feedback, by enhancing the ALK1 signaling, and a negative feedback, by binding and inhibiting BMP-2 and BMP-4 that in turn, declines ALK1 expression and activity [61,63]. This pathway is central for vascular development, disease and homeostasis [63]. BMP-7 were also implicated in this regulatory circuit and Yao and colleagues demonstrated that MGP binds and inhibits BMP-7 and BMP-4 in a similar manner [64].

However, MGP is not the only VKDP that is related with BMP-2. Surmann-Schmitt and colleagues have shown that GRP's mRNA and protein levels are down-regulated in murine chondrocytes treated with rBMP-2. Their results suggest a potential regulatory effect of BMP factors on GRP expression, although without mechanistic any data.

1.5. Vascular calcification

Vascular calcification is a disease described as being present over 5000 years ago, related with increased cardiovascular mortality and morbidity, and considered as a strong and independent risk factor for cardiovascular death [8,9]. Calcification is mainly present in the arterial vessel wall **(Fig. 1.6A)** and results in reduced arterial elasticity

and subsequent altered hemodynamics, contributing to the development of aortic stenosis, cardiac hypertrophy, hypertension, heart failure, and myocardial ischemia [8]. Within the vessel wall there are two different sites of calcification development: intimal and medial calcification [65]. Intimal or atherosclerotic calcification **(Fig. 1.6B)** is caused by a combination of risk-factors such as hypertension, inflammation, or dyslipidemia, and is characterized, in early stage, by a disperse punctate form and, as the disease progresses, by aggregates of calcium phosphate crystals deposits producing larger patchy stippled crystals and associating with the necrotic region of an atheroma [8,66]. Medial calcification **(Fig.1.6C)**, also known as Monckeberg's sclerosis, is characterized by amorphous mineral deposits along the elastin fibres of the tunica media which in an early stage are linear deposits that become circumferential rings of mineral as the disease proceeds [8,66]. This type of calcification is highly associated with aging, CKD and *diabetes mellitus* [8].

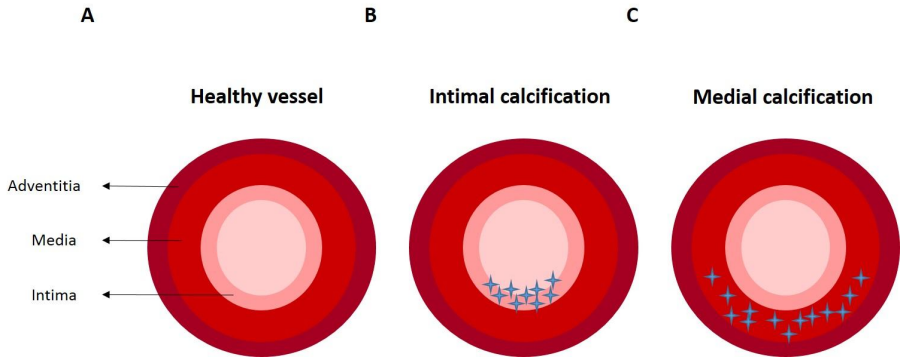

Figure 1.6 - Different types of vascular calcification in the arterial vessel wall. A) Healthy vessel in the absence of calcification. **B)** Intimal calcification with accumulation of calcium deposits in intima. **C)** Medial calcification with calcium deposits along the tunica media. Adapted from Willems *et al* (2014)[8].

1.5.1. Vascular calcification and VKDPs

For decades, vascular calcification development was considered as an inevitable consequence of aging and disease, but it is now considered to be an active process involving different molecular mechanisms where clearly a great importance has been given to VKDPs [8,65,67]. Human body fluids are supersaturated with calcium and phosphate but spontaneous mineralization does not normally occur due to a tight control by calcification inhibitors, namely MGP and eventually GRP [8,66]. In the case of

MGP, it was demonstrated that this protein depends on its Gla residues to bind calcium/phosphate crystals, but when vitamin K supply is inadequate or vitamin K antagonists are administered, the proteins become undercarboxylated, inactive, resulting in arterial calcification [39]. This hypothesis was proved by two independent studies that demonstrated the presence of poorly carboxylated MGP in calcified vasculature of aging rats and rats treated with warfarin, and a study in humans showing deposition of ucMGP in arteries from patients with atherosclerosis and Monckeberg's sclerosis [31,39,58].

Fetuin-A, an extracellular calcium-regulatory protein, is also a potent inhibitor of calcium-phosphate precipitation [68]. Fetuin-A and MGP form a high molecular mass complex with calcium-phosphate mineral, named fetuin-mineral complex (FMC), that is considered to have a role in prevention of vascular calcification avoiding mineral growth and targeting it for clearance from blood [69,70]. MGP γ-carboxylation appears to be essential for the presence of this protein in the complex [8].

1.5.2. Vascular calcification and matrix vesicles

As it occurs during osteogenesis and chondrogenesis, VSMCs release particular membrane structures, known as matrix vesicles (MVs), that serve as the initial *nidus* for calcium phosphate crystal deposition which promotes the onset of extracellular matrix calcification [8,71]. Under normal conditions, MVs are loaded of inhibitory proteins such as fetuin-A and MGP that block hydroxyapatite *nidus* [8,71]. However, in a calcium-rich environment, reported in pathologies associated with increased vascular calcification, these membrane structures are able to calcify. At calcification sites, VSMCs can undergo phenotypic transition to osteoblast/chondrocyte-like cells, upregulating the expression of mineralization-regulating proteins, such as BMPs [8,66,71,72]. BMP-2 is a crucial factor in this transition promoting the upregulation of osteogenic key factors as Cbfa1/Runx2 gene, essential for osteoblast differentiation and bone development, that consequently leads to an osteogenic phenotype [8,73]. As mentioned above, MGP could prevent this phenotypic transition through an

interaction with BMP-2 avoiding its binding to the receptors and consequently blocking gene transcription [59,60].

2. Objectives

Matrix Gla protein is an established inhibitor of soft tissue calcification and, although still not completely understood, it is known that its inhibitory mechanism is regulated by antagonizing BMPs through direct protein-protein interaction. However, these proteins and their mechanisms of action are not enough to explain the several cases of pathological calcification. Gla-rich protein, the newest VKPD, has also been recently associated with ectopic calcification related diseases. This association, together with results that report a potential regulatory effect of BMP factors on GRP expression in murine chondrocytes, lead us to hypothesize this protein as one of the missing regulators involved in the inhibition of ectopic calcification. In this way, the major goal of this work is the study of the possible interaction between GRP and BMPs. To achieve our objectives we established a system of production of human recombinant GRP and MGP in HEK293T cells and used their conditioned media to perform interaction studies between these proteins and BMPs.

Aiming at the better characterization of GRP biological role, it is also our goal to produce and optimize purification methods to isolate human recombinant γ-carboxylated protein. The success of this objective will further enable us, in a near future, to prove our hypothesis on GRP participation on calcification-related events through the development of functional assays.

3. Methods

3.1. Cell culture

Human embrionic kidney 293T (HEK293T) cells were cultured in Dulbecco's modified eagle medium (DMEM, Invitrogen) supplemented with 10% (v/v) of heat-inactivated foetal bovine serum (FBS, Sigma-Aldrich), 200 mM of L-glutamine (Invitrogen) and 1% (v/v) of penicillin/streptomycin (Invitrogen). Cell cultures were maintained in 5% CO_2 at 37º C.

