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"MECANISMOS DE TRANSDUÇÃO DE SINAL ENVOLVIDOS COM A DIFERENCIAÇÃO DE OSTEOBLASTOS E OSTEÓCITOS"

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas para a obtenção do título de Doutor em Biologia Funcional e Molecular, na área de concentração em Bioquímica.

Orientadora: Profa. Dra. Carmen Veríssima Ferreira

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"Nas grandes batalhas da vida,

o primeiro passo para a vitória é o desejo de vencer."

Mahatma Gandhi

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RESUMO

Este trabalho teve como principal objetivo investigar os mecanismos de transdução de sinal disparados durante a diferenciação de células ósseas. Desta forma, vários aspectos moleculares desse processo foram analisados. A modulação da Src kinase pela proteína tirosina fosfatase de baixo peso molecular (LMWPTP) é essencial para a diferenciação dos pré-osteoblastos induzida pelo ácido ascórbico/glicerofosfato. Outro enfoque dado nesse trabalho foi a avaliação, sob o aspecto molecular e morfológico, da diferenciação de pré-osteoblastos em "osteocyte-like cells", processo esse induzido quando o Matrigel foi utilizado como substrato. De forma inédita demonstramos que nessa condição as células produziram a proteína sonic hedgehog (Shh), a qual também foi essencial para o processo de diferenciação. A análise do perfil quinômico dessas células apontou uma prevalência das quinases envolvidas com a comunicação celular. Este fato é coerente com a super-expressão de conexina 43 observada. Além disso, observamos que RECK e TIMP-1 modulam a atividade do rearranjo da matriz extracelular durante a diferenciação de osteoblastos, bem como o requerimento das proteínas PP2A e p38 MAPK durante a adesão de osteoblastos. Adicionalmente observamos uma modulação refinada das PTPs (LMWPTP, SHP2 e PTPα) bem como do status redox celular. Os resultados em conjunto demonstram que o estudo de transdução de sinal pode fornecer informações importantes para o entendimento do funcionamento celular bem como definir alvos moleculares que podem servir como ferramentas para diferentes aplicações.

ABSTRACT

The main goal of this work was to investigate the signal transduction pathways triggered during the bone cells differentiation. In this way, several molecular aspects of this process were analyzed. Src kinase modulation by the low molecular weight protein tyrosine phosphatase (LMWPTP) was essential for the occurrence of the differentiation induced by ascorbic acid and glycerophosphate. We also evaluated, under molecular and morphological patterns, the differentiation of pre-osteoblasts into osteocyte-like cells induced by 3D scaffold (matrigel as substrate). Interestingly, under this condition, the cells produced sonic hedgehog (Shh), which also was essential for stimulating the differentiation signaling pathway. Kinomic profiling of these cells revealed a prevalence of kinases involved in cellular communication, which is in agreement with the overexpression of connexin 43 observed. Besides we observed that RECK and TIMP-1 modulated the extracellular matrix rearrangement and that PP2A and p38 MAPK are required for osteoblasts adhesion. In addition, we observed that during pre-osteoblasts differentiation both PTPs and cellular redox are tightly regulated. Our findings demonstrated that the signal transduction evaluation can provide important information for understanding the cell biology as well as defining molecular targets that can be useful for different applications.

ABREVIATURAS

Akt	Protein kinase B
ALP	Alkaline Phosphatase
Cdks	Proteinas quinases dependents de ciclinas
CDNB	1-chloro-2,4-dinitrobenzene
Cxn	Conexin 43
Dhh	Desert hedgehog
DMF	N,N Dimethylformamide
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemioluminescence
ECM	Extracellular Matrix
MEC	Matrix Extracelular
EGTA	Ethylene glycol tetraacetic acid
EROs	Reactive-oxygen species
FAK	Focal adhesion kinase
FCS	Soro fetal bovino
Fyn	Proteína pertencente à Família de Src
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutationa
GSK3	Glycogen synthase kinase
GST	Glutathione-S-transferase
H_2O_2	Peróxido de Hidrogênio
HB	Homogenization Buffer
hMSC	human mesenchymal stem cell
hMVEC	human Microvascular Endothelial Cells
Ihh	Indian hedgehog
JNK/SAPK	c-jun N-terminal kinase / Stress-activated protein kinase
kDa	Kilodaltons
LMWPTP	Proteina Tirosina Fosfatase de baixo peso molecular
MAPK	Mitogen-avtivated protein kinase

MAPKAPK2	Mitogen-activated protein kinase-activated protein kinase 2
MC3T3-E1	Linhagem de imortalizada de pré-osteoblastos
MDA	Malondialdehyde
MMP	Matrix Metalloproteinase
MPO	N-methyl-2-phenyllindole
Ob	Osteoblastos
Oc	Osteócitos
Ocl	Osteoclastos
OM	Ostogenic Medium
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-Buffer Saline
PDGF	Fator de crescimento derivado de plaquetas
PDGFR	Receptor de PDGF
PDK1	Phosphoinositide-dependent protein kinase 1
PI3K	Phosphoinositide-3 kinase
PK	Protein Kinase
PKA	Protein kinase A
PKC	Protein Kinase C
PMSF	Phenyl-methyl sulphonyl fluoride
PP	Protein Phosphatase
PP2A	Proteína fosfatase 2A
Ptc	Patched
PTK	Protein Tyrosine Kinase
PTP	Proteina tirosina fosfatase
PTPα	Protein Tyrosine Phosphatase alpha
pTyr	Resíduo de fosfotirosina
PVDF	Polyvinylidene difluoride
RankL	Receptor activator NFkappaB-ligand
Rb	Retinoblastoma
RECK	Reversion-inducing cysteine rich protein with Kazal motifs
Rho	GTPase

Sam68	Substrato de Src
Ser	Serina
SH2/3	src homology domain 2/3
Shh	Sonic hedgehog
Shp1	Src hmology 2
Shp2	Src homology 2 domain containing tyrosine phosphatase
Smo	Smoothened
TBS	Tris-Buffer Saline
Thr	Treonina
TIMP	Tissue Inhibitor Metalloproteinase
Y	Tirosina;
Yes	Proteína pertencente à Família de Src
ρNPP	ρ-nitrophenylphosphate

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Capítulo 1

Introdução e Objetivos

INTRODUÇÃO

O tecido ósseo atua proporcionando suporte e proteção mecânica para os órgãos vitais e desempenhando também outras importantes funções, dentre as quais podemos destacar sua atuação como centro de armazenamento/liberação controlada de íons como cálcio, fosfato e magnésio, contribuindo, desta maneira, para a homeostase destes minerais no organismo. Histologicamente trata-se de um tipo especializado de tecido conjuntivo formado por células e material extracelular mineralizado, a matriz óssea. Em termos de constituição celular, o tecido ósseo é formado por: *osteócitos*, os quais se situam em cavidades ou lacunas no interior da matriz mineralizada; *osteoclastos*, que são células gigantes, móveis e multinucleadas, que reabsorvem a matriz mineralizada, participando do processo de remodelação juntamente com os osteoblastos; *osteoblastos*, os quais são capazes de produzir tanto a matriz orgânica do osso como também sua mineralização. A **Figura 1** representa de forma esquemática as células ósseas e como as mesmas estão dispostas no tecido.

O processo de remodelação óssea apresenta intensa relação entre a matriz extracelular (MEC) e células, apresentando forte influência nos mecanismos de transdução de sinal (Anselme, 2000). Dentre os diferentes processos celulares afetados durante a remodelação óssea, o rearranjo de proteínas do citoesqueleto e a expressão diferencial de moléculas de adesão estão em intensa atividade. Em conjunto, estes mecanismos são finamente regulados durante a vida e sua homeostase pode ser alterada por algumas condições fisiológicas, como envelhecimento e estresse químico ou mecânico. Neste contexto, a busca por novas terapias envolvendo o tecido ósseo tem obtido destaque nos

últimos anos, principalmente por causa do aumento da expectativa de vida da população mundial. No caso da terapia de perdas ósseas, a engenharia de tecidos tem oferecido subsídios pelos quais células ósteo-progenitoras possam ser associadas à biomateriais com o objetivo de desenvolver ósteo-substitutos ideais capazes de restaurar e manter as características do tecido (Nerem, 1992; Langer & Vacanti, 1993).

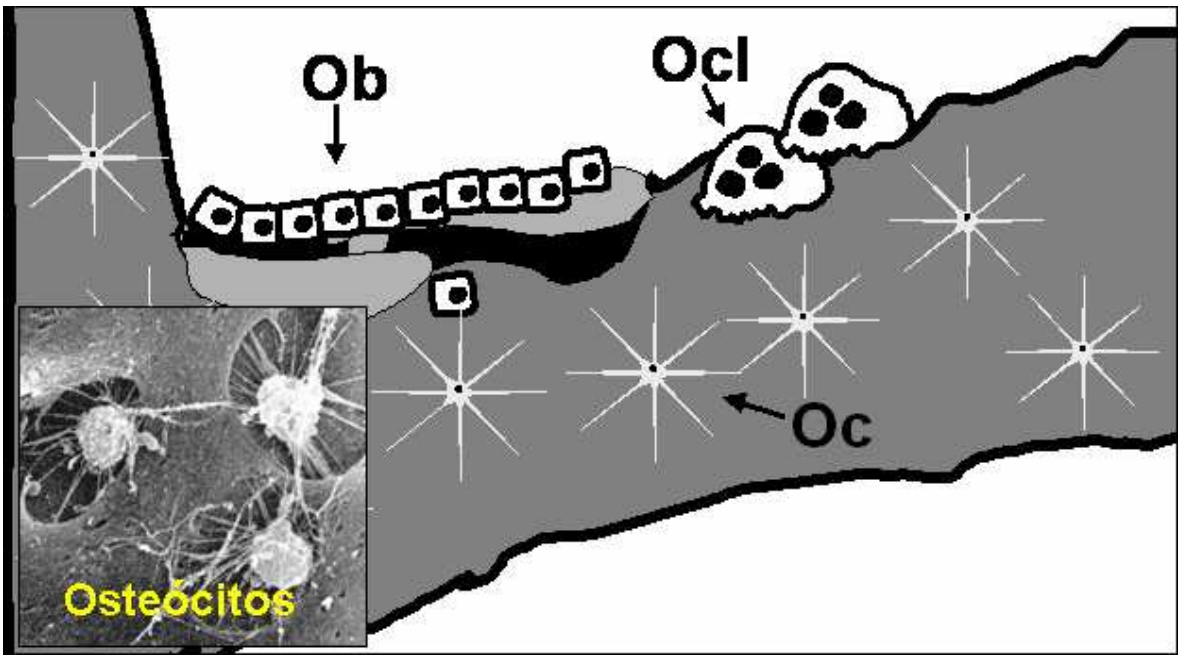

Figura 1: Representação esquemática das células ósseas. Esta figura representa os 3 tipos de células ósseas: Osteoblastos (Ob), Osteoclastos (Ocl) e Osteócitos (Oc). Em destaque note que os osteócitos são células que ficam aprisionadas em lacunas especializadas na matriz mineralizada, de onde os mesmos emitem seus prolongamentos citoplasmáticos.

Embora não existam estatísticas brasileiras, sabe-se que nos Estados Unidos, cerca de 400.000 enxertos ósseos foram realizados no ano 2000, dos quais a maioria foi de enxertos autógenos (Service, 2000). Além de apresentar suas inconvenientes (segundo sítio cirúrgico, morbidade, dor, seqüelas, custo, etc.) os enxertos autógenos nem sempre estão disponíveis em quantidade e qualidade suficiente (Hunt e Jovanovic, 1999; Yashikawa, 2000). Logo, a importância do entendimento dos mecanismos envolvidos na osteogênese tem aumentado. Desta forma, a busca de novas alternativas terapêuticas torna relevante a investigação dos mecanismos de transdução de sinal disparados durante a diferenciação de osteoblastos. Diante deste cenário, neste trabalho será dado destaque ao envolvimento de Proteínas Tirosina Fosfatases como moléculas mediadoras da diferenciação de osteoblastos.

Diferenciação de Osteoblastos *in vitro*: Um Modelo Biológico para o Estudo da Osteogênese

Os osteoblastos são as células responsáveis pela síntese da parte orgânica da matriz óssea (colágeno tipo 1, proteoglicanas e glicoproteínas adesivas) e também são capazes de concentrar fosfato de cálcio, participando da mineralização da matriz. Estas células, *in vivo,* encontram-se emparelhadas nas superfícies ósseas, em um arranjo que lembra um epitélio simples. Quando em intensa atividade sintética, apresentam-se cuboidais e em baixa atividade, tornam-se achatadas.

A diferenciação de osteoblastos *in vivo* envolve, predominantemente, 3 fases distintas: a primeira fase está relacionada com a expressão de genes relacionados ao ciclo celular, aumentando sua taxa proliferativa; a segunda refere-se à diminuição da atividade proliferativa e simultaneamente o aumento da atividade fosfatase alcalina, dando início à

maturação da matriz extracelular; finalmente, o aumento na expressão de osteocalcina e osteopontina inicia o período de mineralização da matriz óssea, a qual induz modificações metabólicas e morfológicas dos osteoblastos. Cabe ressaltar que durante o processo de mineralização biológica *in vivo*, os osteoblastos tendem a se aprisionarem, dando origem aos osteócitos (Stein *et al.*,1990).

As fases de diferenciação dos osteoblastos cultivados *in vitro* assemelham-se bastante aos mecanismos referentes à diferenciação *in vivo*, passando por uma fase de proliferação, maturação e mineralização, que são determinadas por modificações na expressão de genes específicos e acompanhadas pela variação da morfologia.

O processo de mineralização biológica consiste na deposição de íons inorgânicos, principalmente fosfato de cálcio, representando cerca de 50% do peso da matriz óssea. Além destes, são encontrados ainda os magnésio, sódio, potássio, bicarbonato e citrato. O cálcio e o fosfato formam cristais, que estudos de difração de raios-X mostram ter uma estrutura tipo hidroxiapatita, cuja superfície possui íons hidratados formando a chamada camada de hidratação, a qual facilita a troca de íons entre o cristal e o líquido intersticial.

Nas últimas décadas, os osteoblastos vêm sendo amplamente utilizados *in vitro* para o estudo do metabolismo ósseo e interações células/biomaterial. Para este fim são utilizadas linhagens celulares provenientes de ratos (UMR-106), camundongos (MC3T3-E1), fetos humanos (hFOB 1.19) e osteosarcoma (SAOS-2) - (Wang *et al.*, 1999; Declercq *et al.*, 2004; Coelho *et al.*, 2000a Coelho *et al.*, 2000b).

Particularmente, a linhagem celular de pré-osteoblastos MC3T3-E1, obtida por Sudo *et al.* em 1983; (**Figura 2**), tem sido muito utilizada para o estudo do processo de diferenciação de osteoblastos e subseqüente mineralização biológica. Assim, sabe-se que estas células apresentam características fenotípicas de células diferenciadas (osteoblastos)

por volta do 21º dia de tratamento com ácido ascórbico e *β*-glicerofosfato (Quarles *et al.,* 1992; Franceschi & Iyer, 1992). Durante este processo, a primeira semana de cultura é caracterizada pela intensa taxa proliferativa e morfologia fusiforme. Após esse período, as células atingem confluência e exibem morfologia cuboidal, diminuindo a taxa de crescimento (proliferação).

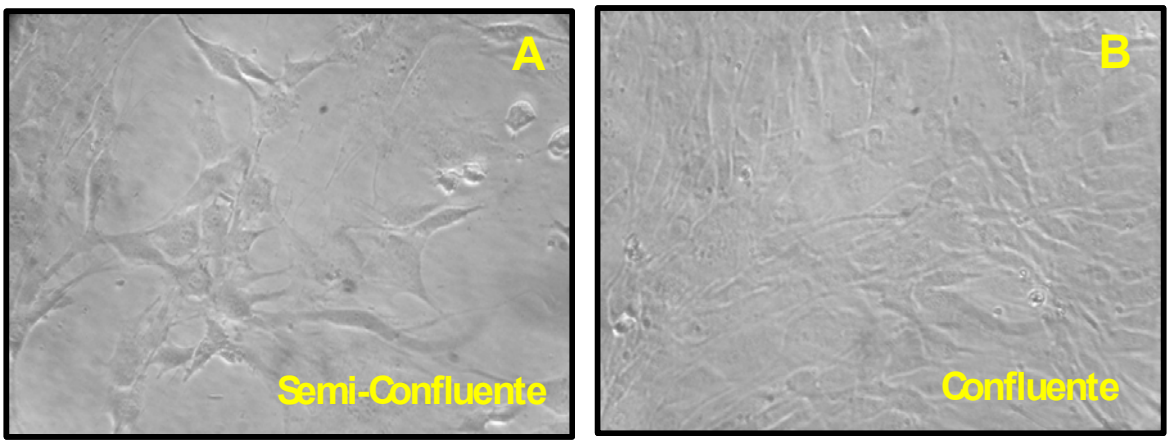

Figura 2: Células MC3T3-E1 em seus estágios de semi-confluência **(A)** e confluência **(B)**. **Aumento:** 40x.

Outra fonte de células osteoprogenitoras e osteoblastos para estudos *in vitro* é a coleta de fragmentos de osso medular para realização da cultura primária. A cultura primária de osteoblastos humanos é um excelente modelo de estudo pelo fato de que, quando cultivadas em meio adequado, mimetizam *in vitro* uma matriz extracelular mineralizada. Porém, a cultura primária apresenta a grande desvantagem de impossibilitar a execução dos estudos com uma mesma amostra de células. De fato, a manutenção deste tipo de cultura por longos períodos não é possível o que está associado à limitada vida útil das células diferenciadas *in*

vitro (Freshney, 2000; Coelho *et al.*, 2000a e b). Outras desvantagens deste modelo de estudo são: heterogeneidade do fenótipo e baixas taxas de crescimento (Subramaniam *et al.*, 2002). Por estas razões, neste trabalho foi utilizada a linhagem MC3T3-E1 como modelo experimental.

Mecanismos de Transdução do Sinal

A habilidade das células em receber e reagir a sinais externos à membrana plasmática é fundamental para a manutenção da homeostase do organismo. Esta capacidade de resposta celular é mediada por mecanismos de transdução de sinal, produzindo respostas intracelulares, às quais normalmente requerem a alteração da expressão de genes específicos.

O balanço entre a fosforilação e desfosforilação de proteínas é a base para o controle de diversos eventos biológicos disparados por efetores extracelulares, como hormônios, mitógenos, oncogenes, citocinas e neurotransmissores. Em particular, a fosforilação e desfosforilação de resíduos de tirosina em proteínas tem emergido como um evento chave na regulação da divisão, diferenciação e desenvolvimento celular, regulação do metabolismo e expressão gênica, contração, transporte, locomoção celular, aprendizado e memória (como revisado em Johnson e Barford, 1993). Em um contexto geral, o requerimento de uma ação coordenada de proteínas tirosinas quinases (PTKs) e proteínas tirosinas fosfatases (PTPs) na transdução do sinal mitogênico tem sido amplamente estudado ao longo dos anos. O uso de cascatas de fosforilação/defosforilação garante a amplificação do sinal, controle negativo e "cross-talk" entre as diversas vias de sinalização (Ferreira *et al.*, 2006; Monteiro *et al.*, 2008; den Hertog *et al.*, 2008).

Proteínas Quinases Ativadas por Mitógenos (MAPKs)

A principal via para regulação do crescimento e da diferenciação celular é através das cascatas envolvendo as Proteínas Quinases Ativadas por Mitógenos (MAPKs), as quais transmitem sinais da membrana plasmática ao núcleo a partir da ligação de hormônios e fatores de crescimento (estímulo extracelular) a receptores específicos na membrana plasmática.

MAPKs são proteínas serina/treonina quinases (representação da via na **Figura 3**) que podem fosforilar vários substratos citoplasmáticos e nucleares, culminando na regulação da expressão de genes que codificam diversas proteínas, incluindo c-Jun, c-myc, ATF2, SAP-1 e MEF2C (Minden e Karin, 1997; Haneda *et al.*, 1999; Nebreda e Porras, 2000). As MAPKs pertencem a uma grande família que inclui a quinase c-Jun N-terminal ou proteína quinase ativada por estresse (JNK/SAPK), a proteína quinase ativada por sinal extracelular (ERK) e a sub-família da MAPK p38, a qual está envolvida na resposta ao estresse desencadeado por vários fatores ambientais. As MAPKs JNK e p38 podem ser ativadas pela radiação UV e alguns quimioterápicos, com a conseqüente indução da apoptose (Berra *et al.*, 2000; Barr e Bogoyevitch, 2001; Kong *et al.*, 2001).

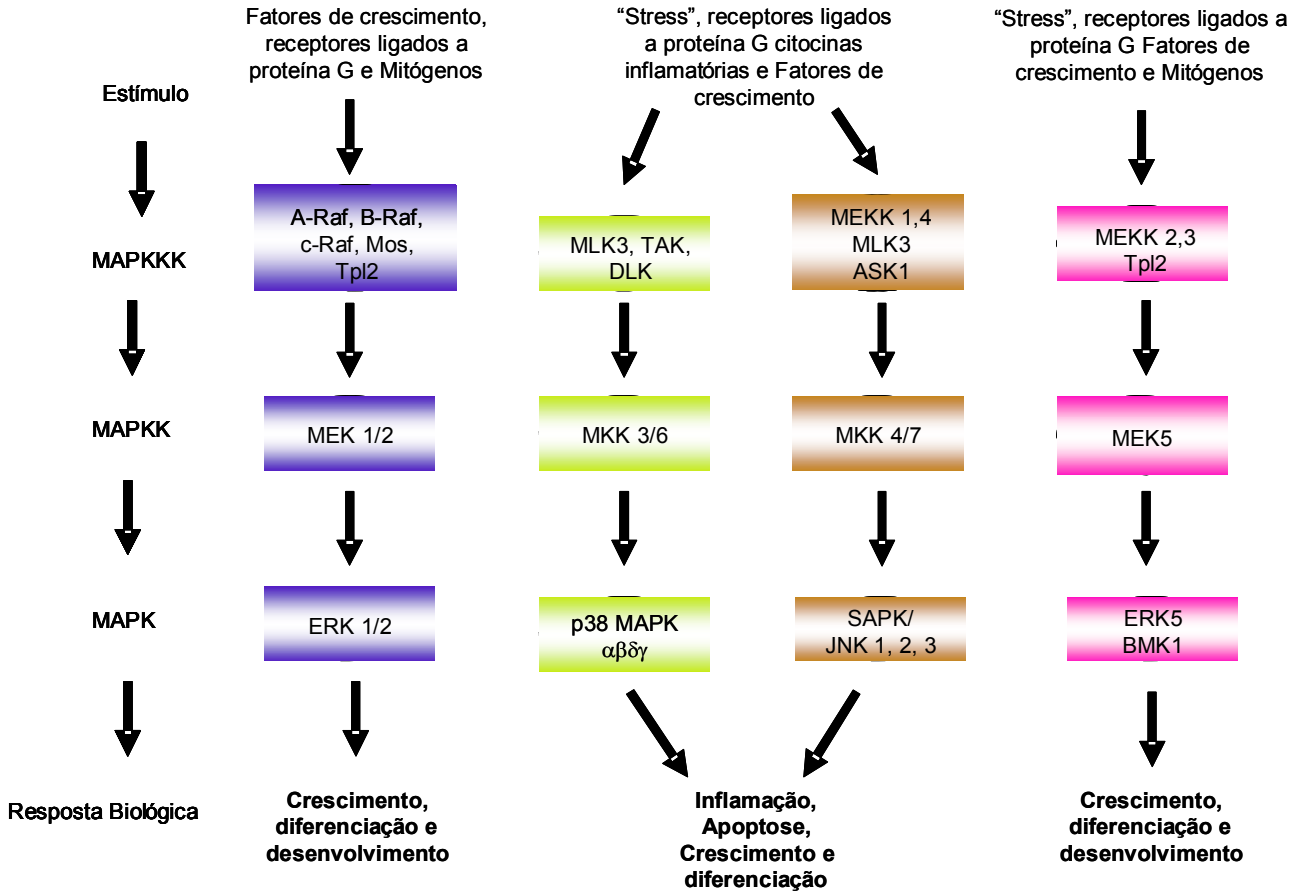

Figura 3: Cascata de sinalização das MAPKs.

Suzuki *et al.* (2002) mostraram que a estimulação da proliferação e diferenciação de osteoblastos envolve uma ativação coordenada de diversas MAPKs que possuem funções distintas e específicas durante a regulação destes eventos celulares. Dentre estas MAPKs, a p38 é ativada quando as células iniciam a diferenciação, controlando, inclusive, a expressão da fosfatase alcalina.

Papel Biológico das Proteínas Tirosina Fosfatases

Com base na função, estrutura, seqüência, especificidade, sensibilidade a ativadores e inibidores, as proteínas fosfatases podem ser divididas em duas grandes famílias: serina/treonina fosfatases e tirosina fosfatases (PTPs) (Ferreira *et al.*, 2006; den Hertog *et al*., 2008). No genoma humano já foram identificados 107 genes que codificam enzimas da família das PTPs (Mustelin *et al.*, 2005; Alonso *et al.*, 2004). Os membros desta família apresentam um domínio catalítico altamente conservado, sendo caracterizadas pela presença no sítio catalítico de uma seqüência consenso [(Ile/Val)-His-Cys-X-Ala-Gly-Arg-(Ser/Thr)-Gly] - (Zhang, 2003). Todas as PTPs apresentam o mesmo mecanismo catalítico comum baseado numa cisteína presente no sítio ativo (Mustelin et al., 2005).

Em relação à função, estrutura, seqüência de aminoácidos e resíduos de aminoácidos presentes em seus domínios catalíticos, as PTPs podem ser divididas em quatro subfamílias: PTPs da classe I, classe II, classe III e IV.

Estas famílias apresentam regiões de consenso na seqüência de aminoácidos, principalmente do sítio ativo, as quais são resumidas na tabela a seguir.

Seqüência de consenso do Sítio Ativo para os quatro principais subgrupos na Superfamília das Tirosina Fosfatases (Fauman e Saper, 1996)				
Subgrupo	**Ácido Geral**		**Padrão do Sítio Ativo**	**Seqüências conhecidas**
PTPase	sWPDh	x_{24}	PIVVHCSAGvGRTG	103
Tipo VH1	PVeDn	x_{24}	rvlVHCqAGISRSa	28
cdc25	yIIDC	x_{40}	liVFhCEFSseRGP	22
LMWPTP	dIeDP	x_{-95}	sVlfVCIGNiCRSP	14

As letras em maiúsculas representam os resíduos altamente conservados (90% das seqüências possuem o resíduo idêntico ou um aminoácido similar). O número Xn se refere ao número de resíduos de aminoácidos entre o ácido geral e o sítio ativo; nas LMWPTPs, o ácido geral vem após a alça do sítio ativo na seqüência de aminoácidos, sendo indicado pelo número negativo.

Influência do *Status Redox* na Atividade das PTPs

A presença de cisteína no sítio ativo é responsável pela característica das PTPs de serem inibidas por agentes oxidantes e substâncias que atuam como aceptores de Michael (Aoyama *et al*, 2003; den Hertog *et al*., 2008). A oxidação da cisteína presente no sítio catalítico bloqueia a capacidade destas enzimas em desfosforilar seus alvos, isto porque a catálise é mediada pela transferência do fosfato a partir do substrato para a cisteína catalítica, seguida pela rápida hidrólise do fosfato. Em geral, oxidação dos resíduos de cisteína a ácido sulfênico é reversível, enquanto ácido sulfínico e sulfônico são

irreversíveis. Fisiologicamente, muitos estímulos, incluindo fatores de crescimento e citocinas, podem induzir a produção de espécies reativas de oxigênio (EROs) e consequentemente, inibição das PTPs. Nesse sentido, a oxidação de cisteína no sítio catalítico de PTPs promove a inibição da atividade dessas fosfatases constituindo importante mecanismos regulatório da atividade catalítica dessas enzimas e, consequentemente potencial estratégia de modulação de vias de sinalização celular sensíveis ao estado redox celular.

Caselli *et al.* (1998), mostraram que a inativação por H_2O_2 é causada pela oxidação específica de ambas as cisteínas do sítio ativo, as quais formam pontes dissulfeto. Devido a estes mecanismos de inativação por EROs e a vulnerabilidade em se oxidar (den Hertog *et al*., 2008), Groen *et al.* (2005) tem colocado as PTPs como um importante sensor do status redox celular.

Proteínas Tirosina Fosfatases de Baixa Massa Molecular

Proteínas tirosina fosfatases de baixa massa molecular (LMWPTP) constituem um grupo de proteínas tirosina fosfatases específicas de 18 kDa (pertencentes à classe II), conhecidas como ACP1, amplamente expressas em diferentes tecidos. Em humanos são codificadas por uma única cópia do gene *acp1*, localizado no cromossomo 2, cuja transcrição origina quatro diferentes RNAs mensageiros através de um complexo sistema de "splicing" alternativo. Das quatro isoformas de LMWPTP apenas duas, isoforma 1 e isoforma 2, demonstram ser cataliticamente ativas exercendo funções até o momento consideradas como idênticas (Modesti *et al.*, 1998).

As LMWPTP podem ser encontradas no citoplasma e também interagindo com proteínas do citoesqueleto. Estas fosfatases interagem especificamente com o receptor de PDGF ativado. De fato, LMWPTP é capaz de se ligar e desfosforilar o receptor de PDGF ativado, modulando a mitogênese induzida por PDGF. De acordo, foi verificada uma diminuição em até 90% na resposta mitogênica ao PDGF e diminuição no nível de auto-fosforilação do seu receptor após a super-expressão de uma das isoformas da LMWPTP (IF2) em células NIH3T3 (Berti *et al.*, 1994).

A função da LMWPTP no controle da proliferação celular é regulada por fosforilação mediada por quinases da família Src (Riggaci *et al.*, 1996; Tailor *et al.*, 1997). Os resíduos fosforilados na LMWPTP são Y131 e Y132. Enquanto a fosforilação do primeiro está associada ao aumento expressivo da atividade catalítica *in vitro* da enzima (aproximadamente 25 vezes), a fosforilação em Y132 parece induzir a inativação da atividade fosfatásica de LMWPTP ao mesmo tempo, criando um importante sítio de interação da enzima com outras proteínas celulares (Bucciantini *et al.*, 1999).

Cirri *et al.* (1998) verificaram que a LMWPTP presente constitutivamente no citoplasma apresenta uma fração associada ao citoesqueleto. Após o estímulo com PDGF, c-Src é capaz de interagir e fosforilar somente a LMWPTP presente na fração do citoesqueleto (Cirri *et al.*, 1998). A fosforilação da LMWPTP por c-Src, após o tratamento por PDGF, influencia fortemente a adesão e migração celular, sendo identificado um novo substrato da LMWPTP localizado no citoesqueleto: p190Rho-GAP (Chiarugi *et al.*, 2000).

Desta maneira, a LMWPTP desempenha múltiplos papéis na mitogênese induzida por PDGF, desde que pode interagir diretamente com o receptor de PDGF e promover sua desfosforilação. Ao mesmo tempo, a fração da LMWPTP associada ao citoesqueleto é

capaz de controlar o rearranjo do citoesqueleto em resposta ao estímulo por PDGF, por meio da regulação do estado de fosforilação de RhoGAP.

A despeito da importância da LMWPTP no controle do metabolismo celular ser inequívoco, como durante a proliferação e diferenciação celular, poucos sistemas celulares controlados (*in vitro*) foram utilizados a fim de avaliar o papel fisiológico desta classe de PTPs. Nesse sentido, o interesse em conhecer sua participação nos mecanismos de transdução de sinal disparados durante a diferenciação de osteoblastos torna-se relevante.

Papel Biológico da Src quinase

Membros da família Src de proteínas tirosinas quinases são caracterizados pela presença de um domínio SH3, um domínio SH2 e um domínio catalítico. Estão incluídas nesta família, além da proteína Src, as proteinas Blk, Brk, Fgr, Frc, Hck, Lck, Lyn, Srm, Fyn e Yes. O aumento de proteínas fosforiladas em tirosina, pelo estímulo do PDGF, por exemplo, é mediado pela interação das proteínas da família Src com o receptor de PDGF, levando a um aumento de 2 a 4 vezes na atividade quinásica de Src, Fyn e Yes (Gould & Hunter, 1988; Kypta *et al.*, 1990). Na sua conformação inativa, o resíduo de tirosina na porção C-terminal da proteína Src está envolvido numa interação intramolecular, que ocorre entre este resíduo de tirosina fosforilado e seu próprio domínio SH2. Cooper e colaboradores (1986) verificaram que o resíduo Y_{527}-Src, na porção C-terminal, é fosforilado *in vivo*. Outro resíduo de tirosina, Y_{416}-Src, também está envolvido na regulação da atividade quinásica dessa proteína. Em sua conformação inativa, o resíduo Y_{416}-Src está na forma desfosforilada interagindo diretamente com o domínio quinásico dessa proteína. Quando este resíduo torna-se fosforilado, o mesmo é deslocado do domínio

quinásico, permitindo o acesso do substrato ao sítio catalítico da enzima (Xu *et al.*, 1999; Bjorge *et al.*, 2000).

A **Figura 4** mostra com maior riqueza de detalhes o processo de ativação e inativação (modulação positiva e negativa) da Src pelo processo de fosforilação dos resíduos Y527 e Y416. A fosforilação/desfosforilação do resíduo Y_{527}-Src, e conseqüentemente regulação da atividade de c-Src, depende do balanço entre a velocidade com que o fosfato é removido por uma PTP e a velocidade com que é adicionado por PTKs. Várias PTPs, como SHP1, SHP2, têm sido caracterizadas como sendo capazes de desfosforilar a Y_{527}-Src e implicadas na regulação da atividade desta tirosina quinase (Bjorge *et al.*, 2000).

Recentemente, foi demonstrado que a inibição de PTKs, particularmente as quinases codificadas pelos proto-oncogenes c-*src* ou c-*fms*, leva à uma diminuição da remodelação óssea, resultando em osteopetrose (Chengalvala, 2001). Estes dados sugerem que a regulação da fosforilação da tirosina parece ser crucial, também, para a manutenção do tecido ósseo. Além disso, a redução na expressão de c-*src* não só inibe a reabsorção óssea como, também estimula a diferenciação de osteoblastos e a formação óssea, devido a alterações no metabolismo dos osteoclastos, como demonstrado em estudos *in vivo* com a deleção de c-*src* em ratos e *in vitro,* em cultura primária (Marzia *et al.*, 2000).

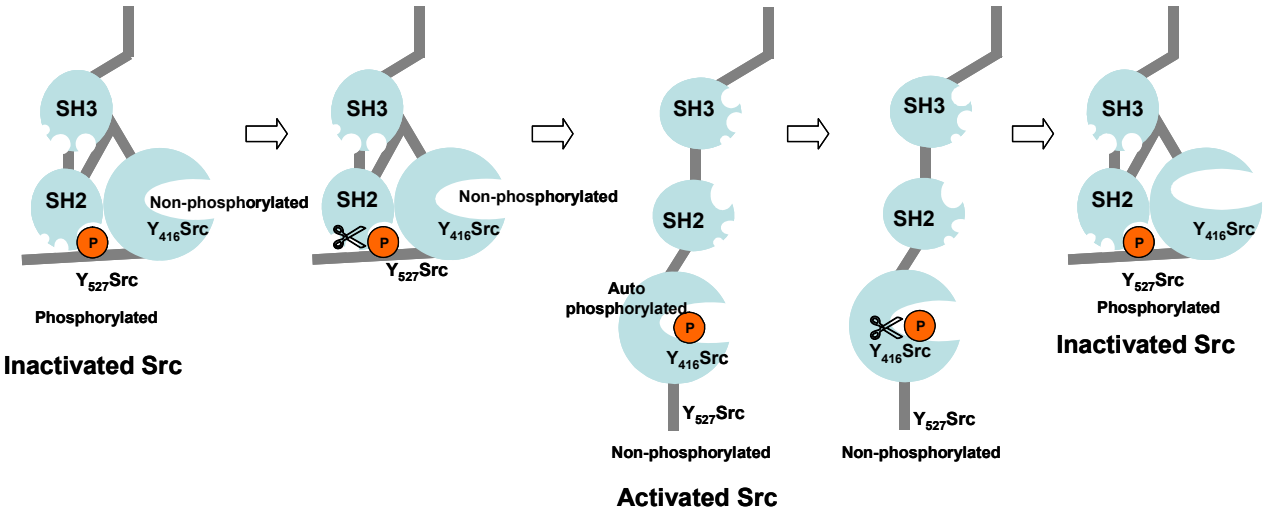

Figura 4: Regulação da atividade catalítica de Src através de fosforilação: A fosforilação de Src em Y527 é crucial para a manutenção de Src em seu estado inativo. Uma vez fosforilada Y527 interage com o domínio SH2 da própria enzima induzindo a formação de novas interações intramoleculares e a manutenção de Src em uma conformação "fechada" caracterizada pela ausência de atividade catalítica e bloqueio da entrada de substratos ao sítio ativo. Uma vez desfosforilada em seu sítio Y527, a proteína adquire nova conformação, a qual favorece a autofosforilação no resíduo Y416, tornando-se ativa.

Sonic Hedgehog

Sinalização pela interação célula-célula é a principal estratégia de coordenação da proliferação, diferenciação e sobrevivência celular durante o desenvolvimento de embriões de vertebrados. Membros da via de sinalização disparada pela sonic hedgehog (Shh) são conservados durante o processo evolucionário e atuam em importantes processos durante o desenvolvimento. No sistema esquelético, mutações em componentes desta via levam as malformações esqueléticas e doenças como osteoporose e artrite (Day e Yang, 2008). Nos mamíferos, há três membros conhecidos da família de hedgehog (Hh): sonic (Shh), desert (Dhh) e indian (Ihh).

Estudos relacionados com a família Hedgehog vêm sendo propostos a cerca de 30 anos e alguns componentes de sua sinalização vem sendo descobertos (revisado em Wang *et al.*, 2007, ver **Figura 5** para maiores detalhes). De uma maneira geral, o receptor primário para Hh é a proteína transmembrana Patched (Ptc) (Marigo *et al.*, 1996) e o segundo receptor, uma outra proteína transmembrana chamada de Smoothened (Smo) (van den Heuvel & Ingham, 1996). Smo está constitutivamente ativa na natureza, mas é mantida em um estado "dormente" pelos receptores primários (Ptc) em células normais. Ao entrar em contato com Hh, o efeito inibitório de Ptc sobre Smo é diminuído, o qual então, ativa a via de Hh (Kalderon, 2000; Taipale *et al.*, 2002).

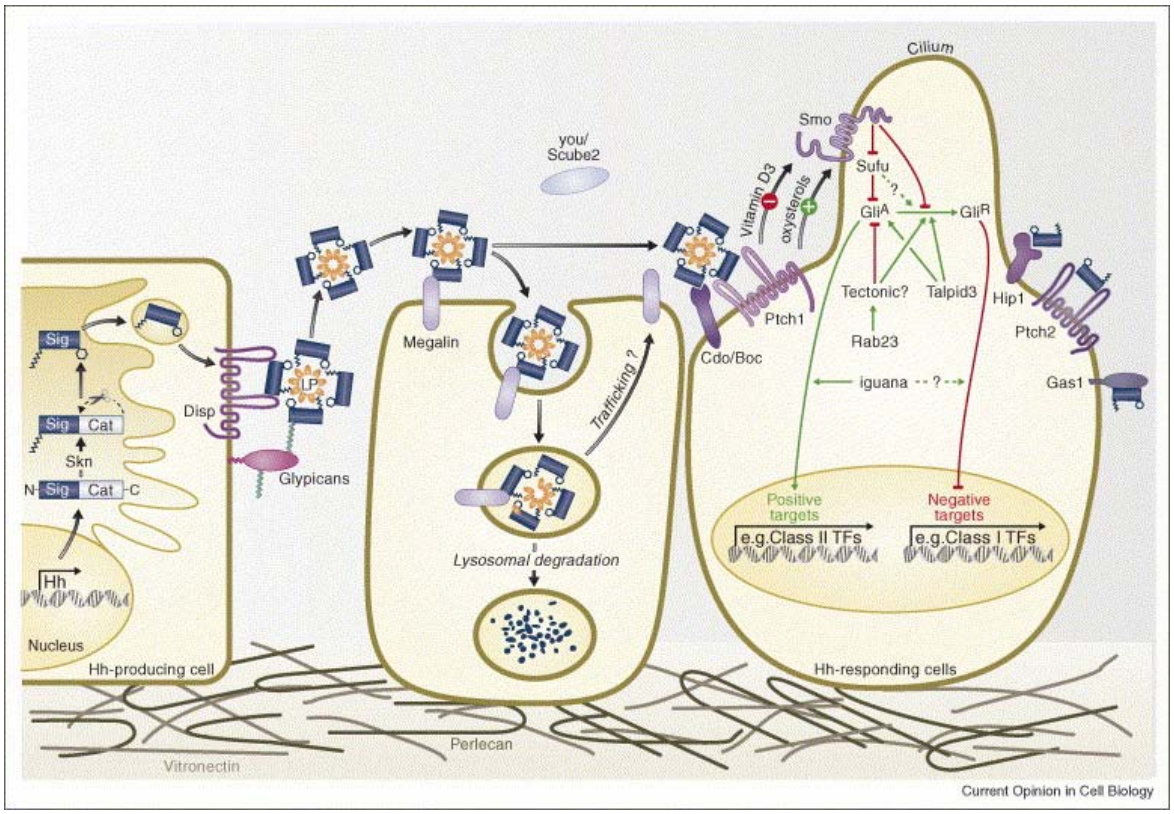

Figura 5: Modelo atual da via de sinalização de Hedgehog em vertebrados. As proteínas Hedgehog (Hh) sintetizadas com 45 kDa (precursoras) são palmitoiladas na porção N-Terminal e clivadas subsequentemente para gerar uma proteína de 19 kDa (proteína sinalizadora). Desta maneira as Hh são enviadas à superfície da célula e secretadas como lipoproteínas. Nas células, Hh interage com um complexo de Ptch 1, ativando Smo. Neste esquema, note que Ptch1 regula a atividade de Smo através da vitamina D3, na ausência do ligante Hh. A ativação de Smo ativa as vias relacionadas que culminam na ativação dos fatores de transcrição Glis (ver **Figura 6**). Retirado de Wang et al., 2007.

