Universidade Federal do Rio Grande do Sul
Instituto de Ciências Básicas da Saúde
Programa de Pós Graduação em Ciências Biológicas: Bioquímica

EFEITOS DA STANNIOCALCINA 1 SOBRE A DIFERENCIAÇÃO OSTEOGÊNICA DAS CÉLULAS TRONCO ADIPOSO-DERIVADAS HUMANAS

SILVIA RESENDE TERRA

Orientadora: Prof. Dra. Fátima Costa Rodrigues Guma

Porto Alegre
2016

Universidade Federal do Rio Grande do Sul
Instituto de Ciências Básicas da Saúde
Programa de Pós Graduação em Ciências Biológicas: Bioquímica

EFEITOS DA STANNIOCALCINA 1 SOBRE A DIFERENCIAÇÃO OSTEOGÊNICA DAS CÉLULAS TRONCO ADIPOSO-DERIVADAS HUMANAS

SILVIA RESENDE TERRA

Orientadora: Prof. Dra. Fátima Costa Rodrigues Guma

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas – Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito para obtenção do grau de Doutor em Bioquímica.

Porto Alegre
2016

...."hoje me sinto mais forte,
mais feliz, quem sabe.
só levo a certeza
de que muito pouco eu sei
ou nada sei"...
..."penso que cumprir a vida
seja .simplesmente
compreender a marcha
e ir tocando em frente"...

(Almir Sater e Renato Teixeira)

AGRADECIMENTOS

A **Profª Fátima Guma** pela orientação, pelo exemplo profissional e pessoal , e, principalmente, pelo incentivo e compreensão.

Ao Prof. José Cláudio pela atenção e auxílio em todos os momentos, amizade e, principalmente, pela confiança no meu trabalho como médica veterinária.

A **Profª Regina Pureur** pela disponibilidade em ser a relatora desse trabalho , pelo doce auxílio e as valiosas sugestões e discussões.

.

A **Profª Vera Treis Trindade** pelo estímulo e esclarecimento de dúvidas sempre que foi necessário.

A **Letícia Pettenuzzo** pela amizade, pela disponibilidade na realização dos experimentos, interpretação e discussão dos resultados e análises estatísticas.

A todos do lab 21 pelo companheirismo, pelo auxílio nos experimentos, pelas discussões científicas e não científicas e pelos momentos de descontração.

A Mariana Maer do lab 23 pela amizade e ajuda na realização deste trabalho.

A todos os meus amigos da vida e amigos colegas de profissão pela amizade, companheirismo e compreensão.

A toda minha família e, em especial, a Maria que é praticamente a minha segunda mãe!

APRESENTAÇÃO

PARTE I

Introdução contendo referências da literatura que fundamentam este trabalho.

Objetivos do presente trabalho.

PARTE II

Capítulo I: Artigo 1 –

Capítulo II: Artigo2 –

PARTE III

Discussão.

Conclusão.

Perspectivas.

REFERÊNCIAS

Referências bibliográficas citadas na introdução, no capítulo III e na discussão.

ANEXOS

Lista de Figuras

SUMÁRIO

PARTE I

I.1. RESUMO

A stanniocalcina-1 (STC1) é uma glicoproteína caracterizada como um fator endócrino com ação anti-hipercalcêmica/hipocalcêmica originalmente descoberta em peixes. Em mamíferos, esse hormônio está expresso em praticamente todos os tecidos, regula diversas funções biológicas e atua como um fator autócrino/ parácrino. Diversas evidências demonstram o envolvimento da STC1 no desenvolvimento ósseo. Durante a embriogênese, a STC1 é expressa nos primeiros estágios de condensação mesenquimal e, posteriormente, se mantém restrita a preosteoblastos e osteoblastos maduros. Além disso, a STC1 estimula a mineralização óssea através do aumento da expressão de transportadores de fosfato e da osteopontina, uma sialoglicoproteína que atua na mineralização óssea. Células-tronco adultas simbolizam atualmente a fonte mais acessível de células progenitoras utilizadas em terapias celulares e engenharia de tecidos. O tecido adiposo contém uma população de células biológica e clinicamente interessantes denominada células tronco adiposo derivadas (CTADs). Atualmente as CTADs são a melhor fonte de células tronco adultas podendo ser obtidas através de procedimentos minimamente invasivos. Um grande número de estudos têm demonstrado o potencial osteogênico dessas células, no entanto, ainda é um desafio a compreensão dos mecanismos envolvidos na diferenciação osteogênica a partir das CTADs. Neste estudo, foi demonstrado que sete dias de indução osteogênica das CTADs na presença de 50 ng/mL de STC1 aumentaram significativamente a expressão gênica e proteica dos marcadores osteogênicos: fosfatase alcalina (FA), *runt related gene* 2 (RUNX2) e osteopontina (OPN).

O aumento na atividade da enzima FAS foi relacionado diretamente com a maior expressão gênica e proteica. Além disso, a STC1 modula a via de sinalização pAKt/pGSK3-β/βcatenina em preosteoblastos de 7 dias sugerindo que seus efeitos sobre a osteogênese sejam mediados por essa via de sinalização. O peptídeo neuroendócrino CGRP (peptídeo relacionado ao gene da calcitonina) possui similaridades com STC1 e desempenha um importante papel nas fases iniciais da diferenciação dos osteoblastos. O CGRP ativa o receptor CALCRL, formando um dímero com a proteína transmembrana acessória RAMP1. Para elucidar o envolvimento da STC1 nas vias de sinalização relacionadas a receptores de calcitonina foi investigado o efeito desse hormônio na modulação

do receptor do CGRP e receptor de calcitonina (CTR) em CTADs diferenciadas para preosteoblastos e células Hek 293 superexpressoras de CALCRL/RAMP1 e CTR. A STC1 não alterou a expressão dos genes CALCRL e *ramp1* durante a osteoblastogênese mas provocou alterações na distribuição espacial do complexo CALCRL/RAMP1 na membrana plasmática de preosteoblastos, induzindo a formação de *clusters*. Além do efeito sobre a sinalização do CGRP a STC1 demonstrou inibir a sinalização da calcitonina diminuindo a produção de cAMP em células transfectadas com CTR. A STC1 não alterou os níveis intracelulares de cálcio e ATP. Esses resultados indicam que, embora não atue diretamente via os receptores CALCRL/RAMP1 e CTR, a STC1 modula a sinalização dos peptídeos CGRP e CT. Estudos mais detalhados sobre os efeitos da STC1 nas diferentes vias de sinalização são necessários para desvendar completamente os mecanismos de diferenciação osteogênicos das CTADs estimuladas por esse hormônio.

Palavras-chaves: Células tronco adiposo derivadas(CTADs) ; STC1; osteogênese; CGRP, calcitonina, CALC/RAMP1;AMPc

I.2. ABSTRACT

The stanniocalcin-1 (STC1) is a glycoprotein characterized as an endocrine factor with anti-hypercalcemic / hypocalcemic action, originally identified in fish. The hormone in mammals is expressed in virtually all tissues and regulates diverse biological functions, acting as an autocrine / paracrine factor. Many evidences demonstrate that STC1 is able to regulate bone development. During embryogenesis the STC1 is expressed in early stages of mesenchymal condensation and thereafter remains restricted to preosteoblast and mature osteoblast. Furthermore, STC1 stimulates bone mineralization by increasing the phosphate transporter expression and osteopontin, a sialoglycoprotein involved in bone mineralization. Adult stem cells currently symbolize the most accessible source of stem cells used in cell therapy and tissue engineering. Adipose tissue contains a population of biological cells clinically interesting called adipose derived stem cells (ASC). Currently, the ASCs are the best source of adult stem cells and can be harvested using minimally invasive procedures. A large number of studies had shown osteogenic potential of these cells, however, it is still a challenge to understand the mechanisms involved in osteogenic differentiation from ASCs. This study demonstrated that 7-day preosteoblast in the presence of 50 ng / ml STC1 significantly increased gene and protein expression of osteogenic markers: alkaline phosphatase (ALP), runt related gene 2 (RUNX2), and osteopontin (OPN). Also, there was an increase in the enzymatic activity of the ALP, possibly related to both gene and protein expression. Furthermore, STC1 modulates pAkt / pGSK3-β / βcatenina signaling in 7-day preosteoblast, suggesting that the STC1 effects on the osteogenesis is mediated by this pathway. The neuroendocrine peptide CGRP (calcitonin gene related peptide) has similarities to STC1 and plays an important role in the early stages of osteoblast differentiation. The active CGRP receptor form a dimer with the receptor activity-modifying protein 1 (RAMP1). To elucidate the involvement of STC1 in signaling pathways related to calcitonin receptors, it was investigated the STC1 effect on peptide receptor modulating the calcitonin gene related peptide (CGRP) and the calcitonin receptor (CTR) in 7-day preosteoblast, and in Hek 293 cells transfected with CALCRL / RAMP1 and CTR. The STC1 did not change the expression of genes CALCRL and *ramp1* during osteoblastogenesis but modified the plasma membrane spatial distribution of

CALCRL/RAMP1 in preosteoblast. Besides the effect on CGRP signaling, STC1 inhibited the calcitonin signaling by decreasing cAMP production in cells transfected with CTR. The STC1 did not alter intracellular calcium levels and ATP. These results indicated that STC1 does not act on the same receptors for calcitonin and CGRP, but modulates the action of these peptides. Studies on the effects of STC1 in different signaling pathways are necessary for understanding the mechanisms underlying the STC1 ability in enhancing osteoblastogenesis from hASCs.

Keywords: Adipose derived stem cell, STC1; osteogenesis; CGRP,calcitonin, CALC/RAMP1;cAMP

I.3 LISTA DE ABREVIATURAS

AKT: proteina quinase B

AMPc: monofosfato cíclico de adenosina

BMP: proteina óssea morfogenética

C/EBPα: CCAAT-enhancer-binding proteins

Ca^{+2}: cálcio ionizado

CALCRL/RAMP1: calcitonin gene related peptide heterodimeric receptor complex

CALCRL: calcitonin Receptor-like

CD: Marcadores de diferenciação de superfície celular

CFU-F: Unidades formadoras de colônia de fibroblastos

CGRP: peptideo relacionado ao gene da calcitonina

CS : corpúsculo de stannius

CT: calcitonina

CTADs: Célula tronco adiposo derivadas

CTE: Célula tronco estromal

CTH: Célula tronco hematopoiética

CTM: Célula tronco mesenquimal

CTMO: Células tronco estromal da medula óssea

CTR: receptor de calcitonina

DMEM: meio de Eagle modificado por Dulbecco

DNA: ácido desoxirribonucléico

DSH :disheveled intracellular proteins

ERK: extracellular-signal-regulated kinases

FA: fosfatase alcalina

FGF: fator de crescimento de fibroblastos

Frz: frizzled receptor

GSK3β: glicogenio quinase sintase 3 beta

HIF: hypoxia-inducible factor 1-alpha

ISCT: Sociedade internacional para terapia celular

JNK: c-Jun N-terminal kinases

LRP5/6: transmembrane low-density lipoprotein receptor

MAPK:Mitogen-activated protein kinase

mRNA: ácido ribonucléico mensageiro

NFκB: factor nuclear kappa B

OPN: osteopontina

OPPG: osteoporose-pseudoglioma

OSX:Osterix

p53: cellular tumor antigen p53

PCR: reação em cadeia da polimerase

Pit1/2: transportador de fosfato dependente de sódio

POH: Heteroplasia progressiva óssea

PPARγ: Receptor ativador da proliferação de peroxissomos gama

RAMP1: receptor activity-modifying protein

RUNX2/ Cbfa1: runt related gene 2

SMAD: Sma- and Mad-related protein

STC1: stanniocalcina 1

SVF- fração vascular estromal.

TBP: Proteína de ligação a TATA-Box

TGF β: Fator de transformação do crescimento beta

TNFα: fator de necrose tumoral alfa

VEGF: fator de crescimento do endotélio vascular

I.4 INTRODUÇÃO

I.4.1 Células Tronco Mesenquimais

O termo mesenquima descreve tecidos embrionários de origem mesodérmicos encontrados em todos os órgãos e que promovem suporte estrutural e regulam o movimento de células através dos tecidos. As células tronco mesenquimais(CTM) ou células tronco estromais (CTE) são células tronco não hematopoiéticas de origem mesodérmica que podem dar origem aos sistemas músculo-esquelético, sanguíneo, vascular, urogenital e ao tecido conjuntivo [1-3].

A medula óssea é uma das fontes mais estudadas de células tronco hematopoiéticas (CTH) e CTM sendo o único órgão conhecido onde duas populações de células tronco adultas não só co-existem, mas cooperam [4]. O microambiente da medula óssea inclui uma população heterogênea de células do estroma composta de macrófagos, fibroblastos, adipócitos, células osteoprogenitoras, células endoteliais e células reticulares que são mantidas por uma população de CTM [5-6]. As CTM de medula óssea foram descritas primeiro por Friedenstein (1968) que padronizou o isolamento, e as caracterizou como células clonogênicas e aderentes em cultura de monocamada, denominadas Unidades Formadoras de Colônia de Fibroblastos (CFU-F). Estudos sobre métodos de cultivo, marcadores de superfície, potencial de diferenciação e expressão gênica realizados posteriormente por Pittenger *et al*. (1999) proporcionaram um maior conhecimento e caracterização das células tronco estromais da medula óssea (CTEMO) .

Pesquisas subsequentes descreveram CTM isoladas de tecidos adultos, como: cordão umbilical, tecido adiposo, fígado, membrana sinovial, pele, líquido amniótico, placenta, polpa dentária de diferentes espécies de animais (roedores, coelhos, suínos, macacos, equinos [7-8]. Na prática as CTM são um grupo heterogêneo de células estromais multipotentes não hematopoiéticas com capacidade de diferenciação em linhagens celulares mesodérmicas como adipócitos, osteóblastos, condrócitos, miócito e não mesodérmicas como hepatócitos, neurônios, astrócitos e outros (Figura 1) [9-10]

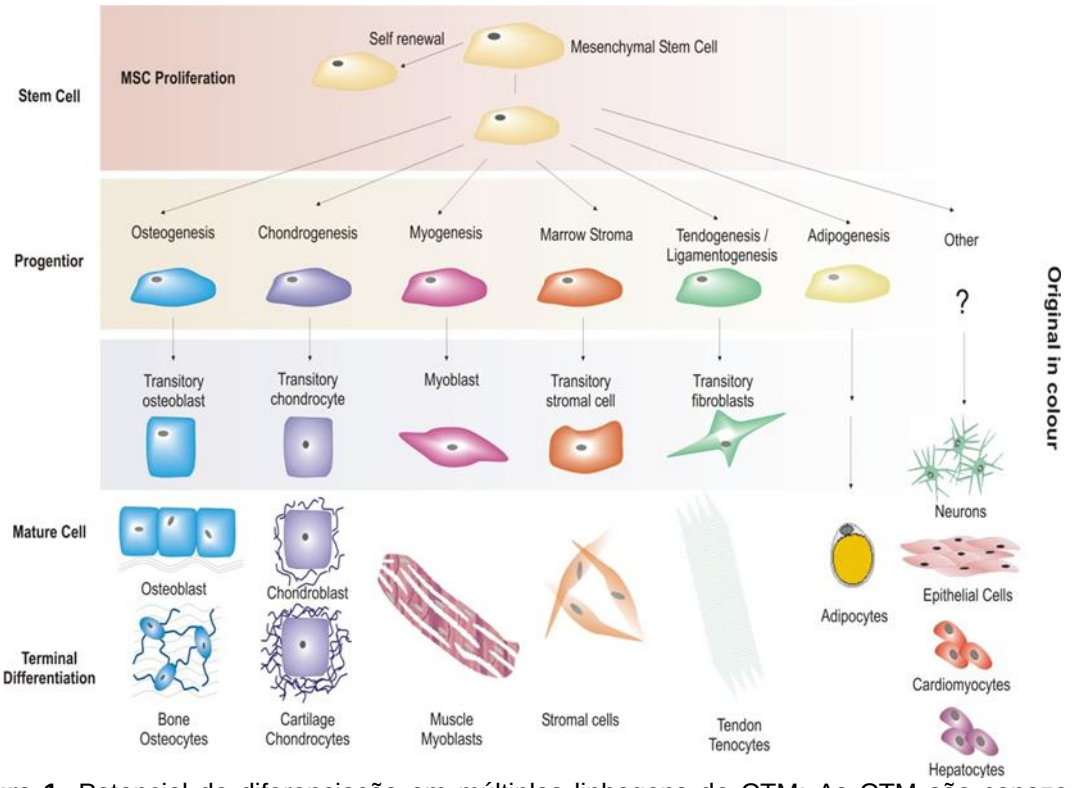

Figura 1: Potencial de diferenciação em múltiplas linhagens de CTM: As CTM são capazes de proliferar antes da diferenciação em vários tipos de tecidos mesenquimais e tipos celulares como: ossos, cartilagem, músculo, estroma, tendão e tecido adiposo (Adaptada de CAPLAN & BRUDER, 2001).

A capacidade de autorrenovação das CTM proporciona uma reposição tecidual constante e, após sinais do microambiente, essas células dão origem a progenitores comprometidos que gradualmente integram o tecido [8]. Lesões teciduais também podem ativar este processo, sendo assim, acredita-se que células tronco presentes nos diferentes tecidos tenham papel regenerativo, principalmente, quando estes sofrem lesão [11].Com relação à capacidade de reparação tecidual é crescente o interesse e a utilização das CTM na terapia celular e engenharia de tecidos [12-13].

Além disso, através da utilização das CTM no tratamento da doença do enxerto *versus* hospedeiro foi demonstrado o seu efeito imunossupressor [14] Experimentos *in vitro* com CTEMO demonstraram diminuição da proliferação e ativação de linfócitos T e uma mudança no perfil de citocinas pró-inflamátorias para citocinas anti-inflamatórias, comprovando a capacidade imunomodulatória dessas células [15].

I.4.2 Células Tronco Adiposo Derivadas Humanas (CTADs)

O tecido adiposo deriva da camada mesodérmica do embrião e possui desenvolvimento tanto pré como pós-natal. A localização das células progenitoras adipogênicas, a origem e a correlação com as células endoteliais, pericitos ou com o compartimento estromal, permanece controversa. Certamente, pré-adipocitos e células endoteliais compartilham antígenos de superfície, consistente com uma origem comum [16].

Os adipócitos são células de grandes dimensões (diâmetro de 50-130 μm) e constituem mais de 90% do volume do tecido adiposo, contudo, representam menos de 50% das células desse tecido [17]. Os adipócitos mantêm contato com capilares, células tronco adiposo derivadas (CTADs), células endoteliais, pericitos, fibroblastos, macrófagos e matriz extracelular devido à rica microvasculatura do tecido adiposo [18].

A Heteroplasia Progressiva Óssea (POH) é uma doença rara causada por mutações no gene responsável pelo acoplamento de receptores hormonais transmembrana com a adelinato ciclase (*GNAS1)*. Apresenta como sintomas a formação de osso ectópico no tecido adiposo subcutâneo. Análises histológicas desta patologia demonstraram a presença de osteoblastos e condrócitos junto à adipócitos evidenciando a existência de células progenitoras multipotentes no tecido adiposo [19]. Lipoma e lipossarcoma são tumores que ocorrem no tecido subcutâneo ou adiposo visceral que fornecem evidências adicionais para a existência de CTADs. O receptor ativador da proliferação de peroxissomos gama (PPARγ), associado à diferenciação terminal de adipócitos, pode ser encontrado em todos os graus histológicos de lipossarcomas. Ligantes de PPARγ incluem compostos naturais (ácidos graxos de cadeia longa e prostaglandinas) e sintéticos (tiazolidinedionas). *In vitro*, esses agentes podem induzir células de lipossarcoma a se diferenciarem em adipócitos [20]. Embora não tenha sido comprovada a eficácia dos ligantes de PPARγ como quimioterápicos para pacientes com lipossarcoma, é consistente a hipótese de que lipossarcomas derivam de uma célula-tronco progenitora [21].

Modelos *in vivo* de adipogênese sugerem que os adipócitos maduros são células plenamente diferenciadas com capacidade limitada de proliferação [22]. O

mecanismo "adipo-homeostático" é responsável por manter constante o volume total de tecido adiposo [23]. Após uma rápida perda de peso resultante de dieta, exercício físico ou lipoaspiração, ocorre o retorno ao nível inicial do volume total de tecido adiposo de um indivíduo [24]. Isso não ocorre somente através de um aumento no volume dos adipócitos pré-existente, mas através da geração de novos adipócitos a partir de um progenitor ou grupo de células-tronco [25].

As células tronco adiposo adiposo derivadas humanas (CTADs) são uma população de células CTM encontradas na fração vascular estromal do tecido adiposo. Essas células constituem 1% das células do tecido adiposo enquanto as células tronco estromal de medula óssea humana (CTEMO) representam 0,001%-0,002% das células da medula. As CTADh podem ser coletadas por procedimentos minimamente invasivos como lipossucção do tecido adiposo subcutâneo [26] Comparações diretas entre CTAD e CTEMO demonstram um imunofenótipo 90% idêntico.[27]. No entanto, a glicoproteína CD34 está presente nas primeiras passagens de CTAD, mas não nas CTEMO [28]. Segundo a ISCT (*International Society for Cellular Therapy)*, as CTAD são semelhantes a fibroblastos e se caracterizam por: aderência ao plástico sob condições padrão de cultura, capacidade de diferenciação *in vitro*, em adipócito, condrócito e osteoblasto e imunofenótipo de superfície positivo para CD13, CD73, CD90 , CD105 e negativo para CD11b ou CD14, CD19 ou CD79α, CD34,CD45 e HLA-DR [29] . No entanto, o perfil de expressão dos marcadores de superfície nas CTAD pode não ser idêntico entre grupos de pesquisa devido a heterogeneidade entre doadores, métodos, qualidade do isolamento e condições de cultura, que incluem: composição do meio, suplementação de oxigênio, tempo de cultura, número de passagens [30].

Assim como as CTEMO, as CTAD são células imunoprevilegiadas capazes de secretar fatores imunomodulatórios, angiogênicos, anti-apoptóticos e hematopoiéticos que facilitam a reparação de lesões teciduais [31]. A combinação dessas propriedades e a grande quantidade de CTAD que pode ser obtida, explica a crescente preferência do tecido adiposo como fonte de CTM adultas para a medicina regenerativa [32].

I.4.3 Diferenciação Osteogênica das CTM

A formação óssea pode acontecer através de ossificação endocondral ou ossificação intramembranosa. A maior parte do esqueleto de mamíferos, incluindo os ossos longos e o esqueleto axial consiste de ossos formados a partir de modelos cartilaginosos no processo de ossificação endocondral [33].

A ossificação endocondral inicia com a condensação de células mesenquimais, que se diferenciam em condrócitos. A diferenciação dos condrócito é seguida por uma maturação hipertrófica com produção de matriz mineralizada, desenvolvimento da placa de crescimento e osteoblastogenese. O fator de crescimento do endotélio vascular (VEGF) produzido por condrócitos hipertróficos estabelece a angiogenese durante o crescimento ósseo longitudinal. A maioria dos ossos da face e do crânio se desenvolvem por ossificação intramembranosa através da condensação e proliferação de células mesenquimais antes da diferenciação para osteoblastos [34]. Na vida pós-natal, as CTEMO desempenham um papel crucial no crescimento ósseo, na remodelação e na reparação óssea após lesão [35]. O processo de diferenciação das CTM para osteoblastos maduros (Figura 2) é caracterizado por três etapas distintas: proliferação,desenvolvimento e maturação da matriz extracelular e a mineralização [36].

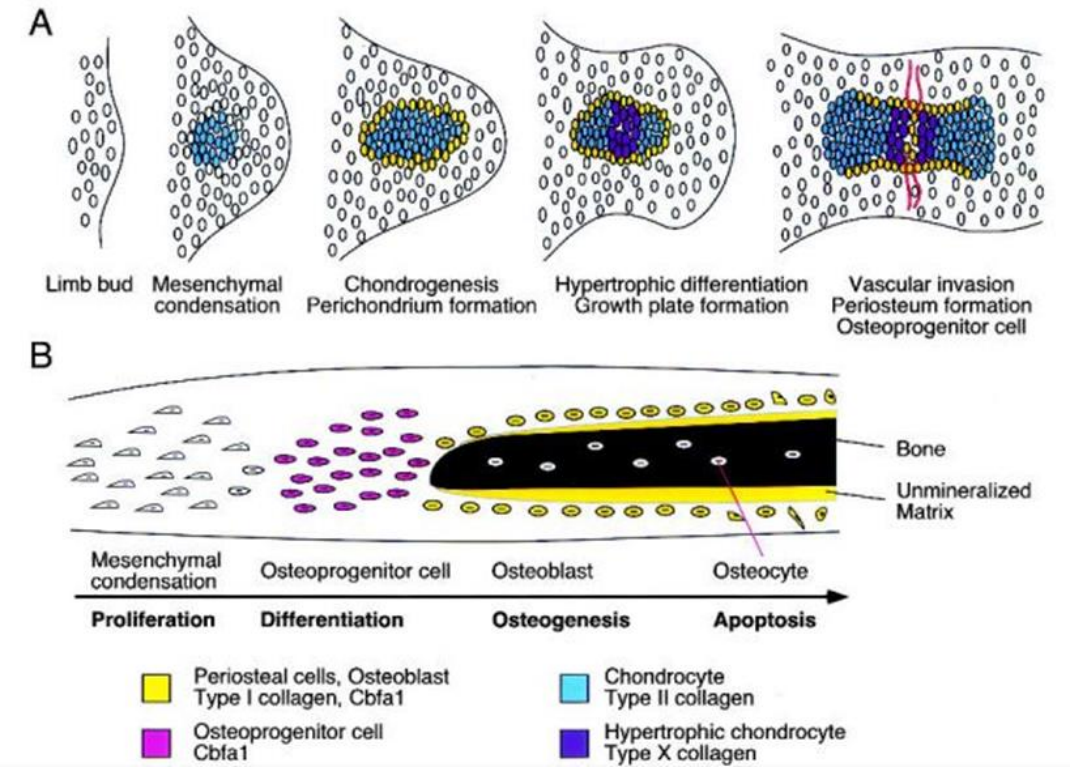

Figura 2: A) A ossificação endocondral inicia com a condensação mesenquimal e expressão do colágeno tipo II (azul). Na área central as células se diferenciam em condrócitos, hipertrofiam e passam a expressar colágeno tipo X (roxo). A progressão da placa de crescimento acompanha o desenvolvimento do pericôndrio (amarelo), invasão vascular e formação de um centro de ossificação contendo osteoblastos que expressam colágeno tipo I, B) A ossificação intramembranosa inicia com a diferenciação de células mesenquimais em células osteoprogenitoras que expressam Cbfa1 (rosa). As células osteoprogenitoras se transformam em osteoblastos maduros que expressam Cbfa1 e colágeno tipo I (amarelo) e são responsáveis pela mineralização da matriz óssea. Na matriz óssea os osteoblastos podem entrar em apoptose ou se transformar em osteócitos (Adaptada de ORNITZ& MARIE 2002).

O comprometimento e diferenciação das CTM para pré-adipócitos ou osteoblastos depende de uma variedade de vias de sinalização e fatores de transcrição. Evidências baseadas em estudos *in vitro* sugerem uma correlação inversa entre adipogênese e osteogênese. O fator de transcrição RUNX2 relacionado à diferenciação osteogênica e o fator de transcrição PPAR relacionado à diferenciação adipogênica desempenham um papel crucial no destino da diferenciação.O aumento da expressão de um é associado à diminuição do outro (Figura 3) [37].

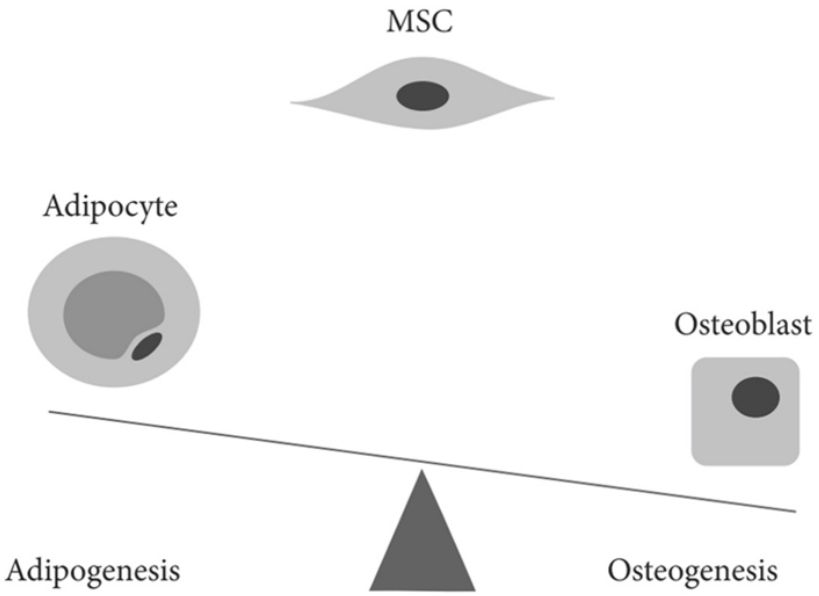

Figura 3: Correlação inversa entre diferenciação osteogenica e adipogenica. A diferenciação das CTM sem adipócitos ou osteoblástos pode ser explicada pela hipótese que sugere que a indução de uma linhagem acontece em detrimento da outra (Adaptado de JAMES *et al*., 2013).