3.2. Human GRP cloning into expression vectors

Two different constructions were prepared to produce either GRP or a fusion of GRP-mKate2 proteins; in both cases, complete coding sequence of GRP (alternative transcript F1, Fig. 1.4) was used. The complete coding sequence of human GRP was cloned into the expression vector pmKate2-C (where mKate2 was removed, Evrogen, **Fig. 3.1A**) and a fusion protein consisting of GRP C-*terminus* fused to the mkate2 N-*terminus* was produced using the plasmid pmKate2-N (Evrogen, **Fig. 3.1B).**

To clone human GRP into pmKate2-C, both respective fragment and plasmid were digested with the restriction enzymes *Nhe*I and *Bgl*II (New England Biolabs). After size-separation onto a 1% (w/v) agarose gel and fragments purification, inserts were cloned into pmKate2-C using T4 DNA ligase (Fermentas) following manufacturer's recommendations. The resulting construction was transformed into DH5α bacteria and selected positive clones were analysed by sequencing at CCMAR Sequencing Facilities. A similar procedure was performed to obtain the fusion construct mentioned above, to clone GRP into pmKate2-N, using in this case the restriction enzymes *Pst*I e *Bam*HI (New England Biolabs) taking into consideration the maintenance of an open reading frame for GRP-mkate2 translation. Specific primers used for the aforementioned cloning constructs are listed in Annex I.

3.3. Production of human recombinant proteins in HEK293T cells

HEK293T cells were seeded out in 10-cm dishes and transiently transfected using the calcium/phosphate method or FuGENE 6 (Promega) with 4 to 16 µg of DNA of interest. In the case of MGP we have used the plasmid pN-FLAG-MGP, a kind gift from Dr. Kristina Böstrom (UCLA, David Geffen School of Medicine, USA). According to experimental plan, total proteins extracts and/or conditioned media were collected. Conditioned media, treated with vitamin K1 (50 nM [74]), were collected 48 or 72h after transfection and aliquots were lyophilized, while total cells extracts were obtained using RIPA buffer. Protein content was determined by spectrophotometry at 280 nm (Nanodrop 1000, Thermo Scientific) following the relation 1 U Abs_{280} = 1 mg/ml.

To produce HEK293T stable clones overexpressing GRP, cells were transfected using the same conditions as described previously but introducing a selective pressure by treating transfected cells with G418 (Sigma-Aldrich) at a concentration of 400 µg/ml. Several days after transfection, individual colonies were expanded and, besides collection of conditioned media and total cells extracts, total RNA were extracted as described by Chomczynski and Sacchi [REF]. RNA concentration was determined by spectrophotometry at 260 nm (Nanodrop 1000).

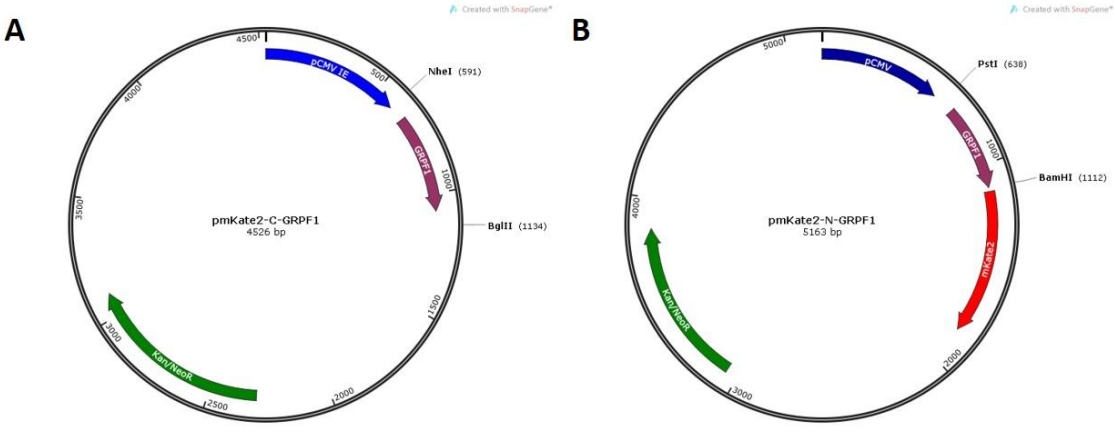

Figure 3.1 - Plasmid constructions used to overexpress GRP and GRP-mKake2 *in vitro*. A) pmKate2-C-GRPF1 (F1 stands for alternative transcript F1). **B)** pmKate2-N-GRPF1. Cytomegalovirus promoter; GRP coding sequence; mKate2 codingsequence; Kanamycin/neomycin resistance gene.

3.4. Genomic DNA extraction

To extract genomic DNA, HEK293T cells were disrupted using lysis buffer (10 mM EDTA, 0.05 M Tris-HCl pH 8.0, 1% (w/v) SDS) and proteinase K (10 mg/ml) and incubated overnight (O/N) at 50ºC with gentle agitation. Genomic DNA was then isolated using 1V of phenol pH 8.0 with gentle agitation followed by an additional extraction step with phenol pH 8.0 / chloroform / isoamyl alcohol (25:24:1). After precipitation and ethanol washing, samples were dried and resuspended in ultrapure water. Centrifugations were performed for 10 min at 13 000xg. DNA concentration was determined by spectrophotometry at 260 nm (Nanodrop 1000).

3.5. RT-qPCR

One microgram of total RNA treated with RQ1 RNase free DNase (Promega) was reverse transcribed using Moloney-murine virus reverse transcriptase (MMLV-RT), RNase Out (both from Invitrogen) and an oligo(dT) adapter, according to manufacturer's recommendations.

To determine which HEK293T stable clone had higher expression of GRP, quantitative PCR (qPCR) amplification was performed using 5 µl of cDNA (1:10 diluted RT) and SsoFast EvaGreen supermix (Bio-Rad) in reactions of 50 cycles. Human ribosomal 18S was used as a housekeeping gene for normalization. Primers used are listed in Annex I.

3.6. Purification of recombinant human GRP through affinity chromatography

To purify the human recombinant GRP, conditioned medium from HEK293T cells transfected with pmKate2-C-GRP or pmKate2-N-GRP (**Fig. 3.1A** and **B**, respectively) were collected and centrifuged at 2 000xg, 5 min to remove cell debris and pellet down any dead cells. Supernatant was then centrifuged at 12 000xg for 15 min, at 4ºC, to remove insoluble proteins and filtered before purification through affinity

chromatography. Briefly, the affinity column was prepared using CNBr-activated Sepharose 4B beads (GE Healthcare), approximately 10 ml of beads, and cross-linked to the rabbit polyclonal CTerm-GRP antibody (GenoGla Diagnostics). First, the column was equilibrated in 10 V of 10 mM Tris pH 7.5 and approximately 50 ml of diluted (1:2) conditioned media, in the same buffer. Conditioned media was repeatedly passed through the column three times. The column was then washed with 10V of 10 mM Tris pH 7.5 and 10 V of 10 mM Tris pH 7.5 / 500 mM NaCl. Acid and basic elutions were performed to fraction affinity-bounded proteins using an acid solution of 100 mM glycine pH 2.5 and a basic solution of 100 mM triethylamine pH 11.5. Fractions of 1 ml were collected and protein content was determined by spectrophotometry at 280 nm (Jasco V630 Spectophotometer) considering the relation 1 U Abs_{280} = 1 mg/ml protein.

3.7. Immunoprecipitation

3.7.1. Immunoprecipitation using magnetic beads

Immunoprecipitation (IP) was performed using the Immunoprecipitation Dynabeads Protein A Kit (Invitrogen). Antibodies, listed in Annex II, and the Dynabeads protein A were incubated, in a ratio of 10 µg of antibody per 50 µl of Dynabeads, for approximately 3 h to promote their binding, followed by incubation with the antigen for 1 h. For antigen preparation, two aliquots of equal volumes of each conditioned media containing MGP-FLAG, GRP-mKate2 and 100 ng/ml of human recombinant BMP-2 protein (Peprotech) were mixed and incubated for 2 h. After incubation, the mixture was equally divided into different tubes, according to IP and respective antibody of interest, and incubated for 1 more hour. All incubations were performed at room temperature (RT), with gentle agitation. Elution was performed in 20 µl of NUPAGE sample buffer followed by immunoblotting as described in section 3.8.