A **Figura 6** mostra um esquema resumido da ativação das Glis, fatores de transcrição requeridos na ativação da via. Na ausência de Hh, o Ptc se encontra ativo e consequentemente inibe a atividade de Smo, deixando os fatores de transcrição (Gli) inativos. Somente quando Hh se liga a Ptc é que a inibição de Smo é desfeita e os diferentes fatores Gli conseguem exercer suas atividades de ativação da transcrição. Em vertebrados, existem pelo menos 3 diferentes Glis (Gli1, Gli2 e Gli3) - (Ruiz e Altaba, 1999; Koebernick e Pieler, 2002). Sabe-se atualmente que Gli3 se liga a um complexo protéico (Fu, Cos2 e SuFu) o qual é responsável pela manutenção deste fator de transcrição no citoplasma. Quando Smo é ativada, este complexo protéico é dissociado e ocorre translocação do Gli para o núcleo, ativando a transcrição de genes específicos **(Figura 6)**.

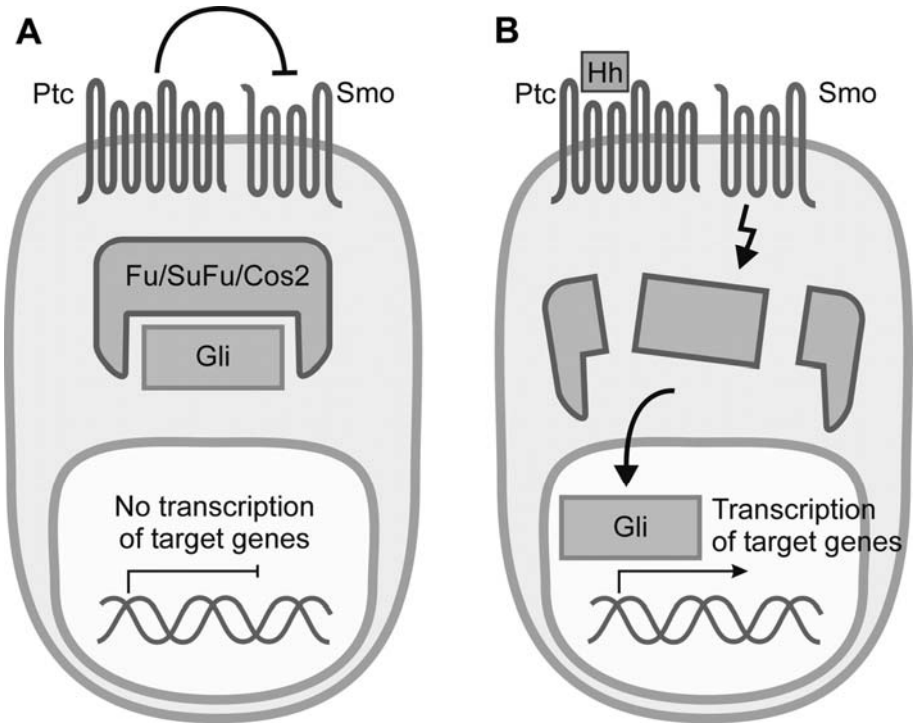

Figura 6. Via de sinalização disparada pela Hh. (A) Em seu estado inativo, Ptc inibe Smo e consequentemente, o complexo protéico (Fu/SuFu/Cos2) seqüestra o fator de transcrição Gli (conhecido apenas para o Gli3 e não para o Gli 1 e 2), prevenindo sua atividade transcricional. **(B)** Na presença de Hh (ligante), Ptc é inativado e Smo fica constitutivamente ativo. Assim, Gli fica livre para se translocar ao núcleo e ativar a transcrição de genes específicos (Bijlsma *et al.* 2006).

Sinalização Intracelular Desencadeada pelo Remodelamento da Matriz Extracelular

O remodelamento ósseo normal (formação e reabsorção) requer mecanismos precisos e interligados os quais são executados de maneira ordenada. A remodelação da MEC é o mecanismo efetivo que mais altera a ancoragem e proliferação celular, bem como algumas vias de sinalização. O estímulo mecânico comprovadamente exerce papel fundamental na remodelação óssea (Turner *et al.*, 1994).

Os osteoblastos e osteócitos contêm um arsenal de proteínas mecano-sensíveis capazes de realizar a mecanotransdução do estímulo mecânico, culminando em uma resposta celular que resultará em produção ou reabsorção óssea (Nomura and Takano-Yamamoto, 2000). Estudos apontam as integrinas e a quinase de adesão focal (FAK - focal adhesion kinase) como os principais mediadores responsáveis pelas etapas iniciais da mecanotransdução do estímulo mecânico (Boutahar *et al*., 2004). A interação entre as integrinas e a FAK deve desencadear a ativação de vias de sinalização intracelular que estimulam a produção de osso e a manutenção da integridade deste tecido (Hughes-Fulford, 2002). Com a fosforilação de FAK há a criação de um sítio de ligação de alta afinidade para moléculas compostas por domínios SH2. A partir deste sítio, interações proteína-proteina são estabelecidas, levando à formação de complexos de sinalização

A porção extracelular das integrinas se conecta com proteínas da matriz extracelular (MEC), enquanto a porção intracelular da subunidade β das integrinas se fixa ao citoesqueleto através de proteínas associadas à actina, como α-actinina, vinculina, paxilina e talina (Duncan e Turner, 1995). Esta interação MEC-integrinas-citoesqueleto forma um verdadeiro sistema de alavancas capaz de transmitir a deformação mecânica sofrida pelo osso até o citoplasma dos osteoblastos e dos osteócitos (**Figura 7**). Somando-se a isso, as

integrinas formam verdadeiras adesões focais, que quando deformadas, organizam-se formando "clusters" e transmitem a deformação mecânica para as proteínas citoplasmáticas (Plopper *et al.*, 1995).

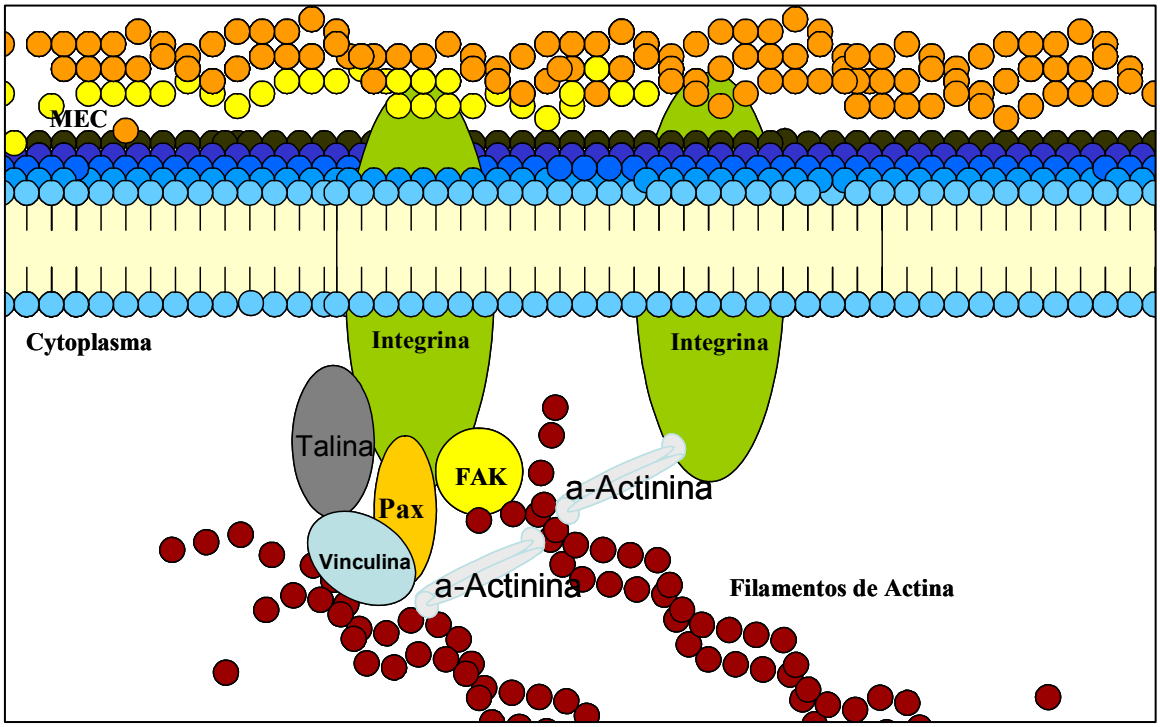

Figura 7: Relação entre a matriz extracelular, integrinas e citoesqueleto. As integrinas interagem com as proteínas da MEC e do citoesqueleto, formando um sistema de alavancas que transmite o estímulo mecânico extracelular para dentro da célula. Talina, paxilina, vinculina, α-actinina e actina são proteínas do citoesqueleto das células eucarióticas.

Alguns dos constituintes de adesões focais participam na ligação estrutural entre os receptores da membrana e o citoesqueleto, enquanto outras são moléculas sinalizadoras, incluindo proteínas fosfatases e quinases, seus substratos e ainda, várias proteínas adaptadoras (Petit e Thiery, 2000).

Neste sentido, durante o remodelamento da MEC, as Metaloproteinases de Matriz (MMPs) desempenham uma importante função. As MMPs constituem uma importante família de endopeptidases metalo-dependentes e representam a principal classe de enzimas responsáveis pela degradação ou reabsorção dos componentes da MEC. Coletivamente, as MMPs são capazes de degradar a maioria das proteínas da MEC, como descrito por Birkedal-Hansen (1995). Todas estas proteases são ativas em pH neutro, requerem Ca^{2+} para ativação e contêm Zn^{2+} em seu sítio ativo (Hannas *et al.*, 2007, **Figura 7**). Atualmente, tem-se focado a importante participação de proteases não somente na solubilização desta matriz, mas, também na sua atuação como componente chave que determina quando e onde a remodelação da MEC deve ocorrer. Por outro lado, tem-se avaliado a atividade intracelular de MMPs, propondo que esta classe de enzimas possa atuar no metabolismo celular (Kwan *et al.*, 2004).

Em mamíferos, esta família é dividida em 2 classes: MMPs solúveis e MMPs ancoradas à membrana celular (MT-MMP), esta última apresentando um domínio transmembrana. As MMPs solúveis são secretadas como pro-enzimas (zimogênio), enquanto que as MT-MMPs são ativadas intracelularmente e expressas na superfície celular na forma ativa (Nabeshima *et al.*, 2002). Dentre as solúveis estão as colagenases, gelatinases, estromelisinas e matrilisinas (**Figura 8**). Já as MT-MMPs são compostas por seis membros diferentes (Nabeshima *et al.*, 2002).

As MMPs são fisiologicamente inibidas por moléculas específicas conhecidas como TIMPs (Inibidores Teciduais de Metaloproteinases). Recentemente, foi relatado que, além dos TIMPs, a glicoproteína ancorada a membrana, RECK, também participa da regulação da atividade de MMPs, através da interação direta com as MMP-2 e -9 (Sasahara *et al.*, 2002).

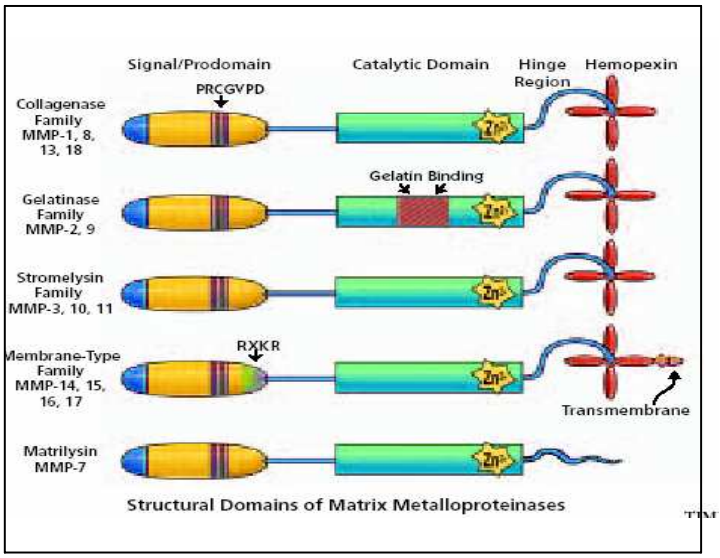

Figura 8: **Classificação das MMPs quanto aos tipos de domínios presentes. Fonte:** Alexander (2002).

Dentre as MMPs, as gelatinases [A e B, MMP-2 (72-KDa) e –9 (92-KDa), respectivamente] estão envolvidas na proteólise e rompimento de membranas basais, pela degradação de colágenos tipo IV, V, colágenos desnaturados (gelatinas), fibronectina e elastina (Thomas *et al.*, 1999; Kahari & Saarialho-Kere, 1999).

Outra família de proteases (cisteíno-proteases), da qual fazem parte as catepsinas, desempenha importante papel na remodelação da MEC e, portanto, na remodelação óssea por degradar os colágenos tipo I e III (Jeong *et al.*, 2004; Karsdal *et al.*, 2005). As catepsinas têm sido apontadas como uma importante classe de enzimas relacionadas com o processo de remodelação óssea, já que participam durante a atividade dos osteoclastos.

No osso, a MMP-2 é expressa constitutivamente por diversos tipos de células ósseas (Murphy *et al.*, 1989; Overall *et al.*, 1989; Rifas *et al.*, 1989, Meikle *et al.*, 1992), sendo uma das principais iniciadoras de ativação das outras MMPs na MEC, inclusive da MMP-9, como sugerido por Curran e Murray (1999).

A produção de MMPs por células ósseas *in vivo* e *in vitro* sugere sua importância no remodelamento ósseo (Otsuka *et al.*, 1984; Hu *et al.*, 2000). Neste processo, colagenases produzidas por osteoblastos são importantes na degradação da matriz orgânica, oferecendo a porção mineralizada para a atividade dos osteoclastos (Tezuka *et al.*, 1994).

A despeito dos numerosos estudos abordando a interação osteoblastos/carreador, pouco se sabe da atividade proteolítica destas células induzida por diferentes materiais. Hu *et al.* (2000) mostraram um aumento na expressão das MMPs-1 e MMP-13 por fragmentos de fibronectina (120 kDa). Até agora, pouca informação é encontrada sobre os mecanismos de transducão de sinal ativada pela remodelação da MEC em osteoblastos.

Outra proteína que vem ganhando destaque nos últimos anos é a proteína Phex, fortemente correlacionada relacionada com a biologia óssea. Esta proteína apresenta homologia à família das endopeptidases neutras (localizado no cromossomo X) e está relacionada à homeostase do fosfato. Sua estrutura consiste de 22 exons que codificam uma proteína com 749 aminoácidos.

Os membros desta família possuem um domínio amino-terminal intracelular, um único domínio transmembrana e um domínio maior, extracelular, carboxi-terminal, com 10 resíduos de cisteína conservados. Cerca de 31 mutações (missense, nonsense ou deleção) foram descritas até o momento nesse gene, associadas ao raquitismo hipofos- fatêmico e 6 casos de polimorfismo (Francis *et al.*, 1995; Holm *et al.*, 1997; Dixon *et al*., 1998; Rowe, 1997).

Microarranjos de peptídeos (kinoma/PepChip) e RNAi como ferramentas para estudos de vias de sinalização celular

Para o estudo de transdução de sinal existia, até pouco tempo, poucas ferramentas capazes de fornecer subsídios técnicos para uma investigação mais detalhada, assim, a técnica de *Western Blotting* ganhou bastante destaque neste sentido. Contudo, a partir da necessidade de se conhecer melhor a atividade de determinadas proteínas, algumas companhias tem se focado no desenvolvimento de recursos mais avançados o que resultou, entre outras coisas, no desenvolvimento e produção de anticorpos capazes de reconhcer fosforilação de sítios específicos em seus antígenos.

Assim, começou-se pela produção de anticorpos contra fosforilação em sítios específicos como ferramentas para identificar a atividade e/ou inibição de determinadas proteínas. Atualmente, com o desenvolvimento e comercialização do RNAi e do PepChip, os dados obtidos pelo *Western Blotting* serão complementados; no caso do arranjo de peptídeos por exemplo, se consegue, com a mesma amostra, identificar em um único experimento, a atividade de mais de 1000 quinases. Por outro lado, a técnica de RNAi oferece a possibilidade de "*knock down"* uma proteína específica, podendo definir de forma mais precisa a real importância biológica da mesma.

PepChip: A predominância da fosforilação de proteínas como regulador do metabolismo celular tem instigado alguns pesquisadores a desenvolverem estratégias que possam fornecer um mapa geral e descritivo da atividade quinásica celular (Krebs, 1993; Versteeg *et al.,* 2000). Classicamente, o estudo da atividade quinásica e da fosforilação de proteínas é realizado por western blot. No entanto, essa técnica não permite a análise de várias

proteínas ao mesmo tempo e, portanto é limitante para estudos clínicos ou que requerem um grande número de análises, como por exemplo, análise de alvos moleculares da ação antitumoral de compostos naturais e seus derivados. Recentemente, o grupo do Prof. Maikel Peppelenbosch desenvolveu uma técnica, chamada kinoma ou pepchip, que basicamente é um microarranjo de peptídeos (substratos de quinases) que permite analisar ao mesmo tempo a atividade de 1.172 quinases (Diks e Peppelenbosch, 2004; Diks *et al.*, 2004).

RNA interferência: A expressão gênica é um processo de fundamental importância para organismos vivos. Nos últimos anos, diversos grupos têm mostrado diferentes funções da molécula de RNA (Mizuno *et al.,* 1984; Lee *et al.,* 1993; Lagos-Quintana *et al.,* 2001). Recentemente, a transfecção do RNA de interferência (RNAi) tem sido utilizada para silenciar genes específicos (Fire *et al.,* 1998; Montgomery *et al.,* 1998; Hamilton e Baulcombe, 1999; Zimmermann *et al.,* 2006). O uso potencial desta tecnologia no campo da genômica funcional e descoberta de novos alvos moleculares e medicamentos é promissor (Eckstein, 2007).

OBJETIVOS

O objetivo geral desta tese foi investigar os mecanismos de transdução de sinal disparados em diferentes modelos biológicos representativos dentro da biologia óssea. Especificamente, este trabalho apresenta um conjunto de informações relacionadas à biologia dos osteoblastos e osteócitos. Neste contexto, os seguintes objetivos específicos foram traçados:

- Demonstrar o papel da LMWPTP na modulação da atividade de Src quinase através da desfosforilação dos resíduos de tirosina 416 e 527 da enzima **(Capítulo 2);**
- Estabelecer um modelo *in vitro* de diferenciação de osteoblastos em osteócitos e caracteriza-lo sob o ponto de vista molecular **(Capítulo 3)**.
- Definir os mecanismos de transdução de sinal envolvidos no processo de diferenciação de osteoblastos *in vitro* induzido pelo ácido ascórbico e glicerofosfato **(Capítulo 4)**;
- Fornecer informações sobre os processos moleculares disparados durante a adesão de osteoblastos **(Capítulo 5)**.

Capítulo 2

LOW MOLECULAR WEIGHT PROTEIN TYROSINE PHOSPHATASE MODULATES SRC ACTIVITY VIA DEPHOSPHORYLATION OF BOTH TYROSINE RESIDUES (Y_{416}Src and Y_{527}Src)

Low molecular weight protein tyrosine phosphatase modulates Src activity via dephosphorylation of both tyrosine residues (Y_{416}-Src and Y_{527}-Src)

Running title: LMWPTP modulates Src activity

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Abstract

The proto-oncogene Src kinase displays complex kinetic regulation of its enzymatic activity following cellular stimulation, involving a very fast activation of the enzyme followed by a slow decrease in its enzymatic activity. The molecular mechanisms governing the dynamics of Src activity remain only partially understood. Src activity is controlled by the phosphorylation status of two different tyrosine residues, Y_{527}-Src whose phosphorylation exerts an inhibitory action on Src kinase and Y_{416}-Src which is autophosphorylated by Src following dephosphorylation of Y_{527}-Src, in turn leading to interaction and phosphorylation of a variety of cellular substrates. Interestingly, Src is in complex with Low Molecular Weight Protein Tyrosine Phosphastase (LMWPTP) and the activity of this phosphatase is enhanced following its phosphorylation by Src. Here we show that LMWPTP dephosphorylates both pY_{527}-Src and pY_{416}-Src, but the specificity of LMWPTP for pY_{527}Src is higher than that for pY_{416}Src. Activation of LMWPTP produces a strong transient activation of Src, mediated by a fast dephosphorylation of pY_{527}-Src, followed by a slower deactivation of this kinase via dephosphorylation of pY_{416}Src, explaining the remarkable kinetic of Src activity following cellular stimulation. Thus our results provide significant novel insight into the cellular mechanisms governing the dynamics of Src kinase activity.

Key words: Src kinase; low molecular weight protein tyrosine phosphatase; covalent modulation; Src Activity.

1. Introduction

Tyrosine phosphorylation status is generally accepted as a critical regulator of a multitude of cell biological processes including cell proliferation, migration, differentiation, and survival [1]. Although the action of tyrosine kinases is now in general fairly well understood, the broad effects of protein tyrosine phosphatases (PTPs) on cellular physiology and on protein tyrosine kinase activity modulation in particular remains largely obscure. Nevertheless, it is clear that close interactions exist, especially with regard to the non-receptor protein tyrosine kinase (PTK) family of Src kinases. Src and Src-family (including Src, Lyn, Fyn, Yes, Lck, Blk and Hck) protein kinases are tightly regulated by their phosphorylation status [2,3]. Members of the protein tyrosine kinase family are kept in an inactive conformation by phosphorylation of Y_{527}-Src, six residues from the c-terminus of c-Src. Under basal conditions, 90–95% of Src is phosphorylated at Y_{527}Src *in vivo* [4,5]. In turn, this phosphorylated residue binds intramolecularly with the Src SH2 domain, this intramolecular association stabilizing a catalytically inactive form of the enzyme [6]. Following dephosphorylation of Y_{527}-Src, Src undergoes an intermolecular autophosphorylation at tyrosine 416; this residue is present in the activation loop, and its phosphorylation promotes kinase activity and association with substrate molecules [6]. Although these studies have provided substantial insight into the regulation of Src activity at the level of the kinase itself, the mechanisms governing the remarkable kinetics of Src activity, which upon cellular stimulation involve a fast upstroke in kinase activity followed by a slower return to baseline levels remain only partially understood.

Various PTPs have been shown to influence Src family tyrosine kinase activity, binding to these tyrosine kinases and mediating dephosphorylation of Y_{527}-Src. Especially

the receptor PTPs CD45 and RPTPα [7] have been shown to activate Src activity in this manner. But also PTP1B, SHP1 (Src homology 2 domain-containing tyrosine phosphatase 1) and SHP2, and transmembrane phosphatases including PTPε, and PTPκ have been proposed to exert such a function. How these phosphatases may mediate the very fast activation of the Src kinase following cellular stimulation remains, however, unresolved.

Also the molecular details governing the slow return to basal Src activity levels following activation require further exploration. Overexpression of Low Molecular Weight PTP (LMWPTP) caused a dramatic decrease of Src kinase activity in response to PDGF stimulation [8]. Importantly, however, clear candidates for deactivation of Src, a process which must occur *in vivo* as increased activity following cellular stimulation is clearly transient in nature, have not been identified. In fact, it is fair to say that the mechanisms governing the transient nature of the Src kinase signal are obscure at best.

In the last decade, evidence has been mounting that LMWPTP is involved in tightly controlling activity of Src and *vice versa* [8,9]. LMWPTP is in the same complex as Src as evidenced by co-immunoprecipitation experiments, and LMWPTP acts as a substrate for Src. Phosphorylation of Y_{131} of LMWPTP by Src increases enzymatic activity of this phosphatase more than twenty-five fold [10,11]. Conversely, however, whether LMWPTP exerts effects on Src remains an open question.

Circumstantial evidence suggests that LMWPTP might be involved in the regulation of Src. LMWPTP is a 18 kDa protein with no particular tissue-specific expression [12], but which have been linked with the negative control of promitogenic pathways, dephosphorylating and thus deactivating the PDGF receptor as well as dephosphorylating p190Rho-GAP and thus increasing its GTPase activity towards Rho family GTPases.

Ephrine-B1 receptor and c-Fms seem to be targets for negative regulation by LMWPTP [13,14]. Thus, it is possible that also c-Src, and especially the critical Y_{416}Src site is a target for negative regulation by this enzyme.

These considerations prompted us to investigate this possibility directly. Unexpectedly, we observed dichotomal regulation by LMWPTP of Src tyrosine kinase activity. It appears that both, the inhibitory Y_{527}Src site as well as the stimulatory Y_{416}Src site of Src are targets for dephosphorylation by LMWPTP. Dephosphorylation of the former site will lead to activation of Src, while subsequent dephosphorylation of the latter site to its subsequent deactivation. Thus LMWPTP emerges as a prime candidate to explain the remarkable dynamics of Src activation following cellular stimulation.

2. Material and methods

2.1. Cell culture and reagents. Pre-osteoblasts (MC3T3-E1 cells) were routinely grown in α-MEM supplemented with 10% FCS and antibiotics. If appropriate, cells were treated with freshly made pervanadate solution (10 μM) for 30 min before transient transfection, immunoprecipitation or western blotting analysis. Polyclonal antibodies against pan-Src, Y_{416}Src, Y_{527}Src, phospho-Y_{416}.Src, phospho-Y_{527}Src, pan-actin, antirabbit, antigoat and antimouse peroxidase-conjugated antibodies were purchased from Cell Signaling Technology (Beverly, MA). Sam68 (Src substrate) and antibody against phospho-tyrosine (α-pY) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against LMWPTP was from Abcam.

2.2. Cellular Fractionation

Cytoskeletal fractions and other cellular fractions were separated using a RIPA lysis. Buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Complete RIPA lysis buffer (cRIPA) is RIPA plus 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate. Cellular fractionation was performed according to Cirri and collaborators [15].

2.3. Transfection of MC3T3-E1 cell with LMWPTP

MC3T3-E1 cells at 60% confluence (6 well plates) were transiently transfected with LMWPTP. Transfections were carried out using Effectene transfection kit (Qiagen) according to the manufacture's instructions. Briefly, cells were transfected with 2.4 ng

pcDNA3.1/V5-His-TOPO vector containing either no insert or the coding sequence of human LMWPTP after a CMV promoter. Overexpression was always verified using Western blotting.

2.4. Transfection of MC3T3-E1 cell with siLMWPTP

MC3T3-E1 cells at 60% confluence (6 well plates) were transiently transfected with siLMWPTP. Transfections were carried out using Hiperfect transfection kit (Qiagen) according to the manufacture's instructions. Briefly, cells were transfected with LMWPTP siRNA reagent (final concentration: 5 nM) or MAPK1 siRNA reagent (positive control) for 72 h. Afterwards, cells were scraped into 100 µl cell lysis buffer (described in western blot analysis), sonicated and spun down. Subsequently, lysates were mixed with loading buffer. Transfection efficiency was assessed by expression of LMWPTP by western blot analysis (on average 58.3 % decreased expression).

2.5. Immunoblotting and immunoprecipitation

Protein extracts were obtained using Lysis Cocktail (50mM Tris–HCl, pH 7.4; 1% Tween 20; 0.25% sodium deoxycholate; 150mM NaCl; 1mM EGTA; 1mM *O*-Vanadate; 1mM NaF; and protease inhibitors [1µg/mL aprotinin, 10µg/mL leupeptin, and 1mM PMSF]) for 2 hours on ice. An equal volume of 2 x SDS gel loading buffer (100mM Tris-HCl, pH 6.8, 200mM DTT, 4% SDS, 0.1% bromophenol blue, and 20% glycerol) was added to samples and boiled for 5 minutes. Proteins extracts were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to PVDF membranes. For *Immunoprecipitation*, the cells were lysed in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 60 mM *n*-octylglucoside, 2 mM EDTA, 1 mM orthovanadate,

100 mM NaF, 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin). After centrifugation, lysates were incubated overnight with anti-pan-Src or phospho-tyrosine at 4°C and then rotated with Protein A-Sepharose at 4°C for 2h. The beads were washed 3 times with lysis buffer and twice with PBS. Next, the immunoprecipitates were used for enzymatic assay and/or resolved by SDS-PAGE and transferred to PVDF membrane as described above

2.6. In vitro phosphatase and kinase activities

In vitro phosphatase assay. cells were treated with 10 µM pervanadate for 30 minutes, and pan Src was immunoprecipitated from the cellular lysate (1.5-3.5 mg of protein/ml). Subsequently, the immunoprecipitate (pan Src) was incubated at 37°C with rhLMWPTP for 15 and 30 minutes. The reaction was stopped by adding pervanadate.

In vitro Kinase activity assay was performed by adding 2 µg Sam68 protein (substrate of Src) and 100 µM ATP to the samples from dephosphorylation *in vitro* (as described above) and the reaction medium was incubated at 37°C. After 30 minutes the reaction was terminated and subsequently the levels of both Sam68 and its phosphorylation status were assessed by Western blot.

2.7. Redox Status

Cells were treated with H_2O_2 (1µM), L-NAME (3µM) and GSH (5mM) for 1-hour and after we collected cRIPA fraction. Afterwards Src phosphorylation (anti-pY_{416}Src) and LMWPTP expression were analyzed by western blotting. LMWPTP phosphorylation status was checked by immunoprecipitation of the enzyme followed by western blotting using an α-pY antibody.

3. Results

3.1. Evidence of pY_{416}-Src dephosphorylation via a tyrosine phosphatase-dependent mechanism

The transient nature of Src activation following various stimuli is still poorly understood. A possibility is that cells contain PTPs that dephosphorylate and thus deactivate pY_{416}-Src. Evidence that such activity really is present in cells was obtained from experiments in which MC3T3-E1 cells were treated with the pan-PTP inhibitor pervanadate (10 μM). The treatment for 30 min with this inhibitor strongly increased pY levels in general, but importantly that of pY_{416}-Src in particular (not shown). As pervanadate would be expected to block dephosphorylation of pY_{527}Src, these data infer that MC3T3-E1 cells contain strong pY_{416}Src dephosphorylating activity. A rational hypothesis for this observation is that a pY_{416}Src PTP activity is present in cells, making such a phosphatase a plausible negative regulator for c-Src activity.

3.2. pY_{416}-Src is a substrate for LMWPTP

As Src is in the same protein complex with LMWPTP and the active pY_{416}-Src phosphorylates and stimulates LMWPTP enzymatic activity, we reasoned that this enzyme was a candidate to act in the feedback of Src activity as one of the hitherto elusive pY_{416}Src dephosphorylating and thus Src deactivating PTPs. This consideration prompted us to investigate whether pSrc is a substrate for LMWPTP in *in vitro* phosphatase assays. Using pervanadate-treated MC3T3-E1 cells as a source for pSrc, we investigated the action of human recombinant LMWPTP on pY_{416}Src following co-incubations of pSrc and rhLMWPTP for different time periods (15 and 30min). Afterwards, western blot analysis

was performed by using anti-Src and anti-phosphotyrosine antibodies. As clearly shown in the **Figure 1**, LMWPTP is able to dephosphorylate immunoprecipitated pSrc and thus this enzyme may act as a negative regulator of this important cytosolic tyrosine kinase. In apparent agreement, also with the treatment of anti-phosphotyrosine immunoprecipitates with rhLMWPTP we observed a decrease of pY levels of a protein correspondent to the pSrc molecular weight (Fig. 1C). We conclude that LMWPTP efficiently dephosphorylates Src on tyrosine residues.

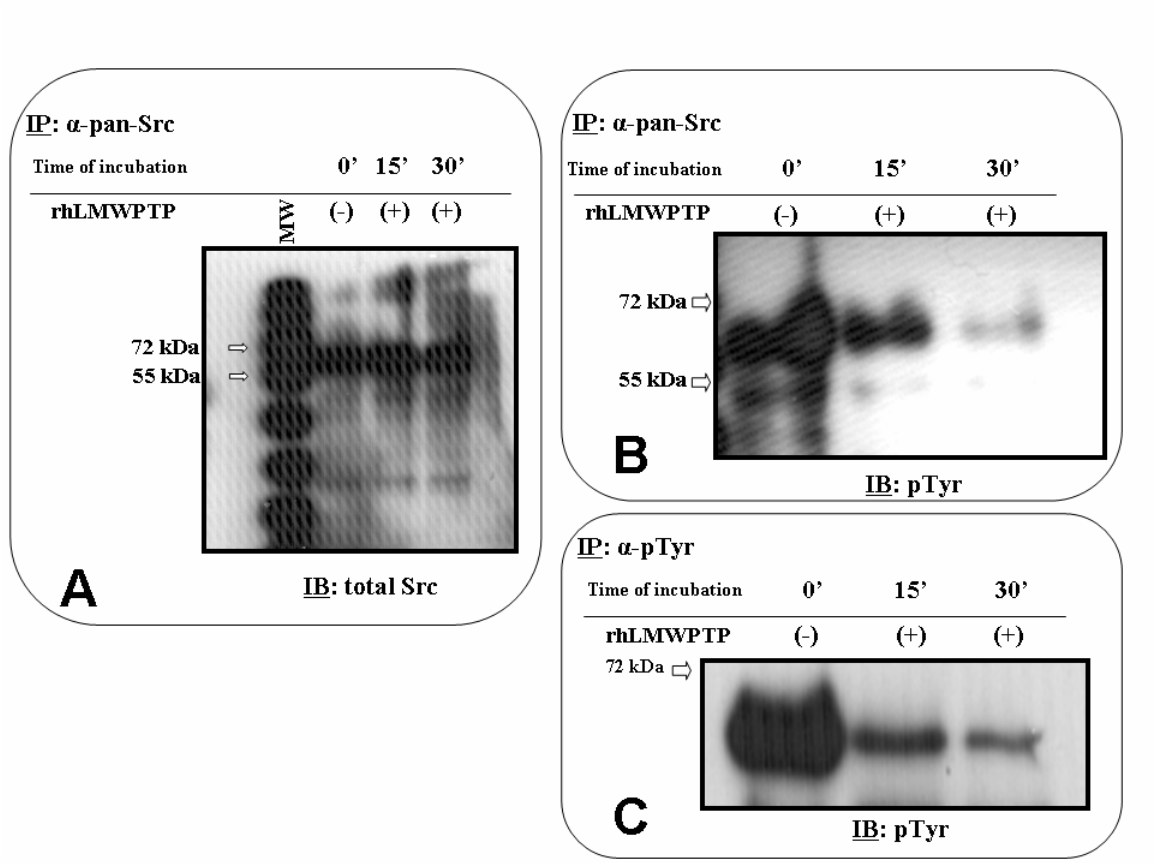


Fig.1. LMWPTP dephosphorylates Src on tyrosine residues. Cells were pretreated with pervanadate for 30 min (to increase pY-Src levels) and afterwards pan-Src (i.e. non-phosphorylated and phosphorylated-Src) was immunoprecipitated and exposed to *in vitro* phosphatase assays by incubation with rhLMWPTP for different time periods. **(A)** Equal amounts of pan-Src are present in the various experimental conditions as assessed by Western blotting using a pan-Src antibody (left panel). MW=Molecular weight marker lane. **(B)** rhLMWPTP incubation reduced pY-Src levels in a time-dependent manner as determined by Western blotting of immunoprecipitated rhLMWPTP-treated Src and probing with pY antibodies. **(C)** Immunoprecipitation of pY proteins from cellular extracts followed by *in vitro* phosphatase of these immunoprecipitates and subsequent reprobing with α-pY antibodies confirms the dephosphorylation of a protein running at the same height as Src by rhLMWPTP.

3.3. LMWPTP dephosphorylates Y_{416}-Src and Y_{527}-Src in vitro

Following the observation that pSrc is a substrate for LMWPTP, we subsequently determined the preference of this PTP for pY_{416} and pY_{527} residues of the kinase respectively. Employing an *in vitro* phosphatase assay similar as that described above, we observe that actually both tyrosine residues of Src clearly act as substrates for LMWPTP (**Figure 2**), pY_{527}-Src rather than pY_{416}-Src being the preferred substrate for this phosphatase. Thus LMWPTP dephosphorylates both the inhibitory pY_{527}Src site as well as the stimulatory pY_{416}Src site and thus exerts opposing effects on Src enzymatic activity.

3.4. LMWPTP dephosphorylates phospho-Src in vivo

To establish whether in a cellular context pSrc can act as a substrate for LMWPTP, next we evaluated the level of phosphorylated Src (pY_{416}) in MC3T3-E1 cells overexpressing LMWPTP or empty vector. As can be seen in the **Figure 3**, cells display only low basal levels of pSrc. To increase pY_{416}-Src levels, cells were given a short term (30 min) pervanadate treatment before transfection with empty vector or a LMWPTP-expressing construct, after which the medium was replaced with a non-pervanadate containing medium. Comparing the upper panel with the middle panel in **Figure 3A** or the Western blot in **Figure 3B** shows that this short-term treatment is sufficient to induce substantial pY_{416}-Src levels in the empty vector-transfected cells, even 24 h later. Upon transfection with a LMWPTP construct (lower panel) no pY_{416}-Src can be detected under these conditions. Thus pSrc can act as a substrate for LMWPTP *in vivo*.

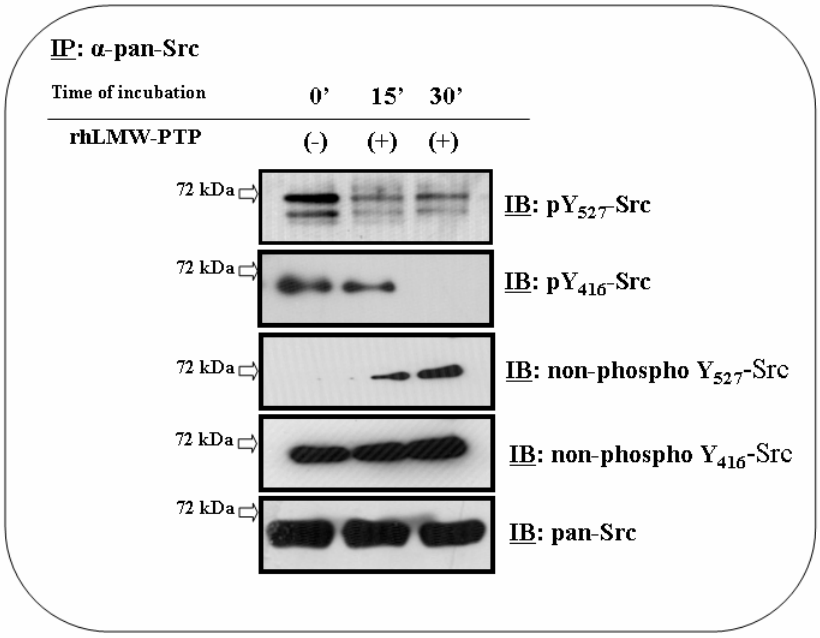


Fig.2. LMWPTP dephosphorylates both pY_{416}-Src and pY_{527}-Src. To analyze which tyrosine residue of Src was dephosphorylated by LMWPTP, total Src was immunoprecipited and incubated with rhLMWPTP for the time periods indicated. Subsequently, pan-Src, phospho-Src (pY_{527}-Src and pY_{416}-Src), and non-phospho-Src (Y_{527}-Src and Y_{416}-Src) were analyzed by western blotting.

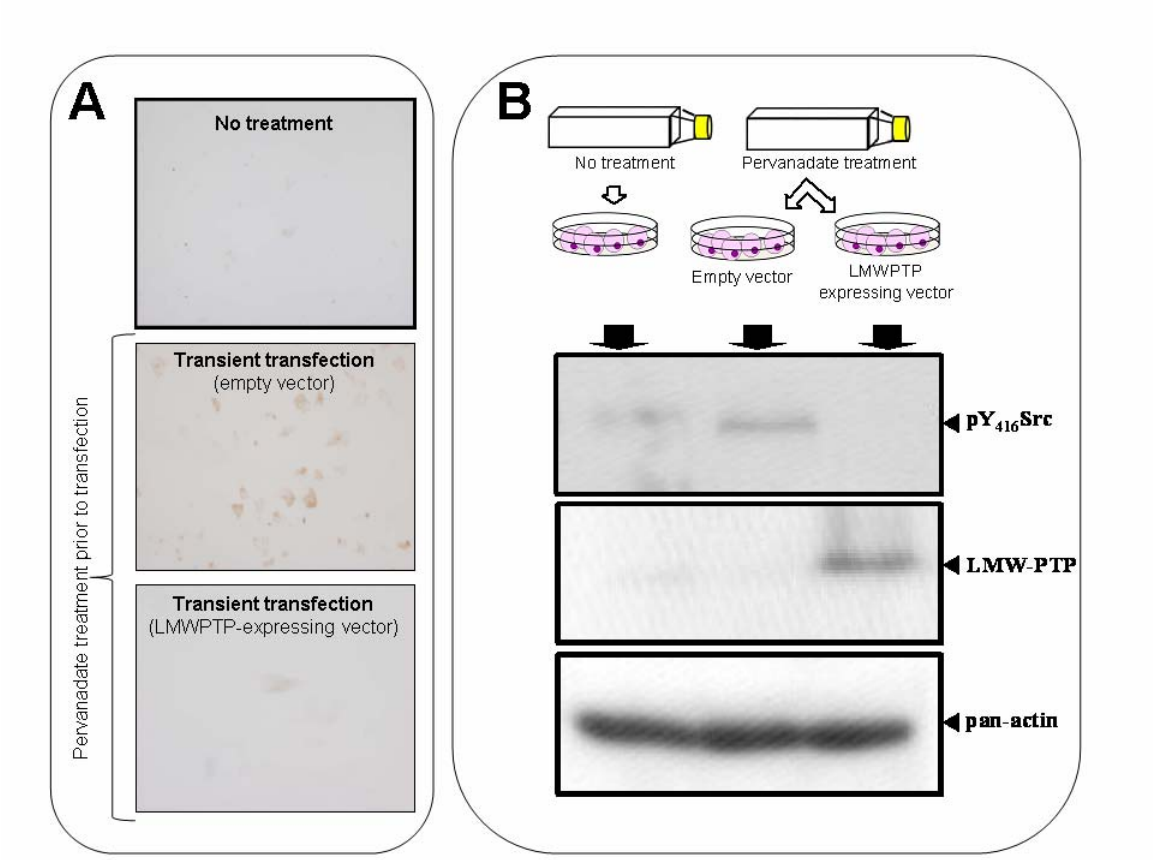


Fig.3. Decreased pY_{416}-Src levels in MC3T3-E1 cells overexpressing LMWPTP. The potential of LMWPTP to dephosphorylate pY_{416}-Src in a cellular context was evaluated by comparing untreated cells to cells transfected with either an empty vector or a CMV promotor-driven LMWPTP-overexpressing construct, followed by assessment of pY_{416}-Src levels. **(A)** Immunocytochemical analysis of pY_{416}-Src levels (*Magnification: 40x*). To increase pY_{416}-Src levels, cells were given a short term (30 min) pervanadate treatment before transfection with empty vector or a LMWPTP-expressing construct, after which the medium was replaced with a non-pervanadate containing medium. Comparing the upper panel with the midle panel shows that this short-term treatment is sufficient to induce substantial pY_{416}-Src levels in the empty vector-transfected cells, even 24 h later. Upon transfection with a LMWPTP construct (lower panel) no pY_{416}-Src can be detected under these conditions **(B)** Western blot analysis confirms increased pY_{416}-Src levels following transient pervanadate treatment, overexpression of LMWPTP following transfections with the CMV promoter-driven LMWPTP coding sequence containing vector as well as dephosphorylation of pY_{416}-Src resulting from LMWPTP overexpression.