Os membros da familia RUNX foram isolados e caracterizados como fatores de transcrição linhagem específico essenciais para diferenciação hematopoiética (RUNX1), neuronal, gatrointestinal (RUNX3), osteogênica e condrogênica (RUNX2). RUNX2 é o fator de transcrição mais expresso em osteoblastos e está envolvido no controle da formação óssea durante o desenvolvimento do esqueleto e na vida pós-natal [38]. A expressão do RUNX2 é necessária para a diferenciação osteogênica das CTM, sendo responsável por ativar genes,como: osteocalcina, fosfatase alcalina, colágeno tipo I, sialoproteína óssea [39]. A deleção homozigótica do RUNX2 resulta em completa ausência de osteoblastos e descréscimo na maturação condrogênica. Camundongos com mutações no *locus* do RUNX2 apresentam uma perda completa da ossificação endocondral e intramembranosa devido ao atraso da maturação dos osteoblastos [37, 40]. Mutações heterozigóticas no gene RUNX2 em humanos causam uma síndrome denominada Displasia Cleidocranial caracterizada por aplasia da clavícula, baixa estatura, dentes extranumerários e outras alterações no padrão de crescimento ósseo [41]. Embora o RUNX2 seja essencial para a formação óssea, o aumento da expressão desse fator de transcrição em camundongos transgênicos leva a osteopenia e decréscimo da densidade óssea e multiplas fraturas. Esses achados

demonstram que a regulação do RUNX2 é essencial para a patobiologia óssea [42].

O Osterix (OSX) também é um fator de transcrição essencial para osteoblastogênese. A deleção do OSX na presença RUNX2 resulta em ausência de osteoblastos em embriões de camundongos e demonstra que sua atuação é posterior a do RUNX2 [32]. O OSX atua como um mediador chave da diferenciação terminal dos osteoblastos humanos [43].

Vários estudos genéticos demonstram a importância da via canônica Wnt/ß-catenina no controle da osteogênese. A interação de proteínas da família Wnt com receptores e co-receptores (LPR5/6) leva à inibição da fosforilação da ß-catenina mediada por GSK3-β e, como consequência, ocorre o acúmulo e translocação dessa proteína para o núcleo formando um complexo com o fator de transcrição TCF/LEF e ativação de genes relacionados à diferenciação e proliferação de osteoblastos [44]. Mutações com perda de função no gene do receptor LPR5 causa a síndrome da Osteoporose-Pseudoglioma (OPPG), caracterizada pela perda da massa óssea e, de maneira oposta, mutações que intensificam a função do LPR5 causam aumento de massa óssea. A formação do complexo ß-catenina e TCF/LEF ativa o fator de transcrição osteogênico RUNX2 e reduz a expressão de fatores de transcrição adipogênicos como C/EBPα e PPARγ. Em CTM precursoras de osteoblastos, a deficiência de ß-catenina atrasa as primeiras etapas de desenvolvimento dos osteoblastos [45].

Os membros da família de proteínas Hedgehog (HH) são classificados em três homólogos estruturais: Sonic Hedgehog (SHH),Indian Hedgehog (IHH), Desert Hedgehog (DHH). As proteínas SHH e IHH estão envolvidas na formação óssea axial, apendicular e facial. A IHH é uma proteína secretada que regula a ossificação endocondral e coordena a diferenciação condrogênica e osteogênica. A ausência da expressão de IHH inibe os osteoblastos na ossificação endocondral [46] A osteoblastogênese induzida pela via de sinalização HH necessita da via de sinalização das proteínas morfogenéticas ósseas (BMPs), para estimular sinergicamente a expressão da fosfatase alcalina [47]. Nas CTM a via SHH demonstrou ter efeitos pró-osteogênicos e anti-adipogênicos [37].

As BMPs compreendem aproximadamente 30 citocinas secretadas que atuam através de receptores de serina/treonina quinase [48]. Membros da superfamília TGF-β, as BMPs atuam no desenvolvimento do esqueleto e formação óssea.

Estudos genéticos demonstram que BMP2 e BMP4 promovem a diferenciação osteogênica por regular a expressão de RUNX2 e OSX. A ativação do receptor BMP desencadeia as vias de sinalização SMAD/1/5/8 e MAPK. Várias proteínas, BMP, incluindo BMP2, BMP4, BMP6, BMP7 e BMP9 promovem a osteogênese *in vitro* e *in vivo.* Diversos modelos *knockout* murinos envolvendo a modificação genética de ligantes, receptores e inibidores de BMP demonstram o papel crítico dessas proteínas na formação óssea [49] .

NELL-1 é uma proteína secretada altamente expressa durante o fechamento prematuro de sutura craniana em pacientes com craniossinostose , uma das deformidades craniofaciais congênitas mais comuns. É normalmente expressa durante a ossificação endocondral e intramembranosa [50]. O aumento da expressão de NELL-1 induz diferenciação e mineralização específica para linhagem osteocondral. A baixa expressão, por outro lado, resulta em inibição da osteoblastogênese em culturas primárias de MC3T3. Diversos modelos *in vivo* demonstraram que a proteína NELL-1 tem capacidade de indução óssea comparável a BMP-2 em modelos de fusão espinhal e defeitos da calota craniana. NELL-1 é uma proteína regulada diretamente pelo fator de transcrição RUNX2, mas a sua ausência diminui significativamente a atividade de RUNX2 *in vitro [51]*. As principais vias de sinalização envolvidas na osteogênese estimuladas por NELL-1 são MAPK, via canônica Wnt/ β-catenina, e Hedgehog (HH). A ativação da MAPK está associada com a fosforilação de RUNX2 [37].

O fator de crescimento de fibroblastos (FGF) e o fator de transformação do crescimento beta (TGF-β) influenciam o desenvolvimento ósseo por seus efeitos pró-osteogênicos e anti-adipogênicos através da ativação e aumento da expressão do fator de transcrição RUNX2 e diminuição da expressão de PPARγ [52-53]. O fator de crescimento semelhante a insulina (IGF) estimula tanto a diferenciação osteogênica, através do aumento da expressão de OSX em osteoblastos, como também a proliferação de precursores de adipócitos através da indução de produtos de glicação avançados (AGES) [54].

I.4.4 A proteína Stanniocalcina-1 (STC1)

Stanniocalcina (STC) é uma glicoproteína descoberta nos corpúsculos de Stannius(CS), minúsculas glândulas endócrinas, localizados na superficie ventral

dos rins dos peixes teleósteos. Devido à proximidade anatômica com os rins, o CS foi confundido com a glândula adrenal (STANNIUS , 1839). Posteriormente, estudos histológicos demonstraram que as células do CS eram distintas do tecido inter-renal e das células cromafins dos peixes [55]. Somente através da microscopia eletrônica de transmissão foi demonstrado que as células do CS possuíam as características citoplasmáticas de uma célula produtora de hormônio peptídico, porém sem atividade esteroidal [56] Figura 4.

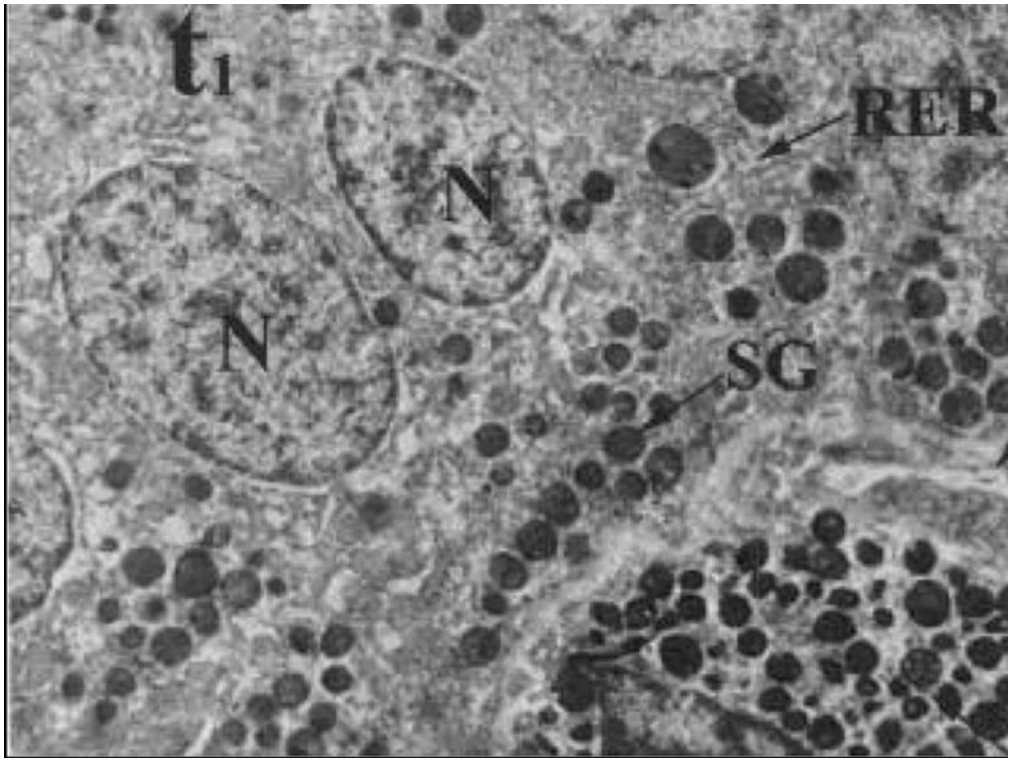

Figura 4: Seção de microscopia eletrônica de transmissão do Corpúsculo de Stannius de um peixe teleósteo, demonstrando uma população celular homogênea tipo 1 (t1), núcleo redondo e distinto (N), retículo endoplasmático rugoso (RER) e grânulos secretórios (SG) (x12600) (Adaptada de AHMAD *et al*., 2002).

A remoção dos Corpúsculos de Stannius produz hipercalcemia comprovando sua influência sobre a homeostase de cálcio em peixes ósseos [57]. Vários trabalhos demonstraram que o efeito anti-hipercalcêmico da STC1 era similar ao da calcitonina atuando, principalmente, nas guelras e no intestino dos peixes [58-61]. A STC não é responsável somente pela diminuição da captação de Ca^{+2}, mas também pelo estímulo da reabsorção de fosfato (Pi) no epitélio tubular

proximal do rim dos peixes [62] Estudos subsequentes demonstraram a presença de STC em outros tecidos como testículo, ovário e rim dos peixes ósseos [63-64].

O mRNA da STC em mamíferos, denominada STC1, foi isolado concomitantemente em pesquisas com fibroblastos e tecido pulmonar fetal humanos [61, 65]. Posteriormente, a STC1 foi identificada em diversos tecidos como coração, pulmão, fígado, adrenais, rins próstata, ovários, tireóide, ossos e sistema nervoso central demonstrando alta similaridade com a STC dos peixes [57, 65-70]. A STC1 está normalmente ausente no sangue e a diferença no padrão de distribuição nos tecidos sugere uma ação parácrina/autócrina dessa proteína [71]. Em 1998, foi identificada a Stanniocalcina-2 (STC2) que apesar de apresentar apenas 30% de homologia com a STC1, manteve conservado os sítios de glicosilação, resíduos de cisteína e as regiões entre íntrons e éxons. Essas evidências indicam um gene ancestral comum entre STC1 e STC2 [72].

A STC1 é uma glicoproteína homodimérica multifuncional, com aproximadamente 50 kD, envolvida em diversos processos fisiológicos e patológicos [66, 73-74]. A estrutura proteica, descrita para a STC de peixes, foi recentemente confirmada para a STC1 humana [75]. É provável que as funções regulatórias dos níveis Ca^{+2} / Pi tenham evoluído de uma ação sistêmica para uma ação celular que resultou em múltiplas funções tecido específicas. Estudos realizados em cardiomiócitos de ratos e macrófagos de camundongos demonstraram a ação moduladora dos níveis do Ca^{+2} intracelular da STC1 recombinante (rSTC1) [76-77]. A STC1 não liga ou sequestra Ca^{+2}, mas aumenta a captação de Pi auxiliando no tamponamento do Ca^{+2} livre intracelular por estimular seu sequestro nas organelas celulares. Células Paju tratadas com rSTC1 ou que superexpressam STC1 são resistentes ao aumento intracelular de cálcio. Além disso, essas células captam mais Pi estimulando a síntese de ATP; o que consequentemente, resulta em maior sobrevivência em condições de hipóxia [78]

McCuden e colaboradores (2002) propuseram a existência de receptores para STC1 (STCR) em tecidos como fígado e rim. No mesmo estudo os autores sugerem que nestes tecidos o STCR estaria localizado na membrana interna das mitocôndrias indicando um possível papel da STC1 no metabolismo celular e na produção de energia em condições de estresse celular.

Diversos trabalhos demonstram que STC1 é um fator de ação pleiotrópico envolvido em diversos processos fisiológicos e patológicos como: embriogênese, adipogênese,carcinogênese, diferenciação neuronal, isquemia cerebral, estresse hipertônico e estruturação óssea e muscular [78-83]. A STC1 também atua sobre os macrófagos diminuindo a sua motilidade, migração e geração de superóxido através da indução da proteína desacopladora -2 (UCP-2) [77]. Em células endoteliais tratadas com TNFα, a STC1 atenua a geração de superóxido, diminui a migração de leucócitos e ativa a vias de sinalização inflamatórias JNK e NFκ-B. Todas essas evidências indicam que além de atuar em mecanismos relacionados ao desenvolvimento, a STC1 tem influência sobre mecanismos de imunidade e inflamação [84]

É cada vez maior o número de pesquisas relacionando a STC1 com câncer, angiogênese e hipóxia. O aumento da expressão de STC1 tem sido identificado em diversos tipos de neoplasias, como: colorretal, hepatocelular, leucemia, ovário, pulmão e mama [85-90]

No câncer de mama os níveis de STC1 foram positivamente relacionados aos níveis de receptores de estrógeno [91] Para investigar o papel da STC1 nos tumores de mama, Chang e colaboradores (2015) silenciaram a STC1 em linhagens agressivas de tumores murinos e humanos. Nos dois modelos tumorais foi observada a diminuição do crescimento e inibição da capacidade metastática [92]. Nesse mesmo estudo, a análise de microarranjos demonstrou 30 genes relacionados à carcinogênese alterados pela inibição da expressão de STC1. Murai e coloboradores (2014) observaram que a superexpressão de STC1 na linhagem humana de tumor de mama MDA-MB-231 resultou em aumento da capacidade de invasão *in vitro* das células [93]. Publicações recentes sugerem a STC1 como biomarcador e alvo terapêutico para tumores de mama, carcinomas renais metastáticos e tumores gástricos humanos [92, 94-95].

O envolvimento da STC1 na vasculatura tumoral também foi comprovado através de modelos *in vitro* e *in vivo.* Em tumores gástricos a STC1 estimulou a angiogênese através da indução de VEGF e da ativação de via de sinalização PKC ERK 1/2 [96].

A STC1 tem sido associada com efeitos pró-apoptóticos e anti-apoptóticos. O efeito anti-apoptótico foi observado em co-culturas de células estromais multipotentes com fibroblastos irradiados com luz ultravioleta, em câncer de

pulmão submetido a apoptose por hipóxia e em células de câncer de ovário [88, 97]. No entanto em tumores nasofaríngeos, colorretais e em fibroblastos de embrião de camundongo, a STC1 demonstrou um efeito pró-apoptótico [98-99].. Os efeitos pró-apoptóticos e anti-apoptóticos são provavelmente dependentes do estresse imposto às células e determinado por alguns fatores de transcrição e vias de sinalização como: HIF, p53, NFκB, ERK1/2 [74, 100-101].Figura 5.

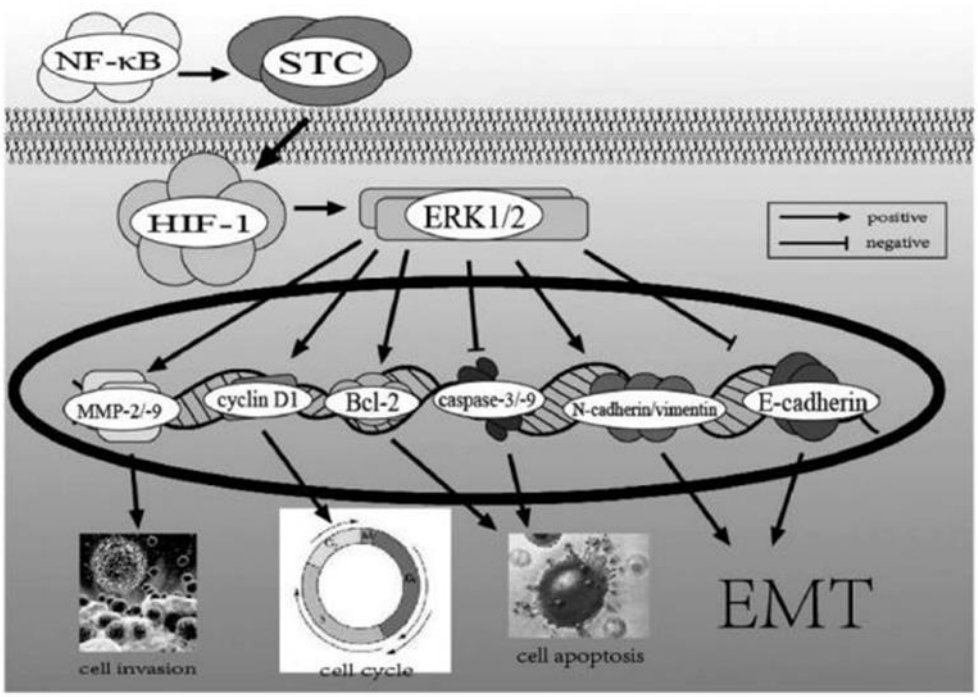

Figura 5: A STC está envolvida na progressão de diversos tipos de câncer promovendo crescimento celular, proliferação, invasão e metástase. O NF-κB aumenta a expressão de STC que ativa a via de sinalização HIF/ERK1/2 e.consequentemente, estimula fatores de transcrição relacionados a carcinogênese como: MMP-2/9, Ciclina D1, Bcl-2, N-caderina (Adaptado de Chu *et al*.,2015).

I.4.5 Stanniocalcina-1 (STC1) e Osteogênese

As primeiras evidências do envolvimento de STC1 no metabolismo ósseo foram observadas através reabsorção de ossos embrionários murinos por osteoclastos estimulados com o extrato do CS [102].

A análise por Northern Blotting de embriões inteiros mostraram que o mRNA da STC1 é altamente expresso durante a embriogênese. Por hibridação *in situ*, o mRNA da STC1 foi detectado no início das condensações mesenquimais, intensamente expresso em células pericondriais, células do periósteo e em

osteoblastos durante a formação ossea endocondral. Em ossos que se formam por ossificação intramembranosa, o mRNA da STC1 não foi detectado até o surgimento dos osteoblastos [103].

Na linhagem de osteoblastos MC3T3-E1, o mRNA e a proteína STC1 foram detectáveis ao longo das etapas de proliferação e de diferenciação, mas os níveis eram relativamente mais elevados tardiamente, durante as fases de formação de nódulos de mineralização [104]. O mRNA da STC1 foi também observado em condrócitos, mas não em osteoclastos [105]. Filvaroff e colaboradores demonstraram em camundongos transgênicos para STC1 apresentavam um aumento na síntese de matriz cartilaginosa, na espessura e densidade do osso cortical e no número de trabéculas ósseas. Todas essas alterações foram acompanhadas pela concomitante diminuição da atividade dos osteoclastos [82].

O controle dos níveis de fosfato inorgânico sistêmico é indispensável para formação óssea, principalmente, para o processo de mineralização osteóide. Yoshiko e colaboradores observaram como resposta ao tratamento com um inibidor do transporte de sódio dependente de fosfato inorgânico (NaPi), o aumento da expressão de STC1 na linhagem de osteoblasto MC3T3-E1 e em subculturas de osteoblastos isolados de crânio de fetos de ratos com 21 dias. A diminuição do transporte de NaPi estimula a expressão da STC1 e da fosfatase alcalina (FA). A ação autócrina / parácrina da STC1,através de um receptor desconhecido, induz a expressão do transportador de fosfato-1(Pit-1) por aumentar o transporte de NaPi. O aumento intracelular de Pi aumenta a expressão de osteopontina (OPN) e estimula a mineralização [106](YOSHIKO *et al*,2007), Figura 6.

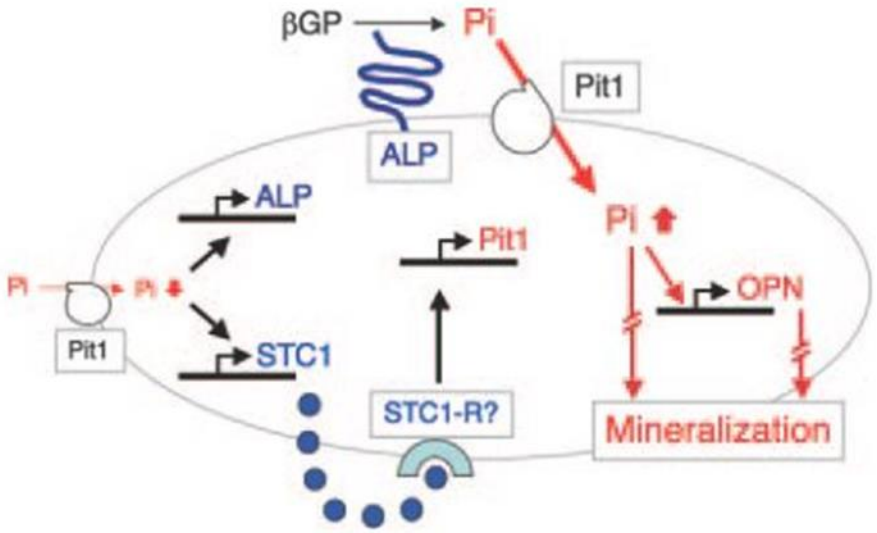

Figura 6: O transporte de NaPi via transportador Pit1 é o passo limitante da mineralização. Baixos níveis de NaPi estimulam a expressão de STC1 e fosfatase alcalina. A STC1 induz a expressão de Pit1 pelo aumento no transporte de NaPi. O aumento intracelular de Pi aumenta a expressão de OPN e estimula a mineralização (Adaptado de Yoshiko *et al*,2007)

Para reforçar ainda mais os efeitos das STCs sobre o desenvolvimento, um estudo demonstrou restrição de crescimento intra-uterino e pós-natal permanente em camundogos *knockout* para STC1 ou STC2 humana. Medições realizadas no esqueleto do axial, apendicular e craniano desses animais evidenciaram um retardo no crescimento ósseo. Foi verificado um grande atraso no fechamento da sutura intramembranosa craniana. As células da calota craniana exibiram viabilidade, proliferação e diferenciação reduzida, indicando que os osteoblastos em desenvolvimento são particularmente sensíveis aos níveis das STCs. Coletivamente, esses dados suportam um papel regulador direto das STCs em osteoblastos e sugerem que a exposição excessiva a esses fatores inibe o desenvolvimento normal do esqueleto [107].

Os membros da família da calcitonina (CT) são um grupo de pequenos hormônios peptídicos envolvidos na homeostase do cálcio, osteogênese e função dos osteoblastos [108]. Esses hormônios compartilham uma semelhança moderada nas sequências de aminoácidos e suas estruturas secundárias estão fortemente correlacionadas. Eles são caracterizados pela presença de dois resíduos conservados de cisteína, formando uma estrutura em anel na extremidade N-terminal da proteína, fundamental para a atividade biológica e a ativação do receptor [109-110].

Nos mamíferos, seis membros da família da CT foram identificados e incluem os peptideos alfa e beta relacionados ao gene da calcitonina (αCGRP e βCGRP), amilina (AMI), adrenomedulina 1 (ADM1), adrenomedulina 2 (ADM2 ou intermedina) e o peptideo estimulante do receptor de calcitonina (CRSP).O peptideo relacionado ao gene da calcitonina (CGRP) é um pequeno peptideo neuroendócrino que possui similaridades com a STC1 e exerce um importante papel na osteogênese e na atividade dos osteoblastos [111-112]. O CGRP é abundantemente expresso em neurônios sensoriais esqueléticos e está envolvido na formação óssea e na inibição da reabsorção óssea estimulando diretamente a diferenciação de células osteoblásticas do estroma e inibindo a ativação de NF-kappaB por RANKL, osteoclastogénese, e reabsorção óssea. [113]. Semelhante a CT e diferente da ADM1, os membros desta família de peptideos têm uma ação predominantemente anabólica e inibem a reabsorção óssea e a formação de osteoclastos [114].

Nos mamíferos, o CGRP foi detectado no tecido ósseo (periósteo, medula óssea e trabeculas ósseas epifisárias) e em culturas de osteoblastos. O CGRP foi responsável por aumentar a proliferação celular e a síntese de citocinas, fatores de crescimento e colágeno em culturas de osteoblastos [115-117]. Também já foi relatado um efeito estimulador importante do CGRP durante as fases iniciais da diferenciação dos osteoblastos [118]. O receptor para a CT (CTR) também foi detectado em osteoblastos, no entanto, o efeito da CT na formação óssea não é consensual [117, 119] .

Todos esses hormônios possuem efeitos distintos sobre as células ósseas A análise detalhada das interações entre os receptores da família da calcitonina com seus ligantes demonstrou reatividade cruzada com algumas das combinações dos receptores com as proteínas modificadoras da atividade do receptor (RAMP1, RAMP2 e RAMP3) [120] .

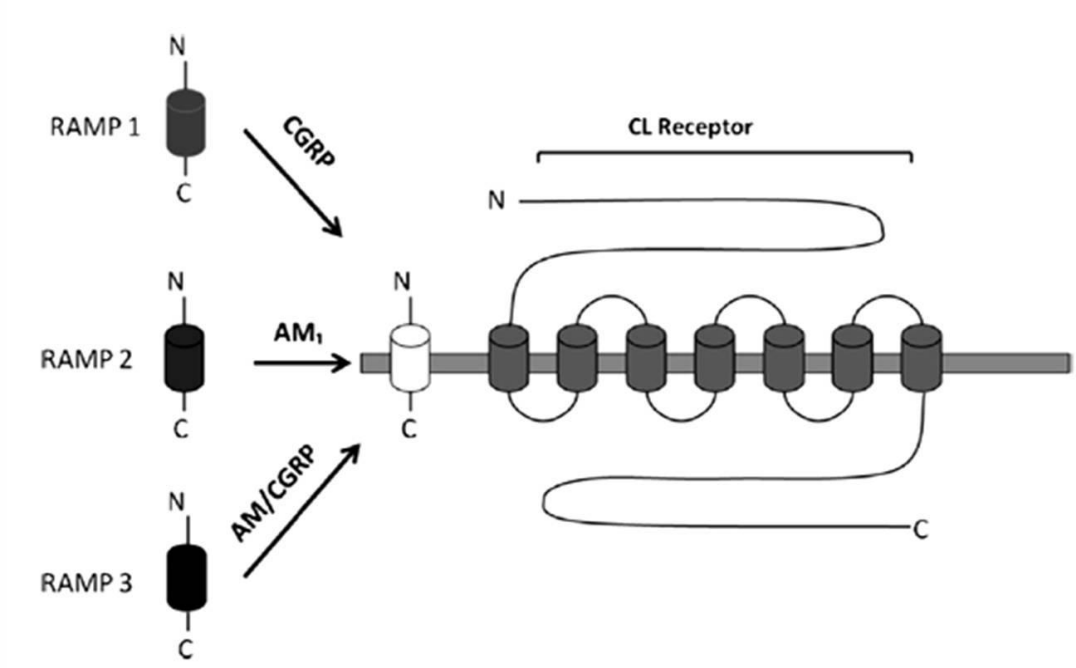

Figura 7: Estrutura do Receptor CGRP: heterodimerização de CALCRL com RAMP1 leva à formação do complexo dimérico funcional do receptor para o peptídeo *Calcitonin Gene-Related Peptide* (CGRP) na superfície celular; a dimerização com RAMP2 forma o receptor para adrenomedulina (AM) e dimerização com RAMP3 forma um complexo para ligação de AM/CGRP (Smillie & Brain, 2011).

O CGRP ativa o receptor, CALCRL, um membro da família de receptores acoplados a proteína G (GPCR) da classe 2 B1, formando um dímero complexo com a proteína transmembrana acessória RAMP1 [121-122]. A análise da expressão de CALCRL e RAMP1 apresentou níveis elevados em linhagens de osteoblasto (MC3T3-E1) e durante a diferenciação osteogênica de células tronco estromais de medula óssea de camundongos (BMSC), sugerindo esse complexo proteico como marcador de diferenciação osteogênica [123]. Na presença de CGRP, a sinalização CALCRL e RAMP1 detectados em pré-osteoblastos e osteoblastos maduros, aumenta o AMPc intracelular, cálcio e fatores de crescimento semelhantes à insulina [124-125].

Em contraste com o CGRP, os mecanismos moleculares que regulam a ação da STC1 permanecem desconhecidos. O receptor específico de STC1 ainda não foi identificado, o que dificulta a caracterização funcional da proteína. Recentemente, no modelo de embrião de *zebra fish*, a superexpressão de CT aumentou três vezes a expressão de STC1 sugerindo um mecanismo de *feedback* entre os dois hormônios [126].

I.5 OBJETIVOS

A presença do mRNA da Stanniocalcina (STC1) durante o desenvolvimento embrionário, principalmente, durante a proliferação e diferenciação de osteoblastos confirma a importância desta proteína nos estágios iniciais do desenvolvimento ósseo. Embora diversos autores já tenham demonstrado a influência da STC1 sobre o metabolismo ósseo através de estudos *in vivo e in vitro,* pouco se sabe sobre sua ação na indução da diferenciação osteogênica de CTADs humanas.

Esta tese de doutorado teve por objetivo investigar a influência da proteína STC1 recombinante humana sobre genes, proteínas e vias de sinalização ativados no processo de diferenciação óssea das CTADs. Assim como, investigar a possível ação da STC1 na sinalização via CGRP durante a diferenciação osteogênica. Para isso desenhamos protocolos experimentais visando determinar :

Capítulo I:

a) O efeito da STC1 sobre a expressão gênica, protéica e atividade da enzima fosfatase alcalina nas CTADs durante sua diferenciação em pré-osteoblastos por 7 e 14 dias.
b) O efeito da STC1 sobre expressão gênica e protéica dos marcadores de osteogênese: RUNX2 e Osteopontina .
c) Via de sinalização desencadeada pela STC1 durante a indução osteogênica das CTADs.