3.7.2. Immunoprecipitation using protein A sepharose beads

For this IP approach, 25 mg of protein A sepharose beads (GE Healthcare) were used per 50 µg of each antibody listed in Annex II. Beads were washed twice with ultrapure water, once with PBS and then blocked with 1 mg/ml of bovine serum albumin (BSA) in PBS for 1 h. After binding of antibodies, beads were washed with excessive PBS. Antibodies were cross-linked to protein A sepharose by adding 200 mmol/L triethanolamine / 20 mmol/L dimethyl pimelimidate in PBS (final pH 8.6). After rotation for 30 min, the beads were washed twice with 200 mmol/L triethanolamine in PBS. The remaining reactive amino groups were quenched by addition of 50 mmol/L ethanolamine in PBS for 1 h. Non-cross-linked antibodies were removed by incubating twice with 1 M glycine pH 3 for 20 min at 56ºC and washed twice with PBS, followed by incubation with antigen O/N. For antigen preparation, two aliquots of equal volumes of each conditioned media containing MGP-FLAG, GRP-mKate2 and 100 ng/ml of human recombinant BMP-2 were mixed and incubated for 1 h. The mixture was then evenly divided into different tubes, according to the antibody used and incubated O/N at 4ºC. Beads were washed six times with PBST, eluted three times with 100 mM glycine pH 2.5 and then washed again three times with PBST. To improve the binding yield, this incubation step was repeated twice. Cross-linked beads were stored at 4 °C in PBS containing 0.2 mL/L Tween-20 (PBST) to be reused. All reactions were performed at RT, except incubation with antigen that was performed at 4ºC; PBS was used at pH 7.0 unless stated otherwise.

3.8. Protein electrophoretic fractioning and immunodetection

Aliquots of conditioned media (usually 100 µl) and total protein extracts (50-100 µg) were size-separated onto a 4-12% (w/v) gradient polyacrylamide precast gel containing 0.1% (w/v) SDS (NuPage, Invitrogen) and ran at 180 V. Transfer onto a nitrocellulose membrane (BioRad) was performed at 80 mA for 1.5 h. Membrane was blocked with 5% (w/v) BSA in phosphate buffer saline tween [PBST: 15 mM NaH_2PO_4, 8.1 mM $Na_2HPO_4.2H_2O$, 1.45 M NaCl, pH 7.4, 0.05% (v/v) Tween20] for 3 h and

incubated O/N with primary antibodies, listed in Annex II, according to the protein of interest. Immunodetection was achieved using species-specific secondary horseradish peroxidase conjugated antibodies (Sigma-Aldrich) and Western Lightning Plus-ECL (PerkinElmer) detection system.

3.9. Total protein content detection

After polyacrylamide gel electrophoresis (SDS-PAGE), as described in section 3.8, gel was washed two times for 5 min with ultrapure water and then fixed with fixing solution [50% (v/v) ethanol, 2% (v/v) phosphoric acid] for 2h. After fixation, gels were washed three times for 25 min and equilibrated with an equilibration solution [34% (v/v) methanol, 17% (w/v) aluminium sulphate, 2% (v/v) phosphoric acid] for 1h. Gels were stained with coomassie brilliant blue (CBB) G-250 [10% (w/v) sulfoacidic acid, 10% (w/v) trichloroacetic acid, 0.2% (w/v) CBB G-250] O/N and destained with ultrapure water. Bands of interest were cut and analysed through LC-MS/MS at the Mass Spectometry Unit, Center for Neurosciences and Cell Biology/Biocant, University of Coimbra.

3.10. Human BMP-2 cDNA amplification

For human BMP-2 (NM_001200) coding sequence amplification, several primers sets were designed (Annex I) using Primer-Blast tool at NCBI. Reverse transcribed RNA from bone and cartilage, from either healthy individuals or osteoarthritis (OA) patients, was used as template for BMP-2 amplification. First and nested PCR reactions were performed using 5 µl of cDNA or previous PCR, respectively, different sets of primers and *Taq* DNA polymerase (Invitrogen), following manufacturer's recommendations. PCR products were size-separated onto a 1% (w/v) agarose gel and selected fragments were purified using GFX Gel Band Purification kit (GE Healthcare), cloned into pCRII-TOPO vector (Invitrogen) and sequenced at CCMAR Sequencing Facilities.

3.11. Immunocytochemistry

HEK293T cells were cultured in 13-mm poly-Lysine pre-coated glass coverslips in 24-well plates at a concentration of $8x10^4$ cells per well. Cells, transiently transfected with pmKate2-C-GRP (expressing only GRP) or co-transfected with pN-FLAG-MGP, were washed two to three times with phosphate-buffered saline (PBS), fixed with 4% (v/v) paraformaldehyde in PBS (PFA) for 30 min, washed three times with PBS, and permeabilized with PBS 0.5% TritonX-100. For immunodetection, cells were incubated with primary antibodies diluted in ADB [1% (v/v) SFB, 0.05% (v/v) of Tween20 in PBS] for 1h at RT in a humidified chamber, followed by an incubation with secondary antibodies, also diluted in ADB, for 30 min in humidified dark chamber. Antibodies are listed in Annex II. Nuclei were stained with 2 µg/ml DAPI and coverslips mounted in ProLong Gold (both from Life Technologies). Images were obtained through stacks imaging using Zeiss Axio-Imager Z2 microscope at the Light Microscopy Unit, Department of Medicine and Biomedical Sciences, UAlg. The images result from a Z projection.

4. Results

4.1. HEK293T cells express and secrete GRP-mKate2 fusion protein into extracellular media

To produce human recombinant GRP, fused to mKate2 fluorescent protein, in order to increase protein solubility, we have started by stably transfecting HEK293T cells with pmKate2-N-GRP construct (mature protein with a theoretical molecular weight of approximately 43 kDa). After treating transfected cells with G418 for positive clone selection, four clones were picked and respective RNA collected to analyse GRP gene expression levels. Results indicate a higher gene expression level for clone 4 **(Fig. 4.1)**, leading us to investigate its protein levels, both extracellularly (conditioned media) and intracellularly (total cell extracts).

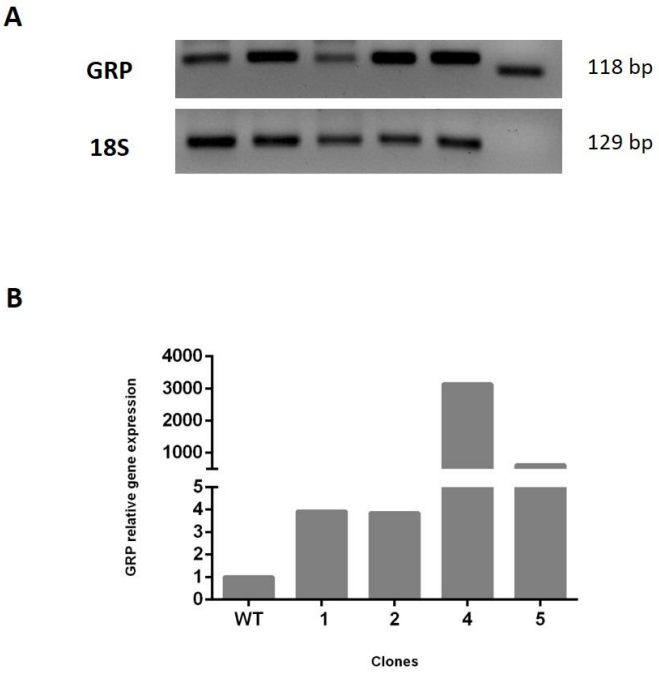

Figure 4.1 - Gene expression levels of HEK293T clones overexpressing GRP. Expression levels of GRP gene in HEK293T stable clones (numbered 1-5) were obtained by (**A**) RT-PCR and quantified by (**B)** qPCR; 18S was used as a housekeeping to normalize expression.