3.5. LMWPTP is essential for suppressing Src hyperphosphorylation in a cellular context

The experiments described above suggest that LMWPTP has the capacity to reduce Src phosphorylation, but do not address the actual importance of action *in vivo*. Hence, we knocked down LMWPTP levels using a siRNA strategy. Importantly, reduced cellular LMWPTP levels strongly enhanced Src phosphorylation (**Figure 4**). Thus LMWPTP is not only sufficient for reducing Src phosphorylation, in a cellular context it is also required for suppressing Src hyperphosphorylation.

3.6. The interaction LMWPTP/Src decreases Src kinase activity

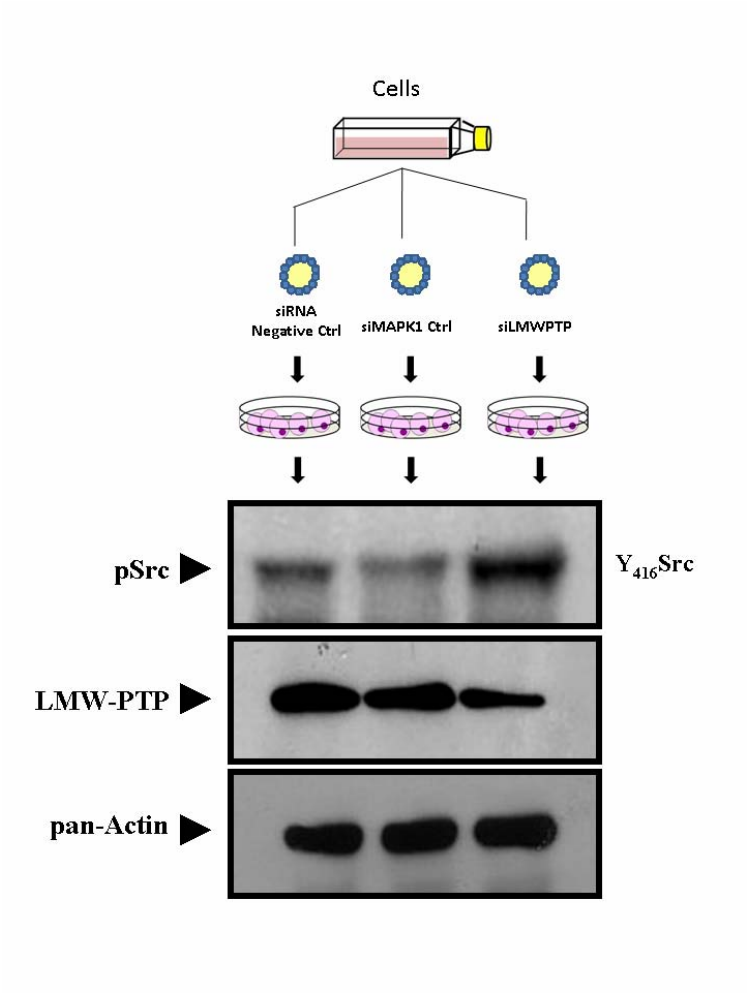
The apparently opposing effects of LMWPTP on both regulatory sites of Src raise questions as to the actual effects of LMWPTP on Src catalytic activity. Hence, we dephosphorylated c-Src by LMWPTP *in vitro* and subsequently performed *in vitro* kinase assays employing the Sam68 protein as substrate to monitor the effect of LMWPTP on the kinase activity of Src. The level of tyrosine phosphorylation of Sam68 was monitored by immunoblotting employing the pY20 anti-phosphotyrosine antibody. As shown in **Figure 5**, Sam68 was efficiently phosphorylated by Src when this kinase was not pre-incubated with LMWPTP. However, after a 30 min pre-incubation with LMWPTP, the capacity of Src to catalyze Sam68 phosphorylation was virtually absent. These experiments unambiguously identify LMWPTP as a negative regulator of c-Src enzymatic activity.

3.5. Redox status affects the LMWPTP and Src subcellular distribution

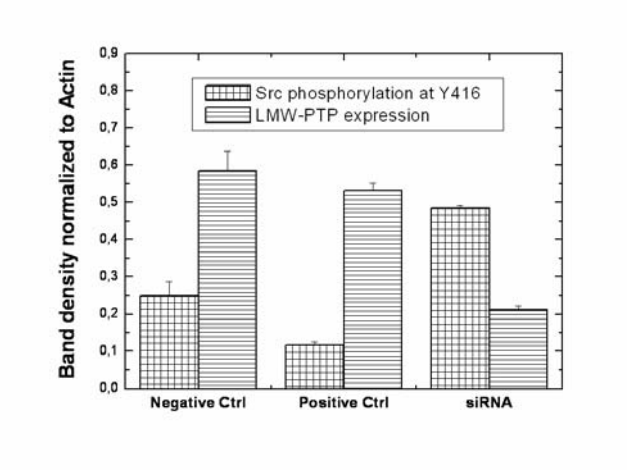
We observed that redox potential changes of the cell caused a translocation of LMWPTP to the RIPA-insoluble cytoskeletal fraction (**Figure 6A**), in agreement with the earlier elegant study of Caselli and collaborators [16] that shows that the redox status of the cell is a

critical determinant of subcellular localization of LMWPTP, an effect that is probably related to Y_{131} phosphorylation of LMWPTP. Strikingly, increasing the redox potential of cells by adding L-NAME or GSH, caused a translocation of LMWPTP to the cRIPA fraction (**Figure 6A**). Under the same conditions, pYSrc levels decreased, in close inverse relation to the increase in LMWPTP levels. Hence, LMWPTP is negatively correlated with pYSrc, also on a subcellular level, providing further evidence for a role of this phosphatase in controlling the phosphorylation status of c-Src. Accordingly cells incubated with GSH presented higher LMWPTP phosphorylation (**Figure 6B**).

A


Cells
siRNA Negative Ctrl
siMAPK1 Ctrl
siLMWPTP
pSrc
Y_{416}Src
LMW-PTP
pan-Actin

B


Band density normalized to Actin
0,0
0,1
0,2
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0,5
0,6
0,7
0,8
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Src phosphorylation at Y416
LMW-PTP expression
Negative Ctrl
Positive Ctrl
siRNA

Fig.4. Effect of LMWPTP on pY_{416}Src assessed using siRNA-mediated knock down. MC3T3-E1 cells at 60% confluence were transiently transfected with siLMWPTP for 72 h. Transfections were carried out using a Hiperfect transfection kit (Qiagen) according to the manufacturer's instructions. Negative control is an irrelevant RNA sequence, the positive control is a sequence directed against MAPK1 (knock down not shown). **A)** Hereafter, cells were scraped into cell lysis buffer and transfection confirmed by western blotting. Western blot analysis shows that Src phosphorylation at Y416 is dependent of LMWPTP activity. ß-actin was used as a loading control. **B)** The bar graph shows the relative amounts of phosphorylated Y_{416}Src and LMWPTP expression corrected for ß-actin. The results are presented as arbitrary units, normalized to the internal control.

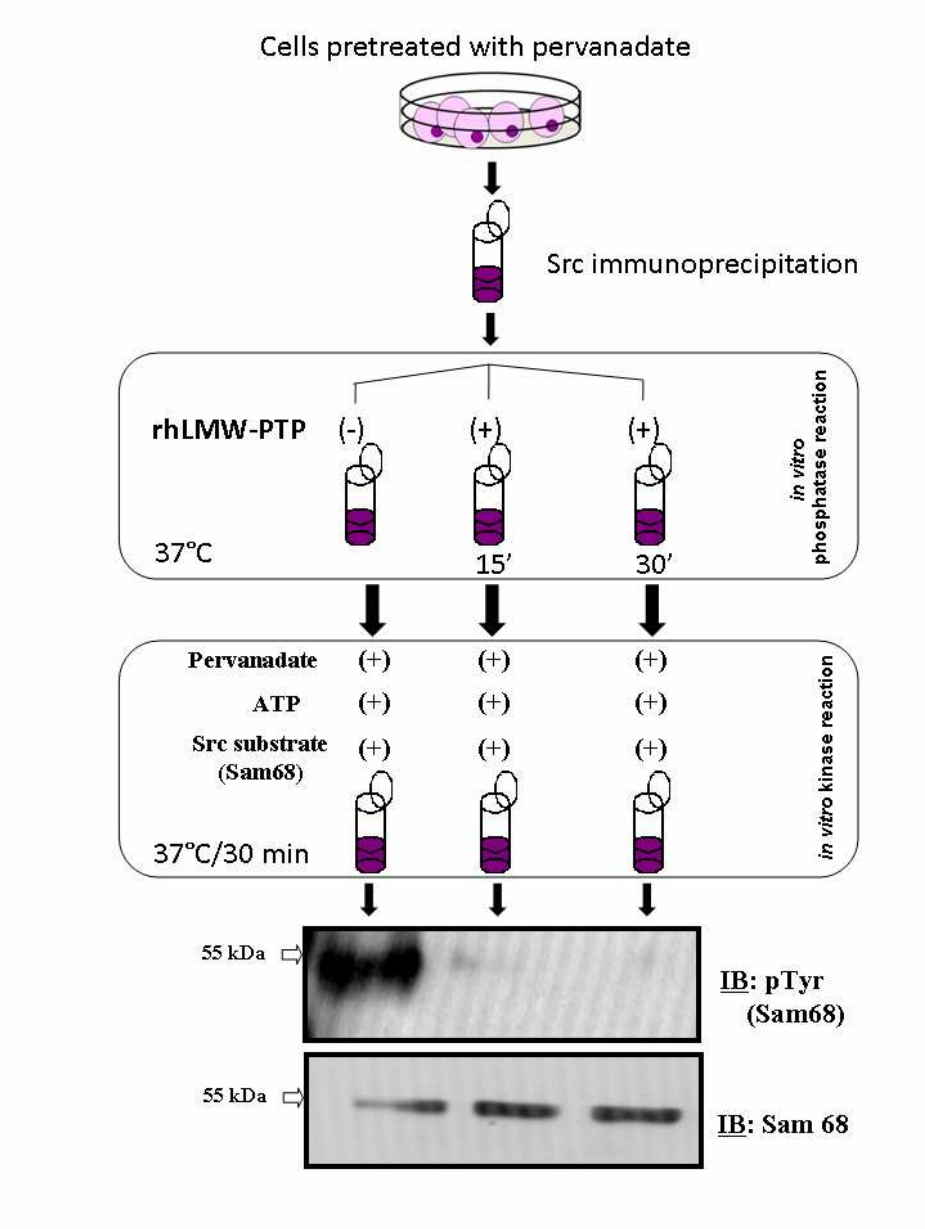

Fig.5. LMWPTP modulates Src kinase activity. Src kinase was immunoprecipitated and incubated with the rhLMWPTP for 15 and 30 min. Afterwards 10 µM of pervanadate was added to this solution together with the Sam68 (Src substrate) and ATP. After 30 min the reaction was terminated and subsequently the levels of both Sam68 and its phosphorylation status were assessed by Western blot.

Figure 6

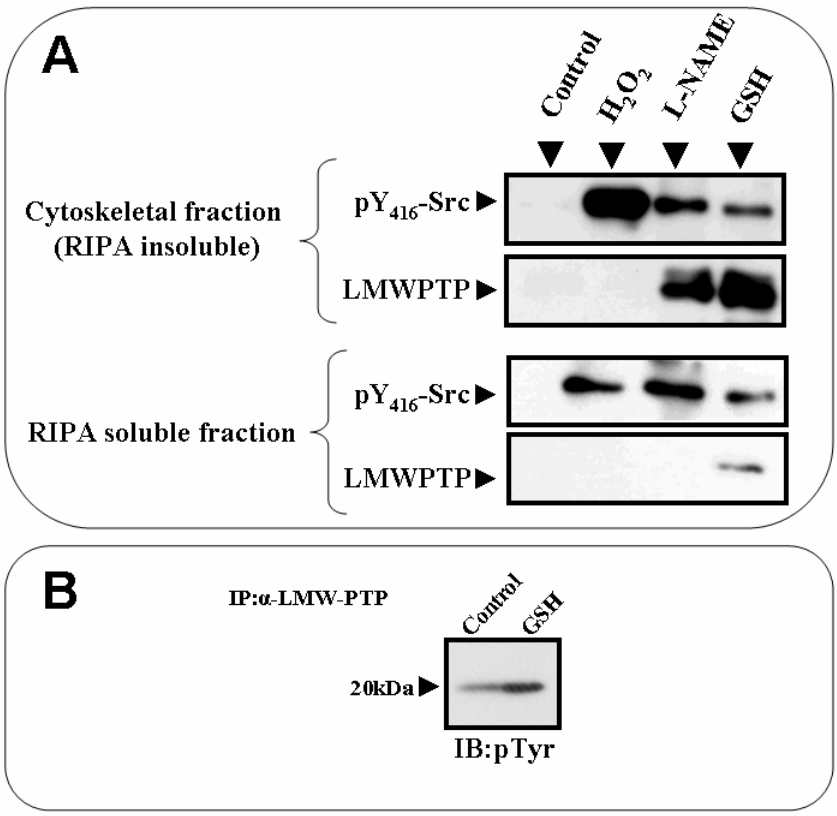

Fig.6. LMWPTP activity is modulated by the cellular redox status. (A) MC3T3 cells were treated with hydrogen peroxide, or L-NAME or GSH for 30 min and cells were fractionated into a RIPA insoluble and RIPA soluble fraction. The presence of LMWPTP and pY_{416}-Src was evaluated in both fractions. **(B)** Cells treated with GSH were lysed and LMWPTP was immunoprecipitated, followed by determination of pY-LMWPTP using α-pY antibody.

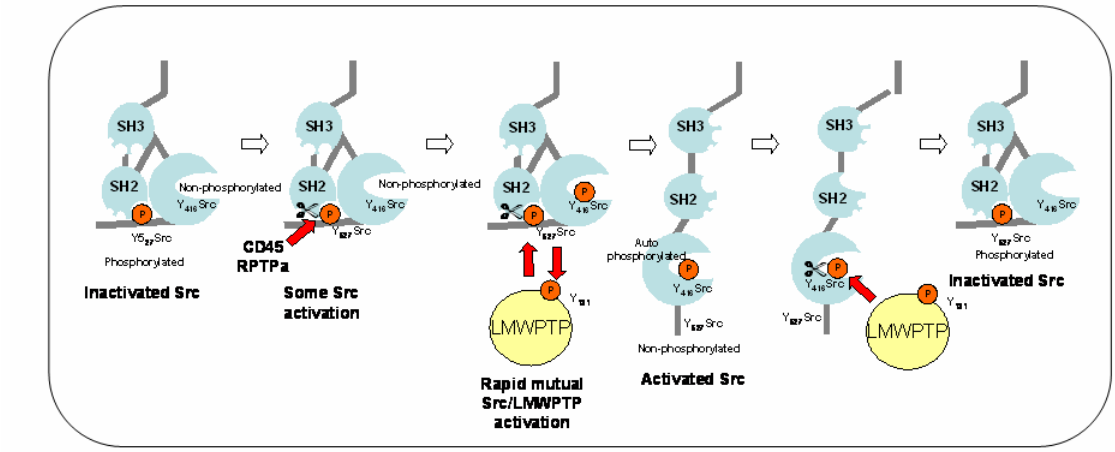

Fig.7. Schematic representation of Src activity modulation by dephosphorylation. Phosphorylation of tyrosine 527 residue of Src is crucial for maintaining this enzyme in its inactive conformation as pY_{527} interacts with the SH2 domain and this intramolecular association stabilizes a catalytically inactive form of the enzyme. Following dephosphorylation of Y_{527}Src by CD45/RPTPα, Src undergoes an intermolecular autophosphorylation at Y_{416}; this residue is present in the activation loop, and its phosphorylation promotes kinase activity and association with substrate molecules, e.g. LMWPTP. LMWPTP dephosphorylates Y_{527}-Src and in turn, this kinase becomes more active, which is responsible for the positive modulation of LMWPTP as well. This process we defined as rapid mutual activation. Additionally, when Y_{416}-Src residue is dephosphorylated by LMWPTP, Src returns to its inactive conformation.

4. Discussion

Despite the fundamental importance of Src non-receptor tyrosine kinase in cellular physiology, many aspects of its regulation, especially with respect to the covalent modulation (negative Y_{527}Src and positive Y_{416}Src residue) remain unclear. Especially, the mechanisms governing the remarkable kinetic of Src activity which upon cellular stimulation involve a fast upstroke in kinase activity followed by a slower return to baseline levels, remain only partially understood. It has become clear that PTPs, especially CD45 [6] and RPTPα [7] can dephosphorylate and activate the cytoplasmic tyrosine kinase, but the subsequent negative regulation remains only partially understood. An obvious possibility is of course that pY_{416}-Src is subject to dephosphorylation by PTPs as well, but the enzymes involved have remained elusive. In the present study support for such phosphatases was obtained from experiments in which MC3T3-E1 cells were treated with pervanadate. Although such treatment would be expected to negatively regulate Y_{527}-Src dephosphorylating enzymes and thus downregulate pY_{416}Src levels, instead increased pY_{416}-Src levels were seen. The only rational explanation for this observation is that pY_{416}-Src PTP activity is present in cells, making such phosphatase a plausible negative regulator for c-Src activity rather than alternative explanations like e.g. proteolytic breakdown of the active kinase. Further experiments indicated that LMWPTP is among enzymes exerting pY_{416}-Src phosphatase activity.

LMWPTP is in complex and is a direct substrate for active Src, phosphorylation of LMWPTP increasing its enzymatic activity [10,15]. Hence, this enzyme is an attractive candidate to act in the negative modulation on Src activity. Support for this notion was obtained from experiments in which the subcellular distribution of LMWPTP was assessed

in parallel with the phosphorylation status of c-Src. Subcellular localization of many enzymes, such as protein kinase C, Raf, Src, is influenced by extracellular stimuli, and detergent phase partitioning is a commonly used separation method [15,17-18]. RIPA buffer is a commonly used lysis buffer containing non-ionic detergents such as Triton X-100 or Nonidet P-40. It is currently accepted that the RIPA-soluble fraction contains cytosolic and many of the plasma membrane structures. Whereas increasing the redox potential of the cell caused a translocation of LMWPTP to the RIPA-insoluble cytoskeletal fraction, in agreement with the earlier elegant study of Caselli and collaborators [16] that shows that the redox status of the cell is a critical determinant of subcellular localization of LMWPTP, an effect that is probably related to Y_{131} phosphorylation of LMWPTP. Importantly, however, appearance of LMWPTP in this fraction correlated strictly with a decrease in pSrc levels. Furthermore, artificially increasing LMWPTP downregulated pSrc levels. Thus our data indicate that pSrc is an *in vivo* substrate for LMWPTP.

The action of LMWPTP is two-fold, this phosphatase might be responsible for transiently modulating Src activity (Fig. 7): a modest activation of LMWPTP dephosphorylates pY_{527}Src, leading to phosphorylation of Y_{416}Src and a subsequent dramatic increase in PTK activity, resulting in more LMWPTP tyrosine phosphorylation and hence more enzymatic phosphatase activity. The inherent positive feedback in this process may explain feedback the fast upstroke in cellular Src activity typically seen following cellular stimulation. Finally, however, the LMWPTP will produce dephosphorylation of pY_{416}Src, thus temporally limiting the Src activity. Thus LMWPTP emerges as a prime candidate to explain the remarkable dynamics of Src activation following cellular stimulation.

However, perhaps more likely is that dephosphorylation of pY_{527}-Src is an epiphenomenon which little *in vivo* relevance. In support of this possibility is that all data obtained in this study suggest that LMWPTP acts as a negative regulator of Src activity. Such a scheme would entail the activation of Src via dephosphorylation of pY_{527}-Src by other PTPs, e.g. CD45 or RPTPα. The increased Src phosphorylation would subsequently produce increased LMWPTP activity via $Y_{131}/_{132}$ phosphorylation. In turn this would provide negative modulation of Src via dephosphorylation of pY_{416}-Src. It is important to remark that both possibilities are not mutually exclusive and may act in parallel and this warrants further investigation. In addition, it would be interesting to establish the extent to which the unphosphorylated Y_{416}/Y_{517}-Src becomes a substrate for Y_{527}-Src phosphorylation by Csk [19]. Disregarding the exact niceties by which LMWPTP regulates Src activity, the present study has shown that this PTP acts a dualistic regulator of Src activity and identifies LMWPTP as the first bonafide negative regulator of Src Y_{416} phosphorylation.

Acknowledgements

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References

[1] C.V. Ferreira, G.Z. Justo, A.C.S. Souza, K.C. Queiroz, W.F. Zambuzzi, H. Aoyama, M.P. Peppelenbosch, Natural compounds as a source of protein tyrosine phosphatase inhibitors: application to the rational design of small-molecule derivatives, Biochimie 88 (2006) 1859-1873.

[2] T.E. Kmiecik, D. Shalloway, Activation and suppression of pp60c-src transforming ability by mutation of its primary sites of tyrosine phosphorylation, Cell 49 (1987) 65-73.

[3] R.L. Geahlen, M.D. Handley, M.L. Harrison, Molecular interdiction of Src-family kinase signaling in hematopoietic cells, Oncogene 23 (2004) 8024-8032.

[4] J.A. Cooper, K.L. Gould, C.A. Cartwright, T. Hunter, Tyr527 is phosphorylated in pp60c-src: implications for regulation, Science 231 (1986) 1431-1434.

[5] X.M. Zheng, R.J. Resnick, D. Shalloway, A phosphotyrosine displacement mechanism for activation of Src by PTPa, EMBO J. 19 (2000) 964-978.

[6] R. Roskoski Jr, Src protein-tyrosine kinase structure and regulation, Biochem. Biophys. Res. Commun. 324 (2004) 1155-1164.

[7] J. den Hertog, C.E. Pals, M.P. Peppelenbosch, L.G. Tertoolen, S.W. de Laat, W. Kruijer, Receptor protein tyrosine phosphatase alpha activates pp60c-src and is involved in neuronal differentiation, EMBO J. 12 (1993) 3789-3798.

[8] P. Chiarugi, P. Cirri, F. Marra, G. Raugei, T. Fiaschi, G. Camici, G. Manao, R.G. Romanelli, G. Ramponi, The Src and signal transducers and activators of transcription pathways as specific targets for low molecular weight phosphotyrosine-protein

phosphatase in platelet-derived growth factor signaling, J. Biol. Chem. 273 (1998) 6776-6785.

[9] P. Chiarugi, P. Cirri, F. Marra, G. Raugei, G. Camici, G. Manao, G. Ramponi, LMWPTP is a negative regulator of insulin-mediated mitotic and metabolic signaling, Biochem Biophys Res Commun. 238 (1997) 676-682.

[10] M. Bucciantini, P. Chiarugi, P. Cirri, L. Taddei, M. Stefani, G. Raugei, P. Nordlund, G. Ramponi, The low Mr phosphotyrosine protein phosphatase behaves differently when phosphorylated at Tyr131 or Tyr132 by Src kinase, FEBS Lett. 456 (1999) 73-78.

[11] P. Chiarugi, M.L. Taddei, P. Cirri, D. Talini, F. Buricchi, G. Camici, G. Manao, G. Raugei, G. Ramponi, Low molecular weight protein-tyrosine phosphatase controls the rate and the strength of NIH-3T3 cells adhesion through its phosphorylation on tyrosine 131 or 132, J. Biol. Chem. 275 (2000) 37619-37627.

[12] G. Ramponi, M. Stefani, Structure and function of the low Mr phosphotyrosine protein phosphatases, Biochim. Biophys. Acta 1341 (1997) 137-156.

[13] S. Rigacci, E. Rovida, S. Bagnoli, P. Dello Sbarba, A. Berti, Low M(r) phosphotyrosine protein phosphatase activity on fibroblast growth factor receptor is not associated with enzyme translocation, FEBS Lett. 459 (1999) 191-194.

[14] E. Rovida, S. Rigacci, A. Paccagnini, P.D. Sbarba, A. Berti, The low-molecular-weight phosphotyrosine protein phosphatase, when overexpressed, reduces the mitogenic response to macrophage colony-stimulating factor and tyrosine phosphorylation of its receptor, Biochem. Biophys. Res. Commun. 253 (1998) 300-304.

[15] P. Cirri, P. Chiarugi, L. Taddei, G. Raugei, G. Camici, G. Manao, G. Ramponi, Low molecular weight protein-tyrosine phosphatase tyrosine phosphorylation by c-Src during platelet-derived growth factor-induced mitogenesis correlates with its subcellular targeting, J. Biol. Chem. 273 (1998) 32522-32527.

[16] A. Caselli, R. Marzocchini, G. Camici, G. Manao, G. Moneti, G. Pieraccini, G. Ramponi, The inactivation mechanism of low molecular weight phosphotyrosine-protein phosphatase by H_2O_2, J. Biol. Chem. 273 (1998) 32554-32560.

[17] S.F. Clark, S. Martin, A.J. Carozzi, M.M. Hill, D.E. James, Intracellular localization of phosphatidylinositide 3-kinase and insulin receptor substrate-1 in adipocytes: potential involvement of a membrane skeleton, J. Cell Biol. 140 (1998) 1211-1225.

[18] E.L. Snapp, S.M. Landfear, Cytoskeletal association is important for differential targeting of glucose transporter isoforms in Leishmania, J. Cell Biol. 139 (1997) 1775-1783.

[19] S.M. Murphy, M. Bergman, D.O. Morgan, Suppression of c-Src activity by C-terminal Src kinase involves the c-Src SH2 and SH3 domains: analysis with Saccharomyces cerevisiae, Mol. Cell Biol. 13 (1993) 5290-5300.

Capítulo 3

HEDGEHOG AUTOCRINE STIMULATES DIFFERENTIATION OF PRE-OSTEOBLASTS INTO OSTEOCYTE-LIKE CELLS

HEDGEHOG AUTOCRINE STIMULATES DIFFERENTIATION OF PRE-OSTEOBLASTS INTO OSTEOCYTE-LIKE CELLS

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Abstract

Background. Osteocytes are deeply embedded within the mineralized bone matrix. It has been difficult to study the functions of these cells because their unique location is far from easy to access, in the compact bone. Therefore, the development of technical approaches to obtain this cell type in culture will provide a broad application.

Methodology/Principal Findings. Intriguingly, we showed that pre-osteoblast cells cultured on matrigel acquire osteocyte-like morphology. In addition, we employed the combination of sonic hedgehog with matrigel as inducers of pre-osteoblasts differention into osteocyte-like cells. In light of the crucial role of protein kinases in the cell metabolism including cell differentiation, we compared the kinomic profiling of osteoblasts cultured on 3D scaffold (matrigel substrate) with those ones on 2D-scaffold (polystyrene substrate). These results indicate that the utilization of matrigel as scaffold offers an easy model for studying several new aspects of the cell biology of osteocytes *in vitro*.

Conclusions/Significance. Our findings provide evidence about the importance of sonic hedgehog (Shh) to stimulate morphological and functional changes during pre-osteoblast differention into osteocytes-like cells. Finally, our data from peptide array revealed some kinases which seem to be important for osteocytes metabolism.

Introduction

Bone is a dynamic, vascular and living tissue under constant formation, resorption and remodelling throughout the entire life. However, these very well organized processes can be affected by some distinct physiological, mechanodamage or pathological conditions. Many cells that participate in the cellular symphony of bone remodelling. Among them, the osteocytes have managed to interest many research groups. Osteocytes potentially to have two distinct functions: ***a)*** to detect microdamage and undergo apoptosis as part of the signals that lead to microdamage repair (Verborgt *et al.*, 2000); and ***b)*** to detect changes in strain and trigger bone gain or bone loss, presumably by regulating osteoblastic and osteoclastic cells function (Aarden *et al.*, 1994). Despite the crucial importance of osteocytes, it has been difficult to study in details, under molecular aspects, their functions because its location, in the compact bone, is far from easy to access **(Figure 1)**. Therefore, an experimental design which makes it possible to obtain osteocyte-like cells can present broad application.

In light of this scenario, in this work we addressed three aspects of pre-osteoblasts differentiation: the influence of three-dimensional (3D) scaffold on these cells differentiation, the efficiency of sonic hedgehog (Shh) to act as a physiological inducer as well as the kinases differentially activated during this process.

Surface topography plays an important role on adhesion and function of numerous cell types and also is an important factor that can define the cellular fate, including final differentiation. Despite many aspects having been described about the molecular processes by which Shh binding to cells influences the cellular final response, the detailed mechanism and function of this signal transduction pathway remains obscure in diverse cell types,

including bone cells. The biological effects of Shh involve its interaction with Patched (Ptch1), thereby releasing Smoothened (Smo) with consequent activation of Gli transcription factor and specific gene transcription (Bijlsma *et al.*, 2006).

In this paper we showed that the matrigel offers ideal conditions for osteoblst-like cells (MC3T3-E1 line) differentiation into osteocyte-like cells. Additionally, the efficiency of this process was improved when Shh was added to both matrigel and culture medium. Importantly, the morphological changes of osteoblasts were prevented by cyclopamine (Shh signaling inhibitor). Our findings revealed that Shh signal pathway was activated in osteocyte-like cells and also pointed out some kinases which seem to be important for osteoblast fate.

Material and Methods

Reagents and antibodies

Cyclopamine, β-Glycerophosphate, Ascorbate and *p*-nitrophenylphosphate (pNPP) were purchased from Sigma; α-MEM medium from Cambrex; Matrigel® and recombinant-Shh were from R&D. The general reagents for cell culture were purchased from Bio-One. The immunoblotting reagents were purchased from Bio-Rad. *Antibodies:* β-actin, Ras, phospho c-Raf, phosphoERK (p42/p44), Gli1, Gli3, Shh, connexin 43, anti-mouse, anti-rabbit and anti-goat were purchased from Cell Signaling Technology (Boston, MA, USA). Primers were obtained from Service X: alkaline phosphatase (sense: 5'-aacccagacacaagcattcc-3' antisense: cgaagggtcagtcaggttgt-3'), osteocalcin (sense: 5'-gcgctctgtctctctgacct-3' antisense: 5'-gccggagtctgttcactacc-3') and β-actin (sense: 5'-cctaaggccaaccgtgaaaag-3' antisense: 5'-tcttcatggtgctaggagcca-3').

Pre-osteoblasts culture condition

MC3T3-E1, a calvaria mouse pre-osteoblast cell line, was obtained from ATCC (Manassas, USA) and grown at 37°C in αMEM medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin under a humidified 5% CO_2 atmosphere. Osteogenic Medium (OM): αMEM + β-glycerophosphate (10mM) + ascorbate (50 μg/mL).

3D for osteocyte-like cells differentiation

Firstly, a gel was prepared by mixturing Matrigel® : αMEM (2:1), and used for coating Petri dishes (100 mm of diameter) or 6-wells plates. These dishes were kept at 37^{o}C for 3 h.

Subsequently, cells were seeded ($3x10^4$ cells/mL) and cultured for 10 days. The medium was replaced each 3 days. In this assay 3 experimental conditions were used: Control group (2D/plastic substrate); Matrigel® (3D) and Matrigel® (3D) + OM.

Treatment with Shh and cyclopamine

Firstly, a gel was prepared by mixturing Matrigel® : αMEM (2:1) plus Shh or cyclopamine, and used for coating Petri dishes (100 mm) or 6-wells plates. These dishes were kept at 37°C for 3 h. Subsequently, cells were seeded ($3x10^4$ cells/mL) and cultured for 5 days. The medium was replaced each 3 days.

Determination of *Alkaline Phosphatase (ALP) activity*

MC3T3-E1 cells ($3x10^4$ cells/mL) were seeded in 24-wells plate (coated with gel) and grown for 10 days. After, the cells were rinsed with ice-cold PBS and incubated for 30 min at room temperature with ALP assay buffer (1.5M of Tris-HCl [pH 9.0], 1mM $ZnCl_2$, and 1mM $MgCl_2$) containing 1% Triton X-100. Cell extracts were collected, centrifuged, and used for the enzyme assay. ALP activity was determined by using 5mM of *p*-nitrophenylphosphate (*p*NPP) as a substrate as described (Hitomi *et al.*, 1992) and expressed as nanomoles of products formed per minute per milligrams of protein. Protein concentrations were determined by Lowry´s method (Hartree 1972).

Cellular Viability assay (MTT)

After 10 days the medium was removed and 1.0 mL of 1 mg/mL MTT (Calbiochem) was added to each well. After incubation for 4h at 37°C, the medium was removed and the formed formazan was released by solubilisation in 1.0 mL of DMSO (Sigma). The plate

was shaken for 10 min on a plate shaker and the absorbance measured at 570 nm in plate reader (BioTek) (Mossman *et al.*, 1983).

Western blotting

MC3T3-E1 cells ($3x10^4$ cells/mL) were seeded in 100mm Pedri dishes coated with gel and grown for 10 days. Afterwards cells were collected, centrifuged and rinsed twice with warm PBS and next cells were lysed with the following buffer (50mM Tris [tris(hydroxymethyl)aminomethane]–HCl [pH 7.4], 1% Tween 20, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EGTA (ethylene glycol tetraacetic acid), 1mM *O*-Vanadate, 1mM NaF, and protease inhibitors [1µg/mL aprotinin, 10µg/mL leupeptin, and 1 mM 4-(2-amino-ethyl)-benzolsulfonyl-fluorid-hydrochloride]) for 2 hours on ice. Protein extracts were centrifuged and the protein concentration was determined. An equal volume of 2 x sodium dodecyl sulfide (SDS) gel loading buffer (100mM Tris-HCl [pH 6.8], 200mM dithiothreitol [DTT], 4% SDS, 0.1% bromophenol blue, and 20% glycerol) was added to samples and boiled for 5 minutes. Cell extracts were resolved by SDS-polyacrylamide gel (10 or 12%) electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Then, membranes were blocked in 1% fat-free dried milk or bovine serum albumin (2.5%) in Tris-buffered saline (TBS)–Tween 20 (0.05%) and incubated overnight at 4° C with appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.05%), membranes were incubated with anti-rabbit, anti-goat or anti-mouse horseradish peroxidase–conjugated secondary antibodies, at 1:2000 dilutions (in all Western blotting assays), in blocking buffer for 1 hour (as described by de de Souza Queiroz *et al.*, 2007; de Jesus *et al.*, 2008). Detection was performed by using enhanced chemiluminescence (ECL).

RT-PCR

Total RNA was isolated from cells cultured on 2D or 3D-scaffold by the Trizol method (Sigma Zwijndrecht, Netherlands) according to manufacturer´s instructions. cDNA was produced by using Oligo-dT primers (Invitrogen, Breda, Netherlands) in a final volume of 30 µL. A PCR was performed for 35 cycles using a 60-second denaturizing step at 94°C, 60-second annealing step at 60°C and a 60-second extension step at 72°C. The product was loaded onto agarose gel and the ethidium bromide stained band was recorded. β-actin was used as internal control. For all primers utilized.

Kinome profile

Cells cultured on 3D and 2D-scaffold were lysed with lysis buffer (Cell Signaling, Beverly, MA) with the addition of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM h-glycerophosphate, 1 mM sodium vanadate, 1 mM sodium fluoride, 1 µg/mL leupeptin, and 1 µg/mL aprotinin. The lysates were centrifuged at 20,000 x g for 10 minutes at 4ºC, and the pellet was discarded. To study kinase activity, 50 µL lysate was added to 12 µL activation mix containing 50% glycerol, 250 µmol/L ATP, 60 mM $MgCl_2$, 0.05% (v/v) Brij-35, 0.25 mg/mL bovine serum albumin, and 2,000 Ci/mL [γ-^{33}P]ATP. The peptide arrays (Pepscan, Lelystad, The Netherlands) containing 1,024 different kinase pseudosubstrates and 12 control sequences, each spotted twice to confirm reproducibility of the results, were

incubated with lysates for 90 minutes in a humidified stove at 37 ºC. Subsequently, the array was washed twice in PBS containing 0.1% Triton X-100, twice with 2 M NaCl containing 0.1% Tween 20, and twice in distilled water. Slides were air dried and exposed to a phosphoimaging screen for 72 hours and scanned on a STORM apparatus (Molecular Dynamics, GE Healthcare, Roosendaal, The Netherlands). As a control for a specific binding of [γ-^{33}P]ATP to peptide motifs, [α-^{33}P]ATP was used: no radioactivity was detected. Furthermore, as a control, we have analyzed frozen versus fresh material using the PepChip because snap freezing could cause degradation in kinase activity. Results indicated that the kinase activity is not influenced by snap freezing of samples.

Peptide array imaging and statistical data analysis

The peptide array data analysis was done as described by Diks *et al.* (2004) and Lowenberg *et al.* (2006). Briefly, ScanAnalyze software was used. Using grid tools, spot density and individual background were corrected and spot intensities and background intensities were analyzed. Data from three individual experiments were exported to an Excel sheet for further analysis. Control spots on the array were analyzed for validation of spot intensities between the different samples. Inconsistent data (i.e., SD between the different data points >1.96 of the mean value) were excluded from further analysis. Spots were averaged and included for dissimilarity measurement to extract kinases of which activity was either significantly induced or reduced. Different kinase activities in lysates between groups of the cells were determined by significant fold change ratios of the combined values of phosphorylated peptides resembling a substrate for kinase activity. Significance analysis was done as described by Lowenberg *et al.* (2006), briefly; a minimum modification for the algorithm originally developed for microarray analysis was used.

Statistical analysis

All experiments were performed in triplicate and the results shown in the graphs represent the means and standard errors. Cell viability (MTT) data were expressed as the means ± standard errors of 3 independent experiments carried out in triplicates. Data from each assay were analyzed statistically by ANOVA. Multiple comparisons among group mean differences were tested with the Tukey post hoc test. Differences were considered significant when the p value was less than 0.05. Western blots represent 3 independent experiments.

Results

3D Scaffold using Matrigel® promotes morphological and biochemical changes in pre-osteoblasts

Pre-osteoblast cells cultured on polystyrene substrate displayed typical morphological features (**Figure 2A**). On the other hand, MC3T3-E1 cells cultured on Matrigel presented morphological changes as it is clearly shown in the **Figure 2B**. In addition, osteogenic medium potentiates the matrigel effect (**Figure 2C**). The morphological characteristics of these cells seem to be very similar to osteocytes, such as star-shaped, and long cytoplasmic extensions. Cell viability was evaluated by assessment of mitochondrial function, which indicates that in all experimental conditions the cells remained viable (**Figure 2D**). It is important to mention that cells cultured on 2D scaffold displayed higher mitochondrial function (cell viability), probably due to the higher cell number, since even during 10 days these cells still can proliferate. Besides the morphological pattern of cells cultured on 3D scaffold that indicates osteoblast differentiation into osteocyte-like cells, the overexpression of connexin 43 is also another indicative of the occurrence of this process (**Figure 2E**).

Osteocalcin and alkaline phosphatase (ALP) expression was evaluated by RT-PCR (**Figure 3A**). Cells cultured on matrigel displayed an expressive increase of osteocalcin expression. In relation to ALP the highest expression was observed when osteogenic medium (OM) was added to the culture. Accordingly, when the activity of ALP was examined, the highest activity was detected when matrigel plus osteogenic medium were employed (**Figure 3B**).