Capítulo II:

a) Os efeitos da STC1 e do CGRP sobre a expressão do complexo heterodimérico CALC/RAMP1 relacionado ao receptor do gene da calcitonina nas CTADh e pre-osteoblastos de 7 dias .
b) Os efeitos da STC1 sobre os níveis intracelulares de AMPc nas CTADs, células HEK 293 e células HEK 293 transfectadas com o complexo heterodimérico CALC/RAMP1.
c) Os efeitos da STC1 sobre os níveis de ATP dos pré-osteoblastos de 7dias.

PARTE II

II-1 STANNIOCALCIN-1 STIMULATES OSTEOGENIC DIFFERENTIATION IN HUMAN ADIPOSE DERIVED STEM CELLS THROUGH ACTIVATION OF pAKT/pGSK3-β/β CATENIN SIGNALING PATHWAY

(manuscrito a ser submetido)

STANNIOCALCIN-1 STIMULATES OSTEOGENIC DIFFERENTIATION IN A HUMAN ADIPOSE DERIVED STEM CELLS THROUGH ACTIVATION OF pAKT/pGSK3-β/β CATENIN SIGNALING PATHWAY

Silvia Resende Terra1*;Fátima Costa Rodrigues Guma1

*Corresponding author: MSc. Silvia Resende Terra

Department of Biochemistry

Rua Ramiro Barcelos, 2600-anexo

CEP 90035-003, Porto Alegre, RS, BRAZIL

Fone:+55 51 3308 5546

FAX:+55 51 3308 5535

e-mail:

1Department of Biochemistry, ICBS, Federal University of Rio Grande do Sul (UFRGS), rua Ramiro Barcelos, 2600-Anexo I, CEP 90035-003, Porto Alegre, RS, Brazil.

ABSTRACT

Adipose-derived stem cells (ASCs) are mesenchymal stem cells (MSCs) that are obtained from adipose tissue and can be expanded in vitro. They have the capacity to differentiate into multiple cell lineages.and can offer the potential to repair, maintain or enhance various tissues. Therefore, ASCs are promising for regenerating tissues and organs damaged by injury and diseases.Stanniocalcin-1 (STC1) is secreted homodimeric glycoprotein that functions in an autocrine or paracrine manner during mammalian bone development. There is a number of reports describing STC1 expression in osteoblasts. However, no study has yet investigated the effect of STC1 on osteogenic differentiation of hASCs .The effects of STC1 on hASCs osteogenic differentiation were evaluated by alkaline phosphatase (ALP) activity assay, immunocytochemistry analysis, flow cytometry analysis and real time-polymerase chain reaction Western blots were performed to examine the role of pAKt/pGSK3-β/β catenin signaling pathway. We demonstrated that recombinant humanSTC1(50ng/ml) stimulate dosteogenic differentiation in 7-day preosteoblast, manifested by an increase in ALP activity, expression of osteogenic markers ALP, runt related gene 2 (RUNX2) and osteopontin (OPN) and modulation of pAKt/pGSK3-β/β catenin pathway These results reveal a new investigation perspective on studying the osteogenic effects of STC1 in hASCs and its possible role in regenerative medicine and bone diseases

Keywords: hASCs, STC1, osteogenic differentiation ,ALP, pAKt/pGSK3-β/β catenin pathway

Abbreviations

STC1:stanniocalcin 1

ASC: adipose-derived stem cells

ALP: alkaline phosphatase

OPN: osteopontin

SPP1: secreted phosphoprotein 1 (OPN)

RUNX2: runt related gene 2

CGRP: calcitonin gene-related peptide

CT: calcitonin

CTR: calcitonin receptor

Pit1/2: NaPi transporter

BMP: bone morphogenetic protein

CALCRL:humancalcitonin receptor

RAMP1:receptor activity-modifying protein 1

CALCRL/RAMP1: calcitonin gene related peptide heterodimeric receptor complex

FGF:fibroblast growth factor

TGF β: Transforming growth factor beta

AKT: protein kinase B (PKB)

GSK3β: glycogen synthase kinase 3 beta

DMEM: Dulbecco's modified Eagle's medium

FBS: fetal bovine serum

TBP:TATA-binding protein

BSA: bovine serum albumin

Smurf 2:E3 ubiquitin-protein ligase

Frz: frizzled receptor

LRP5/6: transmembrane low-density lipoprotein receptor

DSH: disheveled intracellular proteins

1.INTRODUCTION

Human mesenchymal stem cells (MSC) are an attractive target for cell based therapies because of their abundance, ease of isolation, differentiation potential and immuno modulatory effects (Satija et al., 2009). The adult stem cells and in particular adipose-derived stromal cells (ASCs), a heterogeneous group of adult stem cells found in adipose tissue, offer a suitable cell source for bone tissue regeneration (Senarath-Yapa et al., 2014) . ASCs have been used in combination with osteoinductive biomaterial and/or osteogenic molecules, in either static ordynamic culture systems, to improve bone regeneration in several animal models (Barba et al., 2013). Their relatively ease of harvesting and abundance render this cell population an ideal candidate for this purpose (Senarath-Yapa et al., 2014)

The stanniocalcin1 (STC1) is a hypocalcemic glycoprotein hormone of approximately 50 kD discovered in teleost fishes and has been highly conserved from fish to mammals (Wagner e Dimattia, 2006).The protein structure, described for the fish STC1 has been recently confirmed to human STC1(Trindade et al., 2009). Several studies show that STC1 is a pleiotropic action factor involved in various processes as embryogenesis, adipogenesis, neuronal differentiation, cerebral ischemia, hypertonic stress and bone and muscle structure (Varghese et al., 1998; Zhang et al., 1998; Zhang et al., 2000; Filvaroff et al., 2002; Serlachius e Andersson, 2004)

Consistent with its high and ubiquitous expression, STC1 may also play a role in mesenchymal epithelial interactions (Stasko e Wagner, 2001) During bone development, STC-1 is present in the mesenchyme during intramembranous bone formation and is found in chondroprogenitors, chondrocytes and osteoblasts (Jiang et al., 2000) STC1 expression during embryonic mouse osteogenesis is high in cells committed to the osteoblast lineage, especially the mature osteoblasts (Yoshiko et al., 2002).Studies in transgenic mice, the STC1 ectopic expression increased cortical bone thickness, changed trabeculae number and density indicating a concomitant suppression of osteoclast activity (Filvaroff et al., 2002) . Stimulation of confluent calvarial osteoblast subcultures with recombinant hSTC1 can enhance osteoblast mineralization by inducing expression of the NaPi transporter, resulting in increased phosphate uptake (Yoshiko et al., 2007)

In vitro and in vivo models suggest that the use of expanded ASCs improve bone healing through direct differentiation into mature osteoblasts and paracrine effects that facilitate migration and differentiation of resident precursors (Barba et al., 2013). Many signaling pathways converge on key transcription factors to regulate and orchestrate bone development. Osteoblast-specific phenotypic markers are differentially expressed during various stages of development. Some of the markers consist of alkaline phosphatase (ALP), non-collagenous bone matrix proteins, including osteopontin (OPN) and RUNX2 (runt related gene 2). ALP is the most commonly used biomarker of bone formation. ALP is expressed early in development, and is soon observed on the cell surface and in matrix vesicles (Golub e Boesze-Battaglia, 2007).OPN is a secreted glycoprotein expressed in cultured osteoblastic cells early during differentiation, and its expression is maintained at a high level through the later stages of in vitro mineralization (Denhardt et al., 2001) RUNX2 is among the most important transcription factors necessary for the process of osteogenesis and is responsible for the activation of osteoblast differentiation marker genes (Vimalraj et al., 2015).

Our study indicated a cross-talk between calcitonin gene-related peptide (CGRP) and STC1at the earlier stages of the osteoblast differentiation process (Terra et al., 2015). In addition to the previously reported direct effects on osteogenesis present our study shows that STC1 may regulate CGRP and calcitonin (CT) signaling during osteoblastogenesis although the physiological consequences of this remain to be elucidated (Terra et al., 2015).

The signaling involving pathways as BMP, FGF, TGF-β, Notch, Wnt/β-Catenin and Hedgehog have been shown to take part in the differentiation of ASCs into osteoblasts (James, 2013). The osteoblasts are a target for action of STC1 and there is evidence that STC1 modulates bone development in vivo (Johnston et al., 2010).Thus the objective of this study was to evaluate the effects of STC1 in hASC osteoblastic differentiation and whether this effect was regulated by pAKt/pGSK3-β/β catenin signaling pathway.

2. MATERIAL AND METHODS

2.1 Cell culture

Human adipose derived stem cells (hASC) were purchased from Lonza (Lonza Group, Switzerland). Cells were maintained in complete Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS–GIBCO, Carlsbad, CA, USA), 0.1% penicillin: streptomycin antibiotic mix (10.000 U:10 mg/ml, Sigma) and 250µg/ml sterile filtered 1:100 amphotericin B solution (Sigma) at 37°C/5% CO_2. Cells were seeded at 1 x 10^4 cells/cm^2 in 24-well plates and cultured to reach 70-80% of confluence before treatment hASC were induced to osteoblast for 7 and 14 days,using osteoblastogenic differentiation medium consisting of DMEM supplemented with 10% FBS, 50 µM ascorbate-2-phosphate, 100 mM beta-glycerophosphate (Sigma), and 0.1µM dexamethasone (Sigma), according to previously published techniques (Zuk et al, 2002). To confirm the osteogenic phenotype, differentiated cells were fixed in 4% paraformaldehyde (PFA), rinsed with Phosphate Buffered Salin (PBS) and stained with Alizarin Red S for 5 minutes and imaged with light microscopy.

2.2 Quantitative measurement of Alkaline Phosphatase

Alkaline phosphatase (ALP) activity was determined in hASCs cell lysates and hASCs induced to osteoblast for 7 and 14 days. Cells were cultured in 24-well plate in absence or presence of rhSTC1 protein (Biovendor, Czech Republic). The STC1 concentrations ranged from 6.3 ng to 50 ng/ml and medium was replaced every 3 days.

Quantitative measurement of ALP activity was performed by determining the relative amount of p-nitrophenol phosphate substrate using a commercial kit (Labtest Diagnostica SA, MG, Brazil).. Cell lysates in 0.2% Triton X-100 were mixed with alkaline buffer rsolution and gently shaken for 10 min. ALP substrate was added at room temperature for 30 min, and the reaction was subsequently stopped with addition of 0.05 N NaOH. Absorbance at 405 nm was recorded and was used to represent the ALP activity. OD value at a wavelength of 405 nm was

normalized by the cellular protein concentration. The quantitative measurement of cellular proteins was done following the method of Peterson (Peterson, 1979) .

2.3 RNA extraction, cDNA synthesis and Real-time PCR

The RNA was isolated using TRIzol Reagent (Invitrogen). RNA was quantified using the BioPhotometer Plus (Eppendorf, Hamburg, Germany) to measure the absorbance at 260 nm relative to that at 280 nm. It was added 2 µg of total RNA was added to each cDNA synthesis reaction, using SuperScript®-III RT First-Strand Synthesis SuperMix (Invitrogen). The gene sequence information was collected from databases (www.ncbi.nlm.nih.gov and www.ensembl.org). The specifics primers for each gene were designed using IDT Design Software (Integrated DNA Technologies Inc., USA). For osteogenic differentiattion analysis (Zul et al,2002) primers for the following genes were used: RUNX2, osteopontin, alkaline phosphatase . The TATA-binding protein (TBP) gene was used as the internal control gene for all relative expression calculations. All primer sequences are listed in Table 1. q-PCR reactions were carried out in a Step One Plus real-time cycler (Applied-Biosystem, New York, NY, USA) and performed in triplicates and containing 0.2 µM of forward and reverse primers, 0.5 ng/µl cDNA and Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen Corp., USA) according to the thermal cycling conditions: 2 min at 50o C, 2 min at 95oC, followed by 40 cycles of 95oC for 15 s, 60oC for 30 s. The mean Ct values from triplicate measurements were used to calculate expression of the target gene using the 2–ΔCt formula (Schmittgen e Livak, 2008).

Table 1 Primer sequences

Gene	Primer sequences	GenBank Reference
ALP	Forward - 5'GTATGAGAGTGACGAGAAAGCC 3' Reverse - 5' GTTCCAGATGAAGTGGGAGTG 3'	**NM_NM_000478.4**
OSTEOPONTIN	Forward - 5' CCGACCAAGGAAAACTCACTAC 3' Reverse - 5' GGAGATTCTGCTTCTGAGATGG 3'	**NM_001251830.1**
RUNX2	Forward - 5' AGCAAGGTTCAACGATCTGAG 3' Reverse - 5' GGCGGTCAGAGAACAAACTAG 3'	**NM_001015051.3**
TBP	Forward - 5'TGCACAGGAGCCAAGAGTGAA 3' Reverse - 5' CACATCACAGCTCCCCACCA3	**NM_003194.4**

2.4 Immunocytochemistry analysis

Briefly, hASC and hASC induced to osteoblasts during 7 and 14 days were cultured on 4-chamber glass bottom dishes in absence or presence of hSTC1 (50 ng/ml). Cells were washed, fixed with 4% paraformaldehyde in PBS for 20 min before incubation at 4°C with primary rabbit anti-human Alkaline Phosphatase monoclonal antibody (1:200, clone EPR447, ab108337, Abcam, Cambridge, UK) diluted in a PBS/blockage solution with 5% of FBS plus 1% of bovine serum albumin (BSA). The primary rabbit anti-human Osteopontin monoclonal antibody (1:200, clone EPR3688, Abcam, Cambridge, UK) and rabbit anti-human RUNX2 polyclonal antibody (1:1000, Abcam, USA) were used under the same conditions with cells permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. The cells were washed in PBS, and incubated with the secundary anti-rabbit antibody conjugated with the green fluorescent Alexa Fluor® 488 or red fluorescent Alexa Fluor® 555 (both from Life Technologies, USA),. Negative controls reactions were performed by omitting the primary antibody. No reactivity was observed when the primary antibody was excluded. Ten single confocal sections of 0.7 µm were taken parallel to the coverslip (xysections) on the Olympus FV1000 laser-scanning confocal microscopy equipped with an ×60 (numeric aperture 1.35) oil immersion objective (Olympus, U plan-super-apochromat, UPLSAPO 60XO). Imaging analysis is based on 3 independent experiments.

2.5 Western blotting analysis

After treatments, cells were homogenized in lysis buffer (4% sodium dodecyl sulfate (SDS), 2 mM EDTA, 50 mMTris). Protein concentration was determined by the Lowry method. Proteins were analized by 10 or 12% SDS-PAGE (75 µg per lane) and transferred to nitrocellulose membranes (Hybond™ ECL™ nitrocellulose membrane, Amersham Biosciences™, Fryeburg, Germany) using a semi-dry transfer apparatus (Bio- Rad™, Trans-Blot SD, Hercules™, CA, USA). Membranes were incubated for 60 minutes at 4°C in blocking solution (Tris-buffered saline containing 5% powdered milk and 0.1% Tween-20, pH 7.4). and incubated overnight with specific antibodies. Primary antibodies (Cell Signaling

Technology™, Beverly, MA, USA) against the following proteins were used: anti-p-Akt$_{Ser473}$ and anti-Akt (1:1000), anti-p-GSK-3β$_{Ser9}$ and anti-GSK-3β (1:1000), anti-β-actin (1:1000), anti-alkaline phosphatase (1:1000). Membranes were incubated with horseradish peroxidase conjugated anti-rabbit antibody (1:1000; Amersham Pharmacia Biotech, Piscataway, NJ, USA). Chemiluminescence (ECL, Amersham Pharmacia Biotech™) was detected using X-ray films (Kodak X-Omat™, Rochester, NY, USA).

2.6 Flow cytometry analysis

To evaluate the Alkaline Phosphatase (ALP) expression ,hASCs and hASCs induced to osteoblastic differentiation for 7 and 14 days were harvested in 0.25% trypsin/EDTA (Invitrogen, USA). Cellular pellets were suspended and permeabilized with 0.1% Tween-20 in PBS for 20 minutes at room temperature. After blocking for 1 hour with BSA 5% (Sigma Inc., Saint Louis, MO, USA) in PBS, cells were washed and incubated with rabbit anti- alkaline phosphatase monoclonal antibody (1:1000, ab108337, Abcam, Cambridge, UK) for 30 min at 4ºC.Further cells were washed and incubated with secondary goat anti-rabbit Alexa Fluor 488 IgG antibody (1:500, Invitrogen, Grand Island, NY, USA) for 30 min at 4ºC. The cells stained without the primary antibody were used as negative control. At least 10000 events were analyzed by flow cytometry with the CellQuest software (BD Biosciences, USA).

2.7 Statistical analysis

Data from the experiments were analyzed statistically by two-way ANOVA, followed by post hoc Bonferroni test when the F-test was significant. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. All statistical calculations were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc.)

3. RESULTS

3.1 Effect of STC1 on preosteoblast ALP activity and expression

It has been previously described that 50ng/ml rhSTC1 was related with upregulated ALP, indicating that STC1 affected osteoblastogenesis (Ohkouchi et al., 2012) The present results show that ALP activity at day 7 is significantly increased in preosteoblasts treated with 50ng/mL STC1, as compared to the control with or hASCs treated with STC1 with doses ranging from 6.3 to 25 ng/mlSTC1(Fig.1A).It is important to note that hASCs cells failed to respond to STC1 stimulation, since ALP activity in these cells was not altered in response to the increasing hormone doses. In addition, we did not find significantly increased ALP activity in preosteoblasts at day 14 days, as compared to the control (Fig.1A). In this regard, we could infer that 14-day STC1 stimulated preosteoblasts presented ALP activity compatible with cells control, supporting a persistent differentiation profile. Two way ANOVA reveals an interaction between STC1 and day at treatment since STC1 present effect only day 7 whithout effect on day 14(p 0,02). Taking into account these findings, we have chosen 50ng/ml STC1 to explore the mechanisms underlying the action of STC1 on the expression of classical osteogenic markers. The ALP transcript was significantly increased in 7-day preosteoblasts stimulated with STC1 as compared with the control, restoring basal levels in 14-day differentiated cells(Fig. 1B). These results suggest that the increased ALP activity could be ascribed to the upregulated ALP expression. Further supporting the increased expression/activity results, we showed that ALP immunocontent in 7-day induced preosteoblasts stimulated with STC1 was upregulated, as compared to the control. This was evidenced by confocal immunocytochemistry using Alexa Fluor 555 conjugated anti-ALP antibody (Fig.1C). Also, flow cytometry data show that 7-day preosteoblasts stimulated with STC1 increased ALP levels in membrane surface, as compared to the control . Otherwise, ALP levels in STC1-stimulated and non stimulated 14-day preosteoblasts were similar (Figure 1D).

We found no significant effects of STC1 (50ng/ml) on cell viability during osteoblastogenesis using propidium iodide staining (data not shown).

3.2 STC1 upregulate the expression of RUNX2 and Osteopontin in osteoblast differentiation

To further investigate the effect of STC1 on hASC osteoblast differentiation the expression of the two classical osteogenic markers RUNX2 and OPN were determined (Shen e Christakos, 2005). As shown in Figure 2A, 7-day preosteoblasts stimulated with STC1 significantly upregulated the RUNX2 and OPN expression as compared to the control and 14-day preosteoblasts were non responsive to the stimulus of STC1, maintaining the basal expression of these proteins (Fig. 2A). In agreement with these results, the confocal immunocytochemistry with Alexa fluor 555-conjugated anti RUNX2 antibody and Alexa fluor 488-conjugated anti osteopontin antibody showed increased immunocontent of these osteogenic markers in 7-day preosteoblasts stimulated with STC1 (Fig. 2B).

3.3 Effect of rhSTC1 on preosteoblast mineralization

The matrix mineralization in preosteoblasts was evaluated by Alizarin red S staining in 7- and 14-day preosteoblasts stimulated with rhSTC1.Results showed increased matrix mineralization in preosteoblast stimulated with STC1 only after 14 days as compared to the control and 7-day STC1-stimulated preosteoblasts (Fig 3). These results suggest that STC1 functioned as a positive regulator in the osteogenic differentiation, since mineralization is a hallmark of osteogenic differentiation (Sapir-Koren e Livshits, 2011)

3.4 STC1 induces β-catenin accumulation in 7-day preosteoblasts

We evaluated the roles of STC1 on signaling pathways stimulating the osteoblastogenesis. In this regard, we focused in Akt/GSK3β phosphorylation/activation leading to β-catenin accumulation in the cytoplasm, since this is considered a relevant mechanism in the osteogenesis (Chen et al., 2013). The Western blot analysis with anti Akt/pAkt$_{Ser473}$, GSK/pGSK$_{Ser9}$ and β-catenin antibodies showed increased pAkt, pGSK and β-catenin immunocontents

in 7-day STC1-stimulated preosteoblasts as compared to the control. These results indicated that the STC1-induced osteogenic differentiation was related with the activation of pAKt/pGSK3-β/β-catenin signaling pathway (Fig 4).

4. DISCUSSION

Bone formation is a tightly regulated process characterized by a sequence of events starting by the commitment of osteoprogenitor cells, their differentiation into preosteoblast and mature osteoblast (Marie, 2008) STC1 is a glycoprotein hormone expressed in a wide variety of tissues, especially the mature osteoblast during embryonic mouse osteogenesis (Yoshiko et al., 2002). STC1 expression is present as early as the mesenchymal condensations are initiated suggesting a role for the action of STC1 during both endochondral and intramembranous bone formation (Jiang et al., 2000; Stasko e Wagner, 2001)

It has been previously shown that stimulation of confluent calvarial osteoblast subcultures with recombinant STC1 can enhance osteoblast mineralization by inducing the expression of the NaPi transporter Pit1/2, resulting in increased phosphate uptake (Yoshiko et al., 2007). These studies suggest autocrine or paracrine STC1 function during mammalian bone development through modulating phosphate availability to cartilage and bone cells (Yoshiko et al., 2003; Yoshiko e Aubin, 2004)

A tight regulation of STC1 activity is necessary to maintain normal skeletogenesis during bone development. Moreover, persistent exposure to STC1 in hASCs transgenic mice showed embryonic growth restriction on osteoprogenitor cell proliferation or viability both in vivo and in vitro (Varghese et al., 1998; Johnston et al., 2010).

However, the effect the STC1 on osteoblastic differentiation of hASCs has not been thoroughly explored. Our findings show that STC1 leads hASCs to elicit osteogenic differentiation, displayed by increased ALP activity and gene expression of the osteogenic markers ALP, RUNX2 and OPN. Furthermore, we demonstrate for the first time that STC1 could stimulate osteogenic differentiation of hASCs by upregulating the activity of pAkt/GSK3β/β-catenin pathway.

In order to determine a possible role for the STC1 in the potentialization of osteogenenic differentiation of hASCs, the process was performed in the presence

or absence of the hormone. In the presence of 50ng/ml of the hormone for 7 days the activity of ALP was significantly increased as compared with differentiated cell in the absence of the hormone. On the other hand, in the absence of the hormone we have a standard osteogenic differentiation. These findings emphasize the critical role of STC1 in the anticipation of the osteogenic differentiation. Moreover, we could ascribe the increased ALP enzyme activity in the presence of the hormone to the upregulated gene expression and consequent protein levels, as demonstrated by immunocytochemistry and flow cytometry. These results are in agreement with the previously reported effects of 50 ng/ml STC1 in the osteogenic differentiation (Block et al., 2009; Ohkouchi et al., 2012).

In addition, our present results also show the role of STC1 in the activation of other classical markers of osteogenic differentiation, such as RUNX2 and OPN. RUNX2 is among the most important transcription factors undertaking the osteogenesis determining the commitment of the multipotent mesenchymal stem cells to a osteoblast lineage (Ducy et al., 1999; Vimalraj et al., 2015). It has been reported that RUNX2-null mice fail to present osteoblasts and consequently they are not able to develop bone tissue (Ziros et al., 2008). RUNX2 binds to promoter regions initiating the expression of osteoblast marker genes which include osteocalcin, bone sialoprotein, osteopontin, and collagen type I (Ducy, 2000).

OPN, also known as secreted phosphoprotein 1 (SPP1), is a soluble protein expressed early during differentiation, and expression is maintained at a high level throughout the later stages of in vitro mineralization (Denhardt et al., 2001).The concept that OPN is involved in regulation of bone mineralization arose from its tissue distribution, its affinity for calcium, its immunolocalization in regions of mineralization and the regulation of OPN gene expression by calcitrophic hormones (Prince e Butler, 1987; Noda et al., 1988; Ikeda et al., 1992).Interestingly, certain functions of OPN require it to be phosphorylated, since OPN phosphorylation may be controlled by extracellular phosphatases and kinases. Extracellular phosphate induces OPN expression in the osteoblast-like MC3T3 cells and this regulation could be a control mechanism connecting an increased OPN expression to the cessation of osteoblast proliferation. Moreover, the onset of differentiation, is an event that coincide with the induction of alkaline phosphatase (Beck et al., 2000).It has been described that STC1, induces Pit1 expression directly increasing NaPi cotransport. The Pi is required for bone

formation, and growing evidence supports the role of Pi signaling in the induction of OPN (Yoshiko et al., 2007).

The ALP expression is stimulated by RUNX2 and by calciotropic hormones such as PTH and STC1(Weng e Su, 2013). During osteogenic differentiation the ALP ativity increase significantly from day 7 to 14**,** reaching a maximum after 14 days and decreased from day 14 to 21 (Golub e Boesze-Battaglia, 2007). The involvement of osteopontin in the osteogenese take in account its binding into osteoblasts during the bone matrix formation, and by its action in the regulation of the crystal size during bone mineralization (Boskey et al., 2002). The STC1 was able to increase the mineralization and OPN mRNA levels in an osteoblast culture model(Yoshiko et al., 2007). Taken together, our results suggest that the mineralization process is facilitated by STC1, through up-regulation of RUNX2 and consequently ALP and OPN.

In this study, we also found that the treatment with STC1 increases the β-catenin level in7-day preosteoblast compared to the standard preosteoblasts. Our results showing increased levels of $pAKT_{Ser473}$ and $pGSK3\beta_{Ser9}$ suggest that this pathway is negatively regulated, since GSK3β phosphorylated at Ser9 is known to be inactive (Seira e Del Río, 2014) Phosphorylation of GSK3 β is related with the inactive form of the enzyme and consequently its substrate β-catenin is maintained in its dephosphorylated form, accumulating into the cytoplasm. Accumulated β-catenin translocates into the nucleus functioning as an activator of different transcription factors of osteogenesis (Xavier et al., 2014)

Concerning the role of Akt in the osteogenesis, it is important to emphasize that it has been previously reported that PI3K/Akt pathway and its downstream targets play critical effects in bone development and skeletal remodeling (Chen et al., 2013) In particular, Akt1 deficiency in osteoblasts is described to increase the susceptibility to apoptosis, suppress bone differentiation, and decrease osteoclastogenesis. Suppressed differentiation and function in Akt1-/- osteoblasts were shown to be mediated, at least in part, by modulating the RUNX2 activity.(Kawamura et al., 2007). In addition, Akt is also able to regulate RUNX2 by enhancing ubiquitin/proteasome mediated degradation of Smurf2, which consequently reduces Smurf2-induced degradation of RUNX2 (Choi et al., 2014).

GSK3 is a regulator of numerous signaling pathways in mammalian cells and its disregulated activity through phosphorylation/inhibition can be initiated by

diverse stimulus such as PI3K/Akt downstream of receptor tyrosine kinase (RTK),insulin receptor signaling or Wnt–β-catenin signaling (Doble e Woodgett, 2003).The potential of GSK3β inhibition for in vivo differentiation of mesenchymal progenitors into the osteogenic lineage has been recently reported (Gambardella et al., 2011)).

As summarized in Figure 5, our results show that STC1 promote an anticipation in osteogenic differentiation mechanisms in hASCs cultures and that these effects probably occur by stimulation of the pAKt/pGSK3-β/β-catenin signaling pathway.

In conclusion, we propose a role for STC1 in the anticipation of ALP expression and modulation of the activity of pAKt/pGSK3-β/β-catenin signaling pathway and gene activation, represent an important contribution for the understanding of the mechanisms underlying the STC1 ability in enhancing osteoblastogenesis from hASCs.

Acknowledgements

This work was supported by grants from CAPES/CNPq (VS PNPD

fellowship program), CNPq (SRT;FCRG research productivity fellowship program), FAPERGS/CNPq n.008/2009 The authors thank the Centro de MicroscopiaEletrônica (CME-UFRGS) for technical assistance in confocal microscopy.

REFERENCE

BARBA, M. et al. Adipose-derived mesenchymal cells for bone regereneration: state of the art. BioMed research international, v. 2013, 2013. ISSN 2314-6133.

BECK, G. R.; ZERLER, B.; MORAN, E. Phosphate is a specific signal for induction of osteopontin gene expression. Proceedings of the National Academy of Sciences, v. 97, n. 15, p. 8352-8357, 2000. ISSN 0027-8424.

BLOCK, G. J. et al. Multipotent stromal cells are activated to reduce apoptosis in part by upregulation and secretion of stanniocalcin-1. Stem cells, v. 27, n. 3, p. 670-681, 2009. ISSN 1549-4918.

BOSKEY, A. et al. Osteopontin deficiency increases mineral content and mineral crystallinity in mouse bone. Calcified tissue international, v. 71, n. 2, p. 145-154, 2002. ISSN 0171-967X.

CHEN, J. et al. The key regulatory roles of the PI3K/Akt signaling pathway in the functionalities of mesenchymal stem cells and applications in tissue regeneration. Tissue Engineering Part B: Reviews, v. 19, n. 6, p. 516-528, 2013. ISSN 1937-3368.