Western blot results indicate that either GRP-mKate2 fusion protein is not being translated, or that its levels are too low because it was not detected in conditioned media nor in total cell extracts *(data not shown)*. In fact, the absence of protein translation can be explained by the presence of SV40 large T antigen in HEK293T cells. The generation of stable cell lines expressing the SV40 large T antigen is very difficult with any plasmid that contains the SV40 promoter, which is the case of pmKate2-N, once that SV40 large T antigen induces DNA replication at the SV40 promoter in the vector sequence.

To continue our goal of producing human recombinant GRP protein, we decided to perform transient transfections of HEK293T cells with either pmKate2-N-GRP or pmKate2-C-GRP plasmids, to produce recombinant GRP-mKate2 and GRP (with a theoretical molecular weight of approximately 17 kDa), respectively. Conditioned media and total cell extracts were collected from both cell cultures and analysed by western blot. Our results indicate that recombinant proteins were both translated and secreted since could be detected in culture media **(Fig. 4.2A and B)**. In the case of GRP without mKate2 *tag*, the presence of two fragments of different sizes in conditioned media could be due to the presence of different GRP γ-carboxylated forms. In total cell extracts, a fragment with higher molecular weight than the fragments detected in conditioned media is, probably, an unprocessed form of GRP.

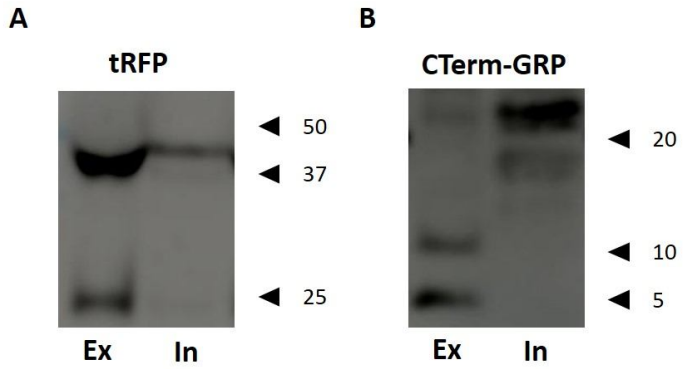

Figure 4.2 - Analysis of GRP protein levels in transiently transfected HEK293T cells. HEK293T cells were transiently transfected with pmKate2-N-GRP or pmKate2-C-GRP plasmids and conditioned extracellular (Ex) and intracellular (In) media were analysed by SDS-PAGE followed by western blot. GRP-mKate2 was immunodetected with **(A)** tRFP and GRP without mKate2 *tag* was immunodetected with **(B)** CTerm-GRP. Relevant molecular mass markers (kDa) are indicated on the right side of the panels.

4.2. GRP-mKate2 is efficiently purified through a CTerm-GRP affinity chromatography

To better characterize GRP biological role, functional studies, that include a comparison between uncarboxylated and carboxylated protein forms, are crucial. The system to produce GRP in *E. Coli*, in large quantities, is currently available in the lab, but provide us only with the uncarboxylated protein form. For functional comparative studies, is urgent to produce also the γ-carboxylated form, believed to be crucial for protein's function. To achieve this goal we have decided to purify the human recombinant GRP or fusion GRP-mKate2 produced in HEK293T cells and secreted into conditioned media, by affinity chromatography. HEK293T cells transfected with pmkate2-N-GRP or pmkate2-C-GRP, and treated with vitamin K1, were collected and fractioned through an affinity column prepared using CNBr-activated Sepharose 4B beads cross-linked to the rabbit polyclonal CTerm-GRP antibody. Acid and basic elutions were performed to fraction affinity-bounded proteins. Fractions were collected and protein content was determined by spectrophotometry at 280 nm for both fusion GRP-mKate2 **(Fig. 4.3A and B)** and GRP without mKate2 *tag* conditioned media **(Fig. 4.5)**. Fractions exhibiting higher absorbance levels were selected **(Fig. 4.3 and 4.4)**, analysed by SDS-PAGE and the protein of interest was detected using specific antibodies. Our results indicate that only GRP-mKate2 fusion protein was efficiently purified **(Fig. 4.3C)**. This is most possibly due to its higher solubility when compared with GRP without mKate2 *tag* for which, although its absorbance levels are high, no signal can be detected using specific antibodies (*data not shown*). The γ-carboxylation status of purified GRP was further investigated using conformational antibodies to carboxylated (cGRP) and undercarboxylated (ucGRP) protein forms. Our results indicate that GRP-mKate2 fusion protein present in the condition media is undercaboxylated **(Fig. 4.3C)**. During GRP-mKate2 conditioned media preparation we have obtained a pellet after centrifugation at 12 000 xg for 15 min that was also analysed. Importantly, SDS-PAGE and western blot results indicate the presence of both forms of GRP, carboxylated and undercarboxylated, which are recognized by ucGRP and cGRP antibodies, respectively **(Fig. 4.3C)**, in this insoluble fraction. Due to

annexin 6 association to matrix vesicles, we have also investigated its presence in the pellet, and a positive signal at 68 kDa was detected in our western blot **(Fig. 4.3C)**.

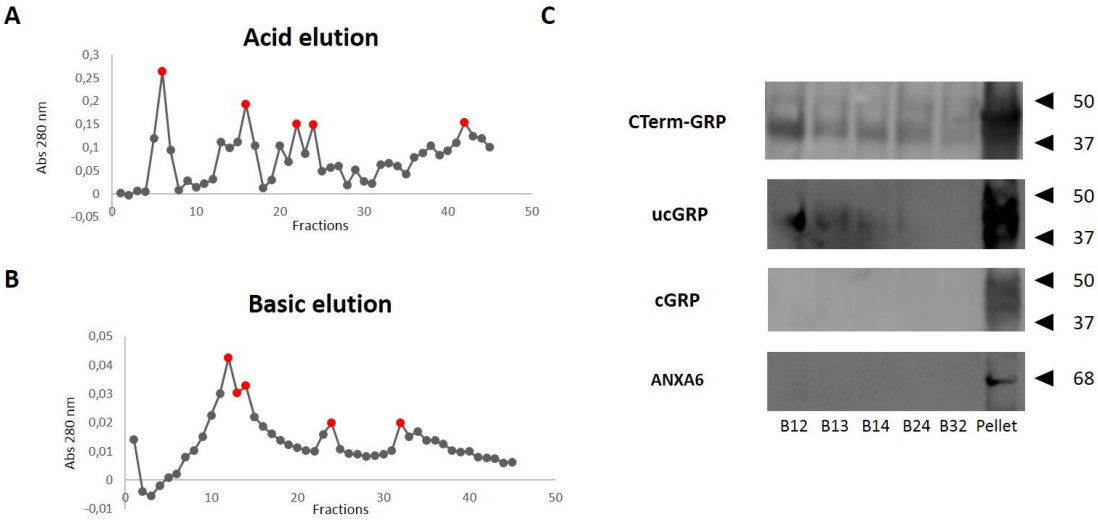

Figure 4.3 - GRP-mKate2 purification through affinity chromatography. Conditioned media from HEK293T cells transfected with pmkate2-N-GRP was collected and fractioned through an affinity column with CTerm-GRP antibody. **(A)** Acid and **(B)** basic elution was performed to fraction affinity-bounded proteins and protein content of each fraction collected was determined by spectrophotometry at 280. **(C)** Fractions exhibiting higher absorbance levels (red points) and conditioned media insoluble fraction (pellet) were size-separated by SDS-PAGE and the protein of interest was immunodetected with CTerm-GRP by western blot. The γ-carboxylation status of purified GRP was investigated using undercarboxylated (ucGRP) and carboxylated (cGRP)antibodies, respectively; the presence of annexin 6 (ANXA6) was also investigated. Relevant molecular mass markers (kDa) are indicated on the right side of the panels.