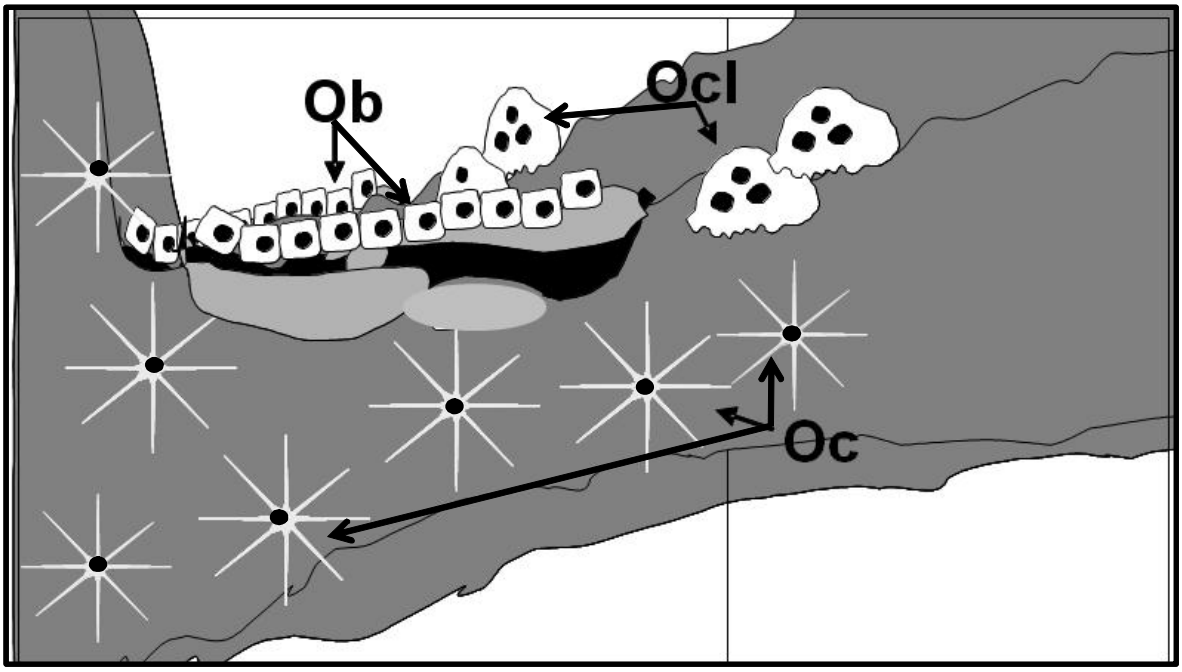

Figure 1: Schematic representation of bone cells. This figure shows the three types of bone cells: Osteoblasts (Ob), Osteoclasts (Ocl) and Osteocytes (Oc). Note that osteocytes are embedded deep within the mineralized bone matrix and connected to each other as well.

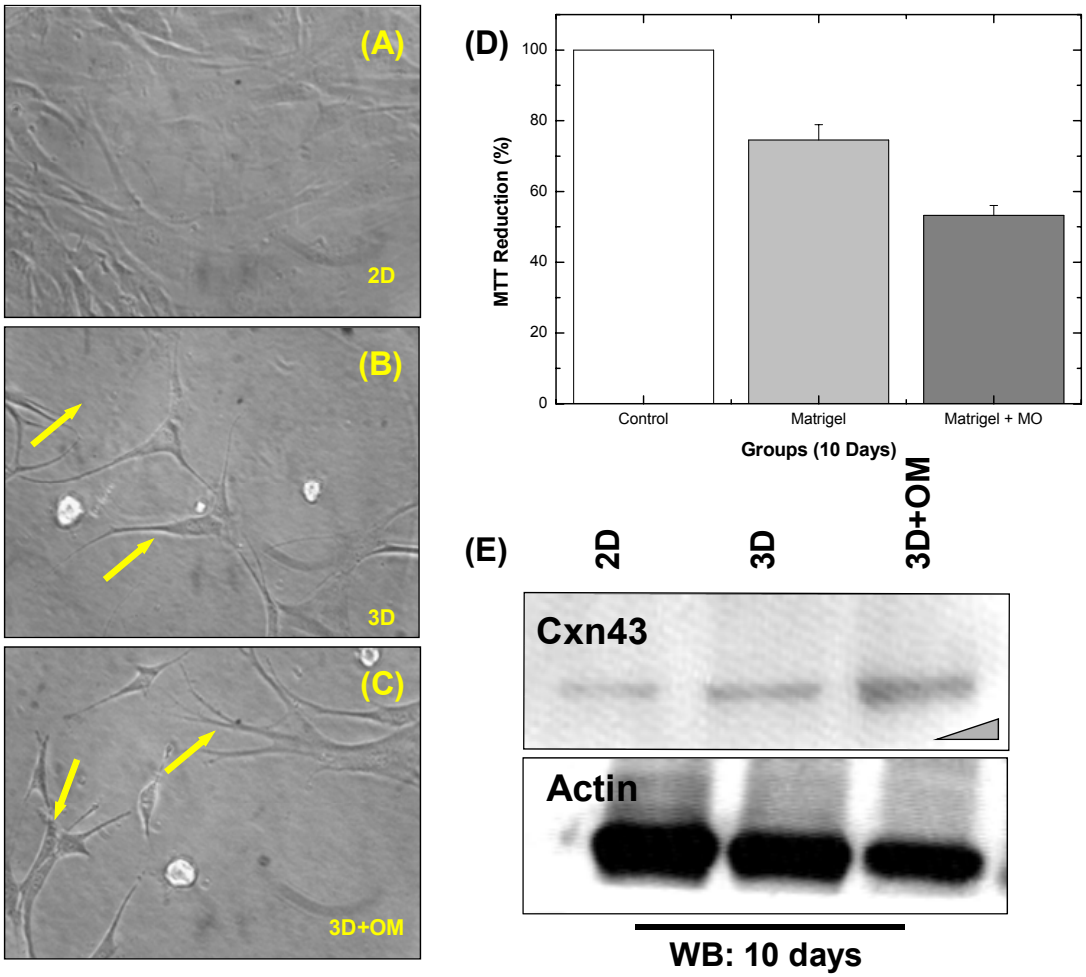

Figure 2: **Indication of osteocyte-like cells obtention after culturing pre-osteoblasts on matrigel.** The left panel shows micrographs obtained from cells cultured at different conditions: **(A)** control - 2D, **(B)** 3D-scaffold and **(C)** 3D-scaffold plus OM (osteogenic medium). Note that there are diverse cytoplasm extensions (indicated by arrows), which are similiar to those ones found in typical osteocytes. The right panel shows the cell viability by MTT assay **(D)** and Conexin-43 expression **(E)**, performed by immunoblotting. **Magnification: 40x (A, B and C).** Actin was used as internal control.

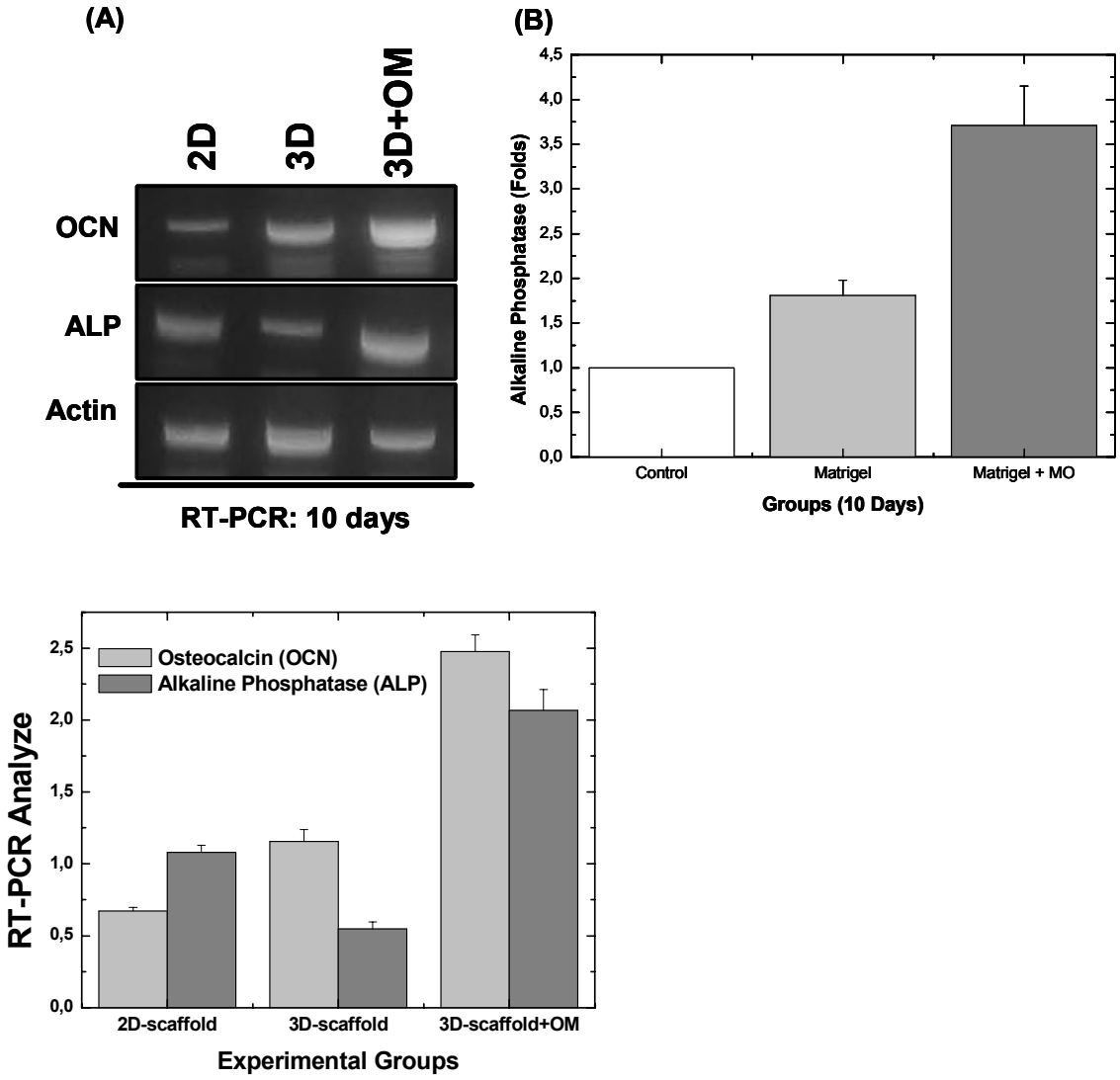

Figure 3: **Evaluation of pre-osteoblasts differentiation. (A)** Pre-osteoblasts were cultured for 10 days and the differentiation process was checked by analyzing osteocalcin and alkaline phosphatase (ALP) expressions by RT-PCR. PCR products were visualized in ethidium bromide stained gels and normalized by actin gene, quantitative expression was analyzed with Quantity One software (version: 4.4.0, Bio-Rad). **(B)** ALP activity assay was performed as described in Methods section.

Sonic hedgehog signaling is required for osteocyte-like cells maturation

Previously, we confirmed the Shh signaling pathway activation on cells cultured on matrigel through examining the expression of Gli 1 and 3. Accordingly, we observed that Gli1 expression increased while Gli3 was down-regulated. Patched expression was increased when the cells were cultured on Matrigel (**Figure 4A**). In the same experiment the conditioned media was collected, concentrated and subsequently the presence of Shh examined by western blotting. We observed an increase of cleaved Shh, mainly of its more active form (**Figure 4B**).

In order to prove this, pre-osteoblast cells were cultured on matrigel in the absence or presence of cyclopamine or recombinant Shh for 5 days (**Figure 4C**). Shh induces osteocyte-like cells obtention earlier than matrigel (3D), and this process was prevented by cyclopamine, as showed in the micrographies.

Kinome profile comparison during osteocyte morphology acquisition.

Reversible phosphorylation catalysed by kinases is probably the most important regulatory mechanism in eukaryotes. Protein kinases are involved in a variety of cellular processes including cytoskeletal reorganization. In order to provide an overview of kinases differentially activated during osteocyte-like cell differentiation, we decided to examine the phosphorylation of peptide arrays (PepChip). Peptide arrays, exhibiting specific consensus sequences for protein kinases, were used to produce a global analysis of cellular kinase activity in osteocyte-like cells-matrigel derived. *In vitro* phosphorylation of peptide arrays by osteocyte-like cell lysates revealed that contain substantial kinase activity; almost all substrate peptides incorporate [γ-33P]ATP (**Figures 5A and 5B**). Subsequent analysis of

the kinome profiles revealed 84 kinase activities showing a significantly differential activity when comparing 2D scaffold ($P < 0.05$; Supplementary Data). **Figure 5C** shows that 46% were involved with cellular communications, indicating that these groups of cell signaling molecules act as determinant in osteocytes biology function. In agreement, some authors have pointed Connexin 43 as good biomarker of osteocytes (Jiang *et al.*, 2007; Gu *et al.*, 2006).

The data obtained from the Pepchip assay revealed that kinases involved with cell survival (MAPK and tyrosine kinase) and cytoskeleton remodeling (Src kinase and LIMK1), were expressively more active on osteocyte-like cells (**Figure 6**).

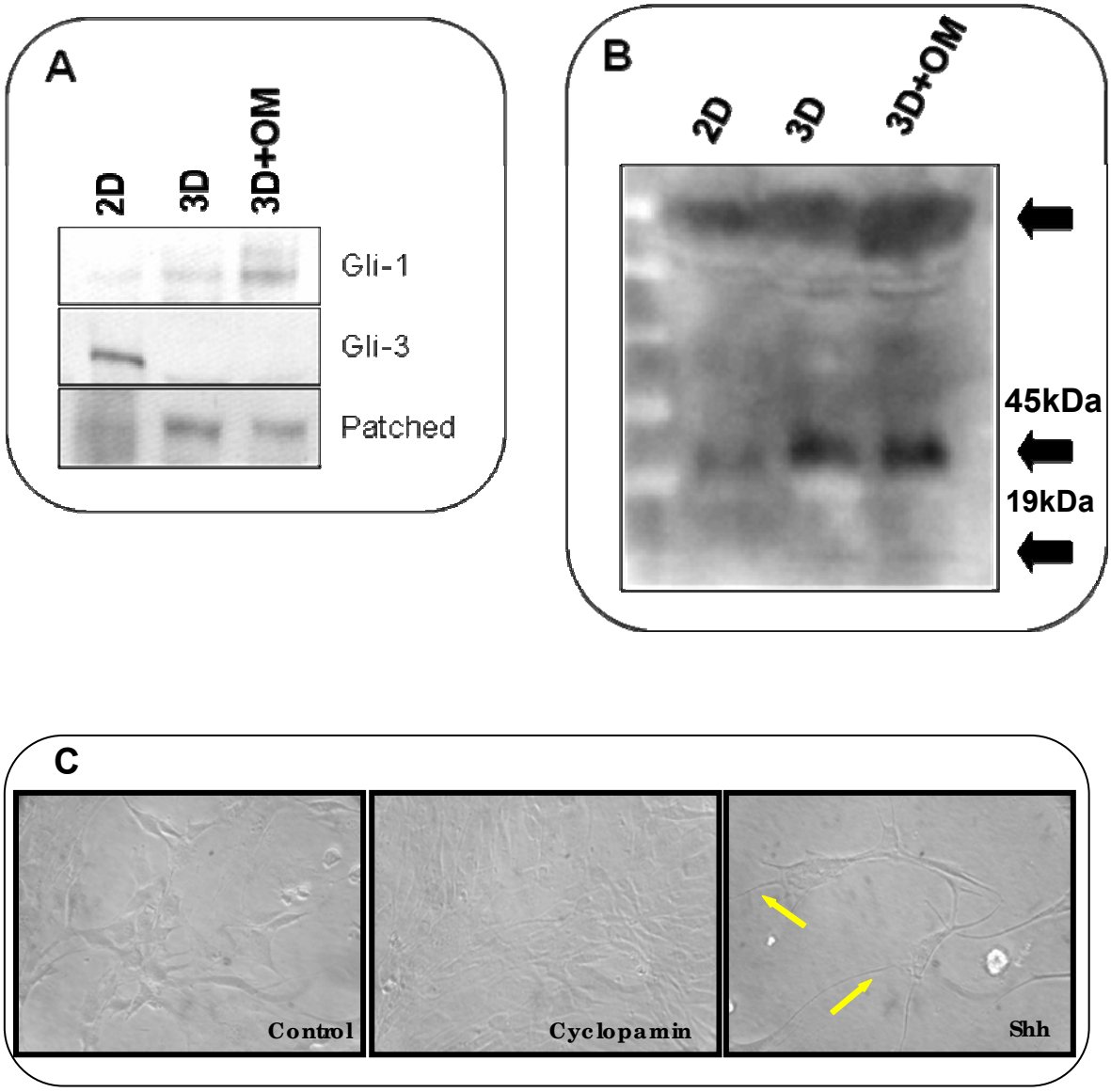

Figure 4: **Shh stimulates pre-osteoblasts differentiation in osteocyte-like cells. (A)** Shh signalling mediators expression in MC3T3-E1 cells cultured on Matrigel (2D, 3D and 3D+OM). **(B)** Cleaved Shh forms were checked in the conditioned medium (arrows indicate different forms of Shh, 45 and 19kDa). **(C)** Pre-osteoblasts were cultured for 5 days on matrigel in the absence or presence of 2 μM cyclopamine or recombinant Shh (10 ng/mL), afterwards the cell morphology was examined. Recombinant Shh promoted osteocyte morphology (yellow arrows). **Magnification: 40x.**

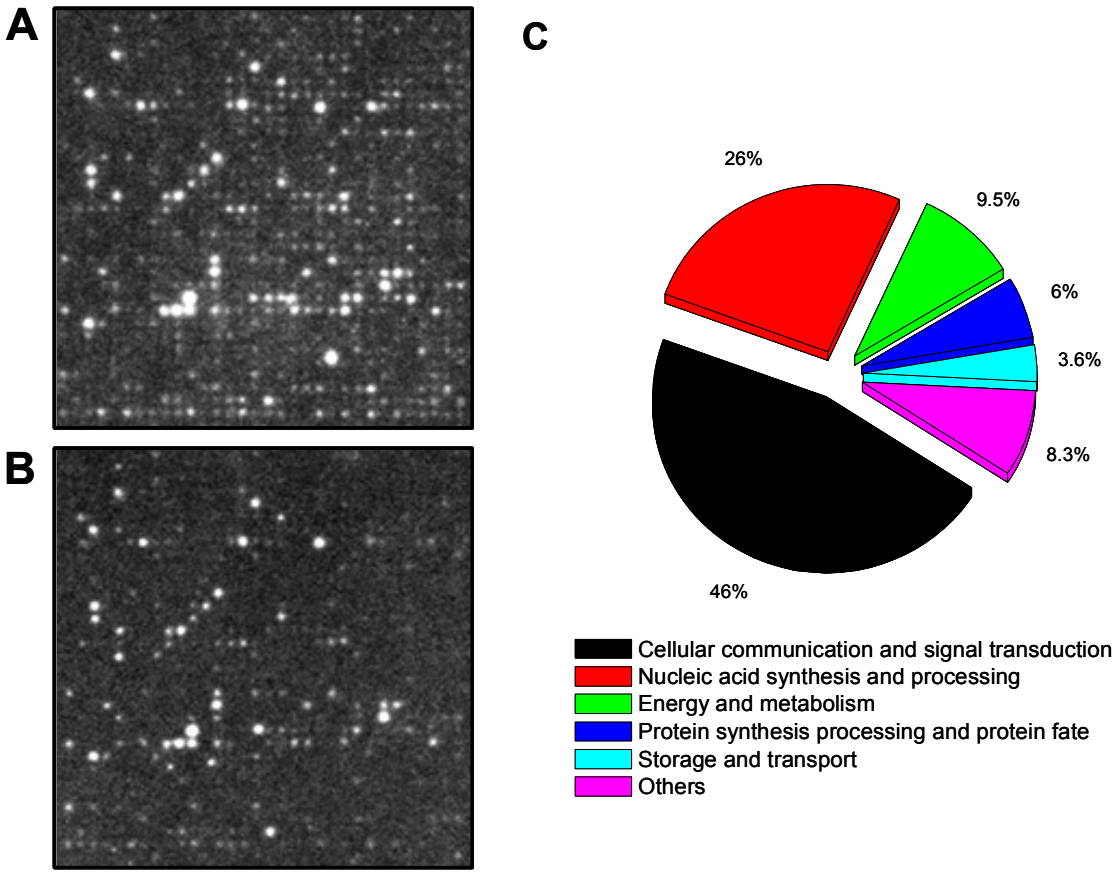

Figure 5. PepChip analysis of pre-osteoblasts differention. Scans of the peptide arrays after incubation with lysates from control culture **(A)** and treated with osteogenic medium (ascorbic acid and *β*-glycerophosphate) and Matrigel **(B)** with [γ-^{33}P] ATP. Each spot represents phosphorylation of a specific substrate through kinase activity (for more details see supplementary material). **(C)** General overview of phosphorylation profile of specific substrates involved with different cellular functions and showed change in the phosphorylation (n= 84, $p<0.05$).

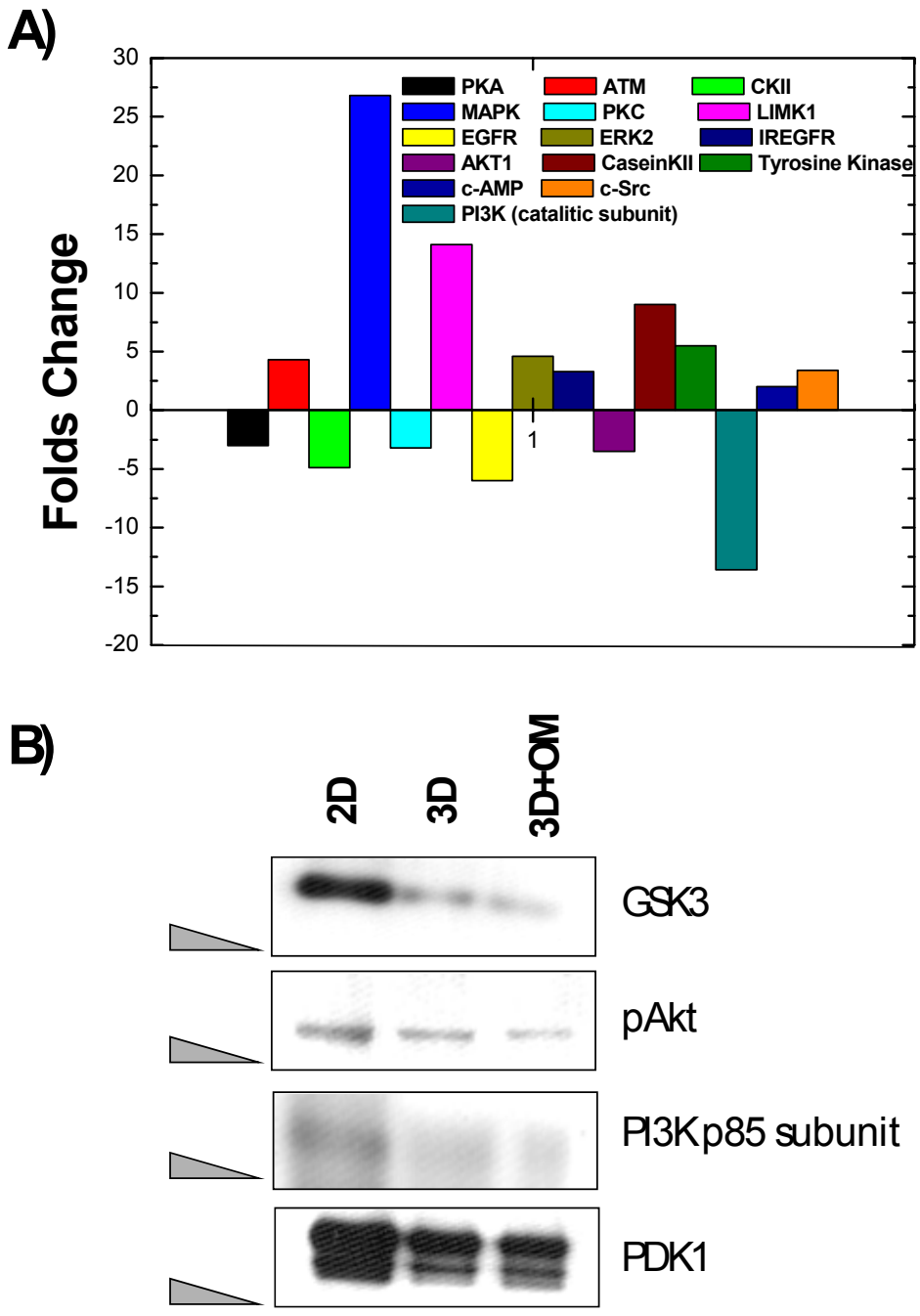

Figure 6. Kinome profile analyzes. (A) Protein kinases differentially activated during pre-oesteoblasts differentiation into osteocytes-like cells. **(B)** Western blotting assay was performed for PepChip validation. We checked GSK3, pAkt, PI3K p85 subunit and PDK1. (for more details see supplementary material).

Discussion

The osteogenesis process represents an interesting research field both for basic and applied investigations. MC3T3-E1 cells have been accepted as an experimental model for studying osteoblast differentiation (Franceschi and Iyer, 1992). In this field little information related to signal transduction pathways involved in osteocyte differentiation is provided in the literature. In this paper we provide evidence that pre-osteoblast cells cultured on matrigel acquired typical morphological and biochemical features of osteocytes. Osteocytes, the most abundant cell type in bone, remain the least characterized, mainly due to the difficulty to isolate them, since in nature these cells are barely accessible because its confinement in the calcified matrix.

Recently, Gu and collaborators (2006) showed that osteocytes can be isolated from cortical bone and maintained *in vitro* for several days or even weeks. However, technically speaking, it is not easy. Therefore, experimental designs which make possible to obtain osteocyte-like cells can present broad application. In regard to provide a model that can be used for studying the osteocytes biology, we showed that the Matrigel offers ideal conditions for MC3T3-E1 differentiation into osteocyte-like cells, which was confirmed under morphological and biochemical aspects. Besides ALP and osteocalcin upregulation (typical osteoblasts differention markers), we also detected an overexpression of connexion 43 in cells cultured on matrigel/3D-scaffold. In general osteocytes express low levels of ALP but are positive for osteocalcin (van der Plas and Nijweide 1992; Aarden *et al.*, 1994; Franz-Odendaal *et al.*, 2006). The higher expression and activity of ALP was in the group treated with OM (osteogenic medium), which is recognized to present this effect on ALP.

Importantly, we verified that Shh signaling pathways was activated during osteocyte-like cells morphology acquisition triggered by 3D scaffold. Initially, the conditioned culture media from 2D and 3D groups were collected and the proteins were resolved in SDS-PAGE for immunoblotting. This result clearly showed that Shh was secreted in both groups cultured on 3D-scaffold. Accordingly, Patched-1 (Ptch1), an important mediator of Shh signaling cascade, was detected in these cells. The biological effects of Shh are mediated through a pathway that involves binding to Ptch1, thereby releasing Smoothened (Smo) with consequent activation of Gli transcription factor and specific gene transcription. In the absence of Shh, Ptch1 represses the activity of the transmembrane protein Smo (Chen *et al.*, 2002; Bijlsma *et al.*, 2006). In order to demonstrate the pivotal role of Shh for the osteocyte-like cells morphology acquisition, we performed an experiment in which the cells cultured on matrigel were treated with recombinant Shh or cyclopamine (Shh signaling inhibitor). The results clearly indicate that Shh participates in the modulation of osteocyte differentiation. This fact was reinforced by the finding that cyclopamine impaired this process. Recently, our group demonstrated that Shh signaling is required in neurite processes (Bijlsma *et al.*, 2008). It is in agreement with our hypothesis that Shh is crucial for the long cytoplasmic extensions in osteocyte-like cells obtained after culturing pre-osteoblasts on 3D scaffold.

Another innovative aspect refers to definition of the kinomic profiling of osteocyte-like cells. Protein phosphatases and protein kinases are key players in regulating intricate mechanisms in signal transduction. The phosphorylation and dephosphorylation of proteins has been found to modify protein function in a multitude of ways. Peppelenbosch´s group have investigated protein kinase content (kinome) of many eukaryotes and their evolutionary relationships in details, revealing both the importance and the diversity of

these proteins (van Baal *et al.*, 2006; Diks *et al.*, 2007). Therefore, the evaluation of the kinome profile might be useful to provide more details about specific cellular process, such as differentiation.

The kinomic analysis revealed that kinases involved with cellular communication signaling are prevalently activated in osteocyte-like cells. This finding is in agreement with those obtained by western blotting that showed an overexpression of connexin 43. Connexin-43 is a good biomarker of osteocyte (Gu *et al.*, 2006). These results suggest that gap junctions play an important role in osteocyte-osteocyte and/or osteocyte-osteoblast communication. The existence of gap junctions between bone cells has previously been described by several authors (Doty 1981; Davidson *et al.* 1986; Palumbo *et al.* 1990; Schiller *et al.* 1992; Yellowley *et al.*, 2000; Kamioka *et al.*, 2007). Also, Plotkin *et al.* (2002) suggested that connexin-43 triggers transduction of cell survival signals. This evidence adds connexin-43 to the list of transmembrane proteins capable of transducing survival signals in response to extracellular cues.

In conclusion, the utilization of Matrigel as scaffold offers an easy model for studying several new aspects of the cell biology of osteocytes *in vitro*. Also, we suggested that Shh exerts crucial role during osteocyte morphology acquisition. Finally, our data from peptide array evidenced that kinases related with cellular communication play pivotal role in osteocyte cells.

Author Contributions

Conceived and designed the experiments: WFZ MPP CVF. Performed the experiments: WFZ KCSQ AJH. Analyzed the data: WFZ JMG AHCN MPP CVF.

Contributed reagents/materials/analysis tools: MPP CVF. Wrote the paper: WFZ JMG MPP CVF.

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References

Aarden EM, Burger EH, and Nijweide PJ. Function of osteocytes in bone. J Cell Biochem 55: 287–299, 1994.

Bijlsma MF, Peppelenbosch MP, Spek CA, Roelink H. Leukotriene Synthesis is Required for Hedgehog-dependent Neurite Projection in Neuralized Embryoid Bodies but not for Motor Neuron Differentiation. Stem Cells. 2008. (In Press).

Bijlsma MF, Peppelenbosch MP, Spek CA. Hedgehog morphogen in cardiovascular disease. Circulation. 2006 Oct 31;114(18):1985-91.

Chen, J. K., Taipale, J., Cooper, M. K., Beachy, P. A. (2002) Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes Dev.* **16**, 2743–2748.

Davidson JS, Baumgarten IM, Harley EH (1986) Reversible inhibition of intercellular junctional communication by glycyrrhetinic acid. Biochem Biophys Res Commun 134:29–36.

de Souza Queiroz KC, Zambuzzi WF, Santos de Souza AC, da Silva RA, Machado D, Justo GZ, Carvalho HF, Peppelenbosch MP, Ferreira CV. (2007) A possible anti-proliferative and anti-metastatic effect of irradiated riboflavin in solid tumours. *Cancer Lett.* **258**(1), 126-134.

de Jesus, M. B., Zambuzzi W. F., Sousa R. R. R., Areche C., Souza A. C. S., Aoyama H., Schmeda-Hirschmann G., Rodríguez J. A. , Brito A. R. M. S., Peppelenbosch M. P., den Hertog J., de Paula E., Ferreira C.V. (2008). Ferruginol suppresses survival signaling pathways in androgen-independent human prostate cancer cells. Biochimie, (*In Pres*, doi: 10.1016/j.biochi.2008.01.011).

Diks SH, Kok K, O'Toole T, *et al.* Kinome profiling for studying lipopolysaccharide signal transduction in human peripheral blood mononuclear cells. J Biol Chem 2004;279:49206–13.

Diks SH, Parikh K, van der Sijde M, Joore J, Ritsema T and Peppelenbosch MP Evidence for a Minimal Eukaryotic Phosphoproteome? PLoS ONE. 2007; 2(8): e777.

Doty SB (1981) Morphological evidence of gap junctions between bone cells. Calcif Tissue Int 33:509–512.

Franceschi RT, Iyer BS 1992 Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. J Bone Miner Res 7:235-246.

Franz-Odendaal TA, Hall BK, Witten PE. Buried alive: how osteoblasts become osteocytes. Dev Dyn. 2006 Jan;235(1):176-90.

Gu G, Nars M, Hentunen TA, Metsikko K, Vaananen H. Isolated primary osteocytes express function Gap junctions *in vitro*. Cell Tissue Res. 2006 Feb;323(2):263-71.

Hartree EF. 1972. Determination of proteins: a modification of Lowry method that give a linear photometric response. Anal Biochem 48:422-27.

Jiang JX, Siller-Jackson AJ, Burra S. Roles of gap junctions and hemichannels in bone cell functions and in signal transmission of mechanical stress. Front Biosci. 2007 Jan 1;12:1450-62.

Kamioka H, Ishihara Y, Ris H, Murshid SA, Sugawara Y, Takano-Yamamoto T, Lim SS. Primary cultures of chick osteocytes retain functional gap junctions between osteocytes and between osteocytes and osteoblasts. Microsc Microanal. 2007 Apr;13(2):108-17.

Lowenberg M, Tuynman J, Scheffer M, *et al.* Kinome analysis reveals nongenomic glucocorticoid receptordependent inhibition of insulin signaling. Endocrinology 2006;147:3555–62.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. J Immunol Meth. 1983;65: 55-63.

Palumbo C, Palazzini S, Marotti G (1990) Morphological study of intercellular junctions during osteocyte differentiation. Bone 11:401–406.

Plas A van der, Nijweide PJ (1992) Isolation and purification of osteocytes. J Bone Miner Res 7:389–396.

Plotkin LI, Manolagas SC, Bellido T. Transduction of cell survival signals by connexin-43 hemichannels. J Biol Chem. 2002 Mar 8;277(10):8648-57

Schiller PC, Mehta PP, Roos BA, Howard GA (1992) Hormonal regulation of intercellular communication: parathyroid hormone increases connexin 43 gene expression and gap-junctional communication in osteoblastic cells. Mol Endocrinol 6:1433–1440.

van Baal JW, Diks SH, Wanders RJ, Rygiel AM, Milano F, Joore J, Bergman JJ, Peppelenbosch MP, Krishnadath KK. Comparison of kinome profiles of Barrett's esophagus with normal squamous esophagus and normal gastric cardia. Cancer Res. 2006 Dec 15;66(24):11605-12.

Verborgt O, Gibson G, and Schaffler MB. Loss of osteocyte integrity in association with microdamage and bone remodeling after fatigue *in vivo*. J Bone Miner Res 15: 60–67, 2000.

Yellowley CE, Li Z, Zhou Z, Jacobs CR, Donahue HJ. Functional gap junctions between osteocytic and osteoblastic cells. J Bone Miner Res. 2000 Feb;15(2):209-17.

Capítulo 4

OSTEOBLAST DIFFERENTIATION SILENCES MULTIPLE SIGNALING PATHWAYS INCLUDING SRC, REQUIRES MMP-2 & -9 AND RECRUITS DISTINCT MMP-INHIBITORS

Osteoblast Differentiation silences multiple signaling pathways including Src, requires MMP-2 & -9 and recruits distinct MMP-inhibitors

Short Title: Signal transduction in the osteogenesis.

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Abstract

Extracellular matrix (ECM) remodelling is important during bone development, repair and pathogenesis (e.g. osteoporosis). Our objective was to evaluate the molecular mechanisms triggered during osteoblast differentiation. In this work, we showed that during the osteoblastic differentiation (induced by treatment with ascorbate and β-glycerophosphate) MMP-2 and MMP-9 activities in the extracellular compartment increased from the 7th day of treatment and intracellular compartment from the 28th day. Importantly, TIMP-2/RECK presented differential expressions along the period analyzed. RECK expression was down-regulated from 14th day in opposition to an increase in TIMP-2. In parallel, our results showed a temporal regulation of two major signaling cascades during osteoblast differentiation, triggered by extracellular matrix remodeling: proliferation cascades in which PI3K p85 subunit and GSK-3β play a pivotal role; and differentiation cascades with participation of Ras, Rho, Rac-1, PKCα/β and TIMP-2. Also, we have evaluated some PTKs during osteoblast differentiation involved with cytoskeleton rearrangement, paxillin was active, but FAK remained unchanged. Most intriguingly, Src phosphorylation (in both Tyrosine residues) was tightly modulated. Thus, Y_{416}Src (activation site) was dephosphorylated while it regulatory site was phosphorylated (Y_{527}Src) during these events. Taken together, our results show new mechanisms of signal transduction triggered during osteoblast differentiation, at least triggered by ECM remodeling, a process rigorously mediated by MMPs and its tissue inhibitors (e.g. TIMP-2 and RECK). Of note, we reinforce that Src inhibition (by dephosphorylation of Tyr416 and phosphorylation of Tyr527) is required for osteoblast differentiation.

Keywords: Osteoblast differentiation; RECK; TIMP-2, MMP, PTKs, SHP2.

Introduction

Bone is a dynamic, vascular and living tissue under constant formation, resorption and remodelling throughout the entire life and being affected by some distinct physiological or pathological conditions such as aging or injuries. Among the bone cells, osteoblasts play a key role in bone formation by secreting and assembling bone matrix components. The osteoblast is a cell of mesenchimal origin that, once terminally differentiated, produces most of the proteins present in the ECM bone and control the mineralization of this ECM. It is likely that differentiation through the osteoblast lineage involves osteoblastic-specific signalling that still needs to be clarified.

ECM is required for various cellular functions including adhesion, migration, proliferation, and differentiation. The degradation of ECM is catalysed by Matrix Metalloproteinases (MMPs), which play a pivotal role in the balance of matrix remodeling (Vu and Werb, 2000; Hannas *et al.*, 2007; Ra and Parks, 2007). In bone, osteoblasts are considered to be the main source of MMP production (Hou *et al.*, 2004) indicating that these cells not only participate in the bone formation process, but also can directly stimulate bone resorption (Heath *et al.*, 1984; Takayanage *et al.*, 2002; Menezes *et al.*, 2006). Moreover, there is an increase in evidences that MMPs are part of a coupling mechanism between osteoblast and osteoclasts (Chambers, 1985; Everts *et al.*, 2002; Accorsi-Mendonça *et al.*, 2005). The MMP activities are tightly regulated by specific tissue inhibitors of metalloproteinases (TIMPs), which can also be produced by osteoblasts (Hatori *et al.*, 2004). MMPs and TIMPs are known to be involved in various physiological processes both in normal and pathological bone remodelling conditions, such as osteoporosis and rheumatoid arthritis (Nagasse and Woessner, 1999; Geofroy *et al.*, 2004). Other class of proteins has been found to inhibit MMPs activity, *Re*version-inducing-

*c*ysteine-rich protein with *K*azal Motifs (*RECK*), which negatively regulates at least three different MMPs, namely: MMP-2, MMP-9, and MT1-MMP (Takahashi *et al.*, 1998; Oh *et al.*, 2001; Sassahara *et al.*, 2002; Clark *et al.*, 2007). ECM remodeling will affect the focal adhesion, which is a specialized region of the plasma membrane. Some of the constituents of focal adhesions participate in the structural link between membrane receptors and the actin cytoskeleton, while others are signalling molecules, including different protein kinases, phosphatases and various adapter proteins (Petit and Thiery, 2000).

Mizutami and collaborators (2001) showed that MMPs-2 and -9 participate during osteoblast differentiation and the changes in their profiles could be attributed to the maturation of collagenous ECM induced by ascorbate. However, a detailed molecular analysis of ECM remodelling in osteoblast differentiation has not been addressed. Very recently, we have demonstrated that inactive and/or active forms of MMP-2, -9, and RECK are differentially expressed on osteogenic cells (osteoblast-like cells) during alveolar bone regeneration in rats (Accorsi-Mendonça *et al.*, 2008). Once determined *in vivo*, our objective here was to examine if MMPs and its inhibitors were responsible for ECM remodeling in osteoblasts cultured *in vitro*, as well as to identify the key mediators in signal transduction mechanisms triggered along osteoblast differentiation. In this way, this work shows that ECM remodeling in MC3T3 cells is modulated by RECK and TIMP-2, as observed *in vivo*. We also observed that the inhibition of Src, by dephosphorylation of Tyr416 and phosphorylation of Tyr527, is an important physiological mechanism to stimulate the osteoblast differentiation and consequently, bone formation.

Materials and Methods

Reagents. β-Glycerophosphate, ascorbate and *p*-nitrophenylphosphate (pNPP) were purchased from Sigma. *Antibodies:* Osteocalcin (sc-18322), caspase-8 (sc-6133), RECK (sc-9689), TIMP-2 (sc-6835), MMP-9 (sc-6841), p21 $^{(WAF1/CIP1)}$ (sc-397), c-myc, cyclinB1 (sc-595) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), pan-actin (#4968), GADPH (#2118), SHP2 (#3751), PTEN (#9552), pRb (#9308), pcdc-2 (#9111), Src (#2109), Non-p-Src (#2107), pSrc (#2105), Non-p-Src (#2102), pSrc (#2101), pPaxillin (#2541), pFAK (#3284), pCofilin (#3311), phosphoHistone-3 (#9701), pc-Raf (#9427), pMEK (#9121), pERK MAP Kinase (p42/p44) (#9101), pPI3Kp85 (#3821), pPDK1 (#3061), Akt (#9272), pAkt (#9275), pGSK-3 (#9336), PKCα/β (#9371), panPKC (#9379), pPKA (#4781), Rac-1 (#2461), anti-mouse (#7076), anti-rabbit (#7074) and were purchased from Cell Signaling Technology (Boston, MA, USA) and CyclinD (C7464) was obtained from Sigma Co. Rho (A,B,C) and phosphoRas were purchased from Upstate. Anti-goat was obtained from DAKO. GADPH, PTPα, SHP2 were purchased from Cell Signaling Technology (Beverly, MA). Antibody against phospho-tyrosine (α-pY) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against LMWPTP was from Abcam.

Cell Culture. MC3T3-E1 (subclone 4), a pre-osteoblast cell line from calvaria of mice, was obtained from ATCC (Manassas, USA) and grown at 37°C in α-MEM medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin under a humidified 5% CO_2 atmosphere. For differentiation, MC3T3-E1 cells were growth at confluence and after these cells were treated with osteogenic medium [β-

glycerophosphate (10mM) + ascorbate (50 μg/mL)]. The medium was changed every 3-4 days.