CHOI, Y. H. et al. Akt enhances Runx2 protein stability by regulating Smurf2 function during osteoblast differentiation. FEBS Journal, v. 281, n. 16, p. 3656-3666, 2014. ISSN 1742-4658.

DENHARDT, D. T. et al. Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. The Journal of clinical investigation, v. 107, n. 9, p. 1055-1061, 2001. ISSN 0021-9738.

DOBLE, B. W.; WOODGETT, J. R. GSK-3: tricks of the trade for a multi-tasking kinase. Journal of cell science, v. 116, n. 7, p. 1175-1186, 2003. ISSN 0021-9533.

DUCY, P. Cbfa1: a molecular switch in osteoblast biology. Developmental Dynamics, v. 219, n. 4, p. 461-471, 2000. ISSN 1097-0177.

DUCY, P. et al. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. Genes & development, v. 13, n. 8, p. 1025-1036, 1999. ISSN 0890-9369.

FILVAROFF, E. H. et al. Stanniocalcin 1 alters muscle and bone structure and function in transgenic mice. Endocrinology, v. 143, n. 9, p. 3681-3690, 2002. ISSN 0013-7227.

GAMBARDELLA, A. et al. Glycogen synthase kinase-3α/β inhibition promotes in vivo amplification of endogenous mesenchymal progenitors with osteogenic and adipogenic potential and their differentiation to the osteogenic lineage. Journal of bone and mineral research, v. 26, n. 4, p. 811-821, 2011. ISSN 1523-4681.

GOLUB, E. E.; BOESZE-BATTAGLIA, K. The role of alkaline phosphatase in mineralization. Current Opinion in Orthopaedics, v. 18, n. 5, p. 444-448, 2007.

IKEDA, T. et al. In situ hybridization of bone matrix proteins in undecalcified adult rat bone sections. Journal of Histochemistry & Cytochemistry, v. 40, n. 8, p. 1079-1088, 1992. ISSN 0022-1554.

JAMES, A. W. Review of signaling pathways governing MSC osteogenic and adipogenic differentiation. Scientifica, v. 2013, 2013.

JIANG, W. et al. The distribution of stanniocalcin 1 protein in fetal mouse tissues suggests a role in bone and muscle development. Journal of Endocrinology, v. 165, n. 2, p. 457-466, 2000. ISSN 0022-0795.

JOHNSTON, J. et al. Human stanniocalcin-1 or-2 expressed in mice reduces bone size and severely inhibits cranial intramembranous bone growth. Transgenic research, v. 19, n. 6, p. 1017-1039, 2010. ISSN 0962-8819.

KAWAMURA, N. et al. Akt1 in osteoblasts and osteoclasts controls bone remodeling. PLoS One, v. 2, n. 10, p. e1058, 2007. ISSN 1932-6203.

MARIE, P. J. Transcription factors controlling osteoblastogenesis. Archives of biochemistry and biophysics, v. 473, n. 2, p. 98-105, 2008. ISSN 0003-9861.

NODA, M. et al. Transcriptional regulation of osteopontin production in rat osteosarcoma cells by type beta transforming growth factor. Journal of Biological Chemistry, v. 263, n. 27, p. 13916-13921, 1988. ISSN 0021-9258.

OHKOUCHI, S. et al. Mesenchymal stromal cells protect cancer cells from ROS-induced apoptosis and enhance the Warburg effect by secreting STC1. Molecular Therapy, v. 20, n. 2, p. 417-423, 2012. ISSN 1525-0016.

PETERSON, G. L. Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Analytical biochemistry, v. 100, n. 2, p. 201-220, 1979. ISSN 0003-2697.

PRINCE, C. W.; BUTLER, W. T. 1, 25-Dihydroxyvitamin D3 regulates the biosynthesis of osteopontin, a bone-derived cell attachment protein, in clonal osteoblast-like osteosarcoma cells. Collagen and related research, v. 7, n. 4, p. 305-313, 1987. ISSN 0174-173X.

SAPIR-KOREN, R.; LIVSHITS, G. Bone mineralization and regulation of phosphate homeostasis. IBMS BoneKEy, v. 8, n. 6, p. 286-300, 2011.

SATIJA, N. K. et al. Mesenchymal stem cell-based therapy: a new paradigm in regenerative medicine. Journal of cellular and molecular medicine, v. 13, n. 11-12, p. 4385-4402, 2009. ISSN 1582-4934.

SCHMITTGEN, T. D.; LIVAK, K. J. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc, v. 3, n. 6, p. 1101-8, 2008. ISSN 1750-2799 (Electronic)
1750-2799 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18546601 >.

SEIRA, O.; DEL RÍO, J. A. Glycogen synthase kinase 3 beta (GSK3β) at the tip of neuronal development and regeneration. Molecular neurobiology, v. 49, n. 2, p. 931-944, 2014. ISSN 0893-7648.

SENARATH-YAPA, K. et al. Adipose-derived stem cells: a review of signaling networks governing cell fate and regenerative potential in the context of craniofacial and long bone skeletal repair. International journal of molecular sciences, v. 15, n. 6, p. 9314-9330, 2014.

SERLACHIUS, M.; ANDERSSON, L. C. Upregulated expression of stanniocalcin-1 during adipogenesis. Experimental cell research, v. 296, n. 2, p. 256-264, 2004. ISSN 0014-4827.

SHARMA, U.; PAL, D.; PRASAD, R. Alkaline phosphatase: an overview. Indian Journal of Clinical Biochemistry, v. 29, n. 3, p. 269-278, 2014. ISSN 0970-1915.

SHEN, Q.; CHRISTAKOS, S. The vitamin D receptor, Runx2, and the Notch signaling pathway cooperate in the transcriptional regulation of osteopontin. Journal of Biological Chemistry, v. 280, n. 49, p. 40589-40598, 2005. ISSN 0021-9258.

STASKO, S.; WAGNER, G. Possible roles for stanniocalcin during early skeletal patterning and joint formation in the mouse. Journal of Endocrinology, v. 171, n. 2, p. 237-248, 2001. ISSN 0022-0795.

TERRA, S. R. et al. STC1 interference on calcitonin family of receptors signaling during osteoblastogenesis via adenylate cyclase inhibition. Molecular and cellular endocrinology, v. 403, p. 78-87, 2015. ISSN 0303-7207.

TRINDADE, D. M. et al. Low-resolution structural studies of human Stanniocalcin-1. BMC structural biology, v. 9, n. 1, p. 1, 2009. ISSN 1472-6807.

VARGHESE, R. et al. Comparative analysis of mammalian stanniocalcin genes 1. Endocrinology, v. 139, n. 11, p. 4714-4725, 1998. ISSN 0013-7227.

VIMALRAJ, S. et al. Runx2: Structure, function, and phosphorylation in osteoblast differentiation. International journal of biological macromolecules, v. 78, p. 202-208, 2015. ISSN 0141-8130.

WAGNER, G. F.; DIMATTIA, G. E. The stanniocalcin family of proteins. Journal of Experimental Zoology Part A: Comparative Experimental Biology, v. 305, n. 9, p. 769-780, 2006. ISSN 1552-499X.

WENG, J.-J.; SU, Y. Nuclear matrix-targeting of the osteogenic factor Runx2 is essential for its recognition and activation of the alkaline phosphatase gene. Biochimica et Biophysica Acta (BBA)-General Subjects, v. 1830, n. 3, p. 2839-2852, 2013. ISSN 0304-4165.

XAVIER, C. P. et al. Secreted Frizzled-related protein potentiation versus inhibition of Wnt3a/β-catenin signaling. Cellular signalling, v. 26, n. 1, p. 94-101, 2014. ISSN 0898-6568.

YOSHIKO, Y.; AUBIN, J. E. Stanniocalcin 1 as a pleiotropic factor in mammals. Peptides, v. 25, n. 10, p. 1663-1669, 2004. ISSN 0196-9781.

YOSHIKO, Y.; AUBIN, J. E.; MAEDA, N. Stanniocalcin 1 (STC1) protein and mRNA are developmentally regulated during embryonic mouse osteogenesis: the potential of stc1 as an autocrine/paracrine factor for osteoblast development and bone formation. Journal of Histochemistry & Cytochemistry, v. 50, n. 4, p. 483-491, 2002. ISSN 0022-1554.

YOSHIKO, Y. et al. Osteoblast autonomous Pi regulation via Pit1 plays a role in bone mineralization. Molecular and cellular biology, v. 27, n. 12, p. 4465-4474, 2007. ISSN 0270-7306.

YOSHIKO, Y.; MAEDA, N.; AUBIN, J. E. Stanniocalcin 1 stimulates osteoblast differentiation in rat calvaria cell cultures. Endocrinology, v. 144, n. 9, p. 4134-4143, 2003. ISSN 0013-7227.

ZHANG, K.-Z. et al. Stanniocalcin: a molecular guard of neurons during cerebral ischemia. Proceedings of the National Academy of Sciences, v. 97, n. 7, p. 3637-3642, 2000. ISSN 0027-8424.

_____. High expression of stanniocalcin in differentiated brain neurons. The American journal of pathology, v. 153, n. 2, p. 439-445, 1998. ISSN 0002-9440.

ZIROS, P. G.; BASDRA, E. K.; PAPAVASSILIOU, A. G. Runx2: of bone and stretch. The international journal of biochemistry & cell biology, v. 40, n. 9, p. 1659-1663, 2008. ISSN 1357-2725. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1357272507002038 >.

FIGURES

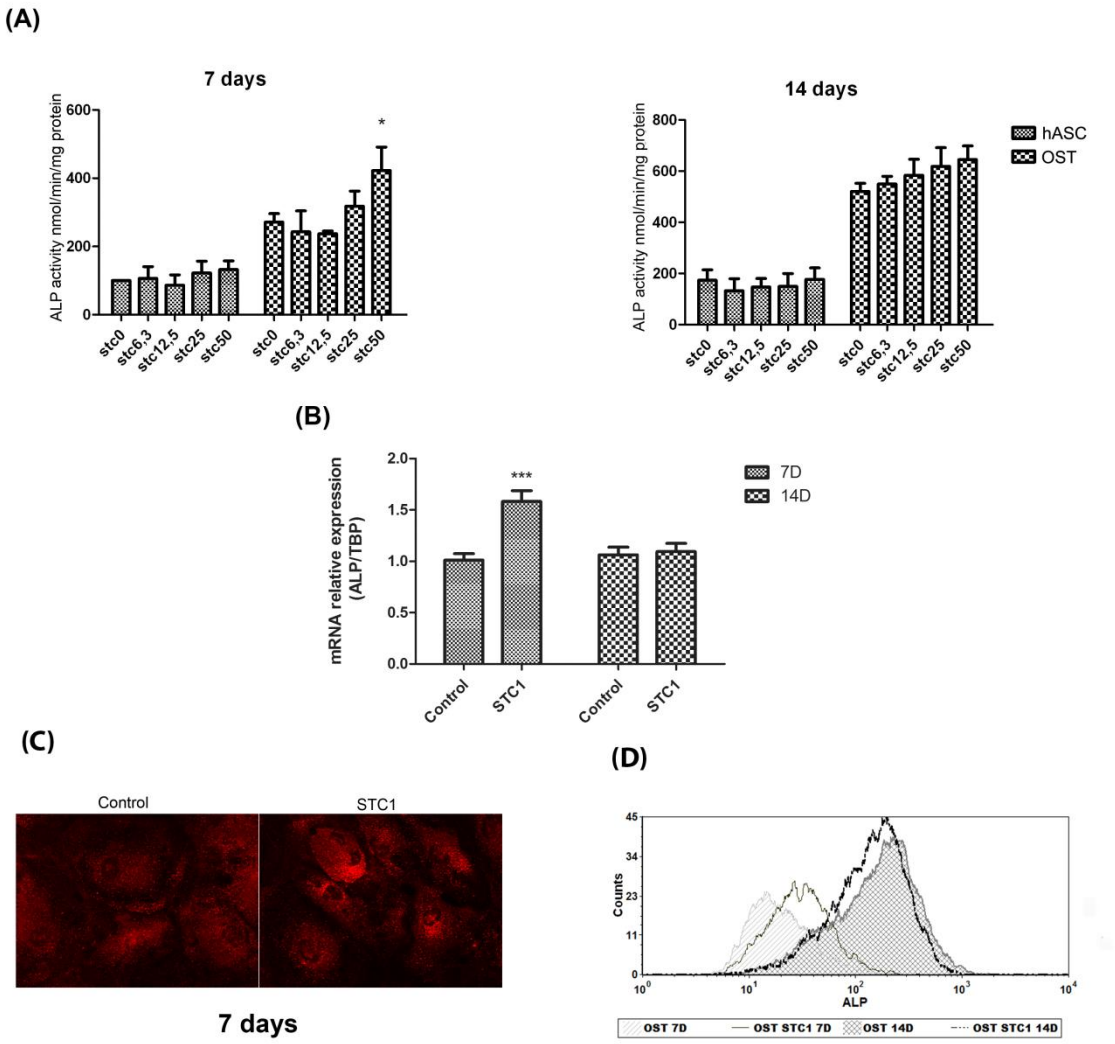

Figure 1: Effects of STC1 on ALP activity and expression.(A) ALP activity was determinate in cell lysates of hASC and preosteoblast after 7 and 14 days of differentiation treated or not with 6.3; 12.5; 25 and 50 ηg/mL of STC1 by measuring the conversion of PNPP to p-nitrophenol. Values are show as mean± SD of three independent experiments. *Statistically different from untreated and treated with small STC1 doses. (B) mRNA analysis of ALP in preosteoblast after 7 and 14 days of differentiation treated with 50 ηg/mL by quantitative polymerase chain reaction (qPCR), the human TBP as an internal standard. Values are show as mean ± SD of three independent experiments. ***Statistically different from 7 days control untreated preosteoblasts and from 14 days treated and untreated cells. (C) Images of confocal microscopy of ALP protein content stained by immunocitochemistry in preosteoblasts treated with 50ng/ml of STC1 and untreated preosteoblast.(D) ALP expression measured by flow cytometry. Representative histograms plots of 7 and 14 days pre-osteoblasts treated or not with STC1.

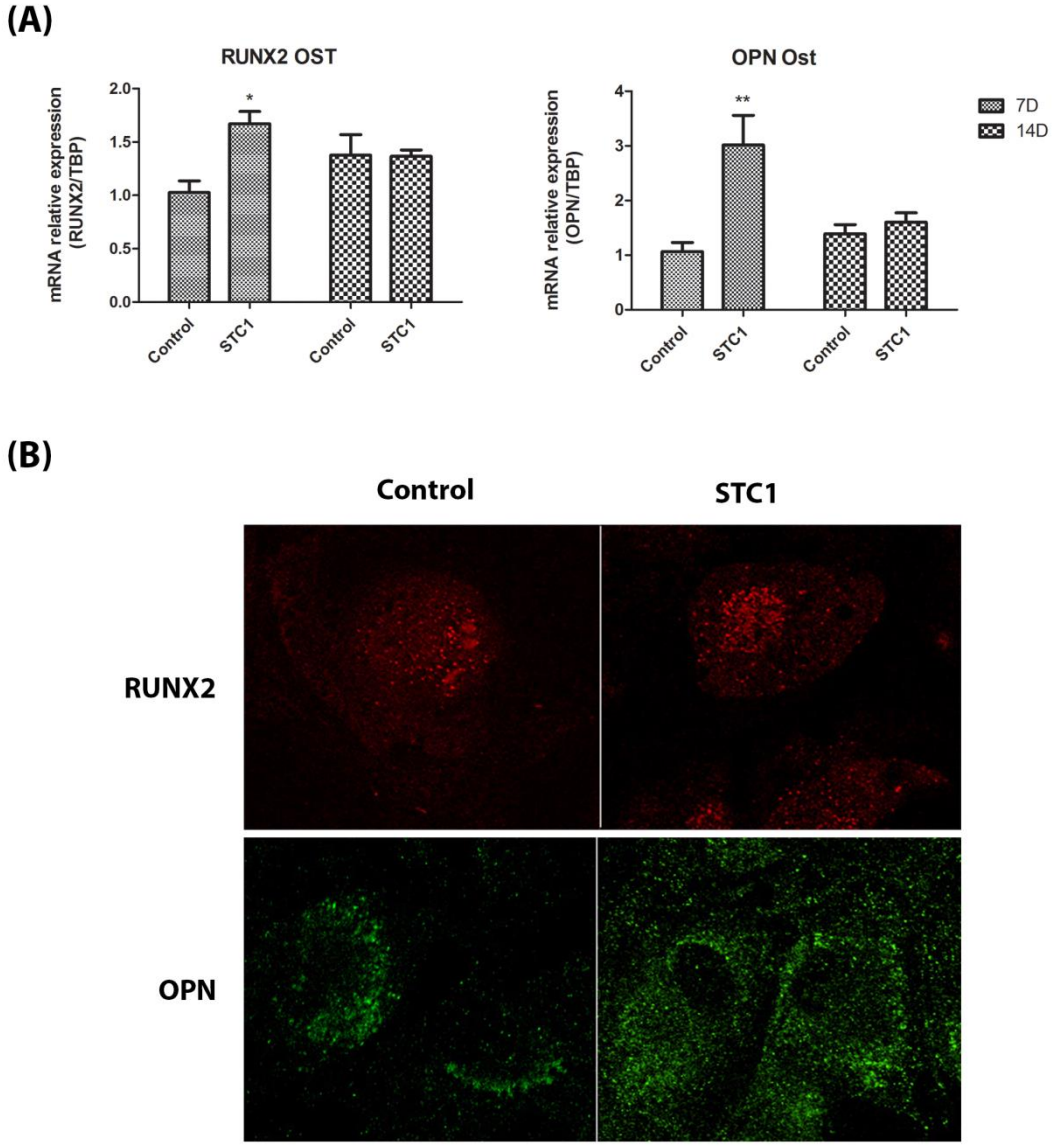

Figure 2: Effects of STC1 treatment on expression of Runx2 and OPN mRNA and protein in hASCs and preosteoblasts. (A) mRNA analysis of Runx2 and OPN by quantitative polymerase chain reaction (qPCR), with human TBP as an internal standard.Values are show as mean± SD of three independent experiments, * and ** statistically different from 7 days control untreated pre-osteoblasts.(B) Images of confocal microscopy of Runx2 and OPN protein content stained by immunocitochemistry in STC1 treated and untreated 7 days induced preosteoblast. Representative images of three experiments with similar results.

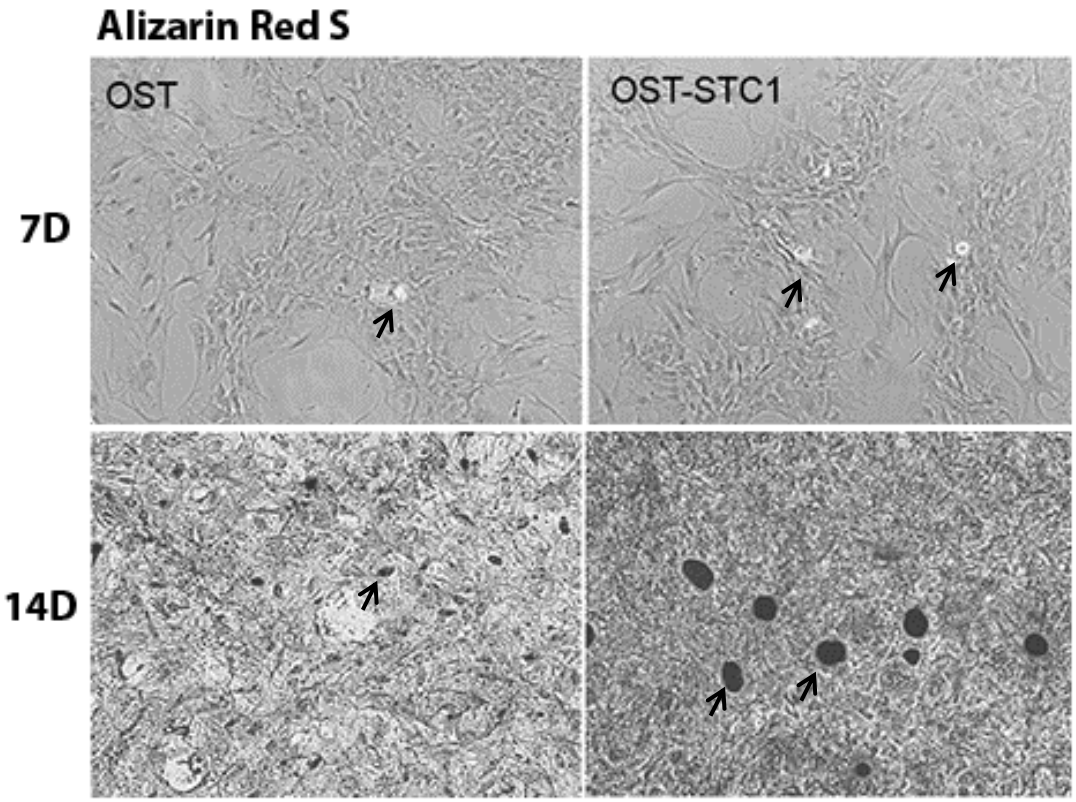

Figure 3: Effect of rhSTC1 on osteoblast mineralization. Phase contrast images of hASCs induced to preosteoblasts in presence (OST-STC1) or absence (OST) of STC1 stained with Alizarin Red S. Arrows point the calcium deposits (x 400).

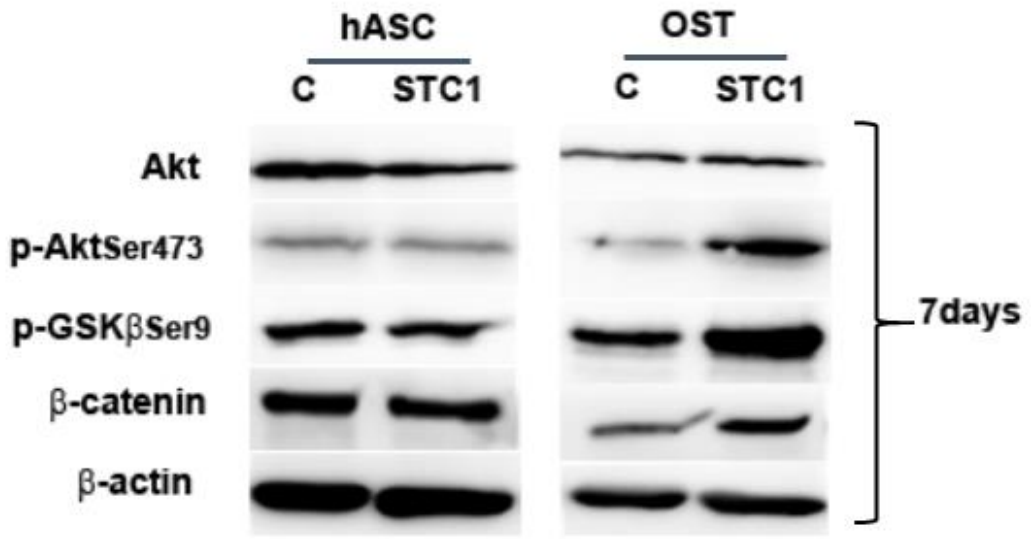

Figure 4: Effect of STC1(50ng/ml) on Akt, p-Akt$_{Ser473}$, p-GSKβ$_{Ser9}$ and β-catenin in hASC and 7-days preosteoblast. Representative Western blots of proteins studied are shown. (C=control.)

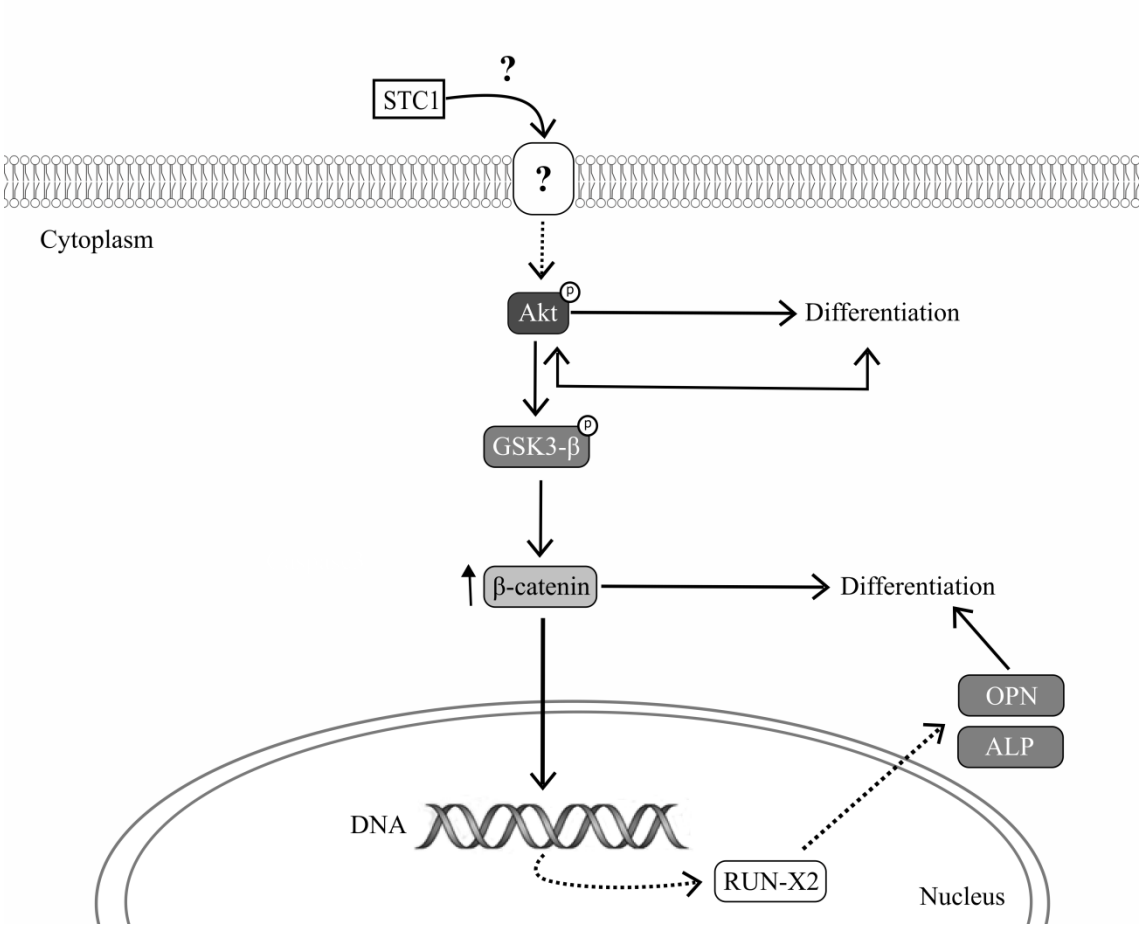

Figure 5: Schematic illustration showing an overall view of the proposed mechanism of STC1 in hASCs osteogenesis. For detailed explanation of this figure, see text. Question (?) represent unknown mechanisms. Upper arrow (↑) represent increased activity. Broken arrows represent proposed, not confirmed, mechanisms. STC1:stanniocalcin; Akt: Protein kinase B GSK3-β: Glycogen synthase kinase 3 beta; RUNX2: Runt-related transcription factor 2; OPN: Osteopontin ; ALP:Alkaline phosphatase.

II. 2 STC1 INTERFERENCE ON CALCITONIN FAMILY OF RECEPTORS SIGNALING DURING OSTEOBLASTOGENESIS VIA ADENYLATE CYCLASE INHIBITION

(Artigo publicado no periodico *Molecular and Cellular Endocrinology*)

Molecular and Cellular Endocrinology 403 (2015) 78–87

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

STC1 interference on calcitonin family of receptors signaling during osteoblastogenesis via adenylate cyclase inhibition

Silvia R. Terra [a,1], João Carlos R. Cardoso [b,1], Rute C. Félix [b,1], Leo Anderson M. Martins [a,1], Diogo Onofre G. Souza [a], Fatima C.R. Guma [a], Adelino Vicente M. Canário [b], Vanessa Schein [a,b,*]

[a] Department of Biochemistry, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 90003-035, Brazil
[b] Comparative Endocrinology and Integrative Biology, Centre of Marine Sciences, Universidade do Algarve, Faro 8005-139, Portugal

ARTICLE INFO

Article history:
Received 12 September 2014
Received in revised form 15 December 2014
Accepted 6 January 2015
Available online 12 January 2015

Keywords:
Stanniocalcin 1
Calcitonin gene-related peptide
Calcitonin gene-related peptide receptor
cAMP
Osteoblastogenesis

ABSTRACT

Stanniocalcin 1 (STC1) and calcitonin gene-related peptide (CGRP) are involved in bone formation/remodeling. Here we investigate the effects of STC1 on functional heterodimer complex CALCRL/RAMP1, expression and activity during osteoblastogenesis. STC1 did not modify *CALCRL* and *ramp1* gene expression during osteoblastogenesis when compared to controls. However, plasma membrane spatial distribution of CALCRL/RAMP1 was modified in 7-day pre-osteoblasts exposed to either CGRP or STC1, and both peptides induced CALCRL and RAMP1 assembly. CGRP, but not STC1 stimulated cAMP accumulation in 7-day osteoblasts and in CALCRL/RAMP1 transfected HEK293 cells. Furthermore, STC1 inhibited forskolin stimulated cAMP accumulation of HEK293 cells, but not in CALCRL/RAMP1 transfected HEK293 cells. However, STC1 inhibited cAMP accumulation in calcitonin receptor (CTR) HEK293 transfected cells stimulated by calcitonin. In conclusion, STC1 signals through inhibitory G-protein modulates CGRP receptor spatial localization during osteoblastogenesis and may function as a regulatory factor interacting with calcitonin peptide members during bone formation.