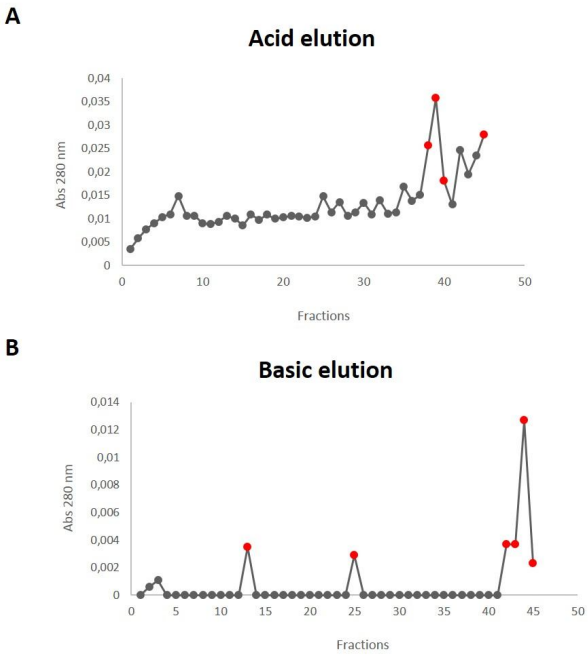

Figure 4.4 - GRP purification through affinity chromatographyy. Conditioned media from HEK293T cells transfected with pmkate2-C-GRP construct were collected and fractioned through an affinity column with the CTerm-GRP antibody. **(A)** Acid and **(B)** basic elution was performed to fraction affinity-bounded proteins and protein content of each fraction collected was determined by spectrophotometry at 280. Fractions exhibiting higher absorbance levels (red points) were size-separated and the presence of protein of interest was investigated by western blot using the CTerm-GRP although no positive signal was obtained (*data not shown*).

4.3. Human MGP recombinant protein is majorly retained intracellularly or in the matrix of HEK293T cells

To overexpress human MGP, HEK293T cells were transiently transfected using pN-FLAG-MGP construct with a respective translated protein with a theoretical molecular weight of approximately 14 kDa. Both total cell extracts and conditioned media obtained from this experiment were analysed by SDS-PAGE and western blot. Results indicate that most of the protein produced is retained intracellularly or in the ECM, while protein in conditioned media is almost barely detected **(Fig. 4.5)**. This result indicates that optimizations are still required, such as altering the amount of DNA for transfection and/or using different transfection agents, adjusting the time point for total cell extracts and conditioned media collection or even using a different protein *tag* that might increase the solubility of the fusion product.

Figure 4.5 - MGP overexpression in HEK293T cells. pN-FLAG-MGP were transiently expressed in HEK293T cells and intracellular (In) and extracellular (Ex) conditioned media were analysed by SDS-PAGE and western blot. MGP was immunodetected with M2 an antibody specific for F tag. Relevant molecular mass (kDa) is indicated on the left side of the panel.

4.4. Identification of a novel BMP-2 alternative transcript

To determine a possible interaction between BMP-2 and GRP as described for MGP, we have started by cloning the human BMP-2 transcript corresponding to the complete coding sequence (CDS) to further use it to overexpress this protein in HEK293T cells. Several pairs of primers were designed to perform both first and nested PCR reactions and bone and cartilage samples from healthy and OA individuals were used as a template. We were not successful on the amplification of BMP-2 complete CDS and instead we have identified a novel fragment of approximately 1000 bp from cartilage of an OA patient **(Fig. 4.6)**. This fragment was confirmed, through sequencing, to be an alternative transcript of the human BMP-2 gene that had not been described previously (GenBank identification number 4670498). This alternative transcript exhibits the loss of exon 2, coding for the initial ATG, resulting in a small truncated protein, if translated. Since full CDS of human BMP-2 was not obtained within this project, we have decided to use a commercial human recombinant protein for further protein-protein interaction studies.

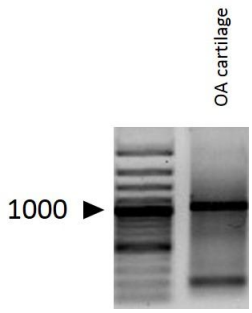

Figure 4.6 - BMP-2 alternative transcript identification. A fragment of approximately 1000 bp corresponding to a novel alternative transcript (GenBank identification number 4670498) was identified in cartilage of an OA patient. Relevant molecular mass (bp) is indicated on the left side of the panel.

4.5. GRP co-immunoprecipitates with BMP-2

To study a possible interaction between human BMP-2, GRP and MGP we have performed IP assays. We have started by mixing equal amounts of conditioned media from HEK 293T cells overexpressing i) GRP-mKate2, ii) MGP-FLAG while iii) 100 ng/ml of human recombinant BMP-2 was also added to the media. In parallel, we have analysed recombinant BMP-2 by SDS-PAGE and western blot to determine electrophoretic migration pattern and optimize conditions for detection. Two bands were detected at 25 and 15 kDa corresponding to dimeric and monomeric forms of BMP-2, respectively **(Fig. 4.7A)**. GRP-mKate2 was first captured with tRFP antibody, specific for mKate2, and the co-immunoprecipitated (co-IP) proteins (IP tRFP) were analysed by western blot using a specific antibody for BMP-2/4. The results show that BMP-2 co-immunoprecipitate with GRP **(Fig. 4.7B)** pointing to an interaction between the two proteins.

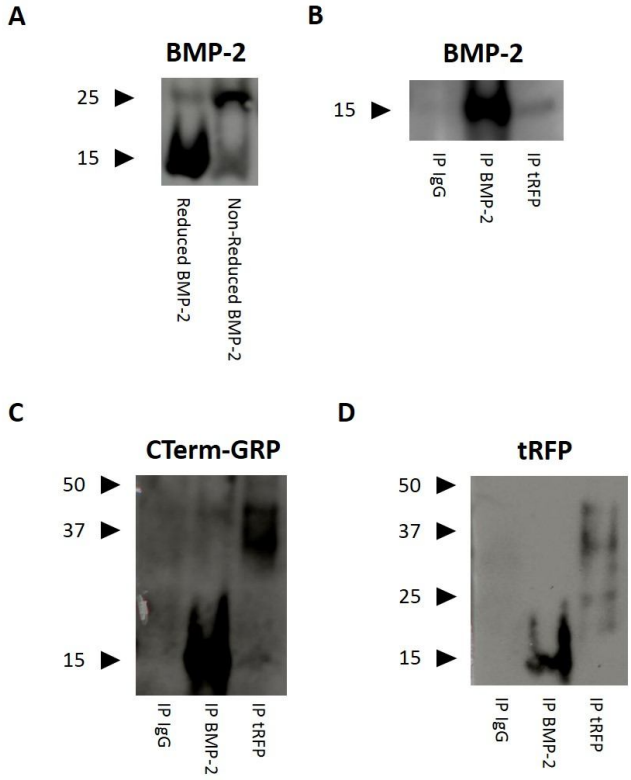

Figure 4.7 - Co-immunoprecipitation of GRP and BMP-2. Conditioned media from cells transfected with pmKate2-N-GRPF1 and pN-MGP-FLAG to which 100 ng/ml of recombinant BMP-2 was added, were used to perform immunoprecipitation assays. **A)** 50 ng of recombinant BMP-2 was analysed by SDS-PAGE and

western blot to determine the appropriate amount for detection and electrophoretic migration pattern. Immunodetection was performed using BMP-2/4 antibody. GRP-mkate2 was captured with tRFP antibody and the co-immunoprecipitated proteins were analysed by western blot using BMP-2/4 antibody. BMP-2 was captured with corresponding antibody and the immunoprecipitated proteins analysed by western blot using **B)** CTerm-GRP and **C)** tRFP antibodies. Relevant molecular mass (kDa) markers are indicated on the left side of panels.