Determination of Alkaline Phosphatase activity. The MC3T3-E1 cells were rinsed with ice-cold PBS and incubated for 30 minutes at room temperature with ALP assay buffer (100 mM of Tris-HCl, pH 9.0, 1mM of $MgCl_2$) containing 1% Triton X-100. The cell extracts were removed from dishes, centrifuged, and used for the enzyme assay. The ALP activity was determined using 5mM of *p*NPP as a substrate as described (Hitomi *et al.*, 1992) and expressed as nanomoles of products formed per minute per miligrams of protein. Protein concentrations were determined by Lowry method (Hartree, 1972).

Alkaline Phosphatase Staining - The MC3T3-E1 cells ($4x10^4$ cells/mL) were plated in 24-wells dish plate and treated with osteogenic medium for 21-days and fixed in ice cool acetone for 5 min, allow the cells air dry for 5 min. After, the cells placed into substrate working solution [Naphthol AS-MX phosphate/DMF solution: Distilled water:Tris buffer, pH 8.74 (0.01:1:1) and fast red violet LB salt was added freshly before use] at 37ºC for 45 min. After, the cells were rinsed in TBS (3x).

Gelatin substrate zymography. The proteolytic activity of MMP-2 and MMP-9 was assayed by gelatin zymography as described previously (de Souza Queiroz *et al.*, 2007). Cells were cultured for various periods (as described by Mizutami *et al.*, 2001 for zymography) and the culture medium was collected and stored at -80°C in the presence of 1 mM PMSF (phenyl-methyl sulphonyl fluoride-serine-protease enzyme inhibitor). Samples were diluted in non-reducing buffer (0.1 M Tris-HCI, pH 6.8, 20% glycerol, 1% SDS and

0.001% bromophenol blue). The protein were resolved by SDS-PAGE (10%) and 4% gelatin. Protein re-naturation was performed in 2% Triton X-100 for 1 h followed by incubation with 50 mM Tris-HCl and 10mM $CaCl_2$ (pH 7.4) at 37^{o}C for 18 h. Gels were stained with 0.5% Comassie Blue G 250 for 30 minutes.

Immunoblotting assay (WB). Protein extracts from MC3T3-E1 cells were obtained using lyses buffer (50mM Tris [tris(hydroxymethyl)aminomethane]–HCl [pH 7.4], 1% Tween 20, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EGTA (ethylene glycol tetraacetic acid), 1mM *O*-Vanadate, 1mM NaF, and protease inhibitors [1μg/mL aprotinin, 10μg/mL leupeptin, and 1 mM 4-(2-amino-ethyl)-benzolsulfonyl-fluorid-hydrochloride]). An equal volume of 2x sodium dodecyl sulfate (SDS) gel loading buffer (100mM Tris-HCl [pH 6.8], 200mM dithiothreitol [DTT], 4% SDS, 0.1% bromophenol blue, and 20% glycerol) was added to samples and boiled for 5 minutes. Proteins extracts were resolved by SDS-PAGE (10 or 12%) and transferred to PVDF membranes. Detection was performed by using ECL.

Redox status analysis - MC3T3-E1 cells were cultured for 28 days under differentiation conditions and after that the cells were washed with cold PBS and collected in homogenization buffer (HB) (20 mM Tris, 1 mM DTT, 2 mM ATP and 5 mM $MgCl_2$, pH 7.2), and centrifuged at 10,000 rpm for 15 min at 4°C (Yano and Marcondes, 2005). Aliquots of homogenate supernatants were analyzed for glutathione-S-transferase (GST) activity based on the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione and the activity was expressed in nanomoles per microgram of protein per minute, using an extinction coefficient of 9.6, as described by Habig *et al.* (1974). The lipid peroxidation product malondialdehyde (MDA) was determined using MPO (*N*-methyl-2-phenylindole)

as the substrate. The resulting absorbance was measured at 590 nm and the results were expressed in nanomoles per milligram protein (Gomes-Marcondes and Tisdale, 2002). The protein content was measured by the method of Lowry.

Statistical analysis. All experiments were performed in triplicate and the results shown in the graphs represent the means and standard errors. Data from each assay were analyzed statistically by ANOVA. Multiple comparisons among group mean differences were checked with Tukey post hoc test. Differences were considered significant when the $p < 0.05$.

Results

Cell cycle progression arrest during pre-osteoblasts differentiation

Generally, eukaryotic cellular differentiation entails the coordination of cell cycle arrest and tissue-specific gene expression. Our results showed that $p21^{(WAF1/CIP1)}$ and *c-myc* were expressed along the pre-osteoblast differentiationon period, but decreased in the latter periods (21^{st} and 28^{th} day) - (**Figure 1A**). Cyclin B1 was present in the earlier periods (1^{st}-, 7^{th}-, and 14^{th}-day), with the largest expression in the 1^{st}-day. Retinoblastoma (Rb) and cdc2 were expressively down-regulated from the 14^{th} day. Also, cdk4 (cyclin-dependent kinase 4) expression decreased during this process. Intriguingly, the cyclin D1 was up-expressed during osteoblast differentiation with the highest expression at 14^{th} and 21^{st} day. The cell cycle arrest observed is in accordance with an increase of both osteocalcin expression and alkaline phosphatase activity (**Figure 1B**).

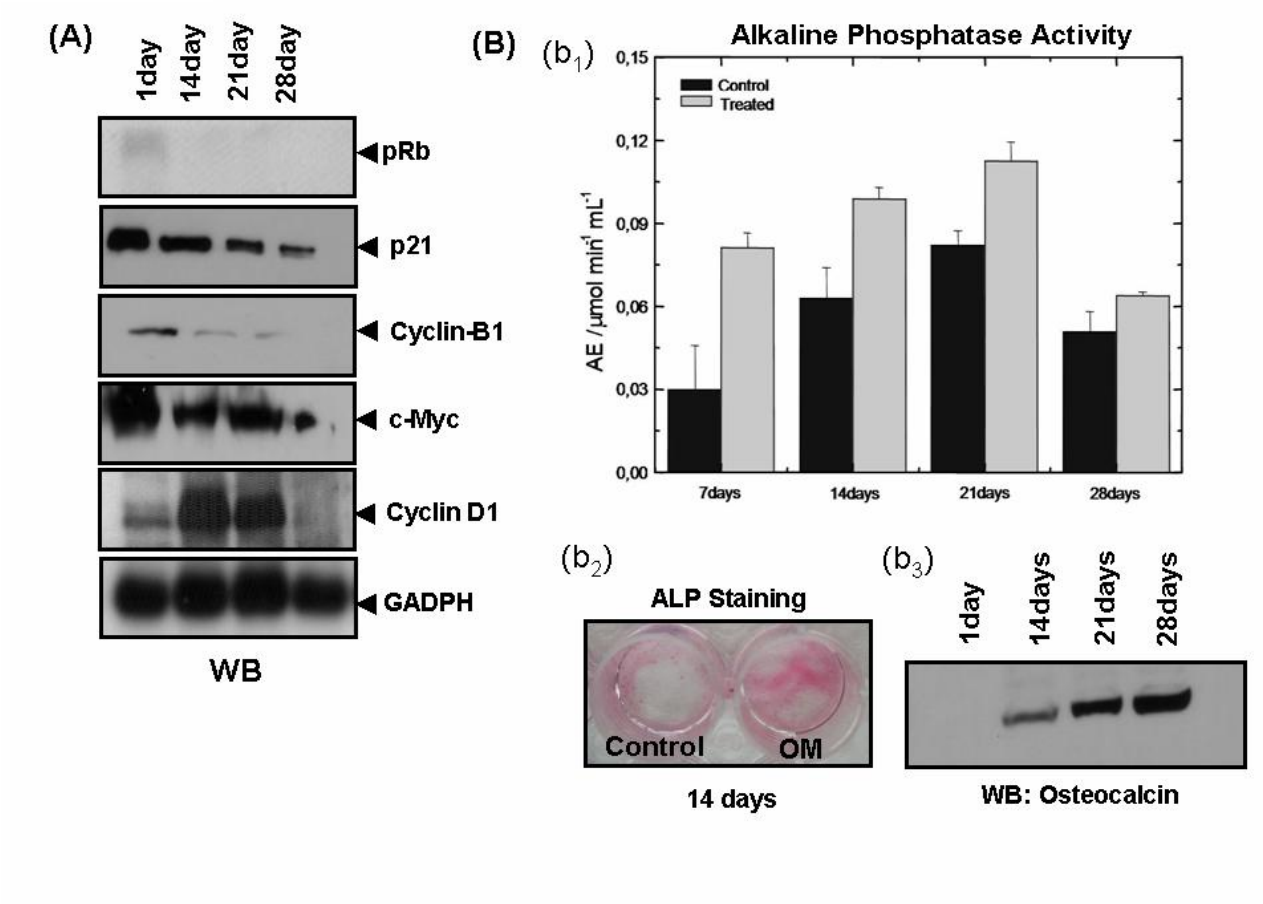

Figure 1. Cell cycle progression arrest during MC3T3-E1 cells differentiation. The expression of proteins involved in the cell cycle progression regulation was analyzed by WB, in the periods indicated **(A).** We evaluated pRb, cyclinD1, p21, cyclinB1 and c-Myc**. (B)** Osteocalcin and Alkaline Phosphatase (ALP) were used as biomarkers of osteoblast differentiation. (*b1* and *b2*; ALP) (*b3;* osteocalcin).

Rac1, Rho, Ras and Protein Kinase C are important mediators of osteoblast differentiation

Taking into consideration the importance of the cell survival during the differentiation process; we have checked a set of protein kinases and G proteins involved in the signal transduction responsible for the control of cell proliferation and survival. **Figure 2A** clearly shows that after 14th day the proliferation process was not the dominant cellular response, since all kinases which activating cell proliferation remained unchanged: ERK pathway [(Raf, MEK1/2 and ERK], PI3K and GSK. On the other hand, Rac1, Rho and PKC were expressively activated at the late step of the differentiation process (**Figure 2A**). Altogether, our results show that up to 14th day, the predominant signal transduction cascade was dependent on MAPK ERK **(Figure 2B)**, leading to cell proliferation. Afterwards, the dominant response was the cell differentiation, which was dependent on Rac1 and PKC activation.

ECM remodeling is modulated by RECK and TIMP-2 along the osteoblast differentiation process

The osteoblast differentiation induced by ascorbic acid and β-glycerophosphate has been well documented in the literature. However, the connection between the matrix remodeling and changing in the cellular signaling pathways is still unclear.

Firstly, we followed the expression of RECK and TIMP-2 during the pre-osteoblast differentiation. We found out that the expression of RECK was down-regulated from 14th day while TIMP-2 was up-regulated (**Figure 3A**). Apparently, TIMP-2 plays an important role during the later step of these cells maturation, since the differentiation markers, osteocalcin and alkaline phosphatase were also significantly affected from the the same

period (**Figure 1B**). In addition, our results showed that the activity of MMP-2 was higher than MMP-9. However, both enzymatic activities have increased along the process (**Figure 3B**).

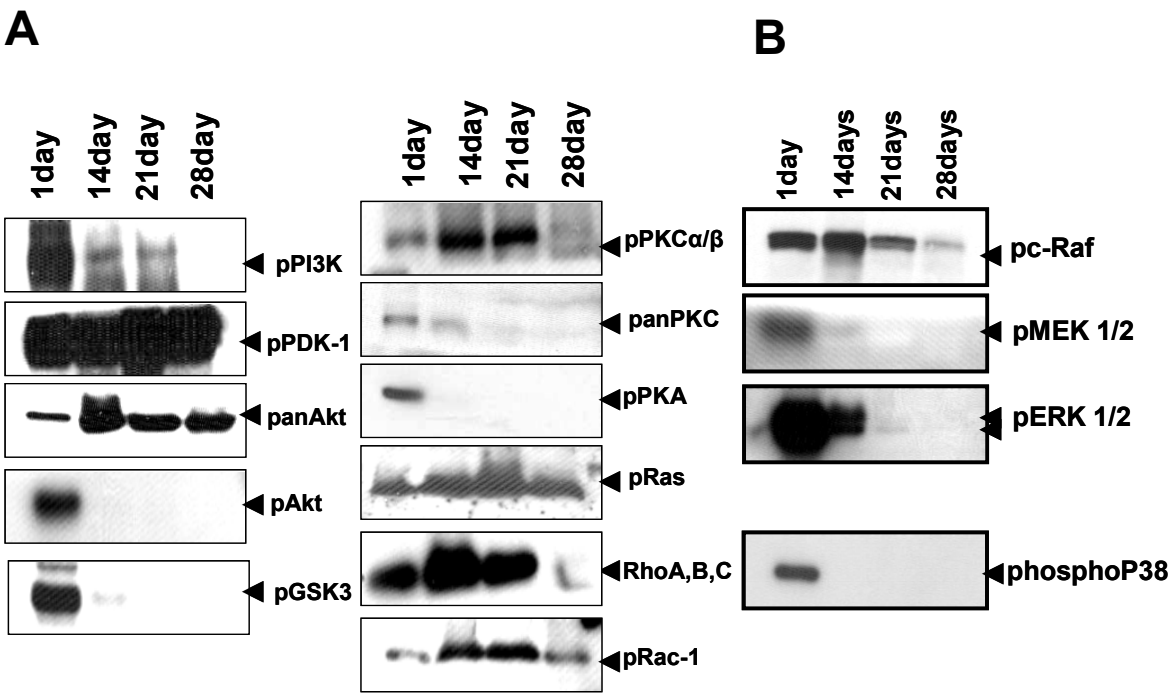

Figure 2. Key signaling proteins during osteoblast differentiation. (A) Panel shows the involvement of some pivotal proteins during this process, such as Akt (total and phosphorylated), PI3Kp85, PDK1, pGSK3, pPKCα/β, panPKC, pPKA, pRas, Rho (ABC), pRac1. The subunit p85 from PI3K is the regulatory subunit. Specifically, PKC (isoforms α/β) was clearly involved along osteoblast differentiation. Also, Rho and Rac-1 presented higher expression pattern in the same period where osteoblast differentiation was highest (14^{th} and 21^{st} days). **(B)** Panel shows that Erk pathway is down-regulated during osteoblasts differentiation. We analyzed several signaling pathways along to osteoblast differentiation. In this painel, we are showing that proteins involved to ERK pathway were down-regulated. Thus, we checked pc-Raf, pERK (p42/p44) and phosphoMEK1/2 by immunoblotting. In this set, we showed also that p38 MAPK phosphorylation was decreased.

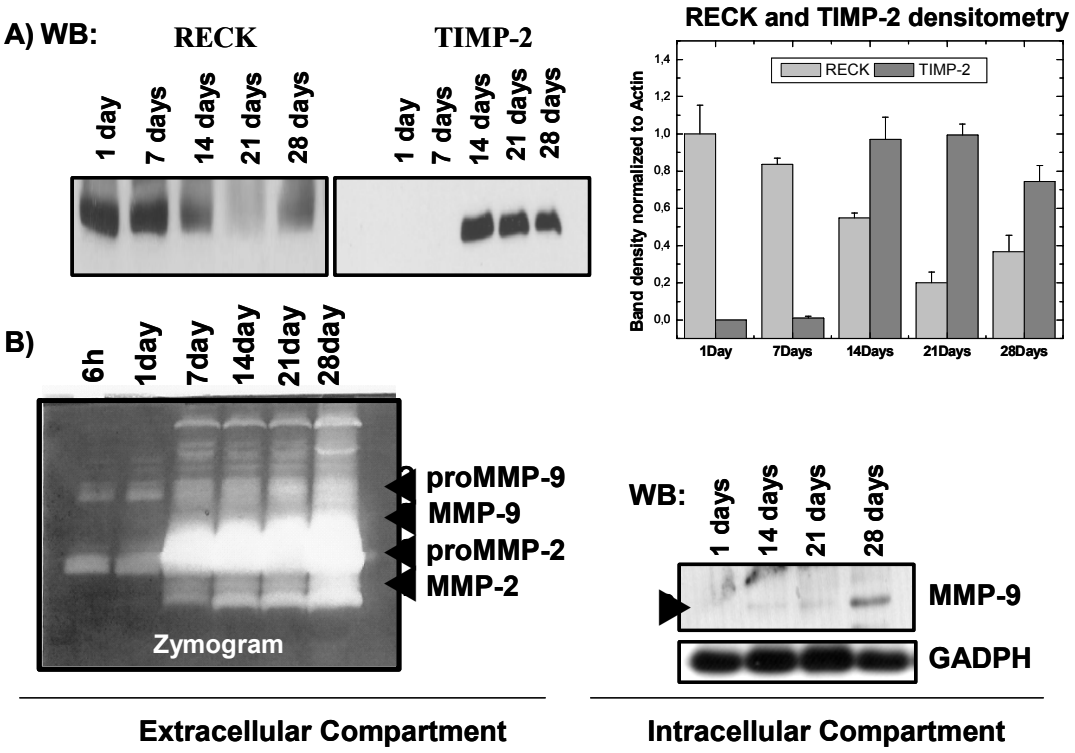

Figure 3. RECK and TIMP-2 are differentially expressed during osteoblastic differentiation. A) MC3T3-E1 cells were cultured in the presence of osteogenic medium and the RECK and TIMP-2 expression was analyzed by immunoblotting. **B)** Conditioned medium from MC3T3-E1 was analyzed by gelatin-substrate zymography (MMPs in the extracellular compartment). MMPs activities (in the intracellular compartment) were evaluated by western blotting as described in Material and Methods.

Differential phosphorylation status of cytoskeleton and focal adhesion proteins during osteoblasts differentiation

The differentiation process caused an increase of phosphorylated paxillin (up to 21st), while FAK expression remained unchanged. To check if cytoskeleton (mainly actin fibers) was rearranged, we analyzed the phosphorylation *status* of cofilin. We verified a decrease of phosphorylated form of cofilin (**Figure 4**).

Phosphorylation "status" in both Tyrosine residues of Src determinates its activity during osteoblasts differentiation

Tyrosine phosphorylation status is generally accepted as a critical regulator of a multitude of cell biological processes including cell proliferation, migration, differentiation, and survival (Ferreira *et al*, 2006). We demonstrated that Src kinase remained inactive during the differentiation of osteoblasts, once its activatior residue (Y_{416}Src) was dephosphorylated and its activatory residue (Y_{527}Src) remained phosphorylated **(Figure 5)**.

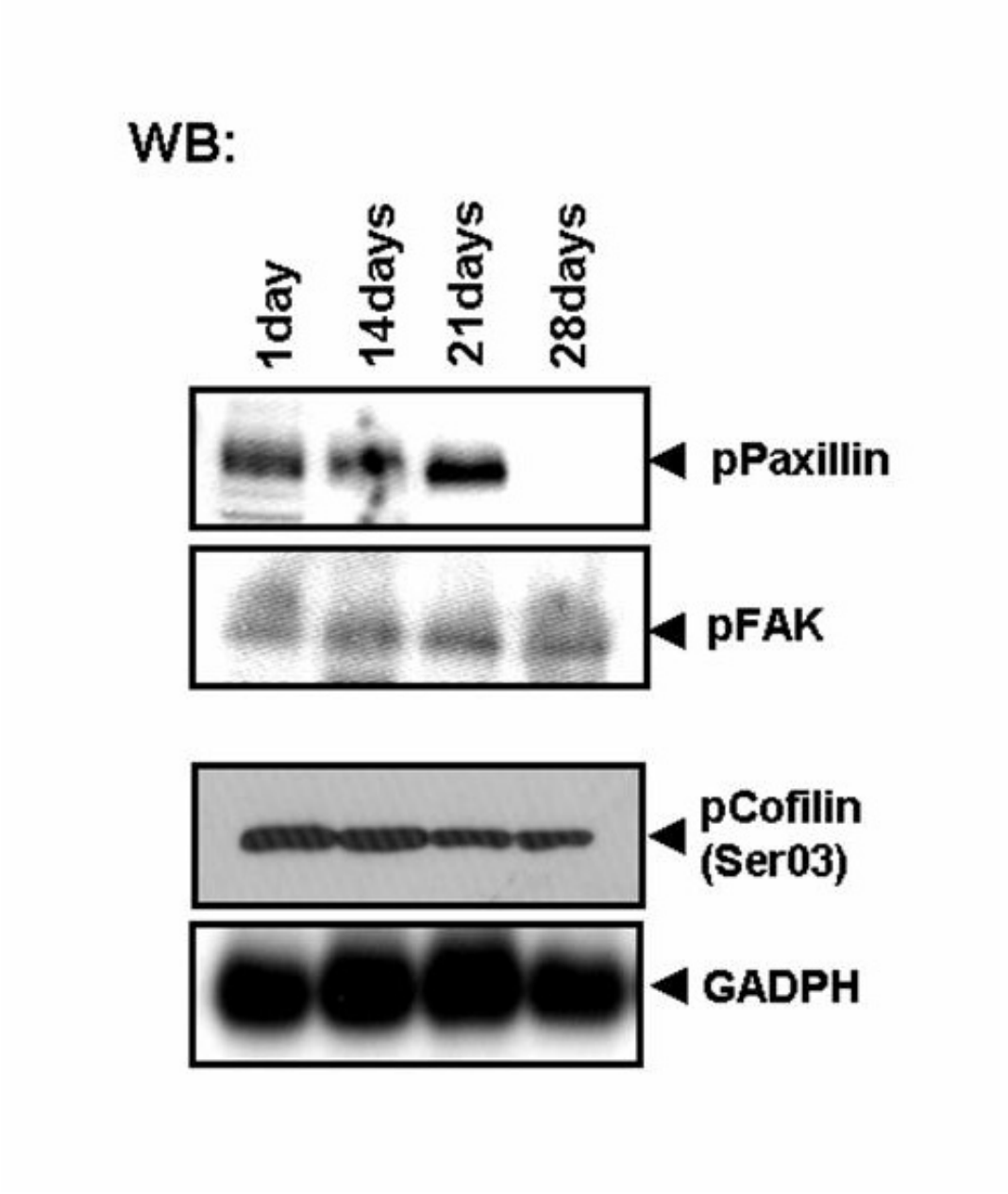

Figure 4. Paxillin, FAK and pCofilin are modulated during osteoblast differentiation. This section shows that phosphorylation Status of PTKs (FAK and Paxillin) and Cofilin is changed during the differentiation process. The Cofilin phosphorylation was determined at Ser03. The proteins were analyzed by immunoblotting, as described in materials and methods. WB: Western Blotting.

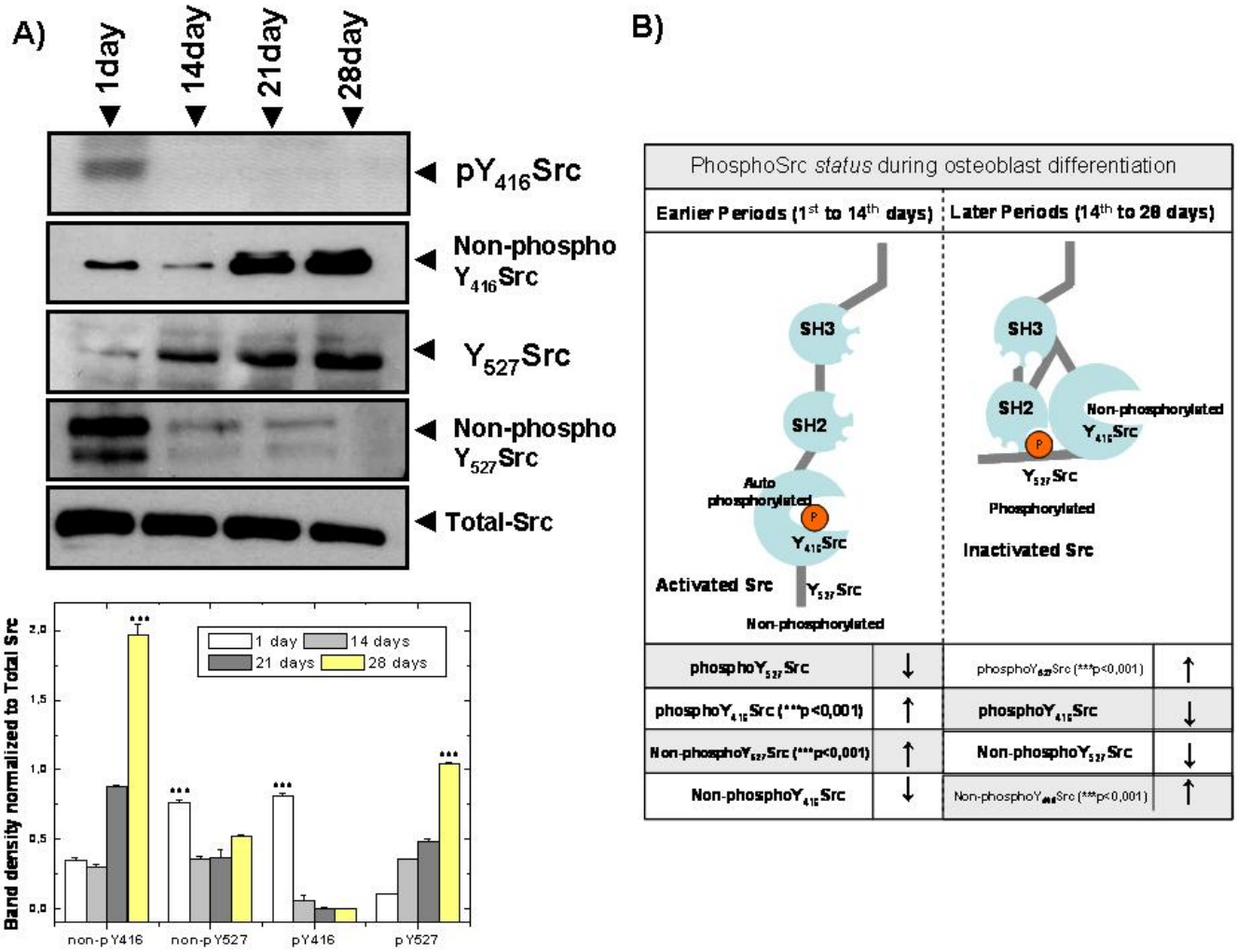

Figure 5. Phosphorylation profile of Src determinates its activity during osteoblast differentiation. Src phospharylation status was evaluated by western blotting **(A).** ***p<0,001. The panel **(B)** brings out a scheme summarizing Src involvement during osteoblast differentiation.

Evaluation of protein tyrosine phosphatase activity/expression during pre-osteoblast differentiation

Given the general importance of PTPs in the regulation of cellular proliferation and differentiation, it is likely that the action of specific members of the PTP superfamily is involved in the control of bone formation, and bone resorption, as well. Therefore, we evaluated some PTPs (LMWPTP, SHP2 and PTPα) along osteoblast differentiation and observed that these proteins are required in latter of differentiation (**Figure 6A**). Also, we showed that LMWPTP activity was changed during osteoblast differentiation (**Figure 6B**). In prove to determinate LMWPTP phosphorylation, we resolved LMWPTP immunoprecipited in SDS-PAGE and verified phosphoTyrosine (pY) (**Figure 6C**). Our results showed that Glutathione-S-transferase activity (nmol μg of protein^{-1} min^{-1}) was increased with 14 and 21 days of differentiation (**Figure 7**). The malondialdehyde content (nmol μg of protein−1) was not significantly affect, which is in agreement with the high activity displayed by GST. In fact, this balance between reducing and oxidizing environment create the ideal conditions for PTP action.

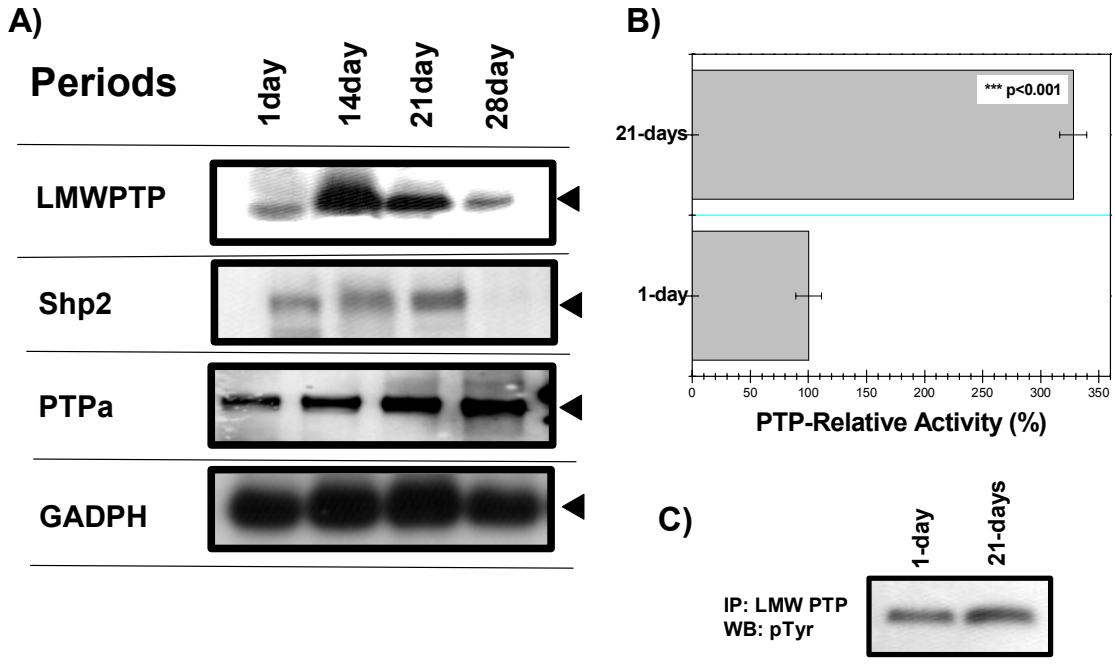

Figure 6. SHP-2, PTP-α and LMWPTP are strongly expressed during osteoblast differentiation. A) Cells were lysed along osteoblast differentiation periods (14d, 21d and 28d) as described in material and method and the protein collected for analysis. The proteins were resolved in SDS-PAGE and transferred to PVDF membranes. Incubation with a SHP-2, PTP-α and LMWPTP specific antibodies reveals significant difference between osteoblast differentiation groups analyzed. Ours results showed clearly that the PTPs were up-expressed along osteoblast differentiation, being PTP-α expression present in differentiated osteoblasts. Also, both of them (SHP-2 and LMWPTP) were highest expressed only in 14d and 21d. In this experiment pan-actin expression was monitored as a control for Western Blotting technique. Bars represent mean + SD (n=3). **B)** To confirm if redox status was negatively PTP activity we immunoprecited LMWPTP in distinct periods of osteoblast differentiation and checked it activity using pNPP as substrate. Unit was expressed in relative activity using 1 day as 100% (***p<0,001). In fact, our result brings out that PTP activity is negatively modulated by cellular redox status along osteoblast differentiation. **C)** After immunoprecipitation, we resolved LMWPTP in SDS-Gel and checked it phosphorylation using α-pY.

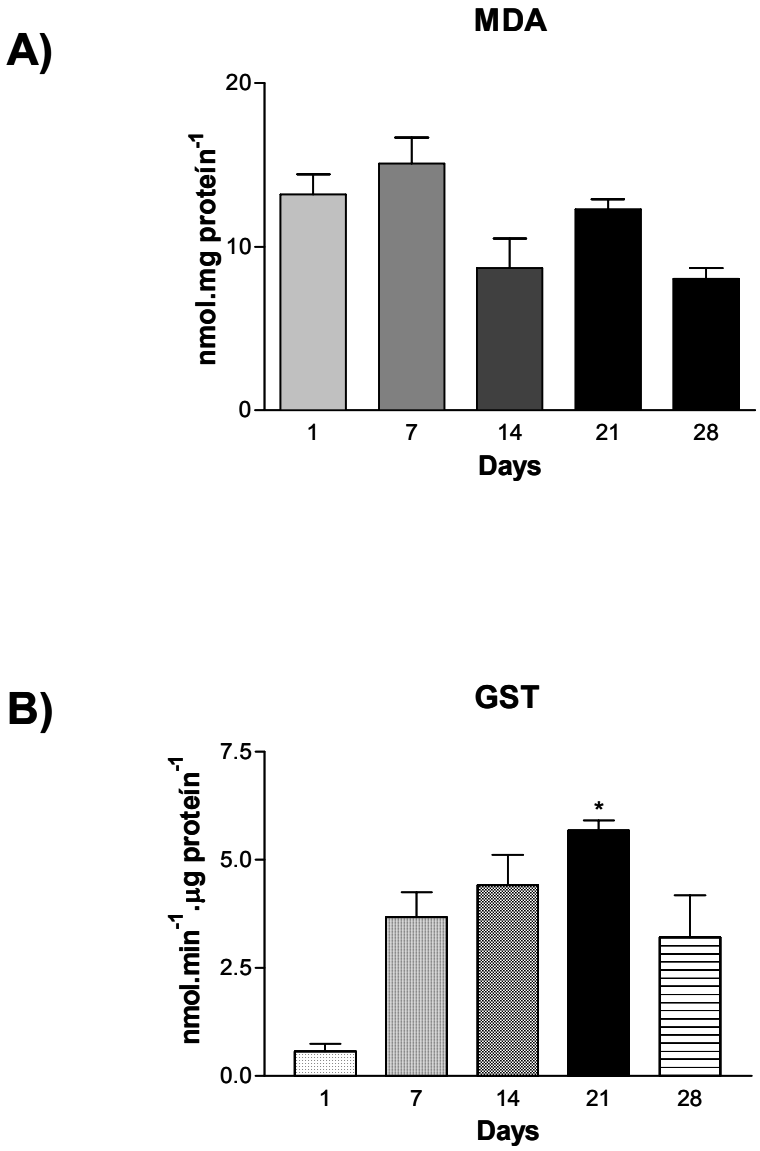

Figure 7. Status redox during osteoblast differentiation. Figure shows malondialdehyde (MDA) levels **(A)** and glutathione-S-transferase (GST) activity (**B**) during osteoblasts differentiation. MC3T3-E1 cells were cultured in αMEM medium and after the specific period cells were lysed using HB buffer (as described in Materials and Methods). Here, our results showed that the status redox along osteoblast differentiation was tightly regulated. The columns are the mean ± SE of triplicate experiments. (*) indicate significant ($P < 0.05$) differences.

Discussion

The osteogenesis mechanism represents an interesting research field both for basic and applied investigations. *In vitro*, MC3T3-E1 cells (pre-osteoblasts from calvaria of mouse) have been accepted as an experimental model for studying osteoblast differentiation (Franceschi and Iyer, 1992). Therefore, in this study, we evaluated in details, some signal transduction pathways, demonstrating that the expression of proteins, as well as their activation by phosphorylation is modulated during osteoblast differentiation.

According to our results we can divide the osteoblasts differentiation process into two major signal transduction occurrences which was temporal dependent: cell proliferation (1^{st} to 14^{th}) and cell differentiation (14^{th} to 21^{th}). At the early step, we observed activation of proliferation mediators including Ras, Raf, ERK, PI3K and GSK3. Ras protein plays an important role in the control of several pathways, including the control of RECK expression and also can affected ERK pathway.

Chang and collaborators (2007) showed that Ras suppressed RECK expression via inhibition of its transcription. This was also apparently true during osteoblast differentiation, at least up to 21 days.

In general, eukaryotic mammalian cellular differentiation entails the coordination of cell cycle arrest and tissue-specific gene expression that is a consequence of a coordinated sequence of biochemical events associated with morphological changes. According to our results $p21^{(WAF1/CIP1)}$, *c-myc*, cyclin B1 and cdk4 were downregulated along the osteoblast differentiation process. Bellosta and collaborators (2003) also showed that $p21^{(WAF1/CIP1)}$ was downregulated during this process. In contrast, we found that cyclin D1 was upregulated during MC3T3-E1 cells differentiation, which can be due to the inhibition of

GSK3. Lee and colleagues (2007) have reported that cyclin D1 expression can be stimulated by osteocalcin.

Despite intensive investigation, the physiological role of the MAPKs in osteoblasts remains controversia. Some studies have indicated a stimulatory role in osteoblast differentiation and others inhibitory (Schindeler and Little *et al.*, 2006). For example, studies using MC3T3-E1 cells suggest that activation of p38 is critical for ALP expression (Rey *et al.*, 2007). Other study using MC3T3-E1 and BMSC indicates that p38 MAPK, but not ERKs, is necessary for osteoblast differentiation (Hu *et al.*, 2003). Our results and from others demonstrated that both ERK (p42/44) and p38 MAP Kinase are important in the early stages of osteoblast differentiation.

Interestingly, our findings revealed a differential expression of the physiological inhibitors of MMPs (RECK and TIMP-2). RECK is required during the initial steps of osteoblasts differentiation (proliferative phase), while TIMP-2 is involved in the later phase (cellular maturation phase). However, it is important to mention that the activities of MMPs-2 and -9 in the extracellular compartment have increased, suggesting their role in the remodeling of osteoid matrix, as described before (Fornoni *et al.*, 2001). Here, we showed that activity of MMP-2 was higher than MMP-9 and both activities have increased along the process. These findings are in accordance to those ones reported by Mizutani and collaborators (2001). Besides modulating MMP activity, it is now widely accepted that TIMPs have other important effects on cell growth, apoptosis, and differentiation (Baker *et al.*, 2002; Jiang *et al.*, 2002). TIMP-2 has been reported to stimulate quiescent cell proliferation, function as a growth factor, and inhibits FGF-stimulated endothelial cell growth [Baker *et al.*, 2002; Jiang *et al.*, 2002]. Our results also indicate that both inhibitors of MMPs might exert an intracellular function such as cytoskeleton reorganization during

osteoblast differentiation. In addition, TIMP-2 can modulate gene expression (i.e. RECK) in an orthovanadate-sensitive manner through binding to α3β1 integrin (Visse and Nagase, 2003), suggesting that TIMP-2 may activate signal transduction pathways important in cell growth regulation, resulting in G1 growth arrest.

Another aspect addressed in this paper was the involviment of proteins related to cytoskeleton and focal adhesion rearrangement. At the later phase of differentiation we detected activation of Ras, Rac1, Rho (A, B, C) and PKC. These results are in agreement with those demonstrated by Lampasso and co-authors (2006) who found that different isoforms of PKC were important components in the signal transduction pathways triggered by estrogen receptor on osteoblasts (Migliaccio *et al.*, 1998). In addition, Huang *et al.*, (2007) have published an elegant review where these authors provided detailed information about signal transduction pathways affected along osteoblast differentiation and they showed that PKC is an upstream mediator of Runx2 activation. Rho, a member of the Ras super-family of GTP-binding proteins, regulates focal adhesion assembly and reassembly. Consequently, other proteins presented in focal adhesions components, such as tensin, paxillin, p130Cas, and focal adhesion kinase, become tyrosine-phosphorylated during integrin and/or growth factor stimulation. Specifically, Paxillin was phosphorylated in the same periods that we found active Rho. In this way, we conclude that these proteins were interacting with cytoskeleton molecules for coordenating cell adhesion as well as morphological changes, required during osteoblast differentiation.

Importantly, our data revealed that Src was inhibited during osteoblast differentiation. Marzia *et al.* (2000) showed that Src-deficient mice ($Src^{-/-}$) stimulates osteoblast differentiation and bone formation. The same observation was made by Soriano *et al.*, 1991, who showed that a disruption of the c-src gene in mice has been demonstrated to

cause osteopetrosis. The analysis of the skeletal phenotype in older Src-/- mice has indicated that bone mass continues to increase with age, suggesting a continued imbalance between bone resorption and formation (Amling *et al.* 2000).

The first PTP that has been demonstrated to play a physiological role in bone remodeling is SHP1 (Umeda *et al.*, 1999; Aoki *et al.*, 1999). SHP1-deficient mice have been described to display an osteopenia caused by increased bone resorption. Thus, SHP1 was identified as a negative regulator of osteoclastogenesis, most likely by affecting Rankl-induced signaling pathways (Zhang et al, 2003). Another PTP involved in bone resorption is PTPe, since the analysis of a PTPe-deficient mouse model revealed an increased trabecular bone mass caused by cell-autonomous defects of osteoclast function (Chiusaroli *et al.*, 2004). Moreover, PTPe has also been suggested to be a target of bisphosphonate action in osteoclasts, based on the finding that its tyrosine phosphatase activity is inhibited by alendronate (Schmidt *et al.*, 1996). Taken together, although the molecular mechanisms underlying the phenotypes in SHP1-deficient and PTPe-deficienct mice are still being investigated, it is obvious that the activity of specific PTPs is also relevant to bone physiology and pathology.

Schinke *et al.* (2008) provide the first evidence for a previously unrecognized role of RPTPzeta in bone formation and expand the knowledge about the genetic mechanisms underlying osteoblast maturation.

Additionally, our results suggest a direct relation between PTPs expression and activity and oxidation levels. Relatively little is known about the regulation of PTPs. Some reports have shown that reversible oxidation of the absolutely conserved catalytic site cysteine is emerging as an important regulatory mechanism (den Hertog, Groen and van der Wijk, 2004, Groen *et al.*, 2005). The catalytic cysteines are susceptible to oxidation because

of their low pKa (Zhang and Dixon, 1993; Peters, Frimurer and Olsen, 1998). Our results showed that Glutathione-S-transferase activity was increased with 14 and 21 days of differentiation. Despite the increase of GST we did not observe and increase o lypid peroxidation. This finding indicates that during the osteoblast differentiation process the balance between reducing and oxidizing environment create an ideal condition for PTP action.

In summary, our results showed a temporal regulation of two major signaling cascades triggered by extracellular matrix remodeling in conjugation with activation of integrins: proliferation cascades in which ERK MAPK, PI3K, GSK3, Src kinase and RECK play pivotal roles; differentiation cascades with participation of Ras, Rho, Rac1, PKC, MMP-2 and TIMP-2. Besides, we also showed that the higher activity of GST along the osteoblasts differentiaiton was crucial for preventing PTP oxidation.