1. Introduction

In vertebrates, bone homeostasis is a dynamic process which includes a balanced calcium and phosphate metabolism, essential to maintain a healthy bone mass. Bone formation and remodeling rely on coordinated functions of bone forming (osteoblasts), resident (osteocytes) and resorbing cells (osteoclasts) that are regulated by several local and endocrine factors that act on calcium and phosphate homeostasis (Bellido et al., 2013; Henriksen et al., 2009; Nakahama, 2010; Stenbeck et al., 2012). Most of these calciotropic hormones mediate their action on osteoblasts regulating the osteoclastic differentiation and activity (Cao, 2011; Costa et al., 2011; Kohli and Kohli, 2011; Naot and Cornish, 2008; Stenbeck et al., 2012).

Stanniocalcin 1 (STC1) is a glycosylated 50 kDa disulfide-linked homodimeric protein (Trindade et al., 2009) that not only acts on bone metabolism, but also is highly abundant in other tissues such as kidney, heart, ovary and brain (Basini et al., 2010; Jiang et al., 2000; Westberg et al., 2007; Worthington et al., 1999; Yoshiko et al., 2002). During embryonic development, STC1 is highly expressed in muscular and skeletal tissues, exhibiting calciotropic effects on osteogenesis by stimulating the major constituents of the mineral phase of bone (Jiang et al., 2000; Stasko and Wagner, 2001; Wu et al., 2006; Yoshiko et al., 2002). The STC1 calciotropic effect is associated with osteoblastic differentiation (Johnston et al., 2010; Yeung et al., 2012; Yoshiko and Aubin, 2004; Yoshiko et al., 2003) and chondrogenic growth inhibition (Wu et al., 2006). Moreover, transgenic studies on mice have shown that human (h) STC1 ectopic expression decreases bone length (Varghese et al., 2002) leading to dwarfism (Filvaroff et al., 2002). The STC1 induced-delay in bone development is associated with its effect on the regulation of growth plate

Abbreviations: AC, adenylate cyclase; ADM1, adrenomedullin1; ADM2, adrenomedullin2 (intermedin); AMY, amylin; *CALCR*, human calcitonin receptor gene; CALCRL/RAMP1, calcitonin gene related peptide heterodimeric receptor complex; CALCRL/RAMP1-HEK293, calcitonin gene related peptide heterodimeric receptor complex expressing HEK293 cells; *CALCRL*, human calcitonin receptor like receptor gene; CGRP, calcitonin gene related peptide; CRSP, calcitonin receptor-stimulating peptide; CT, calcitonin; CTR, calcitonin receptor; CTR-HEK293, calcitonin receptor expressing HEK293 cells; FBS, fetal bovine serum; GPCR, G-protein-coupled receptor(s); hADSC, human adipose derived stem cell; hCGRP, human calcitonin gene related peptide; HEK293, human embryonic kidney cell lineage; hSTC1, human staniocalcin 1; IBMX, 3-isobutyl-1-methylxanthine; iCa^{2+}, intracellular calcium; PBS, phosphate buffered saline; PDE, phosphodiesterase; PKA, protein kinase A; RAMP1, receptor activity-modifying protein 1; *ramp1*, receptor activity-modifying protein 1 gene name; RT-PCR, semi quantitative real time reverse-transcriptase polymerase chain reaction; STC1, stanniocalcin 1.

* Corresponding author. Departamento de Bioquímica, Rua Ramiro Barcelos, 2600-Prédio Anexo, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 90003-035, Brazil. Tel.: +55 51 33085763; fax: +55 51 33165535.
E-mail address: 00107953@ufrgs.br (V. Schein).

[1] Authors that contributed equally to the work.

http://dx.doi.org/10.1016/j.mce.2015.01.010
0303-7207/

chondrogenesis, acting as a paracrine/autocrine growth factor (Wu et al., 2006). Furthermore, STC1 is also involved in angiogenesis (He et al., 2011). Recently, both STC1 mRNA and protein were shown to be up-regulated during synovial membrane inflammation in patients with osteoarthritis and was considered to be a key mediator of synovium neovascularization in osteoarthritis synovitis (Lambert et al., 2014).

Similar to STC, the calcitonin (CT) family members, a group of small peptide hormones, are also involved in calcium homeostasis, vertebrate osteogenesis and osteoblast function (Naot and Cornish, 2008). CT members share moderate sequence similarity but their secondary structures are strongly correlated. They are characterized by the presence of two conserved cysteine residue positions that form a ring structure at the *N*-terminus crucial for biological activity and receptor activation (Feyen et al., 1992; Naot and Cornish, 2008; Pozvek et al., 1997). In mammals, six CT family member peptides have been identified, which include calcitonin gene-related peptide alpha and beta (αCGRP and βCGRP), amylin (AMY), adrenomedullin 1 (ADM1), adrenomedullin 2 (ADM2 or intermedin) and calcitonin receptor-stimulating peptide (CRSP) (Granholm et al., 2011; Naot and Cornish, 2008) with distinct effects on bone cells.

Calcitonin gene-related peptide (CGRP) is a small neuroendocrine peptide (Amara et al., 1982; Goodman and Iversen, 1986) that, similar to STC1, has an important role in osteogenesis and osteoblast activity. CGRP is abundantly expressed in skeletal sensory neurons and is involved on bone formation and inhibition of bone resorption (Granholm et al., 2011; Schinke et al., 2004; Villa et al., 2006; Wang et al., 2010). Similar to CT, and different from ADM1, the members of this peptide family have a predominant anabolic action and inhibit bone resorption and osteoclast formation (Granholm et al., 2011; Naot and Cornish, 2008; Wang et al., 2010). In mammals, CGRP immunoreactivity is detected in bone tissue – in the periosteum, bone marrow and the epiphyseal trabecular bone, and the presence of CGRP in osteoblast cultures was found to increase cell proliferation and the synthesis of cytokines, growth factors, and collagen (Chang and Hsu, 2013; Vignery and McCarthy, 1996; Villa et al., 2006). An important stimulatory effect of CGRP during early stages of the osteoblastic differentiation has been also reported (Kawase et al., 2003; Wang et al., 2010). The receptor for CT (CTR) is also detected in osteoblasts. However the effect of CT in osteoblasts bone formation is not consensual (Monier-Faugere et al., 1996; Villa et al., 2003).

CGRP activates a specific receptor, CALCRL, a member of the class 2 B1 GPCR family which forms a dimer complex with a transmembrane accessory protein, the receptor activity-modifying protein 1 (RAMP1) (Born et al., 2002; Conner et al., 2002). CALCRL and RAMP1 have been detected in pre- and mature osteoblasts and, in the presence of CGRP, the protein complex signals via an increase in intracellular cAMP, calcium and insulin-like growth factors (Naot and Cornish, 2008; Togari et al., 1997; Uzan et al., 2004; Villa et al., 2006).

In contrast to CGRP, the molecular mechanisms that regulate STC1 action remain largely unknown. A specific STC1 receptor has not yet been identified, which makes the functional characterization of the protein more difficult. Based on the fact that calcitonin family peptides and STC1 act on bone metabolism, we hypothesize that their signaling pathways may interact with each other. Recently in the zebrafish embryo model, it was demonstrated that overproduction of CT peptide increased by 3-fold the expression of STC1 suggesting a feedback mechanism between the two hormones (Lafont et al., 2011). Thus, the present study was designed to better elucidate the role of STC1 in human bone formation/remodeling by investigating the potential role of STC1 on CGRP signaling during the differentiation of osteoblast, and to determine whether STC1 modulates CTR and the CGRP functional receptor. The abundance of CALCRL/RAMP1 transcript, the modulation of the receptor complex in cell plasma membrane and the influence of STC1 on CGRP intracellular signaling were studied using human pre-osteoblasts adipose derived stem cells and receptor transfected human embryonic kidney cell lines.

2. Materials and methods

2.1. Cell lines and culture maintenance

Commercially available Poietics™ human adipose derived stem cells (hADSC) isolated from lipoaspirates were purchased from Lonza (Lonza Group, Switzerland). Cells were maintained as adherent cultures in complete Dulbecco's modified Eagle's medium (DMEM, Sigma, Spain) supplemented with 10% fetal bovine serum (FBS), 0.1% penicillin:streptomycin antibiotic mix (10.000 U:10 mg/ml, Sigma, Spain) and 250 μg/ml sterile filtered 1:100 amphotericin B solution (Sigma, Spain) at 37 °C/5% CO_2. Media were changed every 3 days, and the cells were split when they reached 80–90% confluence. Cells were used at early passage (4–8 passages) for all experiments. Human Embryonic Kidney (HEK) 293 cells (ECACC collection, UK) were maintained at 37 °C/5% CO_2 in complete DMEM supplemented with FBS as previously described and divided every 3–4 days 80–90% confluence was reached.

2.2. hADSCs osteoblastic differentiation

hADSC were differentiated to pre-osteoblast for 7, 14, and 21 days, using osteoblastogenic differentiation medium consisting of DMEM supplemented with 10% FBS, 50 μM ascorbate-2-phosphate, 100 mM beta-glycerophosphate, and 0.1 μM dexamethasone, according to previously published techniques (Zuk et al., 2002). To confirm the osteogenic phenotype, differentiated cells were fixed in 4% PFA, rinsed with PBS and stained with Alizarin Red S for 5 minutes and imaged with light microscopy.

2.3. ATP assay

ATP concentration is used as a marker for cell viability because its concentration rapidly declines when cells undergo necrosis or apoptosis. hADSCs were differentiated to pre-osteoblast cells using the osteoblastogenic cocktail supplemented with increased doses of human STC1 (5.0, 50 and 2000 pM; hSTC1, Biovendor, Czech Republic) (Basini et al., 2009) to eliminate the hypothesis that STC1 could induce cell death during osteoblastogenesis. The effects of hSTC1 on the intracellular ATP pool were measured on day 7 after the beginning of the osteoblastic induction. After the experimental period, cells were collected and immediately frozen in liquid nitrogen prior to homogenization in lysis buffer (TCA 6%, NaF 20 mM, Gelatin 0.4 $g.l^{-1}$). ATP was determined using the bioluminescent luciferin kit (Invitrogen, USA) according to the manufacturer's protocol. Results obtained were expressed as intracellular ATP concentration (pM).

2.4. Isolation and cloning of CALCRL and ramp1

The human *CALCRL* and *ramp1* mRNAs were isolated from hADSCs differentiated to osteoblast cells. The human *CALCRL* full-length transcript was amplified from cDNA (see discussion later) using the primers forward 5′ atggagaaaaagtgtaccctgaat and reverse 5′ tcaattatataaattttctggttttaag (annealing temperature 54 °C, 35 cycles) and *ramp1* with primers forward 5′ atggcccgggccctgtgccgcct and reverse 5′ ctacacaatgccctcagtgcgcttg (annealing temperature 64 °C, 40 cycles). All PCR reactions were performed using the Taq DNA Polimerase High Fidelity (Invitrogen, USA) for a final reaction of 50 μl according to the manufacturer's protocol. PCR products obtained were run on 1% agarose/1× TBE gels and products of the correct size

were cloned in pGEM T-easy vector (Promega) and sequenced to confirm identity. The human *CALCRL* and *ramp1* transcripts were amplified from the cloning vector using the iProof DNA polymerase (BioRad, Portugal) according to the previously described conditions and the PCR products obtained were purified and subcloned into the expression pcDNA3.1 vector (Directional TOPO Expression Kit, Invitrogen, USA). The ligation product was used to transform DH5-alpha *E. coli* bacteria. Bacteria containing the correct construct were selected and recombinant plasmids were extracted using the GeneJet TM plasmid Miniprep kit (Fermentas, Portugal). All purified plasmids were used to transfect human HEK293 cells for intracellular signaling assays.

The human calcitonin receptor (CTR; *CALCR gene*) clone in pcDNA 3.1 expression vector was purchased from Missouri S&T cDNA resource Center, USA (catalog number CALCR 00000) and used in the transfection assays (as described earlier) as a model of comparison on cAMP assays (see discussion later).

2.5. RNA extraction, cDNA synthesis and quantitative expression

To analyze the effect of hSTC1 on *CALCRL* and *ramp1* mRNA expression during human osteoblastogenesis, hADSCs cells were differentiated to osteoblasts for up to 21 days using the osteoblastogenic cocktail supplemented with 2 nM hSTC1. Cells were collected at days 7, 14 and 21. Briefly, semi-confluent cultures of undifferentiated hADSCs and pre-osteoblast cells were washed three times with 1× PBS and harvested by centrifugation. Total RNA was extracted with Trizol (Invitrogen, USA) according to the manufacturer's instructions. RNA quantity was assessed by absorbance in the BioPhotometer plus (Eppendorf, USA) and quality was checked by electrophoresis on 1.5% agarose gels.

cDNA was synthesized with 2 μg of total RNA in a reaction mixture containing 5 mM of DTT, 0.5 mM dNTPs mixture (Ludwig Biotech, Brazil), 1.0 μg of random hexamers (pd(N)6), 10 U rRNAsin RNase inhibitor (Promega, USA), 50 U MMuLV reverse transcriptase (Thermo Scientific, USA) for a 20 μl final reaction volume complemented with sterile distilled water. The cDNA synthesis reaction was performed as follows: 10 min at 25 °C followed by 120 min at 37 °C and finally 10 min at 65 °C to inactivate enzyme activity.

Semi quantitative real-time reverse-transcriptase PCR (RT-PCR) analyses were run using specific primer pairs for *CALCRL* and *ramp1*. Primer sequences for RT-PCR were designed using the IDT Design Software (Integrated DNA Technologies Inc., USA) to distinguish exons. Primer sequences were assessed for specificity using non-redundant basic local alignment search tools (www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1990) and target-specific sequence alignment programs (Espinosa et al., 2013). *Tbp* was used as reference gene and the results presented relative to *Tbp* expression. Primer pairs were: *CALCRL* forward 5′ gatttccactgattcctgcttg (exon 11); reverse 5′ ggcaccaagataagagtagctc (exon 13) (amplicon size: 218pb; NM_005795.4); *ramp1* forward 5′ atcacctcttcatgaccactg (exon 2); reverse 5′ cctgtccacctctgcatt (exon 3) (amplicon size: 218pb; NM_005855.2). RT-PCR reactions were performed with 0.1 μM of each specific primer, 0.5 ng/μl cDNA and Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen, USA). The thermal cycling conditions used were: 2 min at 50 °C, 2 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s. Under these conditions, RT-PCR produced a single product at the melting temperature for each analyzed gene. Control reactions (cDNA synthesis reaction without reverse transcriptase) were performed to verify genomic DNA contamination. The mean Ct values from triplicate measurements were used to calculate expression of the target gene, with normalization to internal control (*Tbp*) using the $2^{-\Delta\Delta Ct}$ formula (Schmittgen and Livak, 2008). All gene expression results are based on 3 independent experiments.

2.6. Laser-scanning confocal microscopy

To quantify the CALCRL and RAMP1 protein expression on plasma membrane of hADSCs and 7-days differentiated pre-osteoblasts and to verify the effects of hSTC1 peptide on the distribution pattern of the CGRP heterodimeric receptor complex, an immunocytochemistry assay was performed and confocal microscopy analysis was carried out. Briefly, hADSC were cultured at the aforementioned conditions on 4-chamber glass bottom dishes and were differentiated to pre-osteoblasts during 7 days without hSTC1. Sequentially, hADSCs and pre-osteoblasts were exposed to 2.0 nM of hSTC1 or 2.0 nM of human CGRP for 30 min at 4 °C (hCGRP, cat. number C 0167; Sigma-Aldrich, São Paulo, Brazil). A number of studies that describe the biological effects of STC1 *in vitro* used STC1 at 0.1–15 nM, however, the STC1 effects at doses ranging from 1 nM to 10 nM were found to be very similar (Sazonova et al., 2008; Zlot et al., 2003) . Thus, the concentrations of STC1 and hCGRP used in the experiments (2 nM) were chosen based on studies of Granholm et al. (2011), Zlot et al. (2003), Yoshiko et al. (2003) and Wang et al. (2010). The hCGRP peptide was used as a positive control since it is the native ligand of the heterodimeric CALCRL/RAMP1 receptor complex (Bailey et al., 2012; Hay et al., 2005; McLatchie et al., 1998; Naot and Cornish, 2008).Unexposed cells were used as control. After hSTC1 or hCGRP exposure, cells were washed and incubated with rabbit polyclonal antibody against human CALCRL (clone H-42, cat. no. SC30028) and goat polyclonal antibody against human RAMP1 (clone N-20, cat. no. SC8050) (both from Santa Cruz Biotechnology, USA) diluted in a PBS/blockage solution with 5% of FBS plus 1% of bovine serum albumin (BSA) at 4 °C for 1 h to reduce background staining. Further, cells were washed, fixed with 4% paraformaldehyde in PBS for 20 min before incubation at 4 °C with anti-rabbit antibody conjugated with the green fluorescent Alexa Fluor® 488 and anti-goat antibody conjugated with red fluorescent Alexa Fluor® 555 (both from Life Technologies, USA), which were used to label the primary antibodies for CALCRL and RAMP1, respectively (Vilcaes et al., 2011). Ten single confocal sections of 0.7 μm were taken parallel to the coverslip (*xy* sections) on the Olympus FV1000 laser-scanning confocal microscopy equipped with an ×60 (numeric aperture 1.35) oil-immersion objective (Olympus, U plan-super-apochromat, UPLSAPO60XO). The CALCRL fluorescence was acquired after excitation by a 488 nm laser beam and the emission scan collected at 520 nm. The RAMP1 fluorescence was acquired after laser excitation at 555 nm and emission collected at 647 nm. Imaging analysis is based on 3 independent experiments. Images of three fields of each sample were acquired with Olympus FluoView FV1000 software. Imaging pre-processing (iterative deconvolution) and fluorescence intensity measurement were performed using the public domain Java Image processing software imageJ (http://rsb.info.nih.gov/ij/). Fluorescence emission for CALCRL and RAMP1 was measured by establishing multiple regions of interest (ROI) that surrounded cell boundary thus intending to reduce background interference in all acquired images. To confirm the viewed proximity between CALCRL and RAMP1, co-localization analysis was performed in all images using the JACoP plugin as previously described (Bolte and Cordelieres, 2006; Dunn et al., 2011; Zinchuk et al., 2007). Pearson's coefficients were calculated considering Costes's automatic threshold that seeks to reduce nonspecific correlations (Costes et al., 2004).

2.7. Mammalian cell transfections with the CGRP heterodimeric receptor complex CALCRL/RAMP1

On the day prior to transfection, 2–3 × 10^5 cells were seeded in 6-well plates (Sarstedt, Portugal) and cells were transiently transfected with Fugene 6 transfection reagent (1:6 DNA:Fugene, Roche; USA) according to the manufacturer's protocol. Briefly, the

DNA transfection complex was incubated for 40 min at room temperature before adding to the cells. Cells with the transfection complex were incubated with 500 μl of DMEM medium for 6 h prior to addition of complete medium to a final volume of 2 ml per well.

Human *CALCRL* was transfected in a human RAMP1 stable HEK293 cell line. The stable RAMP1 cell line was generated using the linearized expression construct and 72 h after incubation, cells were selected by supplementing the complete medium with 800 μg/ml of the antibiotic geneticin (G418 sulphate, Gibco® Life Technologies, USA). The human RAMP1-pcDNA3 expression plasmid was constructed using a methodology similar to that previously described for the receptors. Prior to transfection, 5 μg of the plasmid was linearized using the NotI enzyme (Thermo Scientific, Fermentas, Portugal) and purified using the standard phenol:chloroform method. Cell recovery was monitored daily by constant changes of medium until no cell death was observed. RT-PCR using *ramp1* specific primers confirmed gene integration and transcript expression. The efficiencies of the cell transfections were obtained by performing a simultaneous transfection using the pCMV-EGFP vector (Clontech, USA). The human CTR was transiently transfected on wild HEK293 cells using the same protocol described earlier and used for comparisons with *CALCRL*. The receptor pharmacological responses to the hSTC1, hGCRP or human CT (human calcitonin cat no. T3535, Sigma, Spain) peptides to activate the human CALCRL-RAMP1 and CTR on cAMP-signaling pathway were assayed 72 h post transfections.

2.8. cAMP-signaling pathway

The capacity of the human STC1, CGRP and CT peptides to activate transfected CALCRL/RAMP1 and CTR HEK293 transfected cells and human 7 days pre-osteoblast cultures was measured by quantification of intracellular cAMP using the cAMP dynamic 2 kit (Cisbio, France) following the manufacturer's protocol. Approximately 15,000 of transfected cells/well were assayed and peptide incubations were performed in white 384 well small Volume™ HiBase Polystyrene microplates (Greiner, Germany). The osteoblast cell line was also accessed for cAMP production in the presence of the hSTC1 and hCGRP peptides using cells that derived from a 100% confluent culture on a T25 bottle due to their slow growth and difficulty to dislodge from the plastic.

Cell assays were performed for a final reaction volume of 20 μl. Prior to the assay, cells were re-suspended in 1× PBS with 1 mM of 3-isobutyl-1-methylxantine (IBMX, Sigma, Spain) and incubated for 5 min at 37 °C. Peptides diluted to a final concentration of 1–0.1 μM in 1× PBS/1 mM IBMX were added to the cells and incubated for 30 min at 37 °C in the CO_2 incubator. Non-transfected HEK293 cells were used as negative control and were stimulate with the highest STC1, CGRP and CT peptide concentration (1 μM). Forskolin (5 μM, Sigma, Spain) was used as positive control to assess cellular responsiveness of the cAMP pathway. For the calculations, two additional assay controls were also performed according to the manufacturer's instructions: (a) a negative background control without cAMP-d2 and (b) a non-stimulated control (no peptide added). Plates were read using a Biotek Synergy 4 plate reader (Biotek, USA) and results of cAMP stimulation were normalized according to the manufacturer's recommendations for data analysis. To test the peptides capacity to stimulate cell cAMP production, assays were initially performed using 1 μM and 0.1 μM of the hSTC1 and hCGRP on CGRP receptor complex and 1 μM and 0.1 μM of the hSTC1 and 0.1 μM of CT on the CTR transfected cells. The doses selected for the assays were based on the cell responsiveness to peptide stimulation and previous studies (Granholm et al., 2011; Wang et al., 2011).

To assess the effect of hSTC1 on the inhibition of cAMP production, native HEK293 cells were initially stimulated with 5 μM of forskolin for 30 min to achieve maximum cAMP production and then incubated for an additional 30 min with 0.1 μM or 1 μM of hSTC1. Maximum cAMP production of the receptor transfected cells was also stimulated by initially incubating CALCRL/RAMP1 and CTR HEK293 transfected cells for 30 min with 0.1 μM of the hCGRP and CT peptides, respectively followed by 30 min with 0.1 μM or 1 μM of hSTC1. To access if hSTC1 utilizes the CALCRL/RAMP1 complex and inhibits CGRP binding, transfected cells were also initially stimulated with 0.1 μM of hSTC1 for 30 min followed by 0.1 μM or 1 μM of hCGRP peptide for 30 min. Similar assays for the CTR were also performed using 0.1 μM or 1 μM of hCT peptide for 30 min. The amount of cAMP produced by non-transfected receptor cells was subtracted and assay data were normalized as percentage of cAMP accumulation. The effect of the empty receptor vector in cell signaling was also tested and values were equivalent to the peptide assays using non-transfected cells.

2.9. Intracellular calcium release

The effect of hSTC1 to stimulate intracellular Ca^{2+} (iCa^{2+}) release (RFU) was measured using the Ca^{2+} sensitive fluorescent dye Fluo-4 NW (Molecular Probes, Invitrogen, USA) according to the manufacturer's instructions on hADSCs, 7 days pre-osteoblast cultures, receptor transfected cell lines CALCRL/RAMP1 and CTR. Prior to the assay, plates (96 well black/plates, μClear bottom, Greiner, Germany) were coated with sterile poly-L-lysine (0.1 mg/ml, Sigma, Spain) to avoid cell release. Approximately 50,000 cells re-suspended in 100 μl of complete DMEM medium were plated per well and were allowed to attach overnight at 37 °C in a humid 5% CO2 incubator. Prior to the assay, growth medium was removed and cells were washed twice with 1× PBS and incubated for 30 min at 37 °C with 100 μl of Fluo-4 NW dye followed by an additional 30 min incubation period at RT. The background RFU of each well was measured prior to addition of the peptide and receptor response was carried out using 0.1 μM of hSTC1 peptide diluted in assay buffer. Calcium mobilization provoked by the presence of the peptide was measured every 10 s over a total period of 2 min on the plate reader. Positive control assays were performed with 100 nM Carbachol (Sigma-Aldrich, Spain) and negative control assays were performed using non-transfected HEK293 cells stimulated with 0.1 μM of hSTC1. Background fluorescence was measured in each assay by adding 10 μl of assay buffer. Maximal RFU values were used for calculations, which entailed subtraction of background values and data normalization before plotting RFU values against peptide concentration.

2.10. Statistical analysis

Statistical analysis was performed using SigmaStat (v. 3.50, Systat Software, Inc, San Jose, CA, USA). Gene expression and cAMP data were evaluated using one-way or two-way ANOVA when appropriated, followed by the Bonferroni multiple comparison procedures. Before applying the ANOVA test, data were checked for normality (Kolmogorof–Smirnov test) and homogeneity (Levene test). In the absence of homogeneity, data were log-transformed prior to the ANOVA test. Protein expression was analyzed by Student's *t*-test (see Supplementary material). Data are presented as the mean ± standard error of the mean (SEM) and the level of statistical significance was $P < 0.05$. Each experiment was repeated three to six times.

3. Results

3.1. Effect of hSTC1 on osteoblasts ATP levels

Increased concentrations of hSTC1 (5.0, 50 and 2000 pM) during osteoblastogenesis did not modify the intracellular levels of ATP, indicating that hSTC1 peptide incubations did not affect human osteoblast cell viability (see Supplementary material, Fig. S1).

3.2. Effect of hSTC1 on CGRP signaling

3.2.1. Effect on CGRP receptor complex expression

CALCRL gene expression was low in undifferentiated hADSC and did not change over time. However, in differentiating cells and hSTC1-treated cells, the expression of *CALCRL* was significantly elevated at 7 days compared to undifferentiated hADSC and by 21 days levels were similar between the three groups of cells (see Supplementary material, Fig. S2, A). *Ramp1* gene expression was generally low and only elevated in untreated differentiated cells at 7 and 14 days (see Supplementary material, Fig. S2, B). *Ramp1* transcript expression was not affected by STC1 peptide treatment.

CALCRL and RAMP1 proteins were present at the plasma membrane cell surface of hADSCs and 7-day pre-osteoblast cells, as revealed by immunofluorescence (Figs. 1 and 2 and Fig. S3). CALCRL expression was highest at day 7 compared to non-differentiated hADSC, in agreement to the mRNA expression results. There was no statistical difference in RAMP1 protein expression during the same time period (see Supplementary material, Fig. S3).

3.2.2. Effect of hSTC1 or hCGRP on the CGRP heterodimeric receptor complex CALCRL/RAMP1

The expression of the CGRP heterodimeric receptor complex on the cell membrane surface was evaluated in hADSCs (Fig. 1) and in 7-day pre-osteoblast cells (Fig. 2) by confocal microscopy in the absence and presence of hSTC1 and hCGRP peptides. The overall co-localization between the CALCRL/RAMP1 monomers was confirmed by calculating Pearson's coefficient and can be observed in orange in the merged images that were obtained by superimposition of the green- and red-labeled fluorescent images (Figs 1 and 2). Incubation with 2 nM of hSTC1 or hCGRP for 30 min modified the spatial localization of CALCRL and RAMP1, eliciting a cluster of the receptor complex in specific areas at the surface of both types of cells, indicating a similar effect of both hSTC1 and hCGRP peptides.