On the other hand, when these co-IP proteins (IP tRFP) were analysed by western blot using CTerm-GRP and tRFP antibodies, positives bands of different sizes were detected. Bands of approximately 43 and 15 kDa were identified with CTerm-GRP antibody **(Fig. 4.7C)** while tRFP detects 43 and 25 kDa bands **(Fig. 4.7D)**. Based on these results we hypothesized that fragments at approximately 25 and 15 kDa, respectively, could arise from proteolytic cleavage of 43 kDa fusion GRP-mKate2 **(Fig. 4.8)**. To confirm this hypothesis, conditioned media from HEK293T cells overexpressing GRP-mKate2 was immunoprecipitated with tRFP antibody. The IP proteins were size-separated and total proteins were stained with CBB G-250 **(Fig. 4.9)**. Bands corresponding to 43 and 25 kDa were analysed through LC-MS/MS and the identity of GRP-mKate2 at 43 kDa and only mKate2 at 25 kDa was confirmed. These data are consistent with our previously results **(Fig. 4.2A)**.

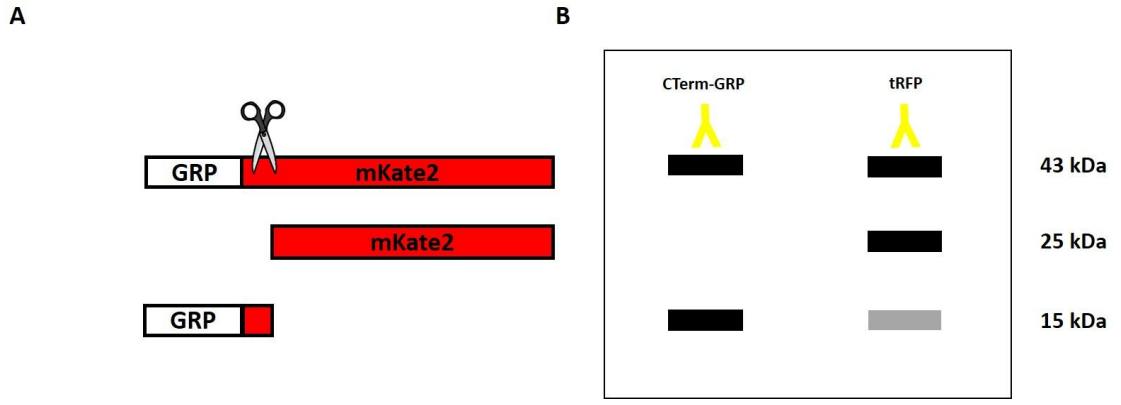

Figure 4.8 - Putative proteolytic cleavage of GRP-mKate2. (A) Putative proteolytic cleavage site in GRP-mKate2 fusion protein and the resulting fragments. **(B)** Our hypothesis of fragments' recognition using CTerm-GRP and tRFP antibodies, according to putative proteolytic cleavage.

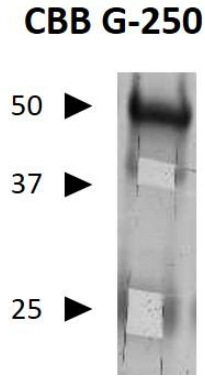

Figure 4.9 – Co-IP and SDS-PAGE analysis for proteins identification. Immunoprecipitated proteins were size-separated through SDS-PAGE and gel was stained with CBB G-250. Bands of interest were cut and analysed by LC-MS/MS. Identification results confirm the band between 50 and 37 kDa as GRP-mKate2 whereas the band at 25 kDa was identified as mKate2. Relevant molecular mass (kDa) markers are indicated on the left side of panel.

BMP-2 was captured using its specific antibody (IP BMP-2) and the co-IP proteins were analysed by western blot using CTerm-GRP and tRFP antibodies. In both cases, a band at 15 kDa was detected, suggesting that the interaction between BMP-2 and GRP could be more efficient with GRP than with the fusion protein **(Fig. 4.7)**.

For both IP assays, co-IP proteins were also analysed using M2 antibody, specific for the FLAG tag but no signal was detected (*data not shown*). Immunoprecipitation with rabbit IgG was always used as a control and in both cases the results were negative **(Fig. 4.7B, C and D)**.

4.6. Co-localization of GRP with MGP

In order to demonstrate a possible interaction between GRP and MGP, HEK293T cell were transiently co-transfected with pmKate2-C-GRP and pN-FLAG-MGP. Immunofluorescence assays were performed 24h after transfection with CTerm-GRP and M2 antibodies. Results show overlapping signal of both MGP and GRP **(Fig. 4.10A)**.

To determine GRP intracellular localization, immunofluorescence experiments were performed 24h after transient transfection of these cells with pmKate2-C-GRP using CTerm-GRP and pan-cadherin antibodies. Colocalization of GRP with endogenous

pan-cadherin, specific for cellular membrane, indicates a predominant localization of GRP in this cellular structure **(Fig. 4.10B)**.

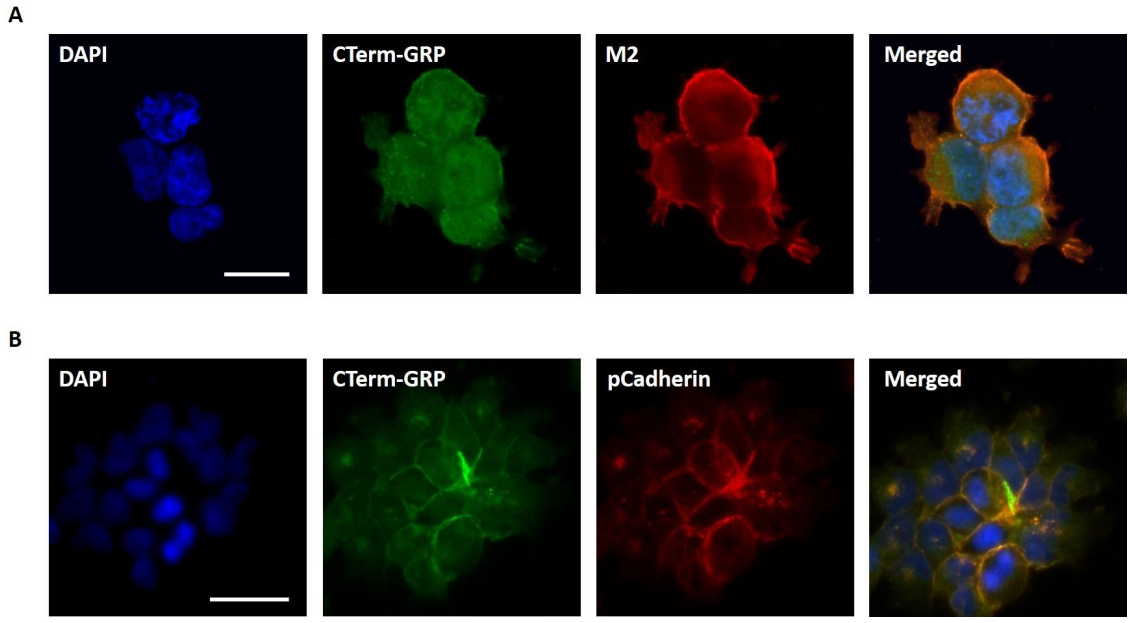

Figure 4.10 - GRP co-localizes with MGP in co-transfected HEK293T cells. HEK293T cells were transiently co-transfected with pmKate2-C-GRP and pN-FLAG-MGP or transfected with pmKate2-C-GRP only. **(A)** GRP detection was achieved using the CTerm-GRP antibody and secondary anti-rabbit-Alexa 488 (green), while MGP was detected with FLAG-tag M2 antibody and anti-mouse-Alexa 594 (red). **(B)** GRP (green) co-localizes with pan-cadherin (red) indicating its presence in cellular membrane. Scale bar represents 20 µm.