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References

Accorsi-Mendonça T, Paiva KB, Zambuzzi WF, Cestari TM, Lara VS, Sogayar MC, Taga R, Granjeiro JM (2008). Expression of matrix metalloproteinases-2 and -9 and RECK during alveolar bone regeneration in rat. J Mol Histol. 39(2):201-8.

Accorsi-Mendonça T, Zambuzzi WF, da Silva Paiva KB, Lauris JRP, Cestari TM, Taga R, Granjeiro JM. (2005). Expression of metalloproteinase 2 in the cell response to porous demineralized bovine bone matrix. J Mol Histol 36:311-6.

Amling M, Neff L, Priemel M, Priemel AF, Schilling AF, RuegerJ.M and Baron R. (2000). Progressive increase in bone mass and development of odontomas in aging osteopetrotic c-Src–deficient mice. Bone. 27:603-10.

Aoki K, Didomenico E, Sims NA, Mukhopadhyay K, Neff L, Houghton A, Amling M, Levy JB, Horne WC, Baron R. (1999). The tyrosine phosphatase SHP-1 is a negative regulator of osteoclastogenesis and osteoclast resorbing activity: increased resorption and osteopenia in me(v)/me(v) mutant mice. Bone 25:261-67.

Baker AH, Edwards DR, Murphy G. (2002). Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J Cell Sci. 115:3719–27.

Bellosta P, Masramon L, Mansukhani A, Basilico C. (2003). p21 (WAF1/CIP1) acts as a brake in osteoblast differentiation. J Bone Miner Res. 18:818-26.

Chambers TJ. (1985). The pathobiology of the osteoclast. J Clin Pathol. 38:241-52.

Chang HC, Cho CY, and Hung WC. (2007). Downregulation of RECK by promoter methylation correlates with lymph node metastasis in non-small cell lung cancer. Cancer Sc. 98:169-73.

Chiusaroli R, Knobler H, Luxenburg C, Sanjay A, Granot-Attas S, and Tiran Z. (2004). Tyrosine phosphatase epsilon is a positive regulator of osteoclast function *in vitro* and *in vivo*. Mol Biol Cell. 15:234-44.

Clark JC, Thomas DM, Choong PF, and Dass CR (2007). RECK-a newly discovered inhibitor of metastasis with prognostic significance in multiple forms of cancer. Cancer Metastasis Rev. 26:675-683.

de Souza Queiroz KC, Zambuzzi WF, Santos de Souza AC, da Silva RA, Machado D, Justo GZ, Carvalho HF, Peppelenbosch MP, and Ferreira CV. (2007). A possible anti-proliferative and anti-metastatic effect of irradiated riboflavin in solid tumours. Cancer Lett. 258:126-34.

den Hertog J, Groen A, and van der Wijk T. (2004). Redox regulation of protein-tyrosine phosphatases. Arch Biochem. Biophys. 434:11–15

Everts V, Delaissé JM, Korper W, Jansen DC, Tigchelaar-Gutter W, Saftig P, and Beertsen W. (2002). The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation. J Bone Miner Res 17:77–90.

Ferreira CV, Justo GZ, Souza AC, Queiroz KC, Zambuzzi WF, Aoyama H, and Peppelenbosch MP. (2006). Natural Compounds as a Source of Protein Tyrosine Phosphatase Inhibitors: Application to the Rational Design of Small-Molecule Derivatives. Biochimie 88:1859-73.

Fornoni A, Cornacchia F, Howard GA, Roos BA, Striker GE, and Striker L. J. (2001). Cyclosporin A affects extracellular matrix synthesis and degradation by mouse MC3T3-E1 osteoblasts *in vitro.* Nephrol Dial Transplant 16:500-5.

Franceschi RT, and Iyer BS. (1992). Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. J Bone Miner Res 7:235-46.

Geoffroy V, Marty-Morieux C, le Goupil N, Clement-Lacroix P, Terraz C, Frain M, Roux S, Rossert J, and de Vernejoul MC. (2004). *In vivo* inhibition of osteoblastic metalloproteinases leads to increased trabecular bone mass. J Bone Miner Res 19:811–822.

Gomes-Marcondes MC, and Tisdale MJ (2002). Induction of protein catabolism and the ubiquitin-proteasome pathway by mild oxidative stress, Cancer Lett. 180:69–74.

Groen A, Lemeer S, van der Wijk T, Overvoorde J, Heck AJ, Ostman A, Barford D, Slijper M, and den Hertog J. (2005). Differential oxidation of protein-tyrosine phosphatases. J Biol Chem. 280:10298-304.

Habig WH, Pabst MJ, and Jakoby WB. (1974). Glutathione S-transferases. The first step in mercapturic acid formation. J. Biol. Chem. 249: 7130–7139.

Hannas AR, Pereira JC, Granjeiro JM, and Tjaderhane L. (2007). The role of matrix metalloproteinases in the oral environment. Acta Odontol Scand. 65:1-13.

Hartree EF. (1972). Determination of proteins: a modification of Lowry method that give a linear photometric response. Anal Biochem 48:422-27.

Hatori K, Sasano Y, Takahashi I, Kamakura S, Kagayama M, Sasaki K. (2004). Osteoblasts and osteocytes express MMP2 and -8 and TIMP1, -2, and -3 along with extracellular matrix molecules during appositional bone formation. Anat Rec Discov Mol Cell Evol Biol. 277:262-71.

Heath JK, Atkinson SJ, Meikle MC, and Reynolds JJ. (1984). Mouse osteoblasts synthesize collagenase in response to bone resorbing agents. Biochim Biophys Acta. 802:151–4.

Hitomi K, Torii Y, and Tsukagoshi N. (1992). Increase in the activity of alkaline phosphatase by L-ascorbic acid 2- phosphate in a human osteoblast cell line, HuO-3N1J. Nutr. Sci. Vitaminol. 38:535-44.

Hou P, Troen T, Ovejero MC, Kirkegaard T, Andersen TL, Byrjalsen I, Ferreras M, Sato T, Shapiro SD, Foged NT, and Delaissé JM. (2004). Matrix metalloproteinase-12 (MMP-12) in osteoclasts: new lesson on the involvement of MMPs in bone resorption. Bone. 34:37–47.

Hu Y, Chan E, Wang SX, and Li B. (2003). Activation of p38 mitogen-activated protein kinase is required for osteoblast differentiation. Endocrinology. 144:2068–74.

Huang W, Yang S, Shao J, Li YP (2007). Signaling and transcriptional regulation in osteoblast commitment and differentiation. Front Biosci. 12:3068-92.

Jiang Y, Goldberg ID, and Shi YE. (2002). Complex roles of tissue inhibitors of metalloproteinases in cancer. Oncogene. 21:2245–52.

Lampasso JD, Chen W, Marzec N. (2006). The expression profile of PKC isoforms during MC3T3-E1 differentiation. Int J Mol Med 17:1125-31.

Lee NK, Sowa H, Hinoi E, Ferron M, Ahn JD, Confavreux C, Dacquin R, Mee PJ, McKee MD, Jung DY, Zhang Z, Kim JK, Mauvais-Jarvis F, Ducy P, Karsenty G. (2007). Endocrine regulation of energy metabolism by the skeleton. Cell. 130:456-69.

Marzia M, Sims NA, Voit S, Migliaccio S, Taranta A, Bernardini S, Faraggiana T, Yoneda T, Mundy GR, Boyce BF, Baron R, and Teti A. Decreased c-Src expression enhances osteoblast differentiation and bone formation. J Cell Biol. 151:311-20.

Menezes R, Bramante CM, da Silva Paiva KB, Letra A, Carneiro E, Zambuzzi WF, Granjeiro JM. (2006). Receptor activator NFkappaB-ligand and osteoprotegerin protein expression in human periapical cysts and granulomas. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 102:404-9.

Migliaccio S, Bernardini S, Wetsel WC, Korach KS, Faraggiana T, and Teti A (1998). Protein kinase C modulates estrogen receptors in differentiated osteoblastic cells *in vitro*. Steroids. 63:352-4.

Mizutani A, Sugiyama I, Kuno E, Matsunaga S, Tsukagoshi N. (2001). Expression of Matrix Metalloproteinases During Ascorbate-Induced Differentiation of Osteoblastic MC3T3-E1 Cells. J Bone Miner Res. 16:2043-9.

Nagase H, and Woessner JF. (1999). Matrix metalloproteinases. J Biol Chem. 274:21491–94.

Oh J, Takahashi R, Kondo S, Mizoguchi A, Adachi E, Sasahara RM, Nishimura S, Imamura Y, Kitayama H, Alexander DB, Ide C, Horan TP, Arakawa T, Yoshida H, Nishikawa S, Itoh Y, Seiki M, Itohara S, Takahashi C, and Noda M. (2001). The membrane-anchored MMP inhibitor RECK is a key regulator of extracelular matrix integrity and angiogenesis. Cell. 107:789-800.

Peters, GH, Frimurer, TM, and Olsen OH. (1998). Electrostatic evaluation of the signature motif (H/V)CX5R(S/T) in protein-tyrosine phosphatases. Biochemistry 37, 5383–5393.

Petit V, and Thiery JP. (2000). Focal adhesions: structure and dynamics. Biol Cell. 92:477-94.

Ra HJ, and Parks WC. Control of matrix metalloproteinase catalytic activity. Matrix Biol. 2007 26(8):587-96.

Rey A, Manen D, Rizzoli R, Ferrari SL, Caverzasio J. (2007). Evidences for a role of p38 MAP kinase in the stimulation of alkaline phosphatase and matrix mineralization induced by parathyroid hormone in osteoblastic cells. Bone. 41:59-67.

Sasahara RM, Brochado SM, Takahashi C, Oh J, Maria-Engler SS, Granjeiro JM, Noda M, Sogayar MC. (2002). Transcriptional control of the RECK metastasis/angiogenesis suppressor gene. Cancer Detect Prev. 26:435-43.

Schindeler A, and Little D. (2006). Ras-MAPK signaling in osteogenic differentiation: friend or foe? J Bone Miner Res. 21:1331–38.

Schinke T, Gebauer M, Schilling AF, Lamprianou S, Priemel M, Mueldner C, Neunaber C, Streichert T, Ignatius A, Harroch S, and Amling M. (2008). The protein tyrosine phosphatase Rptpzeta is expressed in differentiated osteoblasts and affects bone formation in mice.Bone. 42:524-34.

Schmidt A, Rutledge SJ, Endo N, Opas EE, Tanaka H, Wesolowski G, Leu CT, Huang Z, Ramachandaran C, Rodan SB, and Rodan GA. (1996). Protein-tyrosine phosphatase activity regulates osteoclast formation and function: inhibition by alendronate. Proc Natl Acad Sci U S A 93:3068-73.

Soriano P, Montgomery C, Geske ., and Bradley, A. (1991). Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell. 64:693-702.

Takahashi C, Sheng Z, Horan TP, Kitayama H, Maki M, Hitomi XX, Kitaura Y, Takai S, Sasahara RM, Horimoto A, Ikawa Y, Ratzkin BJ, Arakawa T, Noda M. (1998). Regulation of matrix metalloproteinase-9 and inhibition of tumor invasion by the membrane-anchored glycoprotein RECK. Proc Natl Acad Sci. 95:13221-26.

Takayanage H, Kim S, Matsuo K, Suzuki H, Sato K, Yokochi T, Oda H, Nakamura K, Ida N, Wagner EF, Taniguchi T. (2002). RANK-L maintains bone homeostasis through c-Fos-dependent induction of interferon-beta. Nature. 416:744-9.

Umeda S, Beamer WG, Takagi K, Naito M, Hayashi S, Yonemitsu H, Yi T, and Shultz LD. (1999). Deficiency of SHP-1 protein-tyrosine phosphatase activity results in heightened osteoclast function and decreased bone density. Am J Pathol 155:223-33.

Visse R, and Nagasse H. (2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res. 92:827-39.

Vu TH, and Werb Z. (2000). Matrix metalloproteinases, effectors of development and normal physiology. Genes Dev. 14:2123–33.

Yano CL, and Marcondes MC. (2005). Cadmium chloride-induced oxidative stress in skeletal muscle cells *in vitro*. Free Radic Biol Med. 39:1378-84.

Zhang Z, Jimi E, Bothwell AL. (2003). Receptor activator of NF-kappa B ligand stimulates recruitment of SHP-1 to the complex containing TNFR-associated factor 6 that regulates osteoclastogenesis. J Immunol. 171:3620-26.

Zhang, ZY, and Dixon, JE. (1993). Active site labeling of the Yersinia protein tyrosine phosphatase: the determination of the pKa of the active site cysteine and the function of the conserved histidine 402. Biochemistry 32, 9340–9345.

Capítulo 5

PP2A AND P38 MAPK AS PIVOTAL MOLECULES IN THE OSTEOBLASTS ADHESION PROCESS

PP2A AND P38 MAPK AS PIVOTAL MOLECULES IN THE OSTEOBLASTS ADHESION PROCESS

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Abstract

The signal transduction responsible for osteoblasts adhesion and spreading needs to be biochemically clarified and then might provide useful information with broad applications, such as improving oral biomaterials for bone implants. We evaluated phosphoprotein levels during osteoblast adhesion and spreading and also identified some physiological substrates of p38 MAPK and PP2A using specific inhibitors. We spotlighted the importance of the modulation of cofilin, PP2A and p38 MAPK for osteoblast adhesion and spreading, which was correlated with cytoskeleton remodeling. We found that PP2A modulated cofilin activity (by dephosphorylation) and consequently controlled its temporal action during osteoblast adhesion (2h) and spreading (48h). In addition, during adhesion p38 MAPK was an important mediator, since an inhibitor of this kinase caused an expressive decrease of osteoblast adhesion (MTT: 52% of control; crystal violet: 52,79 % of control). Also, we demonstrated that in the initial periods of osteoblast spreading, ATF-2 and MAPKAPK-2 were substrates for p38 MAPK, but not for ERK MAPK. Our findings suggest that the phosphorylation *status* of p38 MAPK, PP2A and cofilin can be useful biomarkers of the quality of osteoblast adhesion and spreading on different surfaces/substrates. This information might have broad applications in the fields of oral biomaterials and bone tissue engineering research.

Key Words: Osteoblasts; Adhesion; PP2A; p38 MAPK; Cytoskeleton.

Introduction

The capacity of osteoblast and other cell types to adhere and spread is a pivotal requirement for their proliferating rate. Recently, we have shown that platelet-rich plasma (PRP) influences human osteoblast growth *in vitro* (1). However, the signal transduction responsible for osteoblasts adhesion and spreading needs to be biochemically clarified and then might provide useful information with broad applications, such as improving biomaterials for bone implants. In this scenario, some proteins from extracellular matrix (ECM) (2), integrins (3,4), focal adhesion (5) and cytoskeleton components (6) might be investigated.

In the cell membrane, integrins function as cell-substrate adhesion molecules, acting as receptors for major ECM molecules including collagens and fibronectin. In addition to their adhesive functions, integrins mediate bi-directional signaling between the ECM and the cell. Upon activation of integrin molecules, cytoskeleton and signaling molecules are recruited into focal adhesion structures, promoting transient FAK (Focal Adhesion Kinase) phosphorylation. Lastly, mechanisms triggered by integrin are responsible for the cytoskeleton rearrangement executed by cofilin, which interact to actin. Cofilin plays pivotal roles in cytokinesis, endocytosis, embryonic development, stress response and tissue regeneration (7). In response to stimuli, cofilin promotes the regeneration of actin filaments by severing preexisting filaments (8). Taking into account that the influence of integrins on the cell signaling is mainly related to activation of protein phosphorylation, the duration of this stimulus/effect will be dependent on protein dephosphorylation.

The ubiquitous nature of protein phosphorylation/dephosphorylation underscores its key role in cell signaling metabolism, growth and differentiation. In fact, cells respond to

internal and external stimulus through integrated networks of intracellular signaling pathways in which phosphorylation or dephosphorylation reactions are governed by protein kinases (PKs) and protein phosphatases (PPs), respectively (9). Therefore, PPs might have essential participation on osteoblasts adhesion and spreading.

The aim of this study was to evaluate signaling pathways involved during osteoblast adhesion and spreading through phosphoproteins *status*, which were correlated with cytoskeleton remodeling. Our findings suggest that the identification of the phosphorylation *status* of p38, PP2A and cofilin can be a useful biomarker of the quality of osteoblast adhesion as well as spreading on different surfaces/substrates. This information can have broadly application in the oral biomaterials and bone tissue engineering research fields.

Material and methods

Materials

Pan-actin, pSHP2 (Tyr542), pFaK (Tyr397), p-p44/p42 MAPK (Thr202/Tyr204), p-p38 MAPK (Thr180), pAkt (Tyr326), pATF-2 (Thr69), pMAPKAPK2 (Thr222), pPKC-αβII (Thr638/641), pCofilin (Ser03), Rho, pcdc2 (Tyr15), anti-mouse, anti-rabbit and anti-goat were purchased from Cell Signaling Technology (Boston, MA, USA). pPP2A and p21 were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Specific Inhibitors (SD203580, PD098059, and Okadaic Acid) were from Sigma Co.

Inhibitors and Concentrations

4-[5-(4-Fluorophenyl)-2-[4-(methylsulfonyl) phenyl]-1H-imidazol-4-yl] pyridine (SD203580 - 5μM), 2'-Amino-3'-methoxyflavone (PD098059 - 5μM), and okadaic acid (10nM). Next, the proteins were resolved by SDS-PAGE and transferred to PVDF membrane as described in "immunoblotting assay" item.

Cell line and culture conditions

MC3T3-E1 cells (subclone 4), a mouse pre-osteoblast cell line, were obtained from ATCC (Manassas, USA) and grown at 37°C in α-MEM medium supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin under a humidified 5% CO_2 atmosphere.

Osteoblast adhesion and proliferative assays

Crystal violet staining - MC3T3-E1 cells were seeded at 50,000 cells/well into 24-well culture plates for 1, 6, 24 and 48 h. Cell proliferation was assessed by a colorimetric assay using crystal violet as described previously (10) with slight modifications. Briefly, viable cells were rinsed in warm PBS and fixed in absolute ethanol-glacial acetic acid (3:1, vol/vol) for 10 min at room temperature and left to air dry (eventually stored at 4°C, wrapped in aluminum paper). The cells were stained with 0.1% crystal violet (wt/vol) for 10 min at room temperature. Excess dye was removed by decantation and washed twice with distilled water. The dye was extracted in 10% acetic acid (vol/vol), and optical density was measured at 550 nm using a microplate reader (Biotek). Data from each experiment were analyzed with six observations in each group.

MTT reduction - MC3T3-E1 cells were seeded at 50,000 cells/well in 24-well culture plates for 1, 6, 24 and 48 h. Cell proliferation was assessed by a colorimetric assay using 3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyltetrazolium bromide (11). Briefly, the culture medium was gently removed and 1mL MTT solution (1mg MTT/mL of culture medium) was added to each well that was incubated for 3 h at 37°C. After incubation, the medium was removed and the formazan released by solubilization in 1mL of DMSO. The plate was shaken for 5 min on a plate shaker and the absorbance measured at 570 nm using a microplate reader (Biotek).

Cell counts - MC3T3-E1 cells were seeded at 30,000 cells/well in 24-well culture plates for 1, 6, 24 and 48 h and the trypsinized cells were counted in Hematologic chamber.

MAP kinases and PP2A activity Inhibition assay

MC3T3-E1 cells (50,000 cells/mL) were seeded on cell culture dishes (10 cm), after 1 hour the cells were incubated with specific inhibitors for 2 hours. Then, the immunoblotting samples were collected.

Immunoblotting assay

Protein extracts from MC3T3-E1 cells were obtained using Lysis Cocktail (50mM Tris [tris(hydroxymethyl)aminomethane]–HCl [pH 7.4], 1% Tween 20, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EGTA (ethylene glycol tetraacetic acid), 1mM *O*-Vanadate, 1mM NaF, and protease inhibitors [1µg/mL aprotinin, 10µg/mL leupeptin, and 1 mM 4-(2-amino-ethyl)-benzolsulfonyl-fluorid-hydrochloride]) for 2 hours on ice, as described by Queiroz *et al.* (12). After clearing by centrifugation, the protein concentration was determined using Lowry method (13). An equal volume of 2 x sodium dodecyl sulfate (SDS) gel loading buffer (100mM Tris-HCl [pH 6.8], 200mM dithiothreitol [DTT], 4% SDS, 0.1% bromophenol blue, and 20% glycerol) was added to samples and boiled for 5 minutes. Proteins extracts were resolved by SDS-polyacrylamide gel (10 or 12%) electrophoresis (PAGE) and transferred to PVDF membranes. Membranes were blocked in 1% fat-free dried milk or bovine serum albumin (2.5%) in Tris-buffered saline (TBS)–Tween 20 (0.05%) and incubated overnight at 4° C with appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.05%), membranes were incubated with anti-rabbit, anti-goat or anti-mouse horseradish peroxidase–conjugated secondary antibodies, at 1:2000 dilutions (in all immunoblotting assays), in blocking buffer for 1 hour. Detection was performed by using enhanced chemiluminescence (ECL).

Statistical analysis

All experiments were performed in triplicates and the results shown in the graphs represent the means and standard errors. Data from each assay were analyzed statistically by ANOVA. Multiple comparisons among group mean differences were checked with Tukey post hoc test. Differences were considered significant when the $p<0.05$. Immublottings

represent 3 independent experiments. We used Image Pro-Plus to analyze the proteins expressions profile (arbitrary values). Each graph shows the means +/- error standard.

Results

Cell cycle inhibition during osteoblasts adhesion and spreading processes.

Initially, we evaluated the adhesion and spreading of osteoblasts as well as cell cycle progression along 48h. Up to 24h the proliferation rate was not linear as demonstrated by different techniques, the cell number increased around 50%. However, after 48h of cell culture the cell number was 2.5-fold higher (Fig. 1).

Evaluation of activity/function of key modulators of cytoskeleton rearrangement

The set of proteins involved in the cytoskeleton remodeling were analyzed up to 48h. In general the pattern of activation of these proteins was time-dependent and we also detected a transient activation of these proteins. FAK was active at 1 and 24h while Rho and *SHP*2 remained active for up to 6h and PKC up to 24h. On the other hand, Akt remained active (phosphorylated) throughout the experimental periods (Fig. 2). Also, p*SHP*-2 and pPTEN were active at 1 and 6h. Figure 2 presents the general profile of phosphoproteins along osteoblast adhesion and spreading, such as: PTEN, p38 MAPK, Akt, MAPKAPK2, PKC, FAK, ERK MAPK, ATF-2, *SHP*-2.

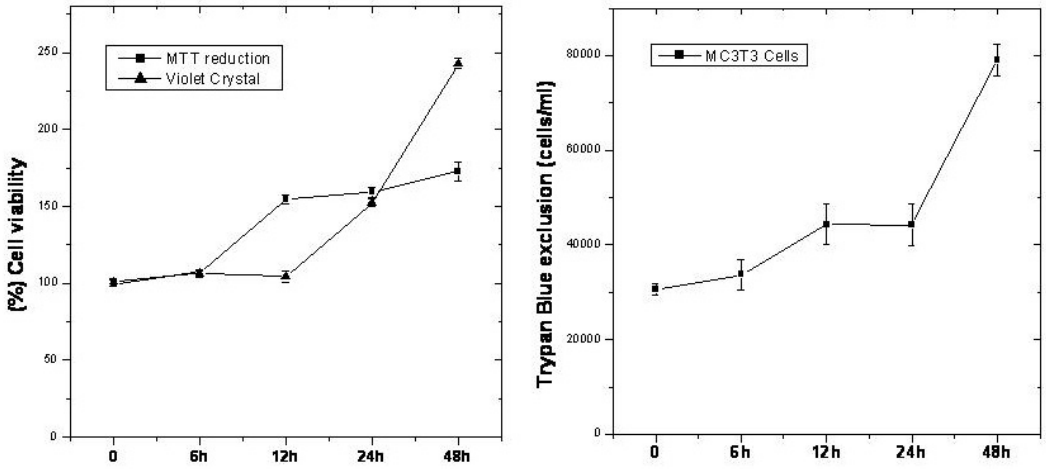

Fig. 1: Adhesion and proliferative assays. Understanding the mechanisms underlying osteoblasts adhesion and spreading should potentially facilitate the search of ideal material for bone fractures, as well as great bone losses. Here, we evaluated osteoblast adhesion and spreading. Cells were cultured up to 48h afterwards mitochondria function, cell number were analyzed. In the time 0, cell viability was considered as 100%. The experiment was performed in a 24 wells plate. Results represent the means ± standard error of 3 experiments run in triplicate ($p < 0.05$). The period studies are indicated in the graphics.

p38 MAP Kinase is required for osteoblasts adhesion and spreading

Our experiments showed that both MAP Kinases (p38 and Erk) are required and probably play an important role in osteoblat adhesion, since these kinases remained active up to 6h after cell seeding (Fig. 2). ERK MAPK remained active even after 24 and 48h. In agreement we also observed phosphorylation of two substrates of p38 MAPK: ATF-2 and MAPKAPK2. To confirm the importance of p38MAPK for osteoblast adhesion, 1h after the seeding of the cells we treated them with classical p38 MAPK inhibitor (SB203580) for 2h and checked the number of cells that were able to adhere, as well as the phosphorylation *status* of ATF2 and MAPKAPK2. Importantly, we noticed that in the presence of p38 MAPK inhibitor affected almost half percent (MTT: 52,37; crystal violet: 52,79), which did not attach on the plastic surface (Fig. 4). In addition, phosphorylation of ATF-2 and MAPKAPK2 levels dropped in the presence of this inhibitor (SB203580). On the other hand, ERK MAPK inhibitor did not affect phosphorylation of both proteins (Fig. 3). The concentration of p38 MAPK inhibitor used here did not present toxic effect in MC3T3-E1 (data not shown).

Phospho-Cofilin (Ser03) is dephosphorylated by PP2A during osteoblasts spreading.

Proteins involved with cytoskeleton rearrangement have been evaluated during osteoblast adhesion and spreading (Fig. 2). Here, we detected an increase of cofilin phosphorylation with concomitant rise of phosphorylation level at the inhibitory site of PP2A (Fig. 5A). To correlate the possible connection between both proteins, we treated the osteoblasts before with 10 nM okadaic acid (concentration non toxic, data not shown). Notably, the inhibition of PP2A guaranteed high levels of phosphorylated cofilin (Fig. 5B).

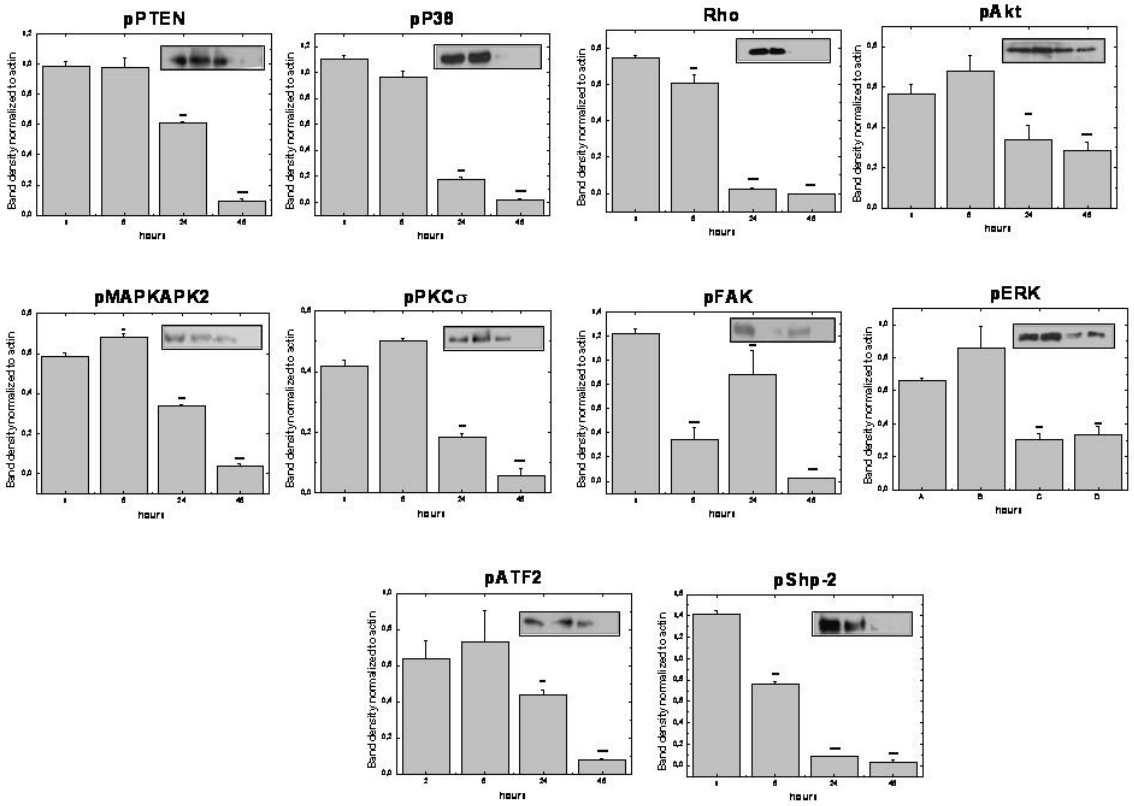

Fig. 2: Protein Expression Performed through Immunoblotting. The expression or phosphorylation levels were assayed using immunoblotting with extracts obtained from osteoblasts cultured for different periods as indicated in the figure, as described in the Material and methods. Protein loading control was assessed by b-actin. $^{*}, ^{**}, ^{***}p < 0,05$. The graphs represent plots of means ± standard error of the means of intensities (minus background) from the region of interest analysis of data from three separate experiments. Band density was normalized to actin.

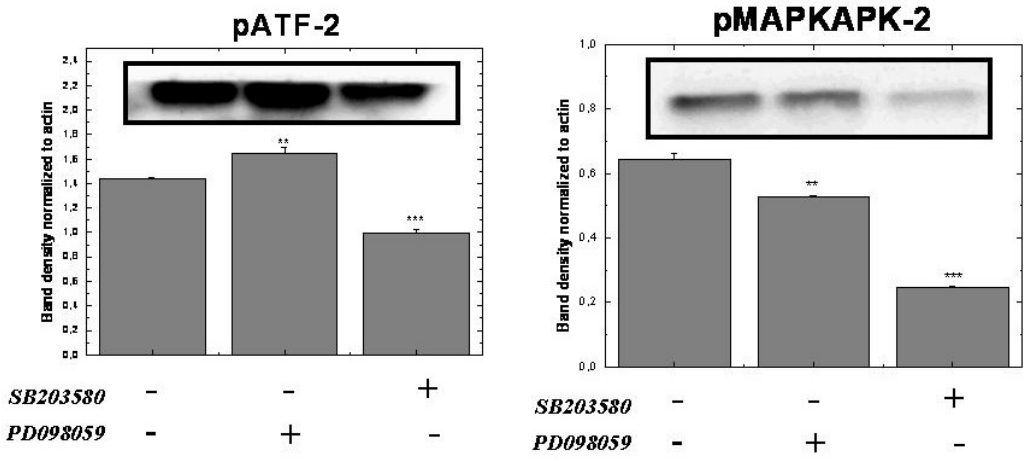

Fig. 3: p38 MAP Kinase is important to promote phosphorylation of ATF 2 and MAPKAPK2 in osteoblasts. *Fig. 2* brought out strong evidence about the importance of p38 MAPK during osteoblast differentiation. In this way, ATF2 and MAPKAPK2 were phosphorylated in the same period pointing out that p38 MAPK was responsible for phosphorylating these proteins. To define the possible MAPK responsible for phosphorylating ATF2 and MAPKAPK-2 during osteoblast differentiation, the cells were previously treated with 5 µM SB203580 (p38 inhibitor) or 5 µM PD098059 (ERK inhibitor) for 2h and the phosphorylation level of ATF-2 and MAPKAPK2 was analyzed by immunoblotting using specific antibodies, as described in the Material and methods. We confirmed that p38 MAPK acts as an upstream modulator of ATF-2 and MAPKAPK2, but not ERK MAPK. **, *** $p < 0,05$. The graphs represent plots of means ± standard error of the means of intensities (minus background) from the region of interest analysis of data from three separate experiments. Protein loading control was assessed by b-actin. Band density was normalized to actin.

The Fig. 6 represents a schematic illustration of signal pathways involved with cytoskeleton rearrangement and required during osteoblast adhesion and spreading.

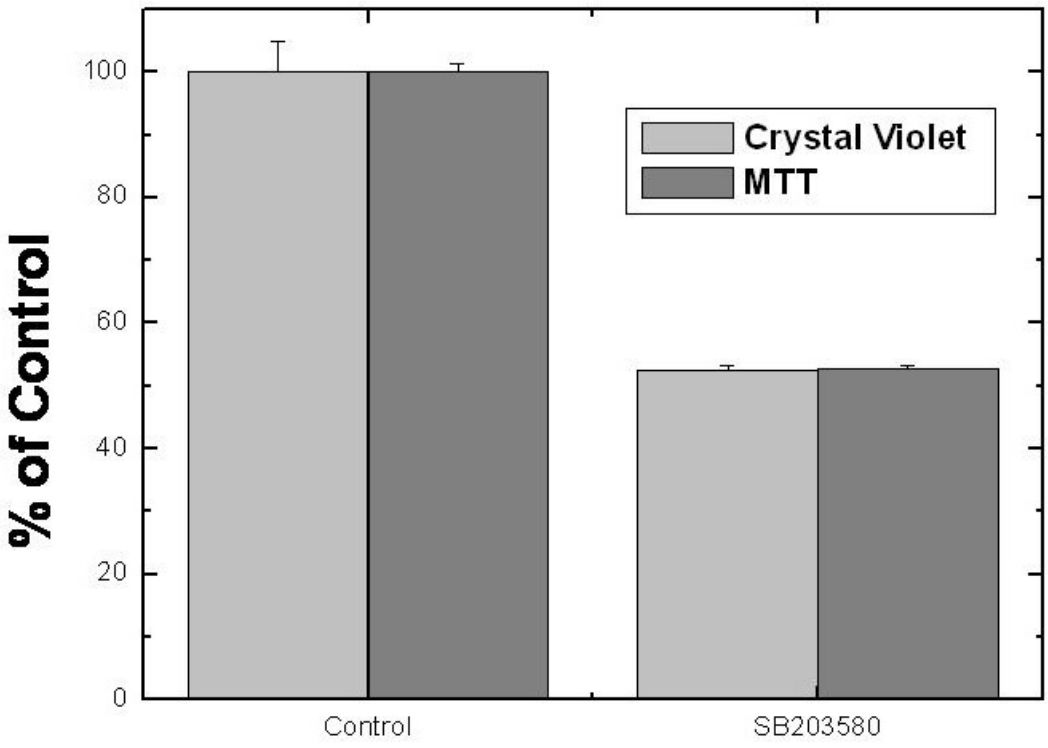

Fig.4: p38 MAP Kinase is important to promote adhesion in osteoblasts. To analyze if p38 MAPK was important to osteoblast adhesion, the cells were treated with SB203580 (p38 inhibitor) for 2h and afterwards, the same cells were trypsinized and seeded again ($5x10^4$cells/mL) in 24-wells plate (1 mL per well). Cell adhesion was checked by MTT reduction and Violet Crystal.

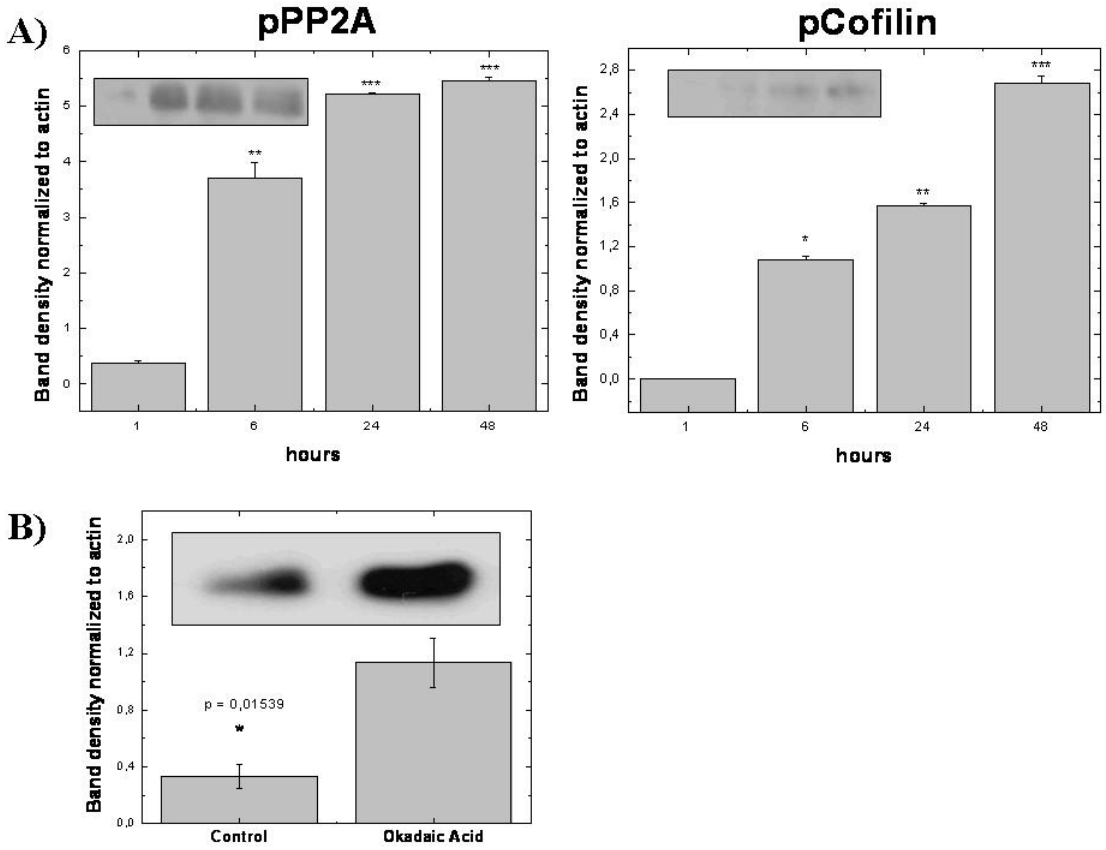

Fig.5: Dephosphorylation of pCofilin by PP2A during osteoblasts adhesion. (A) Expression of PP2A and Cofilin were evaluated. Antibody against PP2A was specific for inhibitory site. Thus, we noted that 1 h after plating the cells PP2A displayed higher activity. (5B) in agreement to (5A) we hypothesized that PP2A modulates Cofilin (Ser03) activity. To confirm it, osteoblasts were plated and after 1h treated with 10nM okadaic acid for 2h, afterwards cofilin phosphorylation *status* was checked by immunoblotting using specific antibody for Ser03, as previously described in Material and method. *, **, *** $p < 0,05$. The graphs represent plots of means ± standard error of the means of intensities (minus background) from the region of interest analysis of data from three separate experiments. Protein loading control was assessed by b-actin. Band density was normalized to actin.

Discussion

The elucidation of the molecular events triggered during adhesion and spreading of osteoblasts is very important to provide new insights into cell-biomaterial interaction improvement which is one of the major aims in the fields of oral implants and bone repair

research (14,15). Immediately after implantation, the implant surface is coated with ECM and host plasma proteins (14). These adsorbed proteins on the implant surface mediate the adhesion of host cells such as fibroblasts and osteoblasts (16-20). Osteoblasts are cells able to produce ECM and to promote its mineralization (21,22). The occurrence of this process requires a rearrangement of the cytoskeleton and therefore (23), identification of key molecular mediators involved in this process might be useful for validating as well as improving the biocompatibility of biomaterials in general.

Herein, we identified the importance of the modulation of cofilin, PP2A and p38 MAPK for attaching and spreading of osteoblasts, which was correlated with cytoskeleton remodeling.

The main changes in the level and/or phosphorylation *status* of protein from focal adhesion and cytoskeleton, and proteins that can interact with these both cellular components was observed up to 24h. Focal adhesions are dynamic structures associated with the actin cytoskeleton that form adhesion plaques of clustered integrin receptors and function in coupling the cell cytoskeleton to the ECM. We clearly detected FAK phosphorylated at 1 and 24h, which can correlate to integrins signaling. In the same way, we showed that FAK activation was highly transient during the cellular events investigated. Integrins are adhesion glycoprotein receptors that regulate a wide variety of dynamic cellular processes such as cell migration, phagocytosis, growth and development. Integrins interaction with extracellular ligands is regulated from inside the cell, through the short cytoplasmic alpha and beta integrin tails, which also mediate biochemical and mechanical signals transmitted to the cytoskeleton by the ligand-occupied integrins, culminating with changes in cell shape, and also regulates a variety of cell functions including adhesion, migration, proliferation, differentiation, and apoptosis (24-26). Although integrin molecules

provide a platform for the signaling complex, they do not have intrinsic enzymatic activities in their cytoplasmic domains (27). Therefore, transmission of downstream signals has to be mediated by non-receptor tyrosine kinases, such as FAK or Src family kinases (28). Integrin-mediated attachment activates FAK by auto-phosphorylation at tyrosine residue, which provides a binding site for Src (29,30). In turn, FAK binds to a number of signaling molecules such as talin and paxillin (31,32), which link the integrin-FAK signaling complex to the actin cytoskeleton (33,34). Furthermore, FAK is required for the phosphorylation of Src and other downstream targets (35,37). Besides FAK, the integrin-mediated intracellular signal transduction is driven by other signaling molecules, such as extracellular signal-regulated protein kinase ERK, protein kinase A (PKA) and Rho. These processes are critical for anchorage-dependent cells as the quality of initial adhesion strongly affects the fate of these cells.