3.2.3. Effect of hSTC1 or hCGRP on cAMP and iCa^{2+} cell signaling

Incubation of 7-day differentiated human osteoblast cells with 0.1 μM hCGRP, but not hSTC1, promoted cAMP accumulation. This indicates that CGRP receptor complex is expressed and functional at the osteoblast cell plasma membrane and suggests that the effect

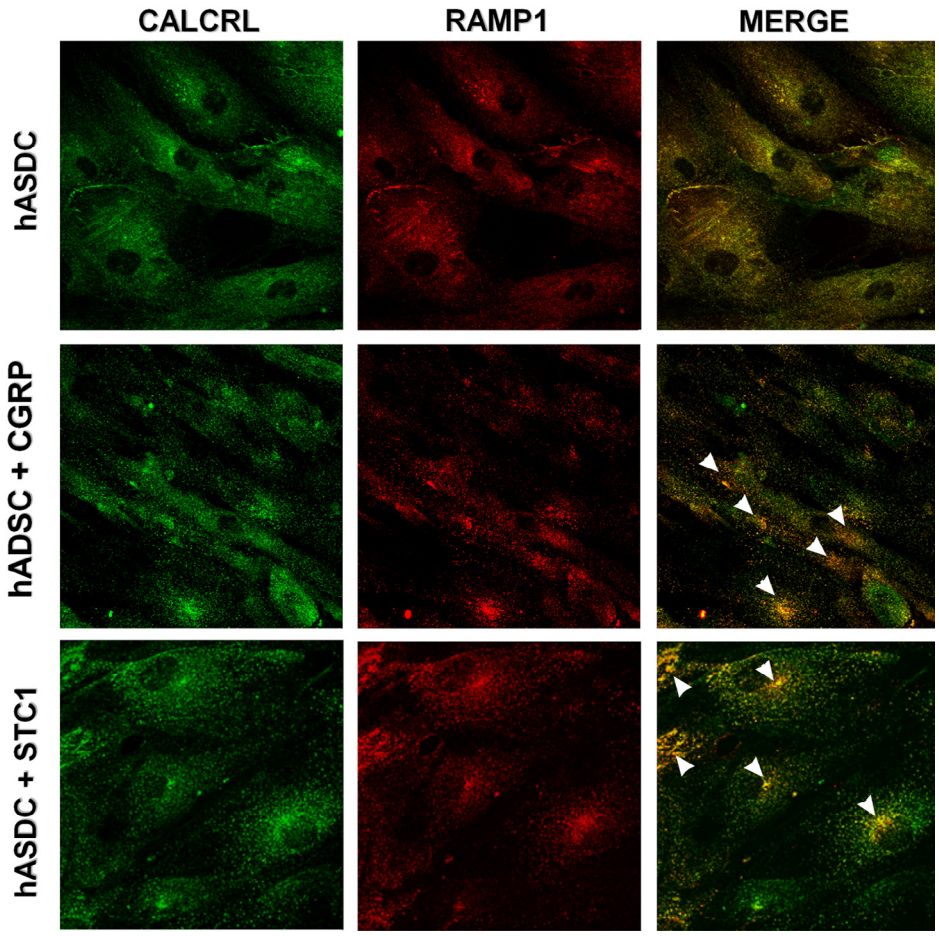

Fig. 1. Spatial distribution of CGRP heterodimeric receptor in the presence of hCGRP and hSTC1 on the cell surface of human adipose derived stem cells (hADSCs) demonstrated by immunocytochemistry. Green fluorescence indicates the presence of CALCRL receptors; red fluorescence indicates RAMP1 accessory protein. Overlapping of CALCRL and RAMP1 images (MERGE) revealed that both proteins are in the same cellular microcompartment. hADSCs were grown on 4-chamber glass bottom dishes, then exposed to hCGRP (2 nM) or hSTC1 (2 nM) diluted in DMEM for 30 min. Unexposed cells were used as negative control (hASDC). Cells were immunostained for CALCRL and RAMP1 as indicated under Section 2. Both proteins seem to be close to each other at the cell surface of unexposed hADSCs. Incubation with STC1 or CGRP modified the spatial localization of CALCRL and RAMP1, eliciting a concentration of the receptor complex in specific areas at the cell surface (white arrows head). Pearson's coefficients of correlation for all the overlapping CALCRL and RAMP1 images were: 0.65 ± 0.02 for control; 0.64 ± 0.01 for hCGRP and 0.55 ± 0.03 for hSTC1. The fluorescence images are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

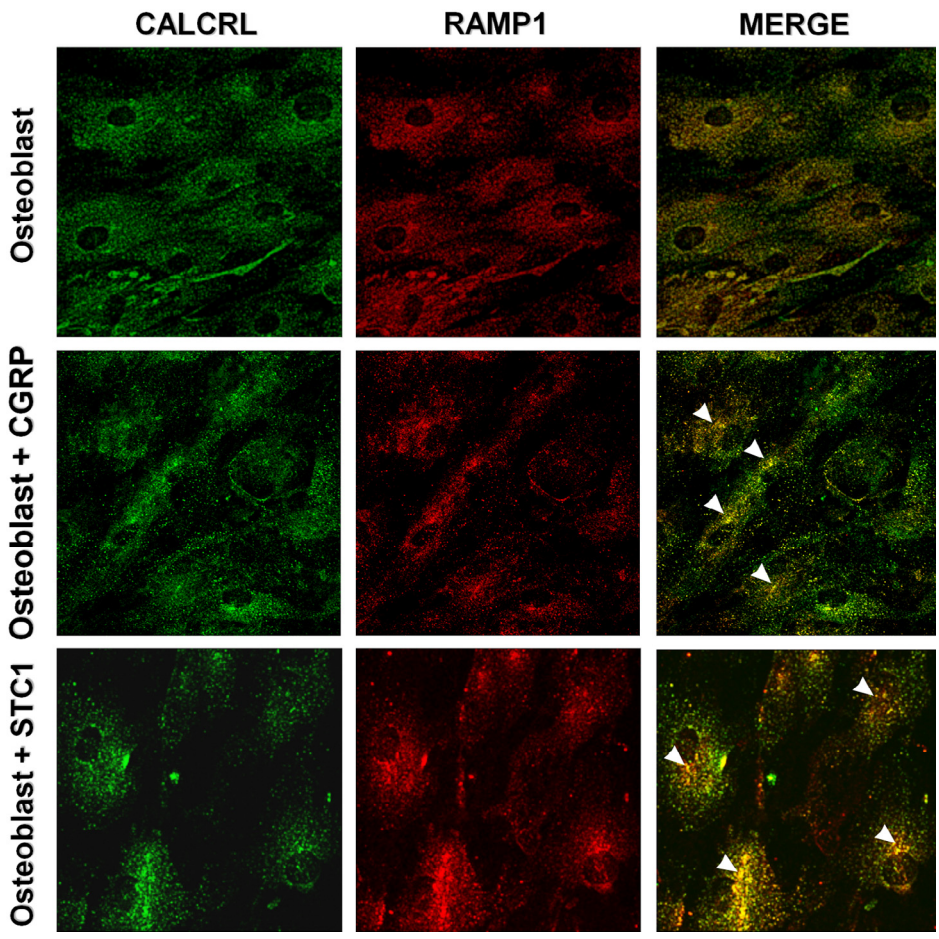

Fig. 2. Spatial distribution of CGRP heterodimeric receptor in the presence of hCGRP and hSTC1 on the cell surface of pre-osteoblasts demonstrated by immunocytochemistry. Green fluorescence indicates the presence of CALCRL receptors; red fluorescence indicates RAMP1 accessory protein. Overlapping of CALCRL and RAMP1 images (MERGE) revealed that both proteins are in the same cellular microcompartment. hADSCs were differentiated to 7-days pre-osteoblasts on 4-chamber glass bottom dishes, then exposed to hCGRP (2 nM) or hSTC1 (2 nM) diluted in osteoblastogenic cocktail for 30 min. Unexposed 7-days pre-osteoblasts were used as negative control. Cells were immunostained for CALCRL and RAMP1 as indicated under Section 2. Both proteins seem to be close to each other at the cell surface of unexposed 7-days pre-osteoblasts. Incubation with hSTC1 or hCGRP modified the spatial localization of CALCRL and RAMP1, eliciting a concentration of the receptor complex in specific areas at the cell surface (white arrows head). Pearson's coefficients of correlation were 0.64 ± 0.02 for control pre-osteoblasts, 0.64 ± 0.02 for the hCGRP peptide incubations and 0.71 ± 0.01 for hSTC1 peptide incubations, suggesting that the presence of hSTC1 favored the assembly of the CALCRL and RAMP1 complex. The fluorescence images are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CGRP in osteoblasts involves the cAMP pathway (Fig. 3A) but the signaling of STC1 remains unknown.

In vitro studies with HEK293 cells were used to clarify the potential interference of hSTC1 in hCGRP signaling. The hSTC1 failed to increase the cAMP levels in native HEK293 cells (Fig. 3B). However, in HEK293 cells first stimulated with forskolin to induce cell maximal cAMP accumulation, hSTC1 decreased cAMP levels in a dose-dependent manner (Fig. 3C). In contrast incubation with CGRP failed to induce cAMP accumulation (not shown), confirming that HEK293 do not express the CGRP functional complex (Atwood et al., 2011). These results indicate that a putative STC1 receptor is naturally expressed in HEK293 cells, suggesting that the observed hSTC1 signaling is not via CGRP functional receptor complex (Fig. 3C).

hCGRP was also able to activate cAMP accumulation in HEK293 CALCRL/RAMP1 transfected cells and an increase of cAMP levels was obtained when 1.0 μM of hCGRP was used in comparison to 0.1 μM (Fig. 4A). In contrast, no signal was obtained when hSTC1 was incubated with HEK293 CALCRL/RAMP1 transfected cell lines. *In vitro* cell assays using HEK293 expressing the CTR were also performed to compare the effect of STC1 in CGRP functional complex. The hCT peptide also stimulated cAMP in CTR transfected cells (Fig. 4B) but not in non-transfected HEK293 (not shown) conforming that HEK293 also do not express the CT receptor (Atwood et al., 2011). Similar to CALCRL/RAMP1, incubations using hSTC1 in HEK293 CTR transfected cells primed with hCT also induced a decrease of the cell cAMP levels (Fig. 4B).

CALCRL/RAMP1 and CTR transfected cells were also stimulated using different combinations of hCGRP/hCT and hSTC1 peptides to investigate the existence of potential peptide interactions by accessing the receptor response on cAMP production. In cells pre-incubated with 0.1 μM of hCGRP, addition of 0.1 μM and 1.0 μM of hSTC1 does not seem to modify cAMP accumulation. However, a dose-dependent decrease in cAMP accumulation was observed in hCT stimulated cells when hSTC1 was added (Fig. 4A and B). Similarly when receptor transfected cells were pre-incubated with hSTC1 (0.1 μM), addition of hCGRP and hCT also show different responses on the cell levels of cAMP. The presence of 1.0 μM of hCGRP significantly increased the cell cAMP ($p < 0.05$) to levels higher than 1.0 μM hCGRP alone (Fig. 4A) but a significant reduction ($p < 0.05$) of cAMP was observed in cells first primed with hSTC1 and subsequently with hCT when compared to hCT at 0.1 μM and 1.0 μM (Fig. 4B).

We also investigated the receptor response to intracellular calcium mobilization in the presence of hSTC1. The 7-day differentiated

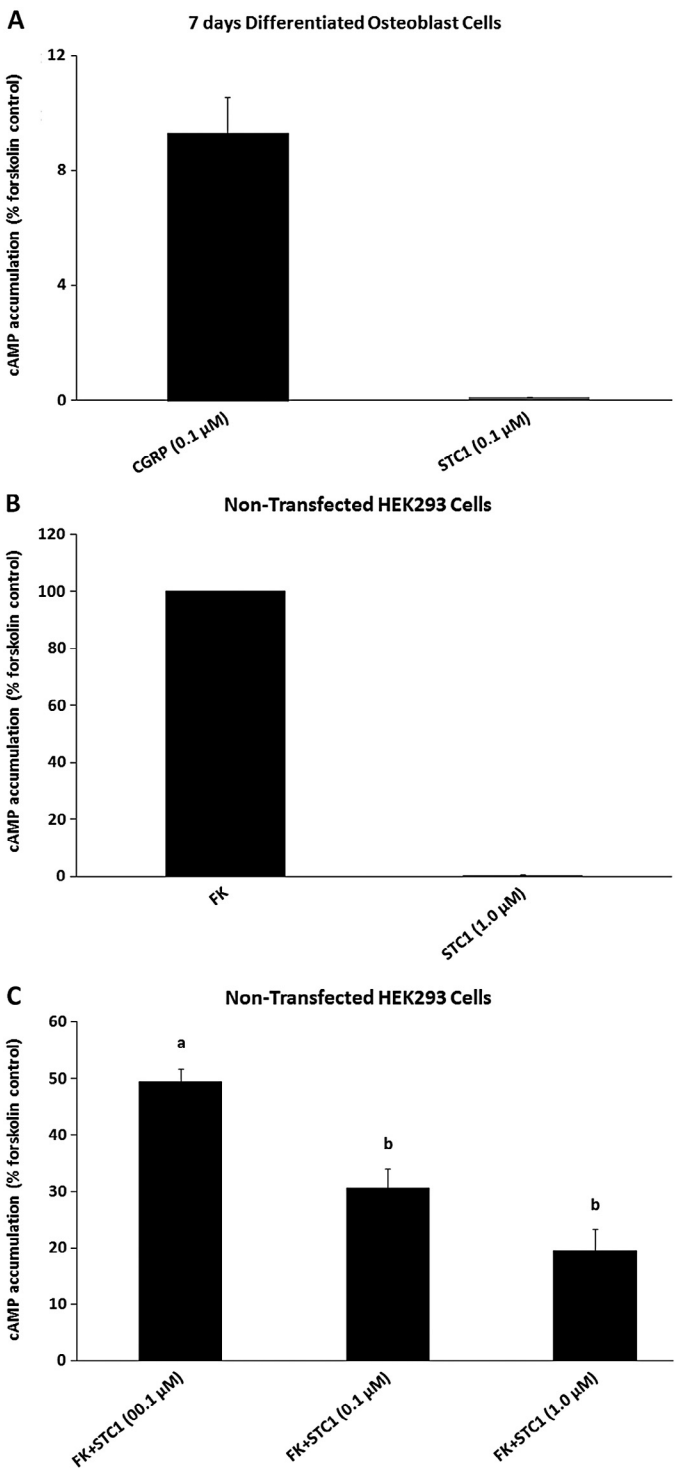

Fig. 3. Intracellular cAMP induced by hCGRP and hSTC1 peptides. (A) cAMP accumulation in pre-osteoblast cells differentiated from human adipose derived stem cells (hADSCs) after incubation with hCGRP (0.1 μM) and hSTC1 (0.1 μM). cAMP was measured after treatment with forskolin (FK, 5 μM), CGRP or STC1 for 30 min. (B) cAMP accumulation in non-transfected HEK293 cell lines stimulated by FK (5 μM) and hSTC1. (C) FK treated cells were subsequently incubated with STC1 (0.01 μM, 0.1 μM and 1.0 μM) for 30 min. Statistical differences were estimated using one-way ANOVA and different letters above bars indicate statistical significance of the 5% level.

human osteoblasts, native and transfected HEK293 cells expressing the human CGRP heterodimeric complex receptor or CTR receptor incubated with 0.1 μM of hSTC1 failed to mobilize calcium.

4. Discussion

The present study demonstrates that during the early period of osteoblastic differentiation, the hADSCs pre-osteoblast derived cells express the highest levels of the CALCRL/RAMP1 heterodimer complex. Furthermore, it shows that hSTC1 is able to elicit clustering of the CALCRL/RAMP1 heterodimer complex receptor in specific areas at the cell membrane. STC1 signal via inhibition of cAMP levels and the presence of the STC1 do not seem to affect cAMP levels induced by hCGRP, but inhibit the action of hCT via CTR.

Several lines of evidence indicate that STC1 (Yoshiko et al., 2002, 2003) and CGRP (Schinke et al., 2004; Wang et al., 2010) are associated with the same processes in osteoblast cells. In the absence of a cognate STC1 receptor we focus on the potential interaction between STC1 and CGRP signaling during osteoblastic differentiation.

The CGRP receptor is a dimeric complex of CALCRL and RAMP1, needing the presence of both for peptide activation (Aiyar et al., 1996; McLatchie et al., 1998; Roh et al., 2004; Smillie and Brain, 2011). The CALCRL/RAMP1 complex was detected in several osteoblastic lineages, including mouse MC3T3-E1, MG63 osteoblastic cell lines, rat primary calvarial osteoblasts and human primary osteoblast cell cultures (Schinke et al., 2004; Togari et al., 1997; Uzan et al., 2004; Villa et al., 2006). The effects of CGRP in osteoprogenitor cells are predominant in the earlier stages of the differentiation process (in the first 2 weeks) prior the mineralization onset (Naot and Cornish, 2008; Wang et al., 2010).

When osteoblast cells are stimulated by CGRP, the CALCRL/RAMP1 heterodimeric complex increases adenylate cyclase (AC) activity and, consequently, intracellular cAMP levels (Chang and Hsu, 2013; Kawase et al., 2003; Villa et al., 2006). In contrast, in the present study, hSTC1 alone had no effect on *CALCRL* and *Ramp1* gene expression and iCa^{2+} release in any of the cell types studied (undifferentiated, differentiating and HEK293 cells). Moreover, it inhibited cAMP buildup in HEK293 cells primed by forskolin but does not seem to modify cAMP levels on CALCRL/RAMP-transfected cells when hCGRP is present. This indicates that HEK293 cells express a functional STC1 receptor and that the presence of STC1 does not seem to modify CGRP functional complex response to CGRP. In contrast, STC1 inhibits CT induced intracellular cAMP formation in CTR over-expressing HEK293 cells. In fact, the results obtained in our study provide evidence that hSTC1 seems to interfere with hCT potency for cAMP production. Moreover we were able to demonstrate that the presence of STC1 inhibits CT peptide function by decreasing the peptide potency to produce cAMP in cells in both combinatory *in vitro* peptide assays performed. The distinct effect of hSTC1 on cAMP levels elicited by hCGRP and hCT peptides may be related with the different capacities that both peptides had on cAMP production *in vitro* and that hCT was more potent than hCGRP in the HEK293 cell assay. Cross regulation of CT and STC has previously been suggested and an increase of CT peptide production in zebrafish eggs was shown to stimulate the increase expression of STC1 transcript resulting in a decrease of total calcium (Lafont et al., 2011). The fish data may indicate that the action of CT and STC1 may be coordinated in tetrapods and that STC1 may inhibit the role of CT *in vivo*, as suggested by our cAMP *in vitro* assays.

Since intracellular cAMP accumulation and calcium mobilization were not detected in response to STC1, it is unlikely that STC1 signaling occurs through a Gαs or Gαq coupled-GPCR in these cell lines. Despite previous suggestions of STC1 activation and signaling via cAMP–PKA pathway in fish tubular proximal cells (Lu et al., 1994), or improved calcium waves evoked by extracellular ATP (Block et al., 2010). Our results indicate that STC1 inhibits cAMP accumulation possibly through a Gαi coupled-receptor signaling or through unrelated GPCR receptor(s). The inhibition of cAMP synthesis by Gαi results in decreased activity of cAMP-dependent protein kinase (Skalhegg and Tasken, 2000). It appears that STC1 may bind to a Gαi

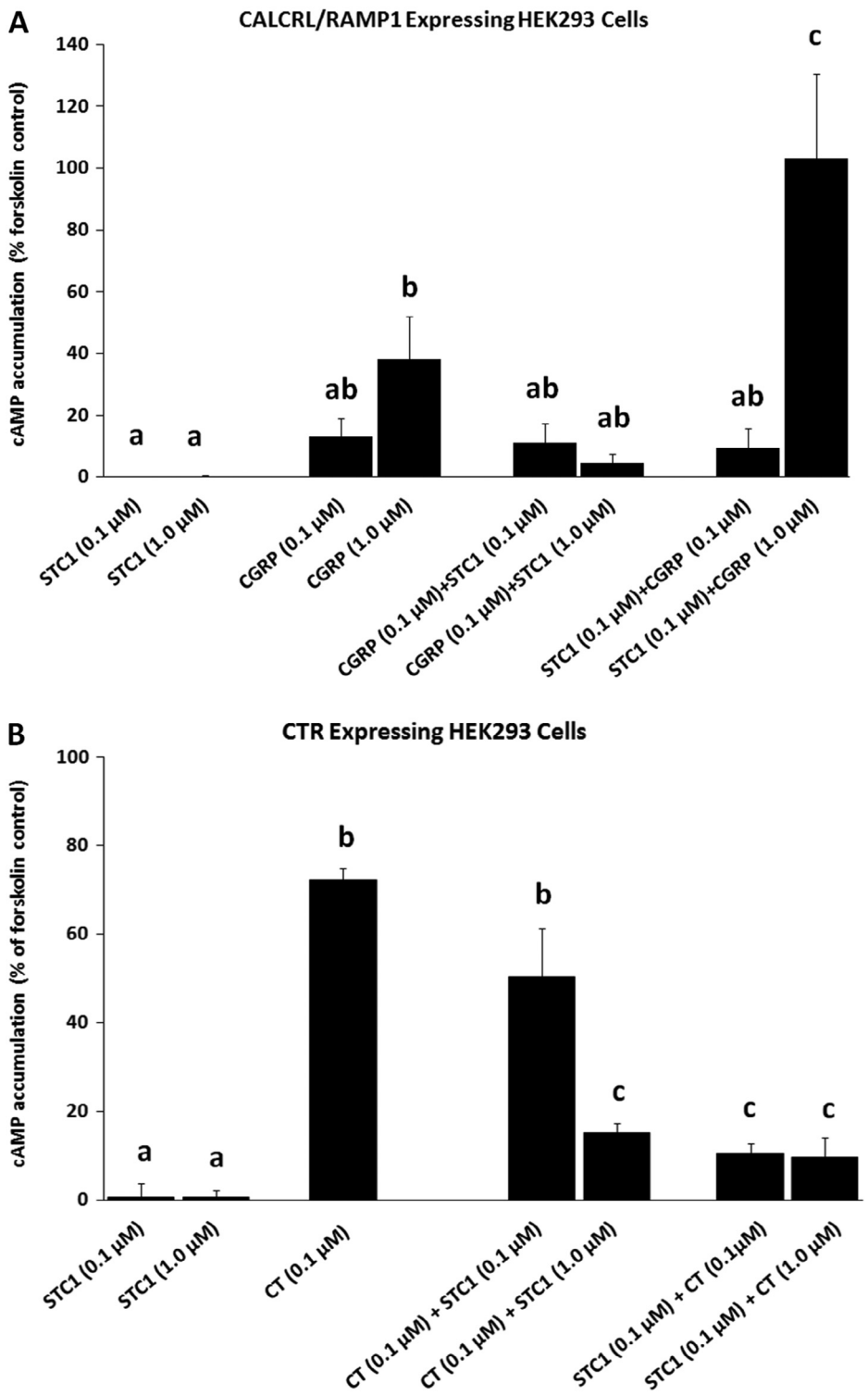

Fig. 4. cAMP accumulation in CALCRL/RAMP1 and CTR expressing HEK293 cell lines stimulated by hSTC1, hCGRP and hCT. (A) CALCRL/RAMP1 transfected cells were treated with hSTC1 (0.1 μM and 1.0 μM) or hCGRP (0.1 μM and 1.0 μM) for 30 min before cAMP measure. To examine if hSTC interferes with hCGRP cAMP production, cells were pre-treated with hCGRP (0.1 μM) or with hSTC1 (0.1 μM) for 30 min and subsequently with hSTC1 (0.1 μM and 1.0 μM) or hCGRP (0.1 μM and 1.0 μM), respectively for 30 min. (B) CTR transfected cells were treated with hSTC1 (0.1 μM and 1.0 μM) or hCT (0.1 μM) for 30 min before cAMP measure. To examine if hSTC interferes with hCGRP cAMP production, cells were pre-treated with hCGRP (0.1 μM) or with hSTC1 (0.1 μM) for 30 min and subsequently with hSTC1 (0.1 μM and 1.0 μM) or hCT (0.1 μM and 1.0 μM), respectively for 30 min. Bars (mean ± SEM) indicate the percentage of cAMP levels in relation to maximal FK stimulation of at least three independent experiments performed in triplicate. Statistical differences were estimated using two-way ANOVA and different letters above bars indicate statistical significance of the 5% level.

coupled-receptor (Gαi coupled-GPCR), which in turn blocks AC activity inside the cell with subsequent decrease in intracellular cAMP formation. This hypothesis is also supported by two other features: firstly, ERK1/2 (Nguyen et al., 2009) and cAMP/CREB pathways (Lu et al., 1994; Yeung et al., 2012; Yoshiko et al., 1996) are involved in STC1 intracellular signaling; secondly, Gαi coupled-GPCR stimulation can activate the ERK1/2 pathway through a downstream phosphorylation cascade, which activates RAS, RAF and

MEK, culminating in migration of the transcription factors ELK1 and/or CREB to the nucleus (Avlani et al., 2013; Goldsmith and Dhanasekaran, 2007).

Similar inhibitory results on cAMP have been obtained using rat osteosarcoma cell lines incubated with human parathyroid hormone (PTH) plus a synthetic teleost *N*-terminal STC1 truncated peptide (Yoshiko et al., 1996). The fish *N*-terminal STC1 had a suppressing effect on cAMP accumulation evoked by PTH. Furthermore, in assays in which granulosa cells were incubated with 10 ng/ml of FSH, or hCG, plus 10 nM of STC1, a significant reduction in cAMP was also detected (Luo et al., 2004). Thus, it seems that STC1 activates similar signaling pathways independently of the cell line used, via inhibition of cAMP accumulation. Moreover, it is unlikely that STC1 inhibits intracellular cAMP levels by stimulating phosphodiesterase (PDE) activity, given that PDE activity was blocked by IBMX before the beginning of the cAMP assays. The measurements of intracellular ATP during osteoblastogenesis indicated that ATP levels are not affected by long-term treatment with hSTC1. Therefore, it is also unlikely that hSTC1 may reduce intracellular ATP, which must be available as a substrate for cAMP formation. Although HEK293 cell viability when incubated with STC1 was not measured, it is unlikely that the peptide affects cell function since vertebrate kidney cell lines normally express high levels of STC1 (Sazonova et al., 2008) and commercially available recombinant STC1 is produced in HEK cells.

Surprisingly, when CALCRL/RAMP1 transfected cells were pre-incubated with hSTC1 and subsequently with CGRP, the hCGRP stimulation of cAMP accumulation was more efficient than hCGRP alone. The increase of cAMP production in the presence of CGRP is intriguing and further studies are required. However, it is most likely to be an example of a paradoxical enhancement or sensitization of AC activity that results in increased cAMP accumulation when the action of the inhibitory receptor is ended (Watts and Neve, 2005). This has been interpreted as an adaptive response to the chronic inhibition. Moreover, confocal immunocytochemistry analysis also suggests that STC1 may interfere in the CGRP receptor localization in differentiated 7-day pre-osteoblasts by an unknown interaction and/or translocation mechanism at the plasma membrane level. However, how this modifies receptor signaling response remains to be established.

Taken together, the present results indicate an interaction of STC1 with the CGRP heterodimeric receptor complex CALCRL/RAMP1 with a possible specific cross-talk between STC1 and CGRP signaling during osteoblastogenesis. Further evaluation is required in order to establish the possible mechanism for this interaction. STC1 was also able to inhibit the CT signaling on CTR transfected cells and since CTR is also expressed on osteoblasts (Monier-Faugere et al., 1996; Villa et al., 2003) this results point out a possible important role of STC1 on the inhibition of the CT signaling during the osteoblastogenesis process.

Although binding assays have not been performed, evidence indicates that STC1 does not seem to share the same receptors than CGRP and CT; nonetheless the presence of STC1 seems to interfere with peptide signaling. Furthermore, future studies are necessary to clarify our new data about STC1 intracellular cAMP inhibition, such as the use of pertussis toxin that inhibit Gi proteins, co-precipitation assays as well as activation and phosphorylation of intracellular signaling molecules including MAP kinase and ERK.

5. Conclusions

To the best of our knowledge, this is the first investigation of a possible link between STC1 and CGRP and CT signaling during osteoblastogenesis. Our study indicates a cross-talk between CGRP and STC1 at the earlier stages of the osteoblast differentiation process. In addition to the previously reported direct effects on osteogenesis, our study shows that STC1 may regulate CGRP and CT signaling during osteoblastogenesis although the physiological consequences of these remain to be elucidated.

Acknowledgement

This work was supported by grants from CAPES/CNPq (VS PNPD fellowship program), CNPq (SRT and LAMM PhD fellowship program; FCRG research productivity fellowship program), FAPERGS/CNPq n. 008/2009 (FCRG) and INCT Exitotoxicity and Neuroprotection (DOGS). AMVC, JCRC and RCF thank the financial support of the Portuguese Foundation for Science and Technology (FCT) (project PTDC/MAR/121279/2010, project PEst-C/MAR/LA0015/2013 and fellowship SFRH/BPD/89811/2012). The authors thank the Centro de Microscopia Eletrônica (CME-UFRGS) for technical assistance in confocal microscopy.

Author contributions

Conceived and designed the experiments: SRT, RCF, JCRC, FCRG, VS. Performed the experiments: SRT, JCRC, LAMM, RCF, VS. Analyzed the data: SRT, JCRC, LAMM, RCF, FCRG, VS, AVMC. Wrote the paper: JCRC, LAMM, FCRG, AVMC, VS. Supervisor: VS. Performed the experiments on RT-PCR, immunocytochemistry and ATP measures: SRT, LAMM, VS. Provided reagents and performed the experiments on transfection cells: JCRC, RCF, VS. Provided reagents and financial support on cell experiments: DOGS, AVMC, FRCG, VS.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.mce.2015.01.010.

References

Aiyar, N., Rand, K., Elshourbagy, N.A., Zeng, Z.Z., Adamou, J.E., Bergsma, D.J., et al., 1996. A cDNA encoding the calcitonin gene-related peptide type 1 receptor. J. Biol. Chem. 271 (19), 11325–11329.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215 (3), 403–410.

Amara, S.G., Jonas, V., Rosenfeld, M.G., Ong, E.S., Evans, R.M., 1982. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. Nature 298 (5871), 240–244.

Atwood, B.K., Lopez, J., Wager-Miller, J., Mackie, K., Straiker, A., 2011. Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. BMC Genomics 12, 14.

Avlani, V.A., Ma, W., Mun, H.C., Leach, K., Delbridge, L., Christopoulos, A., et al., 2013. Calcium-sensing receptor-dependent activation of CREB phosphorylation in HEK293 cells and human parathyroid cells. Am. J. Physiol. Endocrinol. Metab. 304 (10), E1097–E1104.

Bailey, R.J., Walker, C.S., Ferner, A.H., Loomes, K.M., Prijic, G., Halim, A., et al., 2012. Pharmacological characterization of rat amylin receptors: implications for the identification of amylin receptor subtypes. Br. J. Pharmacol. 166 (1), 151–167.

Basini, G., Bussolati, S., Santini, S.E., Grasselli, F., 2009. Stanniocalcin, a potential ovarian angiogenesis regulator, does not affect endothelial cell apoptosis. In: Diederich, M. (Ed.), Natural Compounds and Their Role in Apoptotic Cell Signaling Pathways. Blackwell Publishing, Oxford, pp. 94–99.

Basini, G., Baioni, L., Bussolati, S., Grolli, S., Kramer, L.H., Wagner, G.F., et al., 2010. Expression and localization of stanniocalcin 1 in swine ovary. Gen. Comp. Endocrinol. 166 (2), 404–408.

Bellido, T., Saini, V., Pajevic, P.D., 2013. Effects of PTH on osteocyte function. Bone 54 (2), 250–257.

Block, G.J., DiMattia, G.D., Prockop, D.J., 2010. Stanniocalcin-1 regulates extracellular ATP-induced calcium waves in human epithelial cancer cells by stimulating ATP release from bystander cells. PLoS ONE 5 (4), e10237.