5. Discussion

Gla-rich protein is the newest member of the VKDP family and is characterized by the highest density of Gla residues known for their capacity to bind calcium [28]. It is known that γ-carboxylation of VKDPs is, in most cases, a determining factor for their proper function [2]. In particular, it has been suggested that human GRP carboxylation is a determinant factor for GRP role in ectopic calcification inhibition. The presence of Gla residues in its sequence has been based on its high sequence similarity with sturgeon GRP, previously shown to be γ-carboxylated by amino acid analysis, and the identification of specific domains and motifs conserved in other VKDPs [28]. More recently, using conformational antibodies specific to cGRP and ucGRP, it was also demonstrated that the presence of these protein forms could be further associated to healthy or pathological conditions [45,47].

Since only small amounts of protein can be purified from human tissues or biological fluids, genetic engineering become important to produce a functional recombinant protein, as identical as possible to the human form and for that, all possible post-translational modifications should be considered [75]. This opens up a variety of opportunities of producing proteins with therapeutic applications, such as in diabetes, end-stage renal disease and inborn errors of metabolism [75]. *Escherichia coli* (*E. Coli*) is widely used to produce recombinant proteins due to its great advantages, such as fast growth kinetics and easy transformation with exogenous DNA, but this system fails when eukaryotic post-translational modifications are required [76]. Given the need of GRP to be γ-carboxylated to become functional, we developed a protocol to purify this protein through affinity chromatography using conditioned media from HEK293T cells overexpressing GRP or GRP-mKate2. Our purification was much more efficient in the case of GRP-mKate2 fusion protein, probably due to an increased solubility conferred by the larger mKate2 that is usually used to increase solubility of insoluble proteins. However, the purified protein obtained was mostly undercarboxylated, even treating cells with vitamin K1, suggesting that either vitamin K could have not been uptaken by these cells or the machinery needed for γ-carboxylation could have not been fully functional in HEK293T cells. The requirement

of several protein factors involved in γ-carboxylation, such as GGCX and VKOR, probably hampered the production and secretion of this VKDP as previously described [28]. Stoichiometry of all components has also been described to be a determinant factor for the efficiency of this process [28]. Since this was our first approach, conditions still need to be optimized, therefore, in order to improve the γ-carboxylation status of GRP; for example, the concentration of vitamin K1 should be optimized and VKOR could be simultaneously overexpressed [77]. Nevertheless, Rafael and colleagues demonstrated that γ-carboxylated GRP is produced in this human cell system [45] and their results also point to another possibility that might explain our system. It is possible that we have mainly purified undercarboxylated GRP due to its higher solubility while its γ-carboxylated counterpart, being more insoluble, is mainly detected in an insoluble fraction of conditioned media, pelleted down by centrifugation.

Matrix vesicles are membrane structures known to be responsible for the onset of calcification but under normal conditions, *ie* low Ca^{2+} levels, are enriched in calcification inhibitors [71]. Since annexin 6 has been associated with MVs, we have investigated its presence in this insoluble fraction of condition media [71]. Codetection of annexin 6 and GRP in the same insoluble fraction, further suggests that this protein could be incorporated in vesicles and released into to the extracellular mediua via MVs, as previously demonstrated for MGP, pointing to a possible role of GRP as a calcification regulator through involvement into MVs biogenesis and mineralization competence [71,78].

Matrix Gla protein is widely accepted as an inhibitor of soft tissue calcification being involved in several related human pathologies, but the absence of calcification in the case of Keutel syndrome, where mutations on MGP gene are present, suggests the involvement of alternative calcification regulators [40]. It is our hypothesis that GRP could be one of these missing regulators involved in the inhibition of calcification in soft tissues, once this protein was also found to be associated with ectopic calcification in human skin, vasculature and cartilaginous tissues [45,46].

Although the mechanism by which these proteins inhibit calcification is still not completely understood, for MGP it was proposed that this inhibition is regulated by BMPs, potent inducers of bone formation in both skeletal and vascular tissues [34]. As it

has been mentioned previously, distinct studies have confirmed the direct protein-protein interaction between BMPs and MGP involving its Gla residues. Moreover, BMP-2 was also proposed to be a negative regulator of GRP by down-regulating its gene expression and protein production in murine chondrocytes [41]. Given these previous data on MGP-BMP-2 interaction and BMP-2 effect on GRP gene regulation, we have focused in testing the relevance of GRP in calcification inhibition via interaction with BMP-2. To further investigate these possibilities, we performed IP assays with conditioned media from HEK293T cells overexpressing MGP and GRP, to which recombinant BMP-2 was added. Our results demonstrate that BMP-2 co-immunoprecipitates with GRP, further indicating that GRP could be, in fact, a regulator involved in the inhibition of ectopic calcification and that its mechanism of action, as in MGP, could be by an interaction with BMP-2.

The identification, by LC-MS/MS, of two fragments with 43 and 25 kDa, corresponding to GRP-mKate2 and to mKate2, respectively, confirm our hypothesis that a proteolytic cleavage is most possibly occurring in the GRP-mKate2 fusion protein. This cleavage will probably result in a third fragment of 15 kDa corresponding to GRP. In fact, when BMP-2 is captured with the corresponding antibody and analysed with CTerm-GRP and tRFP antibodies, a positive signal at 15 kDa is detected. These results suggest that the interaction between GRP and BMP-2 could be more efficient with the 15 kDa protein further indicating that the fusion with mKate2 could result in a loss or a decrease of protein's functionality, probably hampering accessibility for BMP-2 binding site.

Immunoprecipitated proteins were also analysed using M2 (FLAG tag specific) antibody and no signal was detected. Levels of available protein in conditioned media are most possibly not enough to co-immunoprecipitate with BMP-2, as it has been previously demonstrated [59,60]. The absence of a positive signal for MGP could be explained by: i) poor solubility of this protein; ii) protein could be trapped in MVs and, in fact, its presence in these structures has been previously demonstrated in VSMCs-derived MVs [79]; or iii) most of the protein is retained in the ECM or even iv) inside the cell. Thus, we were unable to conclude on a putative MGP-GRP interaction. In conclusion, to achieve this goal, several optimization tests must be further performed:

amount of DNA for transfection and different transfection agents, time point for total cell extracts and conditioned media collection, MGP's fusion with a larger protein to overcome solubility issues, as suggested by our results that show higher solubility for GRP-mKate2 in comparison with GRP without mKate2 *tag*. Nevertheless, the existence of an interaction between GRP and BMP-2 in the absence of MGP, suggests an interesting potential role for GRP-BMP-2 interaction, apparently independent of MGP.

An immunocytochemistry approach was used to investigate the interaction between GRP and MGP and to determine its subcellular localization. For that, HEK293T cells were transiently co-transfected with pmKate2-C-GRP and pN-FLAG-MGP or with pmKate2-C-GRP. Since immunofluorescence was performed 24 h after transfection, it is conceivable that both proteins were still intracellularly localized. This is consistent with results from Rafael and co-workers showing that GRP secretion increased from 48 h to 72 h after transfection and demonstrating that GRP secretion in this cell system is time-dependent [45]. Our results showing co-localization of GRP with endogenous pan-cadherin indicate that GRP was most possibly following a membrane-dependent secretory pathway. Co-localization of GRP and MGP signals, suggests that both proteins could interact with each other or, at least, share the same subcellular localization.