Importantly, AKT remained active along the period analyzed, which indicated that besides inhibition of the cell cycle progression, osteoblasts signaling survival pathways were also kept active. On the other hand, we observed a decrease of ERK activity at 24 and 48h, this finding indicates a tendency of the negative modulation of osteoblasts proliferation. In addition we showed that p38 MAP Kinase is important for osteoblast adhesion. Phosphorylation of MAPKAPK2, a physiological substrate of p38 MAPK activity, was increased. Multiples residues of MAPKAPK-2 are phosphorylated *in vivo*. However, only four residues (Thr25, Thr222, Ser272 and Thr334) are phosphorylated by p38 MAPK *in vitro* (38). Accordingly, pre-treatment of the osteoblasts with p38 MAPK inhibitor impaired osteoblasts adhesion (MTT: 52,37%; crystal violet: 52,79%).

In addition to protein tyrosine kinases, several intracellular protein tyrosine phosphatases (PTPs) have been implicated as positive and negative regulators of integrin-

mediated signaling (39). Ours findings suggest that SHP2 (PTP) plays a crucial role during osteoblast adhesion, since the highest activity was observed at 1 and 6h. Therefore, the assembly of α-actin in focal complexes could be directly dependent on SHP2-dependent inhibition of FAK activity (40). In other words, activated SHP2 contributes with focal complex assembly. Interestingly, PP2A (Ser/Thr protein phosphatase) was expressively inhibited from 6h which was in agreement with the increase of phosphorylated cofilin, which was suggested be physiological substrates to PP2A activity. Cofilin is a principal player in regulating the dynamics of actin, via defining the average length of actin filaments. The biological action of cofilin is regulated via phosphorylation by LIM kinases (41,42) and by the binding of phosphoinositide lipids (43). LIM kinase 1 induces actin remodeling by phosphorylating and inactivating cofilin, an actin-depolymerizing factor. Therefore, the results presented here reveal that the activation of PP2A immediately after plating the cells retains cofilin dephosphorylated (in active form) which is essential for osteoblast adhesion. On the other hand, from 6h after plating, PP2A was inhibited and in turn the phosphorylation level of cofilin increased. In this way, we can speculated that phosphorylation of cofilin is crucial for osteoblast spreading.

Our results highlight the importance of the maintenance of cofilin inactivation (phosphorylated form) via inhibition of PP2A, and in turn, triggering actin polymerization. Actin is one of the main components of the cytoskeleton, which consists of globular monomers (G-actin) that polymerize to form helically symmetrical filaments (F-actin). A dynamic cytoskeleton is important in numerous cellular activities including maintenance of cell shape.

In summary, we have investigated some proteins which play crucial role either in osteoblast adhesion or spreading. We can speculate that the identification of the phosphorylation *status* of p38 MAPK, PP2A and cofilin can be useful biomarkers for the quality of osteoblast adhesion and spreading on different surfaces/substrates. This information can have broad applications in the fields of oral biomaterials and bone tissue engineering research, as signaling molecules required during cell adhesion.

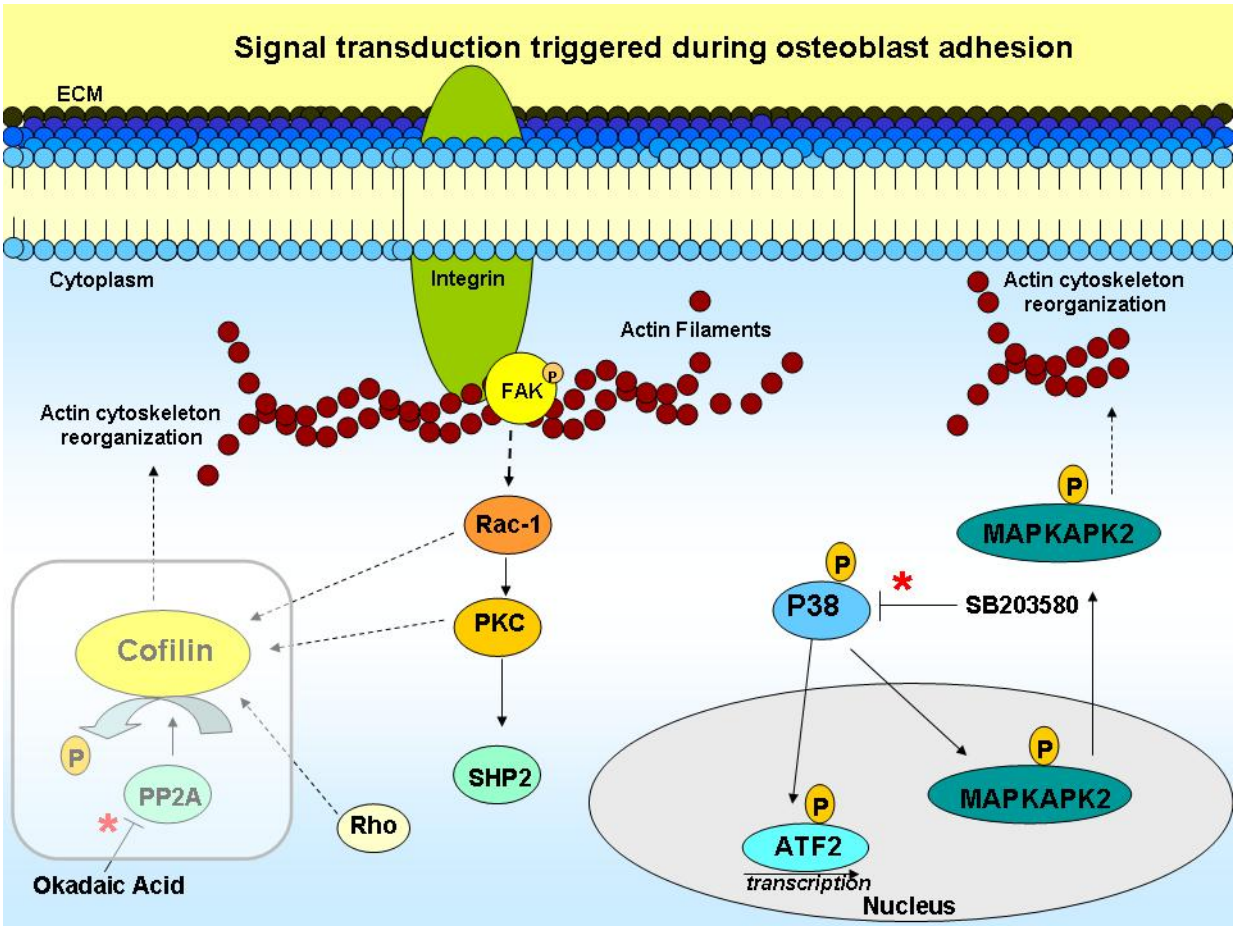

Fig. 6: Schematic representation of Biochemical mechanisms evaluated during osteoblast adhesion and spreading. The most of protein analyzed was involved with cytoskeleton rearrangement; which was affected by two different pathways (Cofilin and MAPKAPK-2; left and right of scheme, respectively).

Acknowledgments

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References

1. Ferreira CF, Carriel Gomes MC, Filho JS, Granjeiro JM, Oliveira Simões CM, Magini RS. Platelet-rich plasma influence on human osteoblasts growth. *Clin Oral Implants Res* 2005; **16**: 456-60.
2. Heino J. The collagen family members as cell adhesion proteins. *Bioessays* 2007; **29**: 1001-10.
3. Klein EA, Yung Y, Castagnino P, Kothapalli D, Assoian RK. Cell adhesion, cellular tension, and cell cycle control. *Methods Enzymol* 2007; **426**: 155-75.
4. Zaidel-Bar R, Itzkovitz S, Ma`Ayan A, Iyengar R, Geiger B. Functional atlas of the integrin adhesome. *Nat Cell Biol* 2007; **9**: 858-67.
5. Lietha D, Cai X, Ceccarelli DF, Li Y, Schaller MD, Eck MJ. Structural basis for the autoinhibition of focal adhesion kinase. *Cell* 2007; **129**: 1177-87.
6. Bhadriraju K, Yang M, Alom Ruiz S, Pirone D, Tan J, Chen CS. Activation of ROCK by RhoA is regulated by cell adhesion, shape, and cytoskeletal tension. *Exp Cell Res* 2007; **313**: 3616-23.
7. Carlier MF, Ressad F, Pantaloni D. Control of actin dynamics in cell motility. Role of ADF/cofilin. *J Biol Chem* 1999; **274**: 33827-30.

8. Condeelis J. How is actin polymerization nucleated *in vivo*? *Trends Cell Biol* 2001; **11**: 288-93.

9. Ferreira CV, Justo GZ, Souza AC, Queiroz KC, Zambuzzi WF, Aoyama H, Peppelenbosch MP. Natural compounds as a source of protein tyrosine phosphatase inhibitors: application to the rational design of small-molecule derivatives. *Biochimie* 2006; **88**: 1859-73.

10. Kaeffer B, Benard C, Blottiere HM, Cherbut C. Treatment of rat proximal and distal colonic cells with sodium orthovanadate enhances their adhesion and survival in primary culture. *Cell Biol Int* 1997; **21**: 303-14.

11. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *J Immunol Meth* 1983; **65**: 55-63.

12. de Souza Queiroz KC, Zambuzzi WF, Santos de Souza AC, da Silva RA, Machado D, Justo GZ, Carvalho HF, Peppelenbosch MP, Ferreira CV. A possible anti-proliferative and anti-metastatic effect of irradiated riboflavin in solid tumours. *Cancer Lett* 2007; **258**: 126-34.

13. Hartree EF. Determination of proteins: a modification of Lowry method that give a linear photometric response. *Anal Biochem* 1972; **48**: 422-427.

14. Wilson CJ, Cleeg RE, Leavesley DI, Pearcy MJ. Mediation of biomaterial-cell interactions by adsorbed proteins: a review. *Tissue Eng* 2005; **11**: 1-18.

15. Barrere F, Van Blitterswijk CA, de Groot K. Bone regeneration: molecular and cellular interactions with calcium phosphate ceramics. *Int J Nanomedicine* 2006; **1**: 317-32.

16. Baier RE, Meyer AE, Natiella Jr, Carter JM. Surface properties determine bioadhesive outcomes: Methods and results. *J Biomed Mater Res* 1984; **18**:327-355.

17. Gristina A. Biomaterial-centered infection: Microbial adhesion versus tissue integration. *Science* 1987; **237**: 1588-1595.

18. Herrmann M, Vaudaux PE, Pittet D, Auckenthaler R, Lew PD, Schumacher-Perdreau F, Peters G, Waldvogel FA. Fibronectin, fibrinogen, and laminin act as mediators of adherence of clinical staphylococcal isolates to foreign material. *J Infect Dis* 1988; **158**: 693-701.

19. Gristina A. Implant failure and the immuno-incompetent fibro-inflammatory zone. *Clin Orthop* 1994; **298**: 106-118.

20. Kuusela P, Hilden P, Savolainen K, Vuento M, Lyytikäinen O, Vuopio-Varkila M. Rapid detection of methicillin-resistant Staphylococcus aureus strains not identified by slide agglutination tests. *J Clin Microbiol* 1994; **32**: 143-147.

21. Robinson RA. Bone tissue: composition and function. *Johns Hopkins Med J* 1979; **145**: 10-24.

22. Hadjidakis DJ, Androulakis II. Bone remodeling. *Ann N Y Acad Sci* 2006; **1092**: 385-96.

23. Salido M, Vilches JI, Gutierrez JL, Vilches J. Actin cytoskeletal organization in human osteoblasts grown on different dental titanium implant surfaces. *Histol Histopathol* 2007; **22**: 1355-64.

24. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell* 2002; **110**: 673-687.

25. Lim JY, Dreiss AD, Zhou Z, Hansen JC, Siedlecki CA, Hengstebeck RW, Cheng J, Winograd N, Donahue HJ. The regulation of integrin-mediated osteoblast focal adhesion and focal adhesion kinase expression by nanoscale topography. *Biomaterials* 2007; **28**: 1787-97.

26. Arnaout MA, Goodman SL, Xiong JP. Structure and mechanics of integrin-based cell adhesion. *Curr Opin Cell Biol* 2007; **19**: 495-507.

27. Liu S, Calderwood DA, Ginsberg MH. Integrin cytoplasmic domain-binding proteins. *J Cell Sci* 2000; **113**: 3563-3571.

28. Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB, Parsons JT. pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc Natl Acad Sci USA* 1992; **89**: 5192–5196.

29. Chan PY, Kanner SB, Whitney G, Aruffo A. A transmembrane-anchored chimeric focal adhesion kinase is constitutively activated and phosphorylated at tyrosine residues identical to pp125FAK. *J Biol Chem* 1994; **269**: 20567-20574.

30. SCHLAEPFER DD, HAUCK CR, SIEG DJ. Signaling through focal adhesion kinase. *Prog Biophys Mol Biol* 1999; **71**: 435-478.

31. Chen HC, Appeddu PA, Parsons JT, Hildebrand JD, Schaller MD, Guan JL. Interaction of focal adhesion kinase with cytoskeletal protein talin. *J Biol Chem* 1995; **270**: 16995-16999.

32. Brown MC, Perrotta JA, Turner CE. Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. *J Cell Biol* 1996; **135**: 1109-1123.

33. Finke B, Luethen F, Schroeder K, Mueller PD, Bergemann C, Frant M, Ohl A, Nebe BJ. The effect of positively charged plasma polymerization on initial osteoblastic focal adhesion on titanium surfaces. *Biomaterials* 2007; **28**: 4521-34.

34. Smilenov LB, Mikhailov A, Pelham RJ, Marcantonio EE, Gundersen GG. Focal adhesion motility revealed in stationary fibroblasts. *Science* 1999; **286**: 1172-1174.

35. Otey CA, Pavalko FM, Burridge K. An interaction between alpha-actinin and the beta 1 integrin subunit *in vitro*. *J Cell Biol* 1990; **111**: 721-729.

36. Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR, Parsons JT. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cell Biol* 1994; **14**:1680-1688.

37. Schlaepfer DD, Jones KC, Hunter T. Multiple Grb2-mediated integrin-stimulated signaling pathways to ERK2/mitogen-activated protein kinase: summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. *Mol Cell Biol* 1998; **18**: 2571-2585.

38. Ben-Levy R, Leighton IA, Doza YN, Attwood P, Morrice N, Marshall CJ, Cohen P. Identification of novel phosphorylation sites required for activation of MAPKAP kinase-2. *EMBO J*. 1995; **14**: 5920-30.

39. Angers-Loustau A, Cote J-F, Tremblay ML. Roles of protein tyrosine phosphatases in cell migration and adhesion. *Biochem Cell Biol* 1999; **77**: 493–505.

40. Von Wichert G, Haimovich B, Feng GG, Sheetz MP. Force-dependent integrin-cytoskeleton linkage formation requires down-regulation of focal complex dynamics by *SHP*2. *EMBO J* 2003; **22**: 5023-35.

41. Morgan TE, Lockerbie RO, Minamide LS, Browning MD, Bamburg JR. Isolation and characterisation of a regulated form of actin depolymerizing factor. *J Cell Biol* 1993; **122**: 623-33.

42. Arber S, Barbayannis FA, Hanser H, Schneider C, Stanyon CA, Bernard O, Caroni P. Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature* 1998; **393**: 805-9.

43. Yonezawa N, Nishida E, Iida K, Yahara I, Sakai H. Inhibition of the interactions of cofilin, destrin and deoxyribonuclease I with actin by phoshoinositides. *J Biol Chem* 1990; **265**: 8382-6.

Capítulo 6

DISCUSSÃO E CONCLUSÕES

DISCUSSÃO

Diante da necessidade atual em se conhecer os mecanismos que orquestram a formação óssea, este trabalho investigou pontos-chave da diferenciação de osteoblastos *in vitro*. As técnicas de cultivo celular têm-se mostrado uma importante ferramenta para o estudo do comportamento celular frente a diversas situações. Em particular, quanto à diferenciação de células osteoprogenitoras em osteoblastos ativos, a cultura primária tem demonstrado ser um excelente modelo de estudo, porém possui limitações: **a)** inadequada para experimentos de longa duração; **b)** heterogeneidade do fenótipo; **c)** baixas taxas de proliferação; **d)** limitado número de passagens em cultura (Subramaniam *et al.*, 2002).

Diante disso, foi escolhida uma linhagem de células estabelecidas para a realização deste projeto. A linhagem celular mais adequada para a proposta deste trabalho foi a linhagem desenvolvida por Sudo *et al.* 1983 (MC3T3-E1), proveniente de calvária de camundongos. Como descrito na literatura trata-se de uma linhagem celular capaz de se diferenciar *in vitro*, comprovada pelos marcadores já estabelecidos: fosfatase alcalina, cujo pico de atividade indica o início do processo de mineralização biológica e osteocalcina.

As células pré-osteoblásticas, MC3T3-E1, na presença de ácido ascórbico e β-Glicerofosfato apresentam ativação de vias de diferenciação que levam a deposição de componentes necessários para formação da matriz óssea mineralizada (Quarles *et al.,* 1992; Raouf e Seth, 2000).

Caracterização da Diferenciação Celular: Análise de biomarcadores clássicos da diferenciação de osteoblastos

Inicialmente, foram realizados experimentos com o intuito de comprovar a diferenciação destas células. Para isso foram monitorados ensaios de atividade de fosfatase alcalina e expressão de osteocalcina. Estes ensaios foram de extrema importância para a continuidade e planejamento dos ensaios posteriores, pois foi definido o tempo exato do grau máximo de diferenciação destas células.

Dentre os biomarcadores, a atividade da fosfatase alcalina está associada com a mineralização biológica pelo fato de que o aumento de sua expressão é necessário antes do começo da mineralização da matriz, fornecendo fosfato inorgânico (PO_4^-) para a nucleação de cristais de hidroxiapatita (Coelho *et al.*, 2000a e b). Além disso, o aumento na expressão de osteocalcina, juntamente com osteopontina, inicia o período de mineralização da matriz (Stein *et al.*,1990; Owen *et al.*, 1990; Owen *et al.*, 1993).

Os resultados obtidos neste trabalho apontam que as células MC3T3-E1 obtiveram um pico de atividade fosfatásica no tempo experimental de 21 dias de tratamento, bem como expressão de osteocalcina. Estes resultados são semelhantes à maioria dos trabalhos envolvidos no estudo da diferenciação de osteoblastos *in vitro.*

Durante a diferenciação osteoblástica a progressão do ciclo celular é interrompida

A entrada para a diferenciação celular é caracterizada pela coordenação da parada do ciclo celular e expressão específica de genes, o qual é uma seqüência coordenada de eventos bioquímicos associados com alterações morfológicas, incluindo a parada na fase G1 e seguida expressão temporal de genes específicos. Nossos resultados mostraram que p21, c-myc, ciclina B1 foram down-reguladas durante a diferenciação de osteoblastos. No

caso da p21 era esperado um aumento, já que esta proteína usualmente é marcador de parada do ciclo por se ligar a Cdks. No entanto Bellosta *et al.* (2003) mostraram que a proteína p21 é de fato fortemente down-regulada durante a diferenciação de osteoblastos *in vitro*. Em contraste, a ciclina D1 estava up-regulada durante a diferenciação da MC3T3-E1, o qual pode se dar, em parte, pela inibição da via da GSK3. Além destas proteínas acima citadas, vimos também que outras envolvidas com o ciclo celular estavam down-reguladas, como foi o caso da Retinoblastoma (Rb) e cdc2.

Análise da remodelação da matriz extracelular durante a diferenciação de osteoblastos

Um processo fisiológico bastante intrigante durante a mineralização biológica é a síntese e degradação de componentes da matriz extracelular pelas células ósseas, as quais regulam finamente este processo. Neste sentido, a relação osteoblasto/matriz é de fundamental importância para viabilidade destas células. No processo de remodelação da MEC, as MMPs assumem um papel muito importante, haja visto que estas proteases fazem parte de uma classe de enzimas zinco dependentes, que são em conjunto, capazes de degradar a maioria dos componentes da matriz extracelular (Bikerdal-Hansen H, 1995).

Durante o ensaio de osteogênese, estabelecido neste trabalho, foi analisado a atividade das MMPs-2 e -9 através de zimografia e expressão da MMP-9. Para isso, uma amostra do meio de cultura foi coletada a partir de cada período experimental (6 horas, 1, 7, 14, 21 e 28 dias de tratamento com ácido ascórbico e β-glicerofosfato) e aplicado em um gel contendo 4% de gelatina (substrato para estas MMPs). O zimograma mostrado neste trabalho mostra que a atividade das MMPs-2 e –9 se eleva gradativamente no decorrer dos períodos, havendo inclusive, diferenças entre a forma pró-enzima e enzima ativa para ambas MMPs. Ressalta-se que mesmo em suas formas de pro-enzimas, estas MMPs

apresentam atividade. Estes resultados estão de acordo com os relatos de Mizutami *et al.*, 2001.

Por outro lado, foi analizada também a expressão de inibidores teciduais de MMPs, TIMP1 e RECK. Aparentemente, RECK é requerido durante os passos iniciais da diferenciação, enquanto TIMP-2 está envolvida somente na fase final. Somado a capacidade de inibir a atividade de MMPs, as TIMPs atualmente têm sido apontadas em outros processos fisiológicos, apresentando efeitos pluripotenciais no crescimento celular, apoptose e diferenciação (Baker *et al.*, 2002; Jiang *et al.*, 2002). Nesse sentido, TIMP-2 tem mostrado ser capaz de estimular a proliferação de células quiescentes, atuar como fator de crescimento e inibir a proliferação de células endoteliais estimuladas por FGF (Baker *et al.*, 2002; Jiang *et al.*, 2002). Nossos resultados indicam que ambos inibidores de MMPs podem exercer função intracelular, tal qual participação na regulação do rearranjo do citoesqueleto durante a diferenciação de osteoblastos.

Proteina tirosina fosfatases são requeridas enquanto níveis de oxidação são finamente modulados durante a diferenciação de osteoblastos

A diferenciação de células eucarióticas está sob controle de uma intrincada rede de processos bioquímicos onde a fosforilação de proteínas em resíduos de tirosina desempenha importante função.

A regulação da fosforilação de resíduos de tirosina é mediada pela ação conjunta e coordenada de PTKs e PTPs (Ferreira *et al.*, 2006). Dada a importância das PTPs na regulação de diferentes eventos celulares, cabe ressaltar que esta super-família está envolvida no controle da formação e reabsorção óssea. A primeira PTP que tem sido demonstrada na fisiologia óssea é a SHP1 (Umeda *et al.*, 1999; Aoki *et al.*, 1999).

Camundongos que tiveram esta proteína "Knock down" desenvolveram osteopenia pelo aumento da reabsorção óssea. Por outro lado, PTPe também tem sido sugerida como um alvo da ação de bifosfonados em osteoclastos, baseados nos resultados que esta PTP é inibida pelo alendronato (Schmidt *et al.*, 1996).

Em 2007, Schinke *et al.* publicou a primeira evidência de que a PTPζ era importante para a formação óssea. Adicionalmente, nós mostramos que outras PTPs (SHP2, LMWPTP e PTPα) são requeridas durante a diferenciação de osteoblastos. Neste sentido ainda, sugerimos que o status redox favorece a atividade de PTPs durante a diferenciação de osteoblastos, uma vez que o compartimento intracelular tende a ficar reduzido nesse processo.

Bioquimicamente, estes resultados estão em concordância com outros trabalhos onde foram descritos que espécies reativas de oxigênio levam a inativação de PTPs. (Sundaresan *et al.*, 1995; Gross *et al.*, 1999; Mahadev *et al.*, 2001; Meng, Fukada e Tonks, 2002; Meng *et al.*, 2004).

Mecanismos intracelulares disparados durante a adesão de osteoblastos

A elucidação dos eventos moleculares disparados durante a adesão de osteoblastos é muito importante para o real conhecimento da relação célula/material e para o avanço de novas tecnologias terapêuticas. Sabe-se que a ocorrência deste processo requer um rearranjo do citoesqueleto (Salido *et al.*, 2007). Novos estudos referentes aos mecanismos disparados durante a adesão de osteobalstos fazem-se necessários para a identificação de mediadores moleculares-chaves envolvidos neste processo. Estas novas descobertas possibilitariam, em um futuro próximo, a validação ou o melhoramento da biocompatibilidade de materiais em geral.

Para estabelecer alguns mecanismos relacionados com a adesão de osteoblastos, foi avaliada a importância da modulação da cofilina, da PP2A, p38 MAPK e FAK neste processo. FAK são proteínas dinâmicas associadas com o citoesqueleto que dão forma às as células, bem como promovem a adesão das mesmas com a matriz extracelular. Foi mostrado que FAK estava fosforilada somente com 1 e 24h de plaqueamento, ficando claro que a atividade de FAK é transiente nas células.

Outro achado importante foi a fosforilação de MAPK p38 nos períodos iniciais de adesão de osteoblastos. Para validar sua importância no processo de adesão destas células, as mesmas foram tratadas com um inibidor específico (concentração sub-tóxica) e depois de 2 horas de tratamento as mesmas foram tripsinizadas e plaqueadas novamente. Surpreendentemente, apenas 50% das células aderiram. Adicionalmente foi verificado que a p38 MAPK estava fosforilando MAPKAPK2 durante a adesão de osteoblastos. A MAPKAPK2 quando fosforilada exerce papel de rearranjo do citoesqueleto interagindo diretamente com os filamentos de actina.

Por outro lado, foi notado que PP2A aumentava sua fosforilação no sítio inibitório ao longo dos períodos avaliados, enquanto cofilina aumentava sua fosforilação no resíduo Ser03. Experimentos posteriores, tratando as células com uma concentração não letal de ácido okadaico (inibidor de PP2A), mostraram que, de fato, PP2A é capaz de desfosforilar cofilina durante a adesão de osteoblastos. Bioquimicamente, cofilina regula o rearranjo dos filamentos de actina por definir o comprimento médio dos mesmos. A ação biológica da cofilina é regulada por fosforilação, principalmente pela LIMK (Yonezawa *et al.*, 1990; Arber *et al.*, 1998).

LMWPTP (Low molecular weight protein tyrosine phosphatase) modula a atividade de Src via desfosforilação de ambos resíduos de tirosina (Y416-Src e Y527-Src).

Src é uma proteína tirosina quinase (não-receptor) com uma grande importância em diversos processos celulares. Pelo fato de poucos relatos mostrarem sua modulação covalente, a proposta deste trabalho foi avaliar a capacidade da LMWPTP em desfosforilar seus dois sítios de fosforilação em tirosina (Y). Bioquimicamente sabe-se que Src apresenta 2 sítios de fosforilação (Y_{527}-Src e Y_{416}-Src, inibitório e ativador, respectivamente), os quais são vulneráveis a ação de PTPs. Atualmente sabe se que apenas duas PTPs (CD45 e RPTPα) podem desfosforilar o resíduo pY_{527}-Src, controlando sua atividade. Neste sentido, fica claro que o resíduo Y_{416}-Src poderia também sofrer a ação de PTPs. Porém a fosfatase responsável por essa desfosforilação ainda é desconhecida. Portanto, utilizadando a MC3T3-E1 como fonte de proteínas fosforiladas em tirosina, pré-tratando estas células com pervanadato (inibidor de PTPs). A interação de Src com LMWPTP já foi relatada, mostrando que estas enzimas formam um complexo protéico onde a Src exerce sua atividade de fosforilação no resíduo Y131 da LMWPTP, evento que aumenta em até 25 vezes a atividade da PTP (Bucciantini *et al.*, 1999). Portanto, essa informação sugere que a LMWPTP é uma importante candidata para atuar na regulação negativa de Src. Desta forma, para avaliar essa hipótese, realizamos imunoprecipitação da fosfo-Src e o mesmo foi incubado, *in vitro,* com a LMWPTP recombinante humana. Os resultados mostraram que esta PTP é capaz de desfosforilar ambos resíduos de Src, controlando sua atividade. Assim, fica claro que há uma modulação mútua entre estas proteínas, onde ambas exercem papeis inversos (PTK e PTP). Além disso, ensaios realizados utilizando fracionamento do conteúdo citoplasmático mostraram que a LMWPTP estava presente na fração do citoesqueleto e que sua expressão era modulada pelo status redox da célula. Estes

resultados foram concordantes com o trabalho publicado por Caselli *et al.* (1998) que mostraram que o status redox da célula é determinante para a localização subcelular da LMWPTP. Portanto, a ação da LMWPTP poderia explicar a atividade transiente da Src, uma vez que esta PTP promove a desfosforilação de pY_{416}-Src.

Sonic Hedgehog estimula a diferenciação de pre-osteoblastos em células tipo osteócitos

Desenvolver um modelo para o estudo da biologia de osteócitos é de extrema importância, haja vista a dificuldade em se obter a amostra e cultivo primário e, ainda, pela pouca informação encontrada sobre esse assunto. O Matrigel é uma preparação da membrana basal solubilizada a partir de sarcoma de rato, um tumor rico em proteínas da matriz extracelular. Seus componentes são: laminina, colágeno, proteoglicanas e entactina. Matrigel® é efetivo para a adesão e diferenciação de células, sendo largamente utilizado em vários estudos, incluindo cultivo celular em *3D*, invasão e migração celular, metabolismo, efeito tóxico de xenobióticos e, por último, ensaios de angiogênese *in vitro* e *in vivo*. Dentre os constituintes do Matrigel, destacam-se a presença de colágeno e laminina, componentes peculiares à biologia do osso e características importantes para um carreador/suporte de pré-osteoblastos na bioengenharia óssea.

Portanto, utilizando o Matrigel como "scaffold" 3D, observamos que os pré-osteoblastos se diferenciaram em células tipo osteócitos. Osteócito é o tipo de célula óssea mais abundante, porém de difícil acesso por residirem na matriz óssea mineralizada. Recentemente Gu e colaboradores (2006) mostraram que osteócitos podem ser isolados da cortical óssea e mantidos *in vitro*. Entretanto, tecnicamente isso não é fácil. Portanto, nossos resultados sugerem que a utilização do Matrigel pode ser uma maneira fácil de obter

e estudar a biologia dos osteócitos. Vale ressaltar que além da avaliação morfológica, foram avaliados outros parâmetros moleculares como análise da expressão de marcadores de diferenciação e avaliação da atividade de proteínas quinases envolvidas em vias de transdução de sinal responsáveis pela sobrevivência e diferenciação celular.

Outro fato que merece destaque foi a identificação da Sonic Hedgehog nos grupos onde as células foram cultivadas sobre o Matrigel. Identificou-se também um aumento da expressão de patched-1 e Gli1, o que comprovou a ativação da via disparada pela Shh. A inibição da diferenciação na presença da ciclopamina confirmou a importância da via Shh para a aquisição da morfologia de osteócitos.

Um outro aspecto importante deste trabalho foi a definição do perfil quinômico dos osteócitos através da técnica de PepChip. Esta técnica mostrou que grande parte das quinases envolvidas no metabolismo de osteócitos estão relacionadas com a comunicação celular. Deste modo, estes resultados estão de acordo com o immunobloting obtido para a proteína conexina 43. É importante frisar que a conexina43 é um bom marcador de osteócitos, como descrito por Gu *et al.*, 2006. Estes resultados sugerem que estas junções de comunicação ("Gap junctions") desempenham como uma importante função na comunicação osteócito/osteócito e/ou osteócito/osteoblastos, uma vez que estas junções têm sido descritas em células ósseas por diferentes autores (Doty 1981; Davidson *et al.* 1986; Palumbo *et al.* 1990; Schiller *et al.* 1992).

CONCLUSÕES

Tendo em vista os resultados obtidos neste trabalho concluímos:

1. MMPs-2 e -9, juntamente aos seus inibidores teciduais RECK e TIMP-1, regulam a remodelação da MEC durante a diferenciação de osteoblastos por controlar a atividade de MMPs;
2. Fosforilação de Src é rigorosamente controlada durante a diferenciação de osteoblastos. Demonstramos de forma inédita, que a LMWPTP atua como modulador dual da Src, apresentando-se como a primeira PTP capaz de modular negativamente a atividade de Src, pela desfosforilação dos resíduos pY416 e pY527;
3. Durante a diferenciação celular o ambiente redutor, proporcionado pelo sistema redutor dependente de glutationa, gera um ambiente adequado para o controle da diferenciação de osteoblastos;
4. A importância das PTPs SHP2, LMWPTP e PTPα no processo de diferenciação dos osteoblastos foi evidenciada neste modelo;
5. O Matrigel, quando usado como suporte, favorece a diferenciação de pré-osteoblastos a osteócitos;
6. Sonic hedgehog exerce função crucial na diferenciação de osteócitos;
7. Os dados obtidos através do microarranjo de peptídeos (PepChip) evidenciaram 84 quinases com diferenças estatisticamente significantes. Destas, 46% são quinases relacionadas com comunicação celular;
8. PP2A, cofilina e p38 MAPK desempenham importantes funções durante a adesão de osteoblastos;

REFERÊNCIAS BIBLIOGRÁFICAS

Aarden, E. M., Burger, E. H., Nijweide, P. J. (1994) Function of osteocytes in bone. *J. Cell Biochem*. **55**, 287–299.

Accorsi-Mendonça, T., Paiva, K. B., Zambuzzi, W. F., Cestari, T. M., Lara, V. S., Sogayar, M. C., Taga, R., Granjeiro, J. M. (2008) Expression of matrix metalloproteinases-2 and -9 and RECK during alveolar bone regeneration in rat. *J. Mol. Histol.* **39**, 201-208.

Accorsi-Mendonça, T., Zambuzzi, W. F., da Silva Paiva, K. B., Lauris, J. R. P., Cestari, T. M., Taga, R., Granjeiro, J. M. (2005) Expression of metalloproteinase 2 in the cell response to porous demineralized bovine bone matrix. *J. Mol. Histol.* **36**, 311-316.

Alexander, P. (2002) Matrix metalloproteinases. *Techinal guide*. **2**, 1-12.

Alonso, A., Sasin, J., Bottini, N., Friedberg, I., Friedberg, I., Osterman, A., Godzik, A., Hunter, T., Dixon, J., Mustelin, T. (2004) Protein tyrosine phosphatases in the human genome. *Cell.* **117**, 699–711.

Amling, M., Neff, L., Priemel, M., Priemel, A. F., Schilling, A. F., Rueger, J. M., Baron, R. (2000) Progressive increase in bone mass and development of odontomas in aging osteopetrotic c-Src–deficient mice. *Bone*. **27**, 603-610.

Angers-Loustau, A., Cote, J. F., Tremblay, M. L. (1999) Roles of protein tyrosine phosphatases in cell migration and adhesion. *Biochem. Cell Biol.* **77**, 493–505.

Anselme, K. (2000) Osteoblast adhesion on biomaterials. *Biomaterials*. **21,** 667-681.

Aoki, K., Didomenico, E., Sims, N. A., Mukhopadhyay, K., Neff, L., Houghton, A., Amling, M., Levy, J. B., Horne, W. C., Baron, R. (1999) The tyrosine phosphatase SHP-1 is a negative regulator of osteoclastogenesis and osteoclast resorbing activity: increased resorption and osteopenia in me(v)/me(v) mutant mice. *Bone.* **25**, 261-267.

Aoyama, H., Silva, T. M. A., Miranda, M. A., Ferreira, C. V. (2003) Protein tyrosine phosphatases: properties and biological functions. *Quím. Nova.* **26**, 896–900.

Arber, S., Barbayannis, F. A., Hanser, H., Schneider, C., Stanyon, C. A., Bernard, O., Caroni, P. (1998) Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature.* **393**, 805-809.

Arnaout, M. A., Goodman, S. L., Xiong, J. P. (2007) Structure and mechanics of integrin-based cell adhesion. *Curr. Opin. Cell Biol.* **19**,495-507.

Baier, R. E., Meyer, A. E., Natiella, J. R., Carter, J. M. (1984) Surface properties determine bioadhesive outcomes: Methods and results. *J. Biomed. Mater. Res.* **18,** 327-355.

Baker, A. H., Edwards, D. R., Murphy, G. (2002) Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J. Cell Sci.* **115**, 3719–3727.

Barr, R. K., Bogoyevitch, M. A. (2001). The c-Jun N-terminal protein kinase family of mitogen-activated protein kinases (JNK MAPKs). *Intern. J. Biochem. Cell Biol*. **33**, 1047-1063.

Barrere, F., Van Blitterswijk C. A., d\e Groot K. (2006) Bone regeneration: molecular and cellular interactions with calcium phosphate ceramics. *Int. J. Nanomedicine.* **1**, 317-332.

Bellosta, P., Masramon, L., Mansukhani, A., Basilico, C. (2003) p21 (WAF1/CIP1) acts as a brake in osteoblast differentiation. *J. Bone Miner. Res.* **18**, 818-826.

Ben-Levy, R., Leighton, I. A., Doza, Y. N., Attwood, P., Morrice, N., Marshall, C. J., Cohen, P. (1995) Identification of novel phosphorylation sites required for activation of MAPKAP kinase-2. *EMBO J.* **14**, 5920-5930.

Berra, E., Milanini, J., Richard, D. E., Gall, M. L., Vinals, F., Gothié, E., Roux, D., Pagès, G. Pouysségur, J. (2000) Signaling angiogenesis via p42/p44 MAP kinase and hypoxia. *Biochem. Pharmacol.* **60**, 1171-1178.

Berti, A., Rigacci, S., Raugei, G., Degl'Innocenti, D., Ramponi, G. (1994) Inhibition of cellular response to platelet-derived growth factor by low Mr phosphotyrosine protein phosphatase overexpression. *FEBS Lett.* **349**, 7-12.

Bhadriraju, K., Yang, M., Alom Ruiz, S., Pirone, D., Tan, J., Chen, C. S. (2007) Activation of ROCK by RhoA is regulated by cell adhesion, shape, and cytoskeletal tension. *Exp. Cell Res.* **313**, 3616-3623.

Bijlsma, M. F., Peppelenbosch, M. P., Spek, C. A. (2006) Hedgehog morphogen in cardiovascular disease. *Circulation.* **114**, 1985-91.

Bijlsma, M. F., Peppelenbosch, M. P., Spek, C. A., Roelink, H. (2008) Leukotriene synthesis is required for hedgehog-dependent Neurite projection in neuralized embryoid bodies but not for motor neuron differentiation. *Stem. Cells.* (In Press).

Bikerdal-Hansen, H. (1995) Proteolytic remodeling of extracellular matrix. *Curr. Opin. Cell Biol.* **7**, 728–35.

Bjorge, J. D., Jakymiw, A., Fujita, D. J. (2000) Selected glimpses into the activation and function of Src kinase. *Oncogene*. **19**, 5620-5635.

Boutahar, N., Guignandon, A., Vico, L., Lafage-Proust, M. H. (2004) Mechanical strain on osteoblasts activates autophosphorylation of Focal adhesion kinase and proline-rich tyrosine kinase 2 tyrosine sites involved in ERK activation. *The Journal of Biological Chemistry*. **279**, 30588-30599.

Brown, M. C., Perrotta, J. A., Turner, C. E. (1996) Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. *J. Cell Biol.* **135,** 1109-1123.

Bucciantini, M., Chiarugi, P., Cirri, P., Taddei, L., Stefani, M., Raugei, G., Nordlund, P., Ramponi, G. (1999) The low Mr phosphotyrosine protein phosphatase behaves differently when phosphorylated at Tyr131 or Tyr132 by Src kinase. *FEBS Lett.* **456**, 73-78.

Carlier, M. F., Ressad, F., Pantaloni, D. (1999) Control of actin dynamics in cell motility. Role of ADF/cofilin. *J. Biol. Chem.* **274,** 33827-33830.

Carvalho, H. F, Recco-Pimentel, S.M. A célula 2001. 1ª edição. São Paulo: Mande. 2001

Caselli, A., Marzocchini, R., Camici, G., Manao, G., Moneti, G., Pieraccini, G., Ramponi, G. (1998) The inactivation mechanism of low molecular weight phosphotyrosine-protein phosphatase by H_2O_2. *J. Biol. Chem*. **273**, 32554-32560.

Chambers, T. J. (1985) The pathobiology of the osteoclast. *J. Clin. Pathol.* **38,** 241-252.

Chan, P. Y., Kanner, S. B., Whitney, G., Aruffo, A. (1994) A transmembrane-anchored chimeric focal adhesion kinase is constitutively activated and phosphorylated at tyrosine residues identical to pp125FAK. *J. Biol. Chem.* **269**, 20567-20574.

Chang, H. C., Cho, C. Y., Hung, W. C. (2007) Downregulation of RECK by promoter methylation correlates with lymph node metastasis in non-small cell lung cancer. *Cancer Sc.* **98**,169-173.

Chen, H. C., Appeddu, P. A., Parsons, J. T., Hildebrand, J. D., Schaller, M. D., Guan, J. L. (1995) Interaction of focal adhesion kinase with cytoskeletal protein talin. *J. Biol. Chem.* **270**, 16995-16999.

Chen, J. K., Taipale, J., Cooper, M. K., Beachy, P. A. (2002) Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes Dev.* **16**, 2743–2748.

Chengalvala, M.V., Bapat, A. R., Hurbult, W. W., Kostek, B., Gonder, D. S., Mastroeni, R. A., Frail, D. E. (2001) Biochemical characterization of osteo-testicular protein tyrosine

phosphatase and its functional significance in rat primary osteoblasts. *Biochemistry*. **40**, 814-21.

Chiarugi, P., Cirri, P., Marra, F., Raugei, G., Camici, G., Manao, G., Ramponi, G. (1997) LMWPTP is a negative regulator of insulin-mediated mitotic and metabolic signaling, *Biochem. Biophys. Res. Commun*. **238**, 676-682.

Chiarugi, P., Cirri, P., Marra, F., Raugei, G., Fiaschi, T., Camici, G., Manao, G., Romanelli, R. G., Ramponi, G. (1998) The Src and signal transducers and activators of transcription pathways as specific targets for low molecular weight phosphotyrosine-protein phosphatase in platelet-derived growth factor signaling. *J. Biol. Chem.* **273**, 6776-6785.

Chiarugi, P., Cirri, P., Taddei, L., Giannoni, E., Camici, G., Manao, G., Raugei, G., Ramponi, G. (2000) The low M(r) protein-tyrosine phosphatase is involved in Rho-mediated cytoskeleton rearrangement after integrin and plateletderived growth factor stimulation. *J. Biol. Chem.* **275**, 4640-4646.