Bolte, S., Cordelieres, F.P., 2006. A guided tour into subcellular colocalization analysis in light microscopy. J. Microsc. 224 (Pt 3), 213–232.

Born, W., Fischer, J.A., Muff, R., 2002. Receptors for calcitonin gene-related peptide, adrenomedullin, and amylin: the contributions of novel receptor-activity-modifying proteins. Receptors Channels 8 (3–4), 201–209.

Cao, J.J., 2011. Effects of obesity on bone metabolism. J. Orthop. Surg. Res. 6 (30), 30.

Chang, C.L., Hsu, S.Y.T., 2013. Roles of CLR/RAMP receptor signaling in reproduction and development. Curr. Protein Pept. Sci. 14 (5), 393–406.

Conner, A.C., Hay, D.L., Howitt, S.G., Kilk, K., Langel, U., Wheatley, M., et al., 2002. Interaction of calcitonin-gene-related peptide with its receptors. Biochem. Soc. Trans. 30 (4), 451–455.
Costa, J.L., Naot, D., Lin, J.M., Watson, M., Callon, K.E., Reid, I.R., et al., 2011. Ghrelin is an osteoblast mitogen and increases osteoclastic bone resorption in vitro. Int. J. Pept. 2011, 605193.
Costes, S.V., Daelemans, D., Cho, E.H., Dobbin, Z., Pavlakis, G., Lockett, S., 2004. Automatic and quantitative measurement of protein-protein colocalization in live cells. Biophys. J. 86 (6), 3993–4003.
Dunn, K.W., Kamocka, M.M., McDonald, J.H., 2011. A practical guide to evaluating colocalization in biological microscopy. Am. J. Physiol. Cell Physiol. 300 (4), C723–C742.
Espinosa, J., Rocha, A., Nunes, F., Costa, M.S., Schein, V., Kazlauckas, V., et al., 2013. Caffeine consumption prevents memory impairment, neuronal damage, and adenosine A(2A) receptors upregulation in the hippocampus of a rat model of sporadic dementia. J. Alzheimers Dis. 34 (2), 509–518.
Feyen, J.H., Cardinaux, F., Gamse, R., Bruns, C., Azria, M., Trechsel, U., 1992. N-terminal truncation of salmon calcitonin leads to calcitonin antagonists. Structure activity relationship of N-terminally truncated salmon calcitonin fragments in vitro and in vivo. Biochem. Biophys. Res. Commun. 187 (1), 8–13.
Filvaroff, E.H., Guillet, S., Zlot, C., Bao, M., Ingle, G., Steinmetz, H., et al., 2002. Stanniocalcin 1 alters muscle and bone structure and function in transgenic mice. Endocrinology 143 (9), 3681–3690.
Goldsmith, Z.G., Dhanasekaran, D.N., 2007. G protein regulation of MAPK networks. Oncogene 26 (22), 3122–3142.
Goodman, E.C., Iversen, L.L., 1986. Calcitonin gene-related peptide: novel neuropeptide. Life Sci. 38 (24), 2169–2178.
Granholm, S., Henning, P., Lerner, U.H., 2011. Comparisons between the effects of calcitonin receptor-stimulating peptide and intermedin and other peptides in the calcitonin family on bone resorption and osteoclastogenesis. J. Cell. Biochem. 112 (11), 3300–3312.
Hay, D.L., Christopoulos, G., Christopoulos, A., Poyner, D.R., Sexton, P.M., 2005. Pharmacological discrimination of calcitonin receptor: receptor activity-modifying protein complexes. Mol. Pharmacol. 67 (5), 1655–1665.
He, L.F., Wang, T.T., Gao, Q.Y., Zhao, G.F., Huang, Y.H., Yu, L.K., et al., 2011. Stanniocalcin-1 promotes tumor angiogenesis through up-regulation of VEGF in gastric cancer cells. J. Biomed. Sci. 14, 18–39.
Henriksen, K., Neutzsky-Wulff, A.V., Bonewald, L.F., Karsdal, M.A., 2009. Local communication on and within bone controls bone remodeling. Bone 44 (6), 1026–1033.
Jiang, W.Q., Chang, A.C., Satoh, M., Furuichi, Y., Tam, P.P., Reddel, R.R., 2000. The distribution of stanniocalcin 1 protein in fetal mouse tissues suggests a role in bone and muscle development. J. Endocrinol. 165 (2), 457–466.
Johnston, J., Ramos-Valdes, Y., Stanton, L.A., Ladhani, S., Beier, F., Dimattia, G.E., 2010. Human stanniocalcin-1 or -2 expressed in mice reduces bone size and severely inhibits cranial intramembranous bone growth. Transgenic Res. 19 (6), 1017–1039.
Kawase, T., Okuda, K., Burns, D.M., 2003. Immature human osteoblastic MG63 cells predominantly express a subtype 1-like CGRP receptor that inactivates extracellular signal response kinase by a cAMP-dependent mechanism. Eur. J. Pharmacol. 470 (3), 125–137.
Kohli, S.S., Kohli, V.S., 2011. Role of RANKL-RANK/osteoprotegerin molecular complex in bone remodeling and its immunopathologic implications. Indian J. Endocrinol. Metab. 15 (3), 175–181.
Lafont, A.G., Wang, Y.F., Chen, G.D., Liao, B.K., Tseng, Y.C., Huang, C.J., et al., 2011. Involvement of calcitonin and its receptor in the control of calcium-regulating genes and calcium homeostasis in zebrafish (Danio rerio). J. Bone Miner. Res. 26 (5), 1072–1083.
Lambert, C., Dubuc, J.E., Montell, E., Vergés, J., Munaut, C., Noël, A., et al., 2014. Gene expression pattern of cells from inflamed and normal areas of osteoarthritis synovial membrane. Arthritis Rheum. 66 (4), 960–968.
Lu, M., Wagner, G.F., Renfro, J.L., 1994. Stanniocalcin stimulates phosphate reabsorption by flounder renal proximal tubule in primary culture. Am. J. Physiol. 267 (5 Pt 2), R1356–R1362.
Luo, C.W., Kawamura, K., Klein, C., Hsueh, A.J., 2004. Paracrine regulation of ovarian granulosa cell differentiation by stanniocalcin (STC) 1: mediation through specific STC1 receptors. Mol. Endocrinol. 18 (8), 2085–2096.
McLatchie, L.M., Fraser, N.J., Main, M.J., Wise, A., Brown, J., Thompson, N., et al., 1998. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 393 (6683), 333–339.
Monier-Faugere, M.C., Geng, Z., Qi, Q., Arnala, I., Malluche, H.H., 1996. Calcitonin prevents bone loss but decreases osteoblastic activity in ovariohysterectomized Beagle dogs. J. Bone Miner. Res. 11 (4), 446–455.
Nakahama, K., 2010. Cellular communications in bone homeostasis and repair. Cell. Mol. Life Sci. 67 (23), 4001–4009.
Naot, D., Cornish, J., 2008. The role of peptides and receptors of the calcitonin family in the regulation of bone metabolism. Bone 43 (5), 813–818.
Nguyen, A., Chang, A.C., Reddel, R.R., 2009. Stanniocalcin-1 acts in a negative feedback loop in the prosurvival ERK1/2 signaling pathway during oxidative stress. Oncogene 28 (18), 1982–1992.
Pozvek, G., Hilton, J.M., Quiza, M., Houssami, S., Sexton, P.M., 1997. Structure/function relationships of calcitonin analogues as agonists, antagonists, or inverse agonists in a constitutively activated receptor cell system. Mol. Pharmacol. 51 (4), 658–665.
Roh, J., Chang, C.L., Bhalla, A., Klein, C., Hsu, S.Y.T., 2004. Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes. J. Biol. Chem. 279 (8), 7264–7274.
Sazonova, O., James, K.A., McCudden, C.R., Segal, D., Talebian, A., Wagner, G.F., 2008. Stanniocalcin-1 secretion and receptor regulation in kidney cells. Am. J. Physiol. Renal Physiol. 294 (4), F788–F794.
Schinke, T., Liese, S., Priemel, M., Haberland, M., Schilling, A.F., Catala-Lehnen, P., et al., 2004. Decreased bone formation and osteopenia in mice lacking alpha-calcitonin gene-related peptide. J. Bone Miner. Res. 19 (12), 2049–2056.
Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. 3, 1101–1108.
Skalhegg, B.S., Tasken, K., 2000. Specificity in the cAMP/PKA signaling pathway. Differential expression, regulation, and subcellular localization of subunits of PKA. Front. Biosci. 5 (5), D678–D693.
Smillie, S.J., Brain, S.D., 2011. Calcitonin gene-related peptide (CGRP) and its role in hypertension. Neuropeptides 45 (2), 93–104.
Stasko, S.E., Wagner, G.F., 2001. Possible roles for stanniocalcin during early skeletal patterning and joint formation in the mouse. J. Endocrinol. 171 (2), 237–248.
Stenbeck, G., Lawrence, K.M., Albert, A.P., 2012. Hormone-stimulated modulation of endocytic trafficking in osteoclasts. Front. Endocrinol. (Lausanne) 3, 103.
Togari, A., Arai, M., Mizutani, S., Koshihara, Y., Nagatsu, T., 1997. Expression of mRNAs for neuropeptide receptors and beta-adrenergic receptors in human osteoblasts and human osteogenic sarcoma cells. Neurosci. Lett. 233 (2–3), 125–128.
Trindade, D.M., Silva, J.C., Navarro, M.S., Torriani, I.C.L., Kobarg, J., 2009. Low-resolution structural studies of human Stanniocalcin-I. BMC Struct. Biol. 9, 57.
Uzan, B., de Vernejoul, M.C., Cressent, M., 2004. RAMPs and CRLR expressions in osteoblastic cells after dexamethasone treatment. Biochem. Biophys. Res. Commun. 321 (4), 802–808.
Varghese, R., Gagliardi, A.D., Bialek, P.E., Yee, S.P., Wagner, G.F., DiMattia, G.E., 2002. Overexpression of human stanniocalcin affects growth and reproduction in transgenic mice. Endocrinology 143 (3), 868–876.
Vignery, A., McCarthy, T.L., 1996. The neuropeptide calcitonin gene-related peptide stimulates insulin-like growth factor I production by primary fetal rat osteoblasts. Bone 18 (4), 331–335.
Vilcaes, A.A., Torres Demichelis, V., Daniotti, J.L., 2011. Trans-activity of plasma membrane-associated ganglioside sialyltransferase in mammalian cells. J. Biol. Chem. 286 (36), 31437–31446.
Villa, I., Dal Fiume, C., Maestroni, A., Rubinacci, A., Ravasi, F., Guidobono, F., 2003. Human osteoblast-like cell proliferation induced by calcitonin-related peptides involves PKC activity. Am. J. Physiol. Endocrinol. Metab. 284 (3), E627–E633.
Villa, I., Mrak, E., Rubinacci, A., Ravasi, F., Guidobono, F., 2006. CGRP inhibits osteoprotegerin production in human osteoblast-like cells via cAMP/PKA-dependent pathway. Am. J. Physiol. Cell Physiol. 291 (3), C529–C537.
Wang, L., Shi, X., Zhao, R., Halloran, B.P., Clark, D.J., Jacobs, C.R., et al., 2010. Calcitonin-gene-related peptide stimulates stromal cell osteogenic differentiation and inhibits RANKL induced NF-kappaB activation, osteoclastogenesis and bone resorption. Bone 46 (5), 1369–1379.
Wang, Y., Kong, Y., Shei, G.J., Kang, L., Cvijic, M.E., 2011. Development of a cyclic adenosine monophosphate assay for Gi-coupled G protein-coupled receptors by utilizing the endogenous calcitonin activity in Chinese hamster ovary cells. Assay Drug Dev. Technol. 9 (5), 522–531.
Watts, V.J., Neve, K.A., 2005. Sensitization of adenylate cyclase by Galpha i/o-coupled receptors. Pharmacol. Ther. 106 (3), 405–421.
Westberg, J.A., Serlachius, M., Lankila, P., Penkowa, M., Hidalgo, J., Andersson, L.C., 2007. Hypoxic preconditioning induces neuroprotective stanniocalcin-1 in brain via IL-6 signaling. Stroke 38 (3), 1025–1030.
Worthington, R.A., Brown, L., Jellinek, D., Chang, A.C.M., Reddel, R.R., Hambly, B.D., et al., 1999. Expression and localisation of stanniocalcin 1 in rat bladder, kidney and ovary. Electrophoresis 20 (10), 2071–2076.
Wu, S., Yoshiko, Y., De Luca, F., 2006. Stanniocalcin 1 acts as a paracrine regulator of growth plate chondrogenesis. J. Biol. Chem. 281 (8), 5120–5127.
Yeung, B.H.Y., Law, A.Y.S., Wong, C.K.C., 2012. Evolution and roles of stanniocalcin. Mol. Cell. Endocrinol. 349 (2), 272–280.
Yoshiko, Y., Aubin, J.E., 2004. Stanniocalcin 1 as a pleiotropic factor in mammals. Peptides 25 (10), 1663–1669.
Yoshiko, Y., Kosugi, T., Koide, Y., 1996. Effects of a synthetic N-terminal fragment of stanniocalcin on the metabolism of mammalian bone in vitro. Biochim. Biophys. Acta 1311 (3), 143–149.
Yoshiko, Y., Aubin, J.E., Maeda, N., 2002. Stanniocalcin 1 (STC1) protein and mRNA are developmentally regulated during embryonic mouse osteogenesis: the potential of stc1 as an autocrine/paracrine factor for osteoblast development and bone formation. J. Histochem. Cytochem. 50 (4), 483–492.
Yoshiko, Y., Maeda, N., Aubin, J.E., 2003. Stanniocalcin 1 stimulates osteoblast differentiation in rat calvaria cell cultures. Endocrinology 144 (9), 4134–4143.
Zinchuk, V., Zinchuk, O., Okada, T., 2007. Quantitative colocalization analysis of multicolor confocal immunofluorescence microscopy images: pushing pixels to explore biological phenomena. Acta Histochem. Cytochem. 40 (4), 101–111.
Zlot, C., Ingle, G., Hongo, J., Yang, S., Sheng, Z., Schwall, R., et al., 2003. Stanniocalcin 1 is an autocrine modulator of endothelial angiogenic responses to hepatocyte growth factor. J. Biol. Chem. 278 (48), 47654–47659.
Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., et al., 2002. Human adipose tissue is a source of multipotent stem cells. Mol. Biol. Cell 13 (12), 4279–4295.

PARTE III

III.1 DISCUSSÃO

A STC1 é uma glicoproteína secretada altamente conservada através da evolução [127]. Nos peixes ósseos essa proteína possui ação hormonal sendo secretada em resposta a hipercalcemia [58, 128]. Em mamíferos a STC1 amplamente expressa em diversos tecidos [79]. Diversos estudos comprovam o envolvimento da STC1 nos processos de ossificação intramembranosa e endocondral e mineralização óssea [106-107]. Apesar deste fato, os efeitos e os mecanismos de ação da STC1 na osteoblastogênese ainda não foram completamente elucidados.

Um grande número de estudos revelam que o tecido adiposo contém uma população celular heterogenea chamada fração vascular estromal (SVF). As células da SVF podem ser empregadas diretamente ou cultivadas para a seleção e expansão de uma população aderente, as ASCs [129]. Nos últimos anos, estudos têm demonstrado a eficácia e segurança da ASCs *in vivo* em modelos animais. A regeneração óssea baseada na utlização das ASCs emergiu como uma abordagem promissora para restaurar a função e estrutura de óssea comprometida por doença ou lesão [130]. A ASCs têm sido utilizadas em combinação com biomateriais e/ou moléculas osteoindutoras [131]**.** Além disso, a utilização de ASCs expandidas em cultura antecipa cicatrização do osso por meio da diferenciação direta em osteoblastos maduros e efeitos parácrinos que facilitam a migração e a diferenciação de precursores residentes [132].

No primeiro capítulo desta tese, foi descrito o efeito do tratamento das ASCs e ASCs diferenciadas para pré-osteoblastos por 7 e 14 dias com doses crescentes (6,3ng a 50ng/ml) de STC1. A dose de 50ng/ml de STC1 foi efetiva no estimulo da atividade da FA em pré- osteoblastos de 7 dias, mas o mesmo não ocorreu em ASCs não tratadas e pré-osteoblastos de 14 dias. Nesta dose, não foi observada a diminuição de proliferação e redução da população de células, sendo eficaz no estimulo da diferenciação osteogênica em pré-osteoblastos de 7 dias. Os resultados obtidos por *q*PCR, imunocitoquímica, Western blott e citometria de fluxo confirmaram a atuação da STC1 no aumento do mRNA, da proteína e, consequentemente, na atividade da FA. Quando se avalia o fenótipo ou maturidade do desenvolvimento de células e tecidos ósseos a FA é um dos

principais marcadores da diferenciação óssea e sua expressão aumenta no início da indução osteogênica [133]..

Além da FA, o tratamento com STC1 (50ng/ml) promoveu o aumento do mRNA e da proteína do RUNX2 e da OPN em pré-osteoblastos de 7 dias. O RUNX2 tem um papel fundamental na diferenciação de osteoblastos, função e, consequentemente, na biologia óssea. Na fase inicial da embriogênese o RUNX2 determina o comprometimento das MSCs para linhagem de osteoblastos. O RUNX2 é um fator de transcrição fundamental na osteogênese, regulando a expressão de outros genes importantes para a formação óssea [134-136]

Múltiplas vias de sinalização que afetam a função dos osteoblastos convergem para o RUNX2 ou são coordenados por ele. Uma variedade de estímulos químicos e físicos, incluindo os iniciados por matriz extracelular (ECM), BMPs e FGF-2, hormônio da paratireóide e do crescimento e carga mecânica (tensão/alongamento) modulam a estado de fosforilação/ativaçãodo RUNX2, bem como a sua interação com uma variedade de proteínas [137].

A OPN também é uma proteína produzida por osteoblastos envolvida na ossificação endocondral e intramembranosa durante o desenvolvimento ósseo [138]. Em linhagens de osteoblastos a OPN está expressa no início da diferenciação e a sua expressão é mantida elevada ao longo das fase de mineralização *in vitro.* Em alguns tipos de células ósseas a expressão OPN é fortemente aumentada por estímulos mecânicos [139]. O envolvimento da OPN na regulação da mineralização óssea surgiu com base em sua distribuição nos tecidos, afinidade pelo cálcio, imunolocalização em regiões de mineralização e regulação da expressão gênica por hormônios calciotrópicos [140-141].. Algumas funções da OPN exigem a sua fosforilação, o que torna essa proteína um alvo para o controle de fosfatases extracelulares e quinases. O fosfato extracellular induz a expressão da OPN na linhagem de osteoblastos MC3T3. Essa regulação pode ser um mecanismo de controle que vincula o aumento na expressão de OPN com a diminuição da proliferação e o início da diferenciação dos osteoblastos, eventos que coincidem com a indução de fosfatase alcalina [142].

O aumento da expressão do fator de transcrição RUNX2, além de OPN e FA é um indicativo de que a STC1 pode estar atuando na antecipação da diferenciação osteogênica das ASCs estimuladas por sete dias. O RUNX2 é uma proteína chave que pode regular genes como: colágeno tipo I ,osteocalcina,

osteopontina , sialoproteína óssea, fosfatase alcalina, [143-146]. Nesse contexto, a STC1 pode estar estimulando a expressão do RUNX2 e este, por sua vez, estimula a expressão de FA e OPN. Além disso, estudos anteriores já demonstraram que a STC1 estimula a expressão de transportadores de Pi aumentando o Pi intracelular, responsável pelo aumento da expressão de OPN [106].

O tratamento com STC1 demonstrou modular a osteoblastogênese através da via de sinalização pAkt/GSK3β/β-catenina. A inativação da GSK3-β por fosforilação pela pAKt, mostrados por Western blott, resulta no acúmulo da β-catenina em pré-osteoblastos quando comparados ao controle.

Estudos realizados em cultura de osteoblastos mostraram que a supressão da expressão da Akt1 resulta em disfunção destas células por aumento da susceptibilidade à apoptose e diminuição da atividade do fator de transcrição relacionados ao RUNX2. Em experimentos *in vivo* realizados em modelo murino, a supressão da Akt1 levou à osteopenia por diminuição do mecanismo de acoplamento entre a reabsorção e formação óssea realizado por osteoclastos e osteoblastos, respectivamente [147].Tem sido relatado que a via de sinalização PI3K/Akt e componentes downstream dessa via são alvos críticos no desenvolvimento ósseo [148]. O fator de transcrição RUNX2 estimula a expressão de proteínas da via de sinalização PI3K/Akt resultando em aumento da habilidade de ligação deste fator no DNA de osteoblastos imaturos [149-150].

Diversos estudos relatam a importância da via de sinalização canônica da Wnt no controle da formação e massa óssea. A interação de proteínas Wnt com receptores Frizzled e co-receptores LRP5/6 leva à inibição da fosforilação da GSK-3β. A inibição da GSK3-β resulta no acúmulo e translocação para o núcleo da β-catenina que, por sua vez, se liga ao fator de transcrição TCF/LEF e ativa genes relacionados à diferenciação osteogênica. A inativação de β-catenina impede a diferenciação de osteoblastos a partir de células progenitoras mesenquimais, indicando que a β-catenina desempenha um papel essencial na diferenciação de osteoblastos *in vivo [151]*. Além disso, os inibidores da via Wnt/βcatenina demonstram a coerência na relação inversa existente entre diferenciação osteogênica e adipogênica [152] .

Essas considerações servem para salientar que os resultados deste estudo nos permitem hipotetizar que a STC1 promove a antecipação da diferenciação

das hASCs em osteoblastos através da via de sinalização pAKt/pGSK3-β/β-catenina e expressão de genes chave envolvidos na diferenciação óssea.

No capítulo II foi a avaliada a interação e modulação da STC1 na via de sinalização mediada pelo peptídeo relacionado ao gene da calcitonina (CGRP), com a calcitonina (CT) e seus respectivos receptores na diferenciação das hASCs para osteoblastos. O CGRP é um neuropeptídeo amplamente distribuído em neurônios sensoriais que inervam o esqueleto e regulam a integridade óssea. Os principais mecanismos de ação do CGRP sobre a osteoblastogenese são a estimulação direta de células tronco estromais em osteoblastos e a inibição da osteoclastogenese e reabsorção óssea [123]. Em células osteoprogenitoras, o CGRP atua no processo incial de diferenciação em osteoblastos [108]. Estudos em osteoblastos de camundongos transgênicos que superexpressam o CGRP demonstram um aumento da atividade de formação óssea e da massa óssea trabecular, enquanto camundongos CGRP deficientes exibem uma taxa de formação óssea menor e perda óssea acelerada com o envelhecimento [153-154].

O receptor de CGRP é um complexo proteíco dimérico formado por CALCRL, um membro da família de receptores acoplados a proteína G (GPCR) e a proteína transmembrana acessória (RAMP1). A interação entre CALCRL e RAMP1 é necessária para a ativação fisiológica pelo CGRP e funcionalidade do receptor [120, 155]. O complexo CALCRL/RAMP1 está presente em várias linhagens de osteoblastos murinos e culturas de osteoblastos primários de ratos e humanos [117, 124]. Em osteoblastos estimulados pelo CGRP ocorre o aumento dos níveis intracelulares de AMPc relacionados ao complexo CALCR/RAMP1 [115, 118].

Estudos anteriores demonstram que a STC1 e o CGRP atuam de forma semelhante no desenvolvimento ósseo [77, 104].

Neste estudo observamos, em hASCs diferenciadas para osteoblastos por 7 dias, que o tratamento com hSTC1 não alterava os níveis de cálcio e AMPc intracelular induzidos por hCGRP. O mesmo resultado foi obtido em células Hek 293 e Hek 293 tranfectadas com CALC/RAMP. No entanto, nas Hek 293, estimuladas por forscolina, a rhSTC1 diminuiu os níveis de AMPc de forma dose dependente. Esses dados indicam que a hSTC1 não interfere na resposta do complexo CALC/RAMP ao CGRP e, só é capaz de diminuir a produção de AMPc, após estímulo por forscolina. Nas Hek 293 transfectadas com CALCR/RAMP não

houve alteração da produção de AMPc em células previamente tratadas com CGRP e posteriormente, com rhSTC1 . Em contraste, a pré incubação com rhSTC1 e, posterior, com CGRP aumentou significadamente a produção do AMPc comparada ao tratamento somente com CGRP. A hSTC1, provavelmente, possui uma ação sensibilizadora sobre as Hek 293 transfectadas com CALCR/RAMP inibindo a adelinato ciclase. Após o término do estímulo inibitório da rhSTC1 ocorreu um aumento acumulativo do AMPc na presença de CGRP. Esta sensibilização da sinalização do AMPc, representa uma resposta celular adaptativa pelo qual a célula compensa a inibição crônica [156].

A inibição da produção intracelular de AMPc foi o principal efeito da rhSTC1 sobre os receptores específicos para calcitonina (CTR) demonstrados em células Hek 293 que superexpressam o CTR. Nessas células, tanto o pré tratamento, como o pós tratamento com rhSTC1, resultaram em inibição dos efeitos da calcitonina (CT). Logo, com base nesses resultados, podemos sugerir que a rhSTC1 inibe a produção de AMPc possivelmente através de um sinalização relacionada a um receptor associado à proteina Gαi (GPCRαi) ou através de outros receptores não relacionados GPCR. A rhSTC1 pode estar ativando o receptor acoplado Gαi e diminuindo a atividade da adenilato ciclase e assim a formação de AMPc intracelular [157]. Diferente do que ocorre em peixes, a hSTC1 inibe a ação da CT em mamíferos [126]. Evidências sobre o efeito inibitório da STC1 sobre o AMPc foram observados em células foliculares tratadas com FSH [158]

No presente trabalho, foi possível demonstrar uma interação da rhSTC1 com o receptor de CGRP (complexo heterodimérico CALCRL / RAMP1) durante a diferenciação das hASCs para pré-osteoblastos. A análise por imunocitoquímica sugere que a rhSTC1 interfere na localização do receptor de CGRP em hASCs diferenciadas para pre-osteoblastos por 7 dias através de uma interação desconhecida e/ou um mecanismo de translocação de proteínas na membrana plasmática. Além disso, a STC1 foi capaz de diminuir a produção de AMPc em células transfectadas com CTR e estimuladas com CT. Nesse contexto, é possível afirmar que a STC1 contribui para inibição da via sinalização mediada pela CT durante o processo de osteoblastogênese.

Todos esses resultados indicam que a STC1, através de uma proteína G inibitória modula a localização espacial do complexo CALCRL/RAMP1,

provavelmente sem interagir diretamente com os receptores de CGRP e CT, mas influenciando a resposta celular durante a diferenciação osteogênica das hASC.

III.2 CONCLUSÕES

Sem dúvida, o reconhecimento do osso como um tecido vivo e com metabolismo e dinâmica celulares próprios, foi necessário para reconhecimento de fatores genéticos, hormônios, vias de sinalização , citocinas e proteínas que governam a formação, manutenção e o reparo ósseo. Nesse contexto, através desse trabalho, podemos concluir que a STC1 foi capaz de estimular a expressão e a atividade da enzima FAS e dos genes RUNX2 e osteopontina e suas respectivas proteínas em hASCs diferenciadas para pré-osteoblastos por 7 dias. Os mecanismos envolvidos no estímulo da osteoblastogenese pela STC1 apontam para a modulação da via de sinalização pAKt/pGSK3-β/β-catenina. O tratamento das hASCs com STC1 não foi capaz de estimular a diferenciação osteogênica, sendo necessário o estímulo através do meio osteoindutor. Através desses resultados podemos afirmar que a STC1 antecipou o processo de diferenciação em pré-osteoblastos de 7 dias.

Além disso, nossos resultados nos possibilitaram uma melhor compreensão do envolvimento da STC1 nas vias de sinalização relacionadas a receptores de calcitonina. A STC1 demonstrou interagir com o complexo heterodimérico CALC/RAMP do receptor de CGRP em pré-osteoblastos de 7 dias e células Hek 293 transfectadas com CALC/RAMP. Nos receptores específicos de calcitonina a STC1 demonstrou inibir a sinalização da calcitonina diminuindo a produção de cAMP em células tranfectadas com o receptor de calcitonina. Esses resultados indicam que, apesar da STC1 não atuar nos mesmos receptores de CGRP e calcitonina , esta proteína interfere na sinalização desses peptídeos

Através desse estudo foi possível compreender melhor as implicações funcionais dos mecanismos de atuação da STC1 no processo de diferenciação osteogênico das hASCs e nas vias de sinalização relacionadas à família de receptores de calcitona. Estes dados podem ser explorados de forma promissora em pesquisas relacionadas a medicina regenerativa do tecido ósseo e doenças osteodegenerativas .