Cardiovascular disease is one of the major causes of death worldwide, thus efforts must be made to decrease this mortality rate. Since vascular calcification is a high risk condition and VKDPs, namely MGP and GRP, have been associated with ectopic calcification-related pathologies, we should try to better understand their molecular mechanism of action. This work contributes to further unveil the role of GRP in calcification's regulation, by pointing to the interaction with BMP-2. Moreover, this interaction seems to be independent of MGP leading us to hypothesize that it can act as an alternative to MGP, although further functional studies must be sought to clarify this subject. Additionally, given the association of GRP with vascular calcification, and other calcification-related diseases such as osteoarthritis and certain cancers, the data presented in this report will further contribute to the establishment of GRP as an additional biomarker for prognostic and diagnostic of ectopic calcification status. Moreover, the discovery of potential GRP interaction partners like BMPs, known to be

involved in osteogenesis, and the study of its mechanism of action, will open new perspectives for treatment of diseases in need of alternatives.

6. Perspectives

Now that an interaction between GRP and BMP-2 was demonstrated, it is necessary to understand if this is a direct or indirect event. In a future work we are interested in identifying GRP additional interacting partners and while further tests must be performed to clarify if the binding between GRP and BMP-2 is independent of the presence of MGP, as our results suggests **(Fig. 6.1)**. To further elucidate the regulatory mechanisms underlying this interaction, we are interested in: i) investigate if this interaction is dependent of the presence of Ca^{2+}; ii) what is the relevance of GRP's γ-carboxylation; and iii) which residues are involved in the binding between these two proteins. It is also crucial to understand the biological role of this interaction. Since BMP-2 is known by its capacity to differentiate cells into an osteogenic lineage, we can further investigate if GRP modulates this differentiation capacity. Additionally, we can perform colocalization of GRP and BMP-2 in human tissues to demonstrate this interaction *in vivo* and comparison between pathological and healthy tissues will help to demystify the mechanism of action of GRP, contributing to the evaluation of its potential as a biomarker.

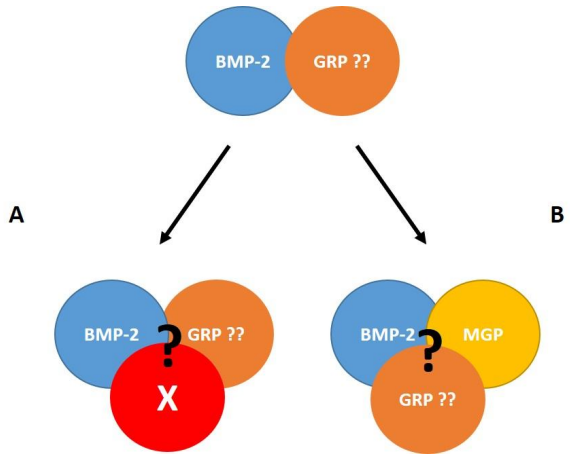

Figure 6.1 - Proposed model of GRP and BMP-2 interaction. Our results demonstrate an interaction between GRP and BMP-2. In a future work we are interested in **(A)** identify additional interacting partners and **(B)** clarify the role of MGP in this interaction.

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Annexes

Annex I – Primers for PCR amplification

	Primer	Sequence (5'-3')	Purpose
SV40 large T antigen	SV40-01FW	TGGAATGTGTGTCAGTTAGGGTGTGGAA	PCR
	SV40-02RV	GGCGGGACTATGGTTGCTGACTAATTGA	
GRP	HuGRP-Ex3-01FW	GTCCCCCAAGTCCCGAGATGAGG	RT-qPCR
	HuGRP-qPCR-Ex4R	CCTCCACGAAGTTCTCAAATTCATTCC	
	HuGRP_mKate2_PstI_Fw	GACTACCTGCAGCGCCACCATGACTTGGAGACAGGCCGTCC	Cloning into pmKate2C
	HuGRP_mKate2_BamHI_Rv	gttataGGATCCGTGTGGTGGCGGTTGTAGAGATA	
	HuGRP_pmKate2_out_NheI	GACTACGCTAGCCGCCACCATGacttggagacaggccgtcctgc	Cloning into pmKate2N
	HuGRP_ pmKate2_out_BglII	gttataAGATCTAggatgccaatggtgctacaagct	
Ribosomal 18S	Hu18S-RT-01FW	GGAGTATGGTTGCAAAGCTGA	RT-qPCR
	Hu18S-RT-02RV	ATCTGTCAATCCTGTCCGTGT	
BMP-2	HuBMP2-01FW	TTTCAATGGACGTGTCCCCG	RT-PCR
	HuBMP2-02FW	TGCTTCTTAGACGGACTGCG	
	HuBMP2-03RV	ACTTCATGTGCTGGGGTTGAA	
	HuBMP2-04RV	AAGCACTTTGCCAGAGTAACCT	
	HuBMP2-05FW	GCGTGAAAAGAGAGACTGCG	
	HuBMP2-07FW	ACGGACTGCGGTCTCCTAAA	
	HuBMP2-08RV	ATTAAAGTGTCAACTGGGGTGGG	
	HuBMP2-09FW	CAGGTCCTTTGACCAGAGTTTTTC	
	HuBMP2-10FW	CCTTTGACCAGAGTTTTTCCATGT	
	HuBMP2-11FW	CAAAGAAAAGGAACGGACATTCGG	
	HuBMP2-12RV	TTTGTTTTCCCAACTTCTTTTCGT	

Annex II – List of antibodies used in this report

Protein	Primary antibody	Secondary antibody	Purpose	Size
Annexin 6	ANXA6 (SantaCruz) 1:200	Anti-mouse 1:100 000	WB	68 kDa
α-Smooth muscle actin (ASMA)	ASMA (SantaCruz) 1:50/1:100	A11062 – Alexa 594 anti-mouse 1:500	IF	42 kDa
GM130	GM130 (SantaCruz) 1:25/1:50	A11062 – Alexa 594 anti-mouse 1:500	IF	112 kDa
GRP	CTerm-GRP (GenoGla) 5 µg/ml	Anti-rabbit 1:70 000 A21441 – Alexa 488 anti-rabbit 1:250	WB, IF	10 kDa
	UCMA (SantaCruz) 1:500	Anti-donkey 1:50 000	WB	10 kDa
	cGRP (GenoGla) 1:1000	Anti-chicken 1:100 000	WB	10 kDa
	ucGRP (VitaK) 1:2000	Anti-mouse 1:100 000	WB	10 kDa
GRP-mKate2N	tRFP (Evrogen) 0,2 µg/ml (WB) 1 µg/µl (IP)	Anti-rabbit 1:70 000	WB, IP	40 kDa
MGP	cMGP (IDS) 1:1000	Anti-mouse 1:100 000	WB	14 kDa
MGP-FLAG	M2 (Sigma-Aldrich) 10 µg/ml (WB) 1:25/1:50/1:100 (IF)	Anti-mouse 1:100 000	WB, IF	13 kDa
Rabbit IgG	IgG (SantaCruz) 1 µg/µl	Anti-rabbit 1:70 000	IP	25 kDa
rBMP-2/4	BMP-2/4 (SantaCruz) 1:100	Anti-mouse 1:100 000	WB	25/36 kDa

pan-Cadherin	pan-Cadherin (SantaCruz)	A11062 – Alexa 594 anti-mouse	IF	135 kDa