REFERÊNCIAS

1. Caplan, A.I. and S.P. Bruder, *Mesenchymal stem cells: building blocks for molecular medicine in the 21st century.* Trends in molecular medicine, 2001. **7**(6): p. 259-264.
2. Sethe, S., A. Scutt, and A. Stolzing, *Aging of mesenchymal stem cells.* Ageing Res Rev, 2006. **5**(1): p. 91-116.
3. Baker, N., L.B. Boyette, and R.S. Tuan, *Characterization of bone marrow-derived mesenchymal stem cells in aging.* Bone, 2015. **70**: p. 37-47.
4. Bianco, P., et al., *Bone marrow stromal stem cells: nature, biology, and potential applications.* Stem Cells, 2001. **19**(3): p. 180-92.
5. Prockop, D.J., *Marrow stromal cells as stem cells for nonhematopoietic tissues.* Science, 1997. **276**(5309): p. 71-4.
6. Murray, I.R., et al., *Natural history of mesenchymal stem cells, from vessel walls to culture vessels.* Cellular and Molecular Life Sciences, 2014. **71**(8): p. 1353-1374.
7. Keating, A., *Mesenchymal stromal cells.* Curr Opin Hematol, 2006. **13**(6): p. 419-25.
8. da Silva Meirelles, L., P.C. Chagastelles, and N.B. Nardi, *Mesenchymal stem cells reside in virtually all post-natal organs and tissues.* Journal of cell science, 2006. **119**(11): p. 2204-2213.
9. da Silva Meirelles, L., et al., *Mechanisms involved in the therapeutic properties of mesenchymal stem cells.* Cytokine & growth factor reviews, 2009. **20**(5): p. 419-427.
10. Zuk, P.A., *The adipose-derived stem cell: looking back and looking ahead.* Molecular biology of the cell, 2010. **21**(11): p. 1783-1787.
11. Fodor, W.L., *Tissue engineering and cell based therapies, from the bench to the clinic: the potential to replace, repair and regenerate.* Reprod Biol Endocrinol, 2003. **1**: p. 102.
12. Chagastelles, P.C. and N.B. Nardi, *Biology of stem cells: an overview.* Kidney Int Suppl (2011), 2011. **1**(3): p. 63-67.
13. Linero, I. and O. Chaparro, *Paracrine effect of mesenchymal stem cells derived from human adipose tissue in bone regeneration.* PLoS One, 2014. **9**(9): p. e107001.

14. Bocelli-Tyndall, C., et al., *Bone marrow mesenchymal stromal cells (BM-MSCs) from healthy donors and auto-immune disease patients reduce the proliferation of autologous-and allogeneic-stimulated lymphocytes in vitro.* Rheumatology, 2007. **46**(3): p. 403-408.
15. Zhao, Q., H. Ren, and Z. Han, *Mesenchymal stem cells: Immunomodulatory capability and clinical potential in immune diseases.* Journal of Cellular Immunotherapy.
16. Gimble, J.M., A.J. Katz, and B.A. Bunnell, *Adipose-derived stem cells for regenerative medicine.* Circ Res, 2007. **100**(9): p. 1249-60.
17. Suga, H., et al., *Numerical measurement of viable and nonviable adipocytes and other cellular components in aspirated fat tissue.* Plast Reconstr Surg, 2008. **122**(1): p. 103-14.
18. Yoshimura, K., et al., *Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates.* J Cell Physiol, 2006. **208**(1): p. 64-76.
19. Kaplan, F.S., G.V. Hahn, and M.A. Zasloff, *Heterotopic Ossification: Two Rare Forms and What They Can Teach Us.* J Am Acad Orthop Surg, 1994. **2**(5): p. 288-296.
20. Debrock, G., et al., *A phase II trial with rosiglitazone in liposarcoma patients.* Br J Cancer, 2003. **89**(8): p. 1409-12.
21. Tontonoz, P., et al., *Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptor gamma and the retinoid X receptor.* Proc Natl Acad Sci U S A, 1997. **94**(1): p. 237-41.
22. Cornelius, P., O.A. MacDougald, and M.D. Lane, *Regulation of adipocyte development.* Annu Rev Nutr, 1994. **14**: p. 99-129.
23. Faust, I.M., P.R. Johnson, and J. Hirsch, *Adipose tissue regeneration following lipectomy.* Science, 1977. **197**(4301): p. 391-3.
24. Cone, R.D., *The Central Melanocortin System and Energy Homeostasis.* Trends Endocrinol Metab, 1999. **10**(6): p. 211-216.
25. Otto, T.C. and M.D. Lane, *Adipose development: from stem cell to adipocyte.* Crit Rev Biochem Mol Biol, 2005. **40**(4): p. 229-42.
26. Fraser, J.K., et al., *Fat tissue: an underappreciated source of stem cells for biotechnology.* Trends in Biotechnology, 2006. **24**(4): p. 150-154.

27. Zuk, P.A., et al., *Human adipose tissue is a source of multipotent stem cells.* Mol Biol Cell, 2002. **13**(12): p. 4279-95.
28. Pittenger, M.F., et al., *Multilineage potential of adult human mesenchymal stem cells.* Science, 1999. **284**(5411): p. 143-7.
29. Horwitz, E.M., et al., *Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement.* Cytotherapy, 2005. **7**(5): p. 393-5.
30. Mitchell, J.B., et al., *Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers.* Stem Cells, 2006. **24**(2): p. 376-85.
31. Ong, W.K. and S. Sugii, *Adipose-derived stem cells: fatty potentials for therapy.* The international journal of biochemistry & cell biology, 2013. **45**(6): p. 1083-1086.
32. Senarath-Yapa, K., et al., *Adipose-derived stem cells: a review of signaling networks governing cell fate and regenerative potential in the context of craniofacial and long bone skeletal repair.* International journal of molecular sciences, 2014. **15**(6): p. 9314-9330.
33. Ornitz, D.M. and P.J. Marie, *FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease.* Genes & development, 2002. **16**(12): p. 1446-1465.
34. Maes, C., et al., *Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels.* Developmental cell, 2010. **19**(2): p. 329-344.
35. Bielby, R., E. Jones, and D. McGonagle, *The role of mesenchymal stem cells in maintenance and repair of bone.* Injury, 2007. **38**(1): p. S26-S32.
36. Javed, A., H. Chen, and F.Y. Ghori, *Genetic and transcriptional control of bone formation.* Oral and maxillofacial surgery clinics of North America, 2010. **22**(3): p. 283-293.
37. James, A.W., *Review of signaling pathways governing MSC osteogenic and adipogenic differentiation.* Scientifica, 2013. **2013**.
38. Levanon, D. and Y. Groner, *Structure and regulated expression of mammalian RUNX genes.* Oncogene, 2004. **23**(24): p. 4211-4219.

39. Vimalraj, S., et al., *Runx2: Structure, function, and phosphorylation in osteoblast differentiation.* International journal of biological macromolecules, 2015. **78**: p. 202-208.
40. Marie, P.J., *Transcription factors controlling osteoblastogenesis.* Archives of biochemistry and biophysics, 2008. **473**(2): p. 98-105.
41. Otto, F., H. Kanegane, and S. Mundlos, *Mutations in the RUNX2 gene in patients with cleidocranial dysplasia.* Human mutation, 2002. **19**(3): p. 209-216.
42. Ziros, P.G., E.K. Basdra, and A.G. Papavassiliou, *Runx2: of bone and stretch.* The international journal of biochemistry & cell biology, 2008. **40**(9): p. 1659-1663.
43. Zhu, F., et al., *The transcription factor osterix (SP7) regulates BMP6-induced human osteoblast differentiation.* Journal of cellular physiology, 2012. **227**(6): p. 2677-2685.
44. Harada, S.-i. and G.A. Rodan, *Control of osteoblast function and regulation of bone mass.* Nature, 2003. **423**(6937): p. 349-355.
45. Okamoto, M., et al., *Noncanonical Wnt5a enhances Wnt/[bgr]-catenin signaling during osteoblastogenesis.* Scientific reports, 2014. **4**.
46. St-Jacques, B., M. Hammerschmidt, and A.P. McMahon, *Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation.* Genes & development, 1999. **13**(16): p. 2072-2086.
47. Yuasa, T., et al., *Sonic hedgehog is involved in osteoblast differentiation by cooperating with BMP-2.* Journal of cellular physiology, 2002. **193**(2): p. 225-232.
48. Wagner, D.O., et al., *BMPs: from bone to body morphogenetic proteins.* Sci. Signal., 2010. **3**(107): p. mr1-mr1.
49. Beederman, M., et al., *BMP signaling in mesenchymal stem cell differentiation and bone formation*.* Journal of Biomedical Science and Engineering, 2013. **6**(8A1): p. 32.
50. Zhang, X., et al., *Craniosynostosis in transgenic mice overexpressing Nell-1.* The Journal of clinical investigation, 2002. **110**(6): p. 861-870.

51. Zhang, X., et al., *Nell-1, a key functional mediator of Runx2, partially rescues calvarial defects in Runx2+/− mice.* Journal of bone and mineral research, 2011. **26**(4): p. 777-791.
52. Ahdjoudj, S., et al., *Transforming growth factor β2 inhibits adipocyte differentiation induced by skeletal unloading in rat bone marrow stroma.* Journal of bone and mineral research, 2002. **17**(4): p. 668-677.
53. Celil, A.B. and P.G. Campbell, *BMP-2 and insulin-like growth factor-I mediate Osterix (Osx) expression in human mesenchymal stem cells via the MAPK and protein kinase D signaling pathways.* Journal of Biological Chemistry, 2005. **280**(36): p. 31353-31359.
54. Yang, S.-J., et al., *Activation of Akt by advanced glycation end products (AGEs): involvement of IGF-1 receptor and caveolin-1.* PLoS One, 2013. **8**(3): p. e58100.
55. Garrett, F.D., *The development and phylogeny of the corpuscle of Stannius in ganoid and teleostean fishes.* Journal of morphology, 1942. **70**(1): p. 41-67.
56. Ogawa, M., *Fine structure of the corpuscles of Stannius and the interrenal tissue in goldfish, Carassius auratus.* Zeitschrift für Zellforschung und Mikroskopische Anatomie, 1967. **81**(2): p. 174-189.
57. Ishibashi, K. and M. Imai, *Prospect of a stanniocalcin endocrine/paracrine system in mammals.* American Journal of Physiology-Renal Physiology, 2002. **282**(3): p. F367-F375.
58. Sundell, K., et al., *Chum salmon (Oncorhynchus keta) stanniocalcin inhibitis in vitro intestinal calcium uptake in Atlantic cod (Gadus morhua).* Journal of Comparative Physiology B, 1992. **162**(6): p. 489-495.
59. Wagner, G.F., E.M. Jaworski, and M. Haddad, *Stanniocalcin in the seawater salmon: structure, function, and regulation.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 1998. **274**(4): p. R1177-R1185.
60. Wagner, G.F., et al., *The gill calcium transport cycle in rainbow trout is correlated with plasma levels of bioactive, not immunoreactive, stanniocalcin.* Molecular and cellular endocrinology, 1993. **93**(2): p. 185-191.

61. Olsen, H.S., et al., *Human stanniocalcin: a possible hormonal regulator of mineral metabolism.* Proceedings of the National Academy of Sciences, 1996. **93**(5): p. 1792-1796.
62. Lu, M., G.F. Wagner, and J.L. Renfro, *Stanniocalcin stimulates phosphate reabsorption by flounder renal proximal tubule in primary culture.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 1994. **267**(5): p. R1356-R1362.
63. McCudden, C., et al., *Novel expression of the stanniocalcin gene in fish.* Journal of Endocrinology, 2001. **171**(1): p. 33-44.
64. Serlachius, M., *Stanniocalcin-1 in cell stress and differentiation.* 2007.
65. Chang, A.C.-M., et al., *A novel human cDNA highly homologous to the fish hormone stanniocalcin.* Molecular and cellular endocrinology, 1995. **112**(2): p. 241-247.
66. Serlachius, M., K.-z. Zhang, and L.C. Andersson, *Stanniocalcin in terminally differentiated mammalian cells.* Peptides, 2004. **25**(10): p. 1657-1662.
67. Amemiya, Y. and J.H. Youson, *Primary structure of stanniocalcin in two basal Actinopterygii.* General and comparative endocrinology, 2004. **135**(2): p. 250-257.
68. Chang, A.C.-M., et al., *The murine stanniocalcin 2 gene is a negative regulator of postnatal growth.* Endocrinology, 2008. **149**(5): p. 2403-2410.
69. Haddad, M., et al., *Immunocytochemical localization of stanniocalcin cells in the rat kidney.* Endocrinology, 1996. **137**(5): p. 2113-2117.
70. Wagner, G.F., et al., *Immunological and biological evidence for a stanniocalcin-like hormone in human kidney.* Proceedings of the National Academy of Sciences, 1995. **92**(6): p. 1871-1875.
71. De Niu, P., et al., *Development of a human stanniocalcin radioimmunoassay: serum and tissue hormone levels and pharmacokinetics in the rat.* Molecular and cellular endocrinology, 2000. **162**(1): p. 131-144.
72. Wagner, G.F. and G.E. Dimattia, *The stanniocalcin family of proteins.* Journal of Experimental Zoology Part A: Comparative Experimental Biology, 2006. **305**(9): p. 769-780.
73. Chang, A.C., D. Jellinek, and R. Reddel, *Mammalian stanniocalcins and cancer.* Endocrine-related cancer, 2003. **10**(3): p. 359-373.

74. Chu, S., et al., *Evolution and functions of stanniocalcins in cancer.* International journal of immunopathology and pharmacology, 2015. **28**(1): p. 14-20.
75. Trindade, D.M., et al., *Low-resolution structural studies of human Stanniocalcin-1.* BMC structural biology, 2009. **9**(1): p. 1.
76. Sheikh-Hamad, D., et al., *Stanniocalcin-1 is a naturally occurring L-channel inhibitor in cardiomyocytes: relevance to human heart failure.* American Journal of Physiology-Heart and Circulatory Physiology, 2003. **285**(1): p. H442-H448.
77. Wang, Y., et al., *Stanniocalcin-1 suppresses superoxide generation in macrophages through induction of mitochondrial UCP2.* Journal of leukocyte biology, 2009. **86**(4): p. 981-988.
78. Zhang, K.-z., et al., *High expression of stanniocalcin in differentiated brain neurons.* The American journal of pathology, 1998. **153**(2): p. 439-445.
79. Varghese, R., et al., *Comparative analysis of mammalian stanniocalcin genes 1.* Endocrinology, 1998. **139**(11): p. 4714-4725.
80. Serlachius, M. and L.C. Andersson, *Upregulated expression of stanniocalcin-1 during adipogenesis.* Experimental cell research, 2004. **296**(2): p. 256-264.
81. Zhang, K.-z., et al., *Stanniocalcin: a molecular guard of neurons during cerebral ischemia.* Proceedings of the National Academy of Sciences, 2000. **97**(7): p. 3637-3642.
82. Filvaroff, E.H., et al., *Stanniocalcin 1 alters muscle and bone structure and function in transgenic mice.* Endocrinology, 2002. **143**(9): p. 3681-3690.
83. Ma, X., et al., *Hypoxia-induced overexpression of stanniocalcin-1 is associated with the metastasis of early stage clear cell renal cell carcinoma.* Journal of translational medicine, 2015. **13**(1): p. 56.
84. Chen, C., et al., *Human Stanniocalcin-1 Blocks TNF-α–Induced Monolayer Permeability in Human Coronary Artery Endothelial Cells.* Arteriosclerosis, thrombosis, and vascular biology, 2008. **28**(5): p. 906-912.
85. Fujiwara, Y., et al., *Assessment of Stanniocalcin-1 mRNA as a molecular marker for micrometastases of various human cancers.* International journal of oncology, 2000. **16**(4): p. 799-1603.

86. Tohmiya, Y., et al., *Stanniocalcin-1 as a novel marker to detect minimal residual disease of human leukemia.* The Tohoku journal of experimental medicine, 2004. **204**(2): p. 125-133.
87. Tamura, S., et al., *Clinical significance of STC1 gene expression in patients with colorectal cancer.* Anticancer research, 2011. **31**(1): p. 325-329.
88. Liu, G., et al., *Stanniocalcin 1 and ovarian tumorigenesis.* Journal of the National Cancer Institute, 2010. **102**(11): p. 812-827.
89. Du, Y.Z., et al., *The diagnostic value of circulating stanniocalcin-1 mRNA in non-small cell lung cancer.* Journal of surgical oncology, 2011. **104**(7): p. 836-840.
90. Joensuu, K., P. Heikkilä, and L.C. Andersson, *Tumor dormancy: elevated expression of stanniocalcins in late relapsing breast cancer.* Cancer letters, 2008. **265**(1): p. 76-83.
91. Bouras, T., et al., *Stanniocalcin 2 is an estrogen-responsive gene coexpressed with the estrogen receptor in human breast cancer.* Cancer Research, 2002. **62**(5): p. 1289-1295.
92. Chang, A.C., et al., *STC1 expression is associated with tumor growth and metastasis in breast cancer.* Clinical & experimental metastasis, 2015. **32**(1): p. 15-27.
93. Murai, R., et al., *Stanniocalcin-1 promotes metastasis in a human breast cancer cell line through activation of PI3K.* Clinical & experimental metastasis, 2014. **31**(7): p. 787-794.
94. Fang, Z., et al., *Clinical significance of stanniocalcin expression in tissue and serum of gastric cancer patients.* Chinese Journal of Cancer Research, 2014. **26**(5): p. 602-610.
95. Peña, C., et al., *STC1 expression by cancer-associated fibroblasts drives metastasis of colorectal cancer.* Cancer Research, 2013. **73**(4): p. 1287-1297.
96. He, L., et al., *Stanniocalcin-1 promotes tumor angiogenesis through up-regulation of VEGF in gastric cancer cells.* J Biomed Sci, 2011. **18**(1): p. 39.
97. Block, G.J., et al., *Multipotent stromal cells are activated to reduce apoptosis in part by upregulation and secretion of stanniocalcin-1.* Stem cells, 2009. **27**(3): p. 670-681.

98. Lai, K.P., et al., *Induction of stanniocalcin-1 expression in apoptotic human nasopharyngeal cancer cells by p53.* Biochemical and biophysical research communications, 2007. **356**(4): p. 968-975.
99. Law, A., et al., *Histone deacetylase inhibitor-induced cellular apoptosis involves stanniocalcin-1 activation.* Experimental cell research, 2008. **314**(16): p. 2975-2984.
100. Law, A., et al., *Identification and characterization of the hypoxia-responsive element in human stanniocalcin-1 gene.* Molecular and cellular endocrinology, 2010. **314**(1): p. 118-127.
101. Nguyen, A., A. Chang, and R. Reddel, *Stanniocalcin-1 acts in a negative feedback loop in the prosurvival ERK1/2 signaling pathway during oxidative stress.* Oncogene, 2009. **28**(18): p. 1982-1992.
102. LAFEBER, F.P., et al., *Parathyroid Hormone-Like Effects of Rainbow Trout Stannius Products on Bone Resorption of Embryonic Mouse Calvaria in Vitro*.* Endocrinology, 1986. **119**(5): p. 2249-2255.
103. Yoshiko, Y., J.E. Aubin, and N. Maeda, *Stanniocalcin 1 (STC1) protein and mRNA are developmentally regulated during embryonic mouse osteogenesis: the potential of stc1 as an autocrine/paracrine factor for osteoblast development and bone formation.* Journal of Histochemistry & Cytochemistry, 2002. **50**(4): p. 483-491.
104. Yoshiko, Y., N. Maeda, and J.E. Aubin, *Stanniocalcin 1 stimulates osteoblast differentiation in rat calvaria cell cultures.* Endocrinology, 2003. **144**(9): p. 4134-4143.
105. Yoshiko, Y., et al., *Evidence for Stanniocalcin Gene Expression in Mammalian Bone 1.* Endocrinology, 1999. **140**(4): p. 1869-1874.
106. Yoshiko, Y., et al., *Osteoblast autonomous Pi regulation via Pit1 plays a role in bone mineralization.* Molecular and cellular biology, 2007. **27**(12): p. 4465-4474.
107. Johnston, J., et al., *Human stanniocalcin-1 or-2 expressed in mice reduces bone size and severely inhibits cranial intramembranous bone growth.* Transgenic research, 2010. **19**(6): p. 1017-1039.
108. Naot, D. and J. Cornish, *The role of peptides and receptors of the calcitonin family in the regulation of bone metabolism.* Bone, 2008. **43**(5): p. 813-818.

109. Feyen, J.H., et al., *N-terminal truncation of salmon calcitonin leads to calcitonin antagonists: Structure activity relationship of N-terminally truncated salmon calcitonin fragments in vitro and in vivo.* Biochemical and biophysical research communications, 1992. **187**(1): p. 8-13.
110. Pozvek, G., et al., *Structure/function relationships of calcitonin analogues as agonists, antagonists, or inverse agonists in a constitutively activated receptor cell system.* Molecular pharmacology, 1997. **51**(4): p. 658-665.
111. Amara, S.G., et al., *Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products.* 1982.
112. Goodman, E. and L. Iversen, *Calcitonin gene-related peptide: novel neuropeptide.* Life sciences, 1986. **38**(24): p. 2169-2178.
113. Granholm, S., P. Henning, and U.H. Lerner, *Comparisons between the effects of calcitonin receptor-stimulating peptide and intermedin and other peptides in the calcitonin family on bone resorption and osteoclastogenesis.* Journal of cellular biochemistry, 2011. **112**(11): p. 3300-3312.
114. Schinke, T., et al., *Decreased Bone Formation and Osteopenia in Mice Lacking α-Calcitonin Gene-Related Peptide.* Journal of bone and mineral research, 2004. **19**(12): p. 2049-2056.
115. Lin Chang, C. and S. Yu Teddy Hsu, *Roles of CLR/RAMP receptor signaling in reproduction and development.* Current Protein and Peptide Science, 2013. **14**(5): p. 393-406.
116. Vignery, A. and T. McCarthy, *The neuropeptide calcitonin gene-related peptide stimulates insulin-like growth factor I production by primary fetal rat osteoblasts.* Bone, 1996. **18**(4): p. 331-335.
117. Villa, I., et al., *CGRP inhibits osteoprotegerin production in human osteoblast-like cells via cAMP/PKA-dependent pathway.* American Journal of Physiology-Cell Physiology, 2006. **291**(3): p. C529-C537.
118. Kawase, T., K. Okuda, and D.M. Burns, *Immature human osteoblastic MG63 cells predominantly express a subtype 1-like CGRP receptor that inactivates extracellular signal response kinase by a cAMP-dependent mechanism.* European journal of pharmacology, 2003. **470**(3): p. 125-137.

119. Monier-Faugere, M.C., et al., *Calcitonin prevents bone loss but decreases osteoblastic activity in ovariohysterectomized beagle dogs.* Journal of bone and mineral research, 1996. **11**(4): p. 446-455.
120. Smillie, S.-J. and S.D. Brain, *Calcitonin gene-related peptide (CGRP) and its role in hypertension.* Neuropeptides, 2011. **45**(2): p. 93-104.
121. Born, W., J. Fischer, and R. Muff, *Receptors for calcitonin gene-related peptide, adrenomedullin, and amylin: the contributions of novel receptor-activity-modifying proteins.* Receptors and Channels, 2002. **8**(3-4): p. 201-209.
122. Conner, A.C., et al., *Interaction of calcitonin-gene-related peptide with its receptors.* Biochemical Society Transactions, 2002. **30**(4): p. 451-455.
123. Wang, L., et al., *Calcitonin-gene-related peptide stimulates stromal cell osteogenic differentiation and inhibits RANKL induced NF-κB activation, osteoclastogenesis and bone resorption.* Bone, 2010. **46**(5): p. 1369-1379.
124. Togari, A., et al., *Expression of mRNAs for neuropeptide receptors and β-adrenergic receptors in human osteoblasts and human osteogenic sarcoma cells.* Neuroscience letters, 1997. **233**(2): p. 125-128.
125. Uzan, B., M.-C. de Vernejoul, and M. Cressent, *RAMPs and CRLR expressions in osteoblastic cells after dexamethasone treatment.* Biochemical and biophysical research communications, 2004. **321**(4): p. 802-808.
126. Lafont, A.G., et al., *Involvement of calcitonin and its receptor in the control of calcium-regulating genes and calcium homeostasis in zebrafish (Danio rerio).* Journal of bone and mineral research, 2011. **26**(5): p. 1072-1083.
127. Roch, G.J. and N.M. Sherwood, *Stanniocalcin has deep evolutionary roots in eukaryotes.* Genome biology and evolution, 2011. **3**: p. 284-294.
128. Schein, V., et al., *Four stanniocalcin genes in teleost fish: Structure, phylogenetic analysis, tissue distribution and expression during hypercalcemic challenge.* General and comparative endocrinology, 2012. **175**(2): p. 344-356.
129. De Francesco, F., et al., *Human Adipose Stem Cells: From Bench to Bedside.* Tissue Engineering Part B: Reviews, 2015. **21**(6): p. 572-584.
130. Barba, M., et al., *Adipose-derived mesenchymal cells for bone regereneration: state of the art.* BioMed research international, 2013. **2013**.

131. Marino, G., et al., *β-Tricalcium phosphate 3D scaffold promote alone osteogenic differentiation of human adipose stem cells: in vitro study.* Journal of Materials Science: Materials in Medicine, 2010. **21**(1): p. 353-363.
132. Bourin, P., et al., *Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT).* Cytotherapy, 2013. **15**(6): p. 641-648.
133. Sharma, U., D. Pal, and R. Prasad, *Alkaline phosphatase: an overview.* Indian Journal of Clinical Biochemistry, 2014. **29**(3): p. 269-278.
134. Ducy, P., et al., *A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development.* Genes & development, 1999. **13**(8): p. 1025-1036.
135. Schroeder, T.M., E.D. Jensen, and J.J. Westendorf, *Runx2: a master organizer of gene transcription in developing and maturing osteoblasts.* Birth Defects Research Part C: Embryo Today: Reviews, 2005. **75**(3): p. 213-225.
136. Jonason, J., et al., *Post-translational regulation of Runx2 in bone and cartilage.* Journal of dental research, 2009. **88**(8): p. 693-703.
137. Franceschi, R.T. and G. Xiao, *Regulation of the osteoblast-specific transcription factor, Runx2: Responsiveness to multiple signal transduction pathways.* Journal of cellular biochemistry, 2003. **88**(3): p. 446-454.
138. Denhardt, D.T., et al., *Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival.* The Journal of clinical investigation, 2001. **107**(9): p. 1055-1061.
139. Klein-Nulend, J., et al., *Mechanical stimulation of osteopontin mRNA expression and synthesis in bone cell cultures.* Journal of cellular physiology, 1997. **170**(2): p. 174-181.
140. Ikeda, T., et al., *In situ hybridization of bone matrix proteins in undecalcified adult rat bone sections.* Journal of Histochemistry & Cytochemistry, 1992. **40**(8): p. 1079-1088.
141. Prince, C.W. and W.T. Butler, *1, 25-Dihydroxyvitamin D3 regulates the biosynthesis of osteopontin, a bone-derived cell attachment protein, in*

clonal osteoblast-like osteosarcoma cells. Collagen and related research, 1987. **7**(4): p. 305-313.

142. Beck, G.R., B. Zerler, and E. Moran, *Phosphate is a specific signal for induction of osteopontin gene expression.* Proceedings of the National Academy of Sciences, 2000. **97**(15): p. 8352-8357.
143. Denhardt, D.T. and X. Guo, *Osteopontin: a protein with diverse functions.* The FASEB journal, 1993. **7**(15): p. 1475-1482.
144. Hauschka, P.V. and F.H. Wians, *Osteocalcin-hydroxyapatite interaction in the extracellular organic matrix of bone.* The Anatomical Record, 1989. **224**(2): p. 180-188.
145. Bianco, P., et al., *Expression of bone sialoprotein (BSP) in developing human tissues.* Calcified tissue international, 1991. **49**(6): p. 421-426.
146. Weng, J.-j. and Y. Su, *Nuclear matrix-targeting of the osteogenic factor Runx2 is essential for its recognition and activation of the alkaline phosphatase gene.* Biochimica et Biophysica Acta (BBA)-General Subjects, 2013. **1830**(3): p. 2839-2852.
147. Kawamura, N., et al., *Akt1 in osteoblasts and osteoclasts controls bone remodeling.* PLoS One, 2007. **2**(10): p. e1058.
148. Chen, J., et al., *The key regulatory roles of the PI3K/Akt signaling pathway in the functionalities of mesenchymal stem cells and applications in tissue regeneration.* Tissue Engineering Part B: Reviews, 2013. **19**(6): p. 516-528.
149. Fujita, T., et al., *Runx2 induces osteoblast and chondrocyte differentiation and enhances their migration by coupling with PI3K-Akt signaling.* The Journal of cell biology, 2004. **166**(1): p. 85-95.
150. Choi, Y.H., et al., *Akt enhances Runx2 protein stability by regulating Smurf2 function during osteoblast differentiation.* FEBS Journal, 2014. **281**(16): p. 3656-3666.
151. Gambardella, A., et al., *Glycogen synthase kinase-3α/β inhibition promotes in vivo amplification of endogenous mesenchymal progenitors with osteogenic and adipogenic potential and their differentiation to the osteogenic lineage.* Journal of bone and mineral research, 2011. **26**(4): p. 811-821.

152. Galli, C., et al., *GSK3b-inhibitor lithium chloride enhances activation of Wnt canonical signaling and osteoblast differentiation on hydrophilic titanium surfaces.* Clinical oral implants research, 2013. **24**(8): p. 921-927.
153. Ballica, R., et al., *Targeted Expression of Calcitonin Gene-Related Peptide to Osteoblasts Increases Bone Density in Mice.* Journal of bone and mineral research, 1999. **14**(7): p. 1067-1074.
154. Huebner, A.K., et al., *Calcitonin deficiency in mice progressively results in high bone turnover.* Journal of bone and mineral research, 2006. **21**(12): p. 1924-1934.
155. Roh, J., et al., *Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes.* Journal of Biological Chemistry, 2004. **279**(8): p. 7264-7274.
156. Watts, V.J. and K.A. Neve, *Sensitization of adenylate cyclase by Gα i/o-coupled receptors.* Pharmacology & therapeutics, 2005. **106**(3): p. 405-421.
157. Skalhegg, B. and K. Tasken, *Specificity in the cAMP/PKA signaling pathway. Differential expression, regulation, and subcellular localization of subunits of PKA.* Front Biosci, 2000. **5**(5): p. D678-D693.
158. Luo, C.-W., et al., *Paracrine regulation of ovarian granulosa cell differentiation by stanniocalcin (STC) 1: mediation through specific STC1 receptors.* Molecular Endocrinology, 2004. **18**(8): p. 2085-2096.

ANEXOS

LISTA DE FIGURAS

I.1 INTRODUÇÃO

II.1 ARTIGO 1

II.2 ARTIGO 2