Chiarugi, P., Taddei, M. L., Cirri, P., Talini, D., Buricchi, F., Camici, G., Manao, G., Raugei, G., Ramponi, G. (2000) Low molecular weight protein-tyrosine phosphatase controls the rate and the strength of NIH-3T3 cells adhesion through its phosphorylation on tyrosine 131 or 132. *J. Biol. Chem.* **275**, 37619-37627.

Chiusaroli, R., Knobler, H., Luxenburg, C., Sanjay, A., Granot-Attas, S., Tiran, Z. (2004) Tyrosine phosphatase epsilon is a positive regulator of osteoclast function *in vitro* and *in vivo*. *Mol. Biol. Cell.* **15**, 234-244.

Cirri, P., Chiarugi, P., Taddei, L., Raugei, G., Camici, G., Manao, G., Ramponi, G. (1998) Low molecular weight protein-tyrosine phosphatase tyrosine phosphorylation by c-Src

during platelet-derived growth factor-induced mitogenesis correlates with its subcellular targeting. *J. Biol. Chem.* **273**, 32522-32527.

Clark, J. C., Thomas, D. M., Choong, P. F., Dass, C. R. (2007) RECK-a newly discovered inhibitor of metastasis with prognostic significance in multiple forms of cancer. *Cancer Metastasis Rev.* **26**, 675-683.

Clark, S. F., Martin, S., Carozzi, A. J., Hill, M. M., James, D. E. (1998) Intracellular localization of phosphatidylinositide 3-kinase and insulin receptor substrate-1 in adipocytes: potential involvement of a membrane skeleton. *J. Cell Biol.* **140**, 1211-1225.

Coelho, M. J., Cabral, A. T., Fernandes, M. H. (2000a) Human bone cell cultures in biocompatibility testing. Part I: osteoblastic differentiation of serially passaged human bone marrow cells cultured in alpha-MEM and in DMEM. *Biomaterials*. **21**, 1087-1094.

Coelho, M. J., Fernandes, M. H. (2000b) Human bone cell cultures in biocompatibility testing. Part II: effect of ascorbic acid, beta-glycerophosphate and dexamethasone on osteoblastic differentiation. *Biomaterials*. **21**, 1095-1102.

Condeelis, J. (2001) How is actin polymerization nucleated *in vivo*? *Trends Cell Biol*. **11**, 288-293.

Cooper, J. A., Gould, K. L., Cartwright, C. A., Hunter, T. (1986) Tyr527 is phosphorylated in pp60c-src: implications for regulation. *Science*. **231**, 1431-1434.

Curran, S., Murray, G. I. (1999) Matrix metalloproteinases in tumour invasion and metastasis. *J. Path.* **189**, 300-308.

Davidson, J. S., Baumgarten, I. M., Harley, E. H. (1986) Reversible inhibition of intercellular junctional communication by glycyrrhetinic acid. *Biochem. Biophys. Res. Commun.* **134**, 29–36.

Davies, J. P., Chen, F. W., Ioannou, Y. A. (2000) Transmembrane molecular pump activity of Niemann-Pick C1 protein. *Science*. **290**, 2295–2298.

Day, T. F., Yang, Y. (2008) Wnt and hedgehog signaling pathways in bone development. *J. Bone Joint Surg. Am.* **90**, 19-24.

de Jesus, M. B., Zambuzzi W. F., Sousa R. R. R., Areche C., Souza A. C. S., Aoyama H., Schmeda-Hirschmann G., Rodríguez J. A. , Brito A. R. M. S., Peppelenbosch M. P., den Hertog J., de Paula E., Ferreira C.V. (2008). Ferruginol suppresses survival signaling pathways in androgen-independent human prostate cancer cells. Biochimie, (*In Pres*, doi: 10.1016/j.biochi.2008.01.011).

de Souza Queiroz, K. C., Zambuzzi, W. F., Santos de Souza, A. C., da Silva, R. A., Machado, D., Justo, G. Z., Carvalho, H. F., Peppelenbosch, M. P., Ferreira, C. V. (2007) A possible anti-proliferative and anti-metastatic effect of irradiated riboflavin in solid tumours. *Cancer Lett.* **258**, 126-134.

Declercq, H., Van de Vreken, N., De Mayer, E., Verbeeck, R., Schacht, E., De Ridder, L., Cornelissen, M. (2004) Isolation, proliferation and differentiation of osteoblastic cells to study cell/biomaterial interactions: comparison of different isolation techniques and source. *Biomaterials*. **25**, 757-768.

den Hertog J., Ostman A., Böhmer F. D. (2008) Protein tyrosine phosphatases: regulatory mechanisms. *FEBS J*. **275**:831-47.

den Hertog, J., Groen, A., van der Wijk, T. (2005) Redox regulation of protein-tyrosine phosphatases. *Arch. Biochem. Biophys.* **434**, 11–15.

den Hertog, J., Pals, C. E., Peppelenbosch, M. P., Tertoolen, L. G., de Laat, S. W., Kruijer, W. (1993) Receptor protein tyrosine phosphatase alpha activates pp60c-src and is involved in neuronal differentiation. *EMBO J.* **12**, 3789-3798.

Diks, S. H., Kok, K., O'Toole, T., Hommes, D. W., van Dijken, P., Joore, J., peppelenbosch, M. P. (2004) Kinome profiling for studying lipopolysaccharide signal transduction in human peripheral blood mononuclear cells. *J. Biol. Chem.* **279**, 49206–49213.

Diks, S. H., Peppelenbosch, M. P. (2004) Single cell proteomics for personalised medicine. *Trends Mol. Med.* **10**, 574-577.

Dixon P. H., Christie P. T., Wooding C., Trump D., Grieff M., Holm I. (1998). Mutational analysisof PHEX gene inX-linked hypophosphatemia. *J Clin Endocrinol Metabol.* **83,** 3615-23.

Doty, S. B. (1981) Morphological evidence of gap junctions between bone cells. *Calcif. Tissue Int.* **33**, 509–512.

Duncan, R. L., Turner, C. H. (1995) Mechanotransduction and the functional response of bone to mechanical strain. *Calcified Tissue Intermational*. **57**, 344-358.

Eckstein, F. (2007) The versatility of oligonucleotides as potential therapeutics. *Expert Opin Biol Ther.* **7**, 1021-1034.

Everts, V., Delaissé, J. M., Korper, W., Jansen, D. C., Tigchelaar-Gutter, W., Saftig, P., Beertsen, W. (2002) The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation. *J. Bone Miner Res.* **17**, 77–90.

Fauman, E. B., Saper, M. A. (1996) Structure and function of the protein tyrosine phosphatases. *Trends Biochem. Sci.* **21**, 413-417.

Ferreira, C. V., Justo, G. Z., Souza, A. C., Queiroz, K. C., Zambuzzi, W. F., Aoyama, H., Peppelenbosch, M. P. (2006) Natural compounds as a source of protein tyrosine phosphatase inhibitors: application to the rational design of small-molecule derivatives. *Biochimie.* **88**, 1859-1873.

Finke, B., Luethen, F., Schroeder, K., Mueller, P. D., Bergemann, C., Frant, M., Ohl, A., Nebe, B. J. (2007) The effect of positively charged plasma polymerization on initial osteoblastic focal adhesion on titanium surfaces. *Biomaterials.* **28**, 4521-4534.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature.* **391**, 806-811.

Fornoni, A., Cornacchia, F., Howard, G. A., Roos, B. A., Striker, G. E., Striker, L. J. (2001) Cyclosporin A affects extracellular matrix synthesis and degradation by mouse MC3T3-E1 osteoblasts *in vitro. Nephrol. Dial Transplant.* **16**, 500-505.

Franceschi, R. T., Iyer, B. S. (1992) Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *J. Bone Miner. Res.* **7**, 235-246.

Francis F., Rowe P. S. N., Econs M. J., Oudet C, Strom T (1995). M. A gene (PEX) with homologiesto endopeptides is mutated in patients with X-linked hypophosphatemic rickets. *Nature Genetics*. **11,** 130-6.

Frank-Kamenetsky, M., Zhang, X. M., Bottega, S., Guicherit, O., Wichterle, H., Dudek, H., Bumcrot, D., Wang, F. Y., Jones, S., Shulok, J., Rubin, L. L., Porter, J. A. (2002) Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. *J Biol.* **1**, 10.

Franz-Odendaal, T. A., Hall, B. K., Witten, P. E. (2006) Buried alive: how osteoblasts become osteocytes. *Dev. Dyn.* **235**, 176-190.

Freshney, R. I. (2000) Culture of animal cells: a manual of basic technique. 4ed. New York, Wiley-liss.

Geahlen, R. L., Handley, M. D., Harrison, M. L. (2004) Molecular interdiction of Src-family kinase signaling in hematopoietic cells. *Oncogene*. **23**, 8024-8032.

Geoffroy, V., Marty-Morieux, C., le Goupil, N., Clement-Lacroix, P., Terraz, C., Frain, M., Roux, S., Rossert, J., de Vernejoul, M. C. (2004) *In vivo* inhibition of osteoblastic metalloproteinases leads to increased trabecular bone mass. *J. Bone Miner. Res*. **19**, 811– 822.

Gomes-Marcondes, M. C., Tisdale, M. J. (2002) Induction of protein catabolism and the ubiquitin-proteasome pathway by mild oxidative stress. *Cancer Lett.* **180**, 69–74.

Gould, K. L., Hunter, T. (1988) Platelet-dericed growth factor induces multisite phosphorylation of p660 src and increases its protein tyrosine kinase activity. *Mol. Cell. Biol.* **8**, 3345-3356.

Granjeiro, J. M., Ferreira, C. V., Jucá, M. B., Taga, E. M., Aoyama, H. (1997b) Bovine kidney low molecular weight acid phosphatase: FMN-dependent kinetics. *Biochem. Mol. Biol. Int.* **41**, 1201-1208.

Granjeiro, J. M., Taga, E. M., Aoyama, H. (1997a) Purification and characterization of a low-molecular-weight bovine kidney acid phosphatase. *An. Acad. Bras. Ci.* **69**, 451-460.

Gristina, A. (1987) Biomaterial-centered infection: Microbial adhesion versus tissue integration. *Science.* **237**, 1588-1595.

Gristina, A. (1994) Implant failure and the immuno-incompetent fibro-inflammatory zone. *Clin. Orthop.* **298**, 106-118.

Groen, A., Lemeer, S., van der Wijk, T., Overvoorde, J., Heck, A. J., Ostman, A., Barford, D., Slijper, M., den Hertog, J. (2005) Differential oxidation of protein-tyrosine phosphatases. *J. Biol. Chem.* **280**, 10298-10304.

Gross, S., Knebel, A., Tenev, T., Neininger, A., Gaestel, M., Herrlich, P., Bohmer, F. D. (1999) Inactivation of protein-tyrosine phosphatases as mechanism of UV-induced signal transduction. *J. Biol. Chem.* **274**, 26378–26386.

Gu, G., Nars, M., Hentunen, T. A., Metsikko, K., Vaananen, H. (2006) Isolated primary osteocytes express function Gap junctions *in vitro*. *Cell Tissue Res.* **323**, 263-271.

Habig, W. H., Pabst, M. J., Jakoby, W. B. (1974) Glutathione S-transferases. The first step in mercapturic acid formation. *J. Biol. Chem.* **249**, 7130–7139.

Hadjidakis, D. J., Androulakis, I. I. (2006) Bone remodeling. *Ann. N. Y. Acad. Sci.* **1092**, 385-396.

Hamilton, A. J., Baulcombe, D. C. (1999) Species of small antisense RNA in posttranscriptional gene silencing in plants. *Science.* **286**, 950-952.

Haneda, M., Sugimoto, T., Kikkawa, R. (1999) Mitogen-activated protein kinase phosphatase: a negative regulator of the mitogen-activated protein kinase cascade. *Eur. J. Pharmacol.* **365**, 1-7.

Hannas, A. R., Pereira, J. C., Granjeiro, J. M., Tjaderhane, L. (2007) The role of matrix metalloproteinases in the oral environment. *Acta Odontol. Scand.* **65**, 1-13.

Hartree, E. F. (1972) Determination of proteins: a modification of Lowry method that give a linear photometric response. *Anal. Biochem.* **48**, 422-427.

Hatori, K., Sasano, Y., Takahashi, I., Kamakura, S., Kagayama, M., Sasaki, K. (2004) Osteoblasts and osteocytes express MMP2 and -8 and TIMP1, -2, and -3 along with extracellular matrix molecules during appositional bone formation. *Anat. Rec. Discov. Mol. Cell Evol. Biol.* **277**, 262-271.

Heath, J. K., Atkinson, S. J., Meikle, M. C., Reynolds, J. J. (1984) Mouse osteoblasts synthesize collagenase in response to bone resorbing agents. *Biochim. Biophys. Acta.* **802**, 151–154.

Heino, J. (2007) The collagen family members as cell adhesion proteins. *Bioessays.* **29**, 1001-1010.

Herrmann, M., Vaudaux, P. E., Pittet, D., Auckenthaler, R., Lew, P. D., Schumacher-Perdreau, F., Peters, G., Waldvogel, F. A. (1988) Fibronectin, fibrinogen, and laminin act as mediators of adherence of clinical staphylococcal isolates to foreign material. *J. Infect. Dis*. **158**, 693-701.

Hitomi, K., Torii, Y., Tsukagoshi, N. (1992) Increase in the activity of alkaline phosphatase by L-ascorbic acid 2- phosphate in a human osteoblast cell line, HuO-3N1J. *Nutr. Sci. Vitaminol.* **38**, 535-544.

Holm I. A., Huang X., Kunkel L. M. (1997). Mutational analysis of the PEX gene in patients with X-linked hypophosphatemic rickets. *Am J Hum Gene.t* **60,** 790-7.

Hou, P., Troen, T., Ovejero, M. C., Kirkegaard, T., Andersen, T. L., Byrjalsen, I., Ferreras, M., Sato, T., Shapiro, S. D., Foged, N. T., Delaissé, J. M. (2004) Matrix metalloproteinase-12 (MMP-12) in osteoclasts: new lesson on the involvement of MMPs in bone resorption. *Bone*. **34**, 37–47.

Hu, B., Kapila, Y. L., Buddhikot, M., Shiga, M., Kapila, S. (2000) Coordinate induction of collagenase-1, stromelysin-1 and urokinase plasminogen activator (uPA) by the 120-

kDa cell-binding fibronectin fragment in fibrocartilaginous cells: uPA contributes to activation of procollagenase-1. *Matrix Biol.* **19**, 657-669.

Huang, W., Yang, S., Shao, J., Li, Y. P. (2007) Signaling and transcriptional regulation in osteoblast commitment and differentiation. *Front. Biosci.* **12**, 3068-3092.

Hughes-Fulford, M. (2002) The role of signaling pathways in osteoblast gravity perception. *J Gravit Phys*. **9**, 257-260.

Hunt, D. R., Javanovic, S. A. (1999) Autogenous bone harvesting: a chin graft technique for particulate and monocortical bone blocks. *Int. J. Period Rest Dent.* **19**, 165-173.

Hynes, R. O. (2002) Integrins: bidirectional, allosteric signaling machines. *Cell.* **110**, 673-687.

Jeong, J. C., Lee, J. W., Yoon, C. H., Kim, H. M., Kim, C. H. (2004) Drynariae Rhizoma promotes osteoblast differentiation and mineralization in MC3T3-E1 cells through regulation of bone morphogenetic protein-2, alkaline phosphatase, type I collagen and collagenase-1. *Toxicol. In vitro*. **18**, 829-834.

Jia, Z., Barford, D., Flint, A. J., Tonks, N. K. (1995) Structural basis for phosphotyrosine peptide recognition by protein tyrosine phosphatase 1B. *Science*. **268**, 1754-1758.

Jiang, J. X., Siller-Jackson, A. J., Burra, S. (2007) Roles of gap junctions and hemichannels in bone cell functions and in signal transmission of mechanical stress. *Front Biosci.* **12**, 1450-1462.

Jiang, Y., Goldberg, I. D., Shi, Y. E. (2002) Complex roles of tissue inhibitors of metalloproteinases in cancer. *Oncogene*. **21**, 2245–2252.

Johnson, L. N., Barford, D. (1993) The effect of phosphorylation on the structure and function of proteins. *Ann. Rev. Biophys. Biomol. Struct.* **22**, 199-232.

Josset, Y., Oum'Hamed, Z., Zarrinpour, A., Lorenzato, M., Adnet, J. J., Laurent-Maquin, D. (1999) *In vitro* reactions of human osteoblasts in culture with zirconia and alumina ceramics. *J. Biomed. Mater. Res.* **47**, 481-493.

Kaeffer, B., Benard, C., Blottiere, H. M., Cherbut, C. (1997) Treatment of rat proximal and distal colonic cells with sodium orthovanadate enhances their adhesion and survival in primary culture. *Cell Biol Int*. **21**, 303-314.

Kahari, V. M., Saarialho-Kere, U. (1999) Matrix metalloproteinases and their inhibitors In tumour growth and invasion. *Ann. Med.* **31**, 34.

Kalderon, D. (2000) Transducing the hedgehog signal. *Cell*. **103**, 371–374.

Kamioka, H., Ishihara, Y., Ris, H., Murshid, S. A., Sugawara, Y., Takano-Yamamoto, T., Lim, S. S. (2007) Primary cultures of chick osteocytes retain functional gap junctions between osteocytes and between osteocytes and osteoblasts. *Microsc. Microanal.* **13**, 108-117.

Karsdal, M. A., Henriksen, K., Sorensen, M. G., Gram, J., Schaller, S., Dziegiel, M. H., Heegaard, A. M., Christophersen, P., Martin, T. J., Christiansen, C., Bollerslev, J. (2005) Acidification of the osteoclastic resorption compartment provides insight into the coupling of bone formation to bone resorption. *Am. J. Pathol.* **166**, 467-476.

Klein, E. A., Yung, Y., Castagnino, P., Kothapalli, D., Assoian, R. K. (2007) Cell adhesion, cellular tension, and cell cycle control. *Methods Enzymol.* **426**, 155-175.

Kmieci, T. E., Shalloway, K. D. (1987) Activation and suppression of pp60c-src transforming ability by mutation of its primary sites of tyrosine phosphorylation. *Cell*. **49**, 65-73.

Koebernick, K., Pieler, T. (2002) Gli-type zinc finger proteins as bipotential transducers of Hedgehog signaling. *Differentiation*. **70**, 69–76.

Kong, A. T., Yu, R., Hebbar, V., Chen, C., Owuor, E., Hu, R., Raquel, E., Mandlekar S. (2001) Signal transduction events elicited by cancer prevention compounds. *Mut. Res.* **480-481**, 231-241.

Krebs, E. G. (1993) Nobel Lecture. Protein phosphorylation and cellular regulation. *I. Biosci. Rep.* **13**, 127-142.

Kuusela, P., Hilden, P., Savolainen, K., Vuento, M., Lyytikäinen, O., Vuopio-Varkila, M. (1994) Rapid detection of methicillin-resistant Staphylococcus aureus strains not identified by slide agglutination tests. *J. Clin. Microbiol.* **32**, 143-147.

Kwan, J. A., Schulze, C. J., Wang, W., Leon, H., Sariahmetoglu, M., Sung, M., Sawicka, j., Sims, D. E., Sawicki, I., Schulz, R. (2004) Matrix metalloproteinase-2 (MMP-2) is present in the nucleus of cardiac myocytes and is capable of cleaving poly (ADP-ribose) polymerase (PARP) *in vitro*. *FASEB J.* **18**, 690-702.

Kypta, R. M., Goldberg, Y., Ulug, E. T., Courtneidge, S. A. (1990) Association between the PDEGF receptor and members of the Src family of tyrosine kinases. *Cell*. **62**, 481-492.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., Tuschl, T. (2001) Identification of novel genes coding for small expressed RNAs. *Science*. **294**, 853-58.

Lampasso, J. D., Chen, W., Marzec, N. (2006) The expression profile of PKC isoforms during MC3T3-E1 differentiation. *Int. J. Mol. Med.* **17**, 1125-1131.

Langer, R., Vacanti, J. P. (1993) Tissue engineering. *Science*. **260**, 920-926.

Lee, N. K., Sowa, H., Hinoi, E., Ferron, M., Ahn, J. D., Confavreux, C., Dacquin, R., Mee, P. J., McKee, M. D., Jung, D. Y., Zhang, Z., Kim, J. K., Mauvais-Jarvis, F., Ducy, P., Karsenty, G. (2007) Endocrine regulation of energy metabolism by the skeleton. *Cell*. **130**, 456-469.

Lee, R. C., Feinbaum, R. L., Ambros, V. (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*. **75**, 843-854.

Lietha, D., Cai, X., Ceccarelli, D. F., Li, Y., Schaller, M. D., Eck, M. J. (2007) Structural basis for the autoinhibition of focal adhesion kinase. *Cell.* **129**, 1177-1187.

Lim, J. Y., Dreiss, A. D., Zhou, Z., Hansen, J. C., Siedlecki C. A., Hengstebeck R. W., Cheng J., Winograd, N., Donahue, H. J. (2007) The regulation of integrin-mediated osteoblast focal adhesion and focal adhesion kinase expression by nanoscale topography. *Biomaterials*. **28**, 1787-1797.

Liu, S., Calderwood, D. A., Ginsberg, M. H. (2000) Integrin cytoplasmic domain-binding proteins. *J. Cell Sci.* **113**, 3563-3571.

Lowenberg, M., Tuynman, J., Scheffer, M., Verhaar, V., Vermeulen, L., van Deventer, S., Hommes, D., Peppelenbosch, M. (2006) Kinome analysis reveals nongenomic glucocorticoid receptordependent inhibition of insulin signaling. *Endocrinology.* **147**, 3555–3562.

Mahadev, K., Zilbering, A., Zhu, L., Goldstein, B. J. (2001) Insulin-stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1b *in vivo* and enhances the early insulin action cascade. *J. Biol. Chem.* **276**, 21938–21942.

Maniatopoulos, C., Sodek, J., Melcher, A.H. (1988) Bone formation *in vitro* by stromal cells obtained from bone marrow of Young adult rats. *Cell Tissue Res.* **254**, 317-330.

Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M., Tabin, C. J. (1996) Biochemical evidence that patched is the Hedgehog receptor. *Nature.* **384**, 176–179.

Marzia, M., Sims, N. A., Voit, S., Migliaccio, S., Taranta, A., Bernardini, S., Faraggiana, T., Yoneda, T., Mundy, G. R., Boyce, B. F., Baron, R., Teti, A. (2000) Decreased c-Src

expression enhances osteoblast differentiation and bone formation. *J Cell Biol.* **151**, 311-320.

Meikle, M. C., Bord, S., Hembry, R. M., Compston, J., Croucher, P. I., Reynolds, J. J. (1992) Human osteoblasts in culture synthesize collagenase and other matrix metalloproteinases in response to osteotropic hormones and cytokines. *J. Cell Sci*. **103**, 1093–1099.

Menezes, R., Bramante, C. M., da Silva Paiva, K. B., Letra, A., Carneiro, E., Zambuzzi, W. F., Granjeiro, J. M. (2006) Receptor activator NFkappaB-ligand and osteoprotegerin protein expression in human periapical cysts and granulomas. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* **102**, 404-409.

Meng, T. C., Buckley, D. A., Galic, S., Tiganis, T., Tonks, N. K. (2004) Regulation of insulin signaling through reversible oxidation of the protein-tyrosine phosphatases TC45 and PTP1B. *J. Biol. Chem.* **279**, 37716–37725.

Meng, T. C., Fukada, T., Tonks, N. K. (2002) Reversible oxidation and inactivation of protein tyrosine phosphatases *in vivo*. *Mol. Cell*. **9**, 387–399.

Migliaccio, S., Bernardini, S., Wetsel, W. C., Korach, K. S., Faraggiana, T., Teti, A. (1998) Protein kinase C modulates estrogen receptors in differentiated osteoblastic cells *in vitro*. *Steroids*. **63**, 352-354.

Minden, A., Karin, M. (1997) Regulation and function of the JNK subgroup of MAP kinases. *Biochim. Biophys. Acta.* **1333**, 85-104.

Mizuno, T., Chou, M-Y., Inouye, M. (1984) A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). *Proc. Natl Acad. Sci.* **81**, 1966-1970.

Mizutani, A., Sugiyama, I., Kuno, E., Matsunaga, S., Tsukagoshi, N. (2001) Expression of matrix metalloproteinases during ascorbate-induced differentiation of osteoblastic MC3T3-E1 cells. *J. Bone Miner. Res.* **16**, 2043-2049.

Modesti, A., Marzocchini, R., Raugei, G., Chiti, F., Sereni, A., Magherini, F., Ramponi, G. (1998) Cloning, expression and characterisation of a new human low Mr phosphotyrosine protein phosphatase originating by alternative splicing. *FEBS Lett.* **431**, 111-115.

Mogi, M., Togari, A. (2003) Activation of caspases is required for osteoblastic differentiation. *J. Biol. Chem.* **278**, 47477-47482.

Monteiro, H. P., Arai, R. J., Travassos, L. R. (2008) Protein tyrosine phosphorylation and protein tyrosine nitration in redox signaling. *Antioxid Redox Signal.* **10**, 843-890.

Montgomery, M. K., Xu, S., Fire, A. (1998) RNA as a target of double-stranded RNA-mediated genetic interference in Caenorhabditis elegans. *Proc. Natl Acad. Sci.* **95**, 15502-15507.

Morgan, T. E., Lockerbie, R. O., Minamide, L. S., Browning, M. D., Bamburg, J. R. (1993) Isolation and characterisation of a regulated form of actin depolymerizing factor. *J. Cell Biol.* **122**, 623-633.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *J. Immunol. Meth.* **65**, 55-63.

Murphy, G., Hembry, R. M., McGarrity, A. M., Reynolds, J. J. (1989) Gelatinase (type IV collagenase) immunolocalization in cells and tissues: use of an antiserum to rabbit bone gelatinase that identifies high and low Mr forms. *J. Cell Sci.* **92**, 487-495.

Murphy, S. M., Bergman, M., Morgan, D. O. (1993) Suppression of c-Src activity by C-terminal Src kinase involves the c-Src SH2 and SH3 domains: analysis with Saccharomyces cerevisiae. *Mol. Cell Biol.* **13**, 5290-5300.

Mustelin, T., Vang, T., Bottini, N. (2005) Protein tyrosine phosphatases and the immune response. *Nat. Rev. Immunol.* **5**, 43-57.

Nabeshima, K., Inoue, T., Shimao, Y. T. (2002) Matrix metalloproteinases in tumor invasion: role for cell migration. *Pathol. Int.* **52**, 255-264.

Nagase, H., Woessner, J. F. (1999) Matrix metalloproteinases. *J. Biol. Chem.* **274**, 21491–21494.

Nebreda, A. R., Porras, A. (2000) p38 MAP kinases: beyond the stress response. *Trends Biochem. Sci.* **25**, 257-260.

Nerem, R. M. (1992) Tissue engineering in the USA. *Med. Biol. Eng. Comput*. **30**, CE8-12.

Nomura, S., Takano-Yamamoto, T. (2000) Molecular events caused by mechanical stress in bone. *Matrix Biology*. **19**, 91-96.

Oh, J., Takahashi, R., Kondo, S., Mizoguchi, A., Adachi, E., Sasahara, R. M., Nishimura, S., Imamura, Y., Kitayama, H., Alexander, D. B., Ide, C., Horan, T. P., Arakawa, T., Yoshida, H., Nishikawa, S., Itoh, Y., Seiki. M., Itohara, S., Takahashi, C., Noda, M. (2001) The membrane-anchored MMP inhibitor RECK is a key regulator of extracelular matrix integrity and angiogenesis. *Cell*. **107**, 789-800.

Otey, C. A., Pavalko, F. M., Burridge, K. (1990) An interaction between alpha-actinin and the beta 1 integrin subunit *in vitro*. *J Cell Biol.* **111**, 721-729.

Otsuka, K., Sodek, J., Limeback, H. (1984) Synthesis of collagenase and collagenase inhibitors by osteoblast-like cells in culture. *Eur. J. Biochem.* **145**, 123-129.

Overall, C. M., Wrana, J. F, Sodek, J. (1989) Transforming growth factor-beta regulation of collagenase, 72 kDa-progelatinase, TIMP and PAI-1 expression in rat bone cell populations and human fibroblasts. *Connect Tissue Res.* **20**, 289-294.

Owen, T. A., Aronow, M., Shalhoub, V., Barone, L. M., Wilming, L., Tassinari, M. S., Kennedy, M. B., Pockwinse, S., Lian, J. B., Stein, G. S. (1990) Progressive development of the rat osteoblast phenotype *in vitro*: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J. Cell. Physiol.* **143**, 420-430.

Owen, T. A., Bortell, R., Shahoub, V., Heinrichs, A., Stein, G. S., Lian, J. B. (1993) Postproliferative transcription of the rat osteocalcin gene is reflected by vitamin D-responsive developmental modifications in protein-DNA interactions at basal and enhancer promoter elements. *Proc. Natl. Acad. Sci. USA*. **90**, 1503-1507.

Palumbo, C., Palazzini, S., Marotti, G. (1990) Morphological study of intercellular junctions during osteocyte differentiation. *Bone*. **11**, 401–406.

Peters, G. H., Frimurer, T. M., Olsen, O. H. (1998) Electrostatic evaluation of the signature motif (H/V)CX5R(S/T) in protein-tyrosine phosphatases. *Biochemistry*. **37**, 5383–5393.

Petit, V., Thiery, J. P. (2000) Focal adhesions: structure and dynamics. *Biol. Cell*. **92**, 477-494.

Plas, A. van der, Nijweide, P. J. (1992) Isolation and purification of osteocytes. *J. Bone Miner. Res.* **7**, 389–396.

Plopper, G. E., McNamee, H. P., Dike, L. E., Bojanowski, K., Ingber, D. E. (1995) Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. *Mol. Biol. Cell*. **6**, 1349-1365.

Plotkin, L. I., Manolagas, S. C., Bellido, T. (2002) Transduction of cell survival signals by connexin-43 hemichannels. *J. Biol. Chem.* **277**, 8648-8657.

Quarles, L. D., Yohay, D. A., Lever, L. W., Caton, R., Wenstrup, R. J. (1992) Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an *in vitro* model of osteoblast development. *J. Bone Miner. Res.* **7**, 683-692.

Ra, H. J., Parks, W. C. (2007) Control of matrix metalloproteinase catalytic activity. *Matrix Biol.* **26**,587-596.

Ramponi, G., Stefani, M. (1997) Structural, catalytic, and functional properties of low Mr phosphotyrosine protein phosphatases. Evidence of a long evolutionary history. *Int. J. Biochem. Cell Biol.* **29**, 279-292.

Ramponi, G., Stefani, M. (1997) Structure and function of the low Mr phosphotyrosine protein phosphatases. *Biochim. Biophys. Acta*. **1341**, 137-156.

Raouf, A., Seth, A. (2000) Ets transcription factors and targets in osteogenesis. *Oncogene.* **19**, 6455-6463.

Rey, A., Manen, D., Rizzoli, R., Ferrari, S. L., Caverzasio, J. (2007) Evidences for a role of p38 MAP kinase in the stimulation of alkaline phosphatase and matrix mineralization induced by parathyroid hormone in osteoblastic cells. *Bone*. **41**, 59-67.

Rifas, L., Halstead, L. R., Peck, W. A., Avioli, L. V., Welgus, H. G. (1989) Human osteoblasts *in vitro* secrete tissue inhibitor of metalloproteinases and gelatinase but not interstitial collagenase as major cellular products. *J. Clin. Invest.* **84**, 686-694.

Rigacci, S., Degl'Innocenti, D., Bucciantini, M., Cirri, P., Berti, A., Ramponi, G. (1996) pp60v-src phosphorylates and activates low molecular weight phosphotyrosine protein phosphatase. *J. Biol. Chem.* **271**, 1278-1281.

Rigacci, S., Rovida, E., Bagnoli, S., Dello Sbarba, P., Berti, A. (1999) Low M(r) phosphotyrosine protein phosphatase activity on fibroblast growth factor receptor is not associated with enzyme translocation. *FEBS Lett.* **459**, 191-194.

Robinson, R. A. (1979) Bone tissue: composition and function. *Johns Hopkins Med. J.* **145**, 10-24.

Roskoski, Jr R. (2004) Src protein-tyrosine kinase structure and regulation. *Biochem. Biophys. Res. Commun.* **324**, 1155-1164.

Rovida, E., Rigacci, S., Paccagnini, A., Sbarba, P. D., Berti, A. (1998) The low-molecular-weight phosphotyrosine protein phosphatase, when overexpressed, reduces the mitogenic response to macrophage colony-stimulating factor and tyrosine phosphorylation of its receptor. *Biochem. Biophys. Res. Commun.* **253,** 300-304.

Rowe P. S. N. (1997). The PEX gene: isrole in X-linked rickets, osteomalacia, and bone mineral metabolism. *Exp Nephrol* **5,** 355-63.

Ruiz, I., Altaba, A. (1999) Gli proteins encode context-dependent positive and negative functions: implications for development and disease. *Development*. **126,** 3205–3216.

Salido, M., Vilches, Ji, Gutierrez, J. l., Vilches, J. (2007) Actin cytoskeletal organization in human osteoblasts grown on different dental titanium implant surfaces. *Histol. Histopathol.* **22**, 1355-1364.

Sasahara, R. M., Brochado, S. M., Takahashi, C., Oh, J., Maria-Engler, S. S., Granjeiro, J. M., Noda, M., Sogayar, N. C. (2002) Transcriptional control of the RECK metastasis/angiogenesis suppressor gene. *Cancer Defect Rev.* **26**, 435-443.

Schaller, M. D., Borgaman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., Parsons, J. T. (1992) pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. USA.* **89,** 5192–5196.

Schilaepfer, D. D., Hauck, C. R., Sieg, D. J. (1999) Signaling through focal adhesion kinase. *Prog. Biophys. Mol. Biol*. **71**, 435-478.

Schilaepfer, D. D., Jones, K. C., Hunter, T. (1998) Multiple Grb2-mediated integrin-stimulated signaling pathways to ERK2/mitogen-activated protein kinase: summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. *Mol. Cell Biol*. **18**, 2571-2585.

Schiller, P. C., Mehta, P. P., Roos, B. A., Howard, G. A. (1992) Hormonal regulation of intercellular communication: parathyroid hormone increases connexin 43 gene expression and gap-junctional communication in osteoblastic cells. *Mol. Endocrinol.* **6**, 1433–1440.

Schindeler, A., Little, D. (2006) Ras-MAPK signaling in osteogenic differentiation: friend or foe? *J. Bone Miner. Res.* **21**, 1331–1338.

Schinke, T., Gebauer, M., Schilling, A. F., Lamprianou, S., Priemel, M., Mueldner, C., Neunaber, C., Streichert, T., Ignatius, A., Harroch, S., Amling, M. (2008) The protein tyrosine phosphatase Rptpzeta is expressed in differentiated osteoblasts and affects bone formation in mice. *Bone*. **42**, 524-534.

Schmidt, A., Rutledge, S. J., Endo, N., Opas, E. E., Tanaka, H., Wesolowski, G., Leu, C. T., Huang, Z., Ramachandaran, C., Rodan, S. B., Rodan, G. A. (1996) Protein-tyrosine phosphatase activity regulates osteoclast formation and function: inhibition by alendronate. *Proc. Natl. Acad. Sci. USA*. **93**, 3068-3073.

Service, R. F. (2000) Tissue engineers build new bone. *Science.* **289,** 1498-1500.

Smilenov, L. B., Mikhailov, A., Pelma, R. J., Marcantonio, E. E., Gundersen, G. G. (1999) Focal adhesion motility revealed in stationary fibroblasts. *Science.* **286,** 1172-1174.

Snapp, E. L., Landfear, S. M. (1997) Cytoskeletal association is important for differential targeting of glucose transporter isoforms in Leishmania. *J. Cell Biol.* **139**, 1775-1783.

Soriano, P., Montgomery, C., Geske, R., Bradley, A. (1991) Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell*. **64**, 693-702.

Stein, G. S., Lian, J. B., Owen, T. O. (1990) Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. *FASEB J.* **4**, 3111-3123.

Subramaniam, M., Jalal, S. M., Rickard, D. J., Harris, S. A., Bolander, M. E., Spelsberg, T. C. (2002) Further characterization of human fetal osteoblastic hFOB 1.19 and hFOB/ERα cells: bone formation *in vivo* and karyotype analysis using multicolor fluorescent in situ hybridization. *J. Cell Biochem.* **87,** 9-15.

Sudo, H., Kodama, H. A., Amagai, Y., Yamamoto, S., Kasai, S. (1983) *In vitro* differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J. Cell Biol.* **96**, 191-198.

Sundaresan, M., Yu, Z. X., Ferrans, V. J., Irani, K., Finkel, T. (1995) Requirement for generation of H2O2 for platelet-derived growth factor signal transduction. *Science*. **270**, 296-299.

Suzuki, A., Guicheux, J., Palmer, G., Miura, Y., Oiso, Y., Bonjour, J. P., Caverzasio, J. (2002) Evidence for a role of p38 MAP kinase in expression of alkaline phosphatase during osteoblastic cell differentiation. *Bone*. **30**, 91-98.

Tabernero, L., Evans, B. N., Tishmack, P. A., Van Etten, R. L., Stauffacher, C. V. (1999) The structure of the bovine protein tyrosine phosphatase dimer reveals a potential self-regulation mechanism. *Biochemistry*. **38,** 11651-11658.

Tailor, P., Gilman, J., Williams, S., Couture, C., Mustelin, T. (1997) Regulation of the low molecular weight phosphotyrosine phosphatase by phosphorylation at tyrosines 131 and 132. *J. Biol. Chem.* **272,** 5371-5374.

Taipale, J., Cooper, M. K., Maiti, T., Beachy, P A. (2002) Patched acts catalytically to suppress the activity of Smoothened. *Nature*. **418**, 892–897.

Takahashi, C., Sheng, Z., Horan, T. P., Kitayama, H., Maki, M., Hitomi, X. X., Kitaura, Y., Takai, S., Sasahara, R. M., Horimoto, A., Ikawa, Y., Ratzkin, B. J., Arakawa, T., Noda. M. (1998) Regulation of matrix metalloproteinase-9 and inhibition of tumor invasion by the membrane-anchored glycoprotein RECK. *Proc. Natl. Acad. Sci.* **95**, 13221-13226.

Takayanage, H., Kim, S., Matsuo, K., Suzuki, H., Sato, K., Yokochi, T., Oda, H., Nakamura, K., Ida, N., Wagner, E. F., Taniguchi, T. (2002) RANK-L maintains bone homeostasis through c-Fos-dependent induction of interferon-beta. *Nature*. **416**, 744-749.

Tezuka, K., Nemoto, K., Tezuka, Y., Sato, T., Ikeda, Y., Kobori, M., Kawashima, H., Eguchi, H., Hakeda, Y., Kumegawa, M. (1994) Identification of matrix metalloproteinase 9 in rabbit osteoclasts. *J. Biol. Chem.* **269,** 15006-15009.

Thomas, G. T., Lewis, M. P., Speight, P. M. (1999) Matrix metalloproteinases and oral cancer. *Oral Oncol.* **35**, 227-233.

Turner, C. H., Forwood, M. R., Otter, M. W. (1994) Mechanotransduction in bone: do bone cells act as sensors of fluid flow? *FASEB J*. **8**, 875-878.

Umeda, S., Beamer, W. G., Takagi, K., Naito, M., Hayashi, S., Yonemitsu, H., Yi, T., Shultz, L. D. (1999) Deficiency of SHP-1 protein-tyrosine phosphatase activity results in heightened osteoclast function and decreased bone density. *Am. J. Pathol*. **155,** 223-233.

van Baal, J. W., Diks, S. H., Wanders, R. J., Rygiel, A. M., Milano, F., Joore, J., Bergman, J. J., Peppelenbosch, M. P., Krishnadath, K. K. (2006) Comparison of kinome profiles of Barrett's esophagus with normal squamous esophagus and normal gastric cardia. *Cancer Res.* **66**, 11605-11612.

van den Heuvel, M., Ingham, P. W. (1996) Smoothened encodes a receptor-like serpentine protein required for hedgehog signalling. *Nature*. **382,** 547–551.

Verborgt, O., Gibson, G., Schaffler, M. B. (2000) Loss of osteocyte integrity in association with microdamage and bone remodeling after fatigue *in vivo*. *J. Bone Miner. Res*. **15**, 60–67.

Versteeg, H. H., Nijhuis, E., Van Den Brink, G. R., Evertzen, M., Pynaert, G. N., Deventer, S. J. W., Coffer, P. J., PePPelenbosch, M. P. (2000) A new phosphospecific cell-based ELISA for p42/p44 mitogen-activated protein kinase (MAPK), p38 MAPK, protein kinase B and cAMP-response-element-binding protein. *Biochem. J.* **350,** 717-722.

Visse, R., Nagasse, H. (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ. Res.* **92**, 827-839.

Von Wichert, G., Haimovich, B., Feng, G. S., Sheetz, M. P. (2003) Force-dependent integrin-cytoskeleton linkage formation requires down-regulation of focal complex dynamics by *SHP*2. *EMBO J*. **22**, 5023-5035.

Vu, T. H., Werb, Z. (2000) Matrix metalloproteinases, effectors of development and normal physiology. *Genes Dev*. **14,** 2123–2133.

Walton, K. M., Dixon, J. E. (1993) Protein tyrosine phosphatases. Annu. *Rev. Biochem.* **62**, 101-120.

Wang Y., McMahon A. P., Allen B. L. (2007). Shifting paradigms in Hedgehog signaling. *Curr Opin Cell Biol*. **9**, 159-65.

Wang, D., Christensen, K., Chawla, K., Xiao, G., Krebsbach, P. H., Fanceschi, R. T. (1999) Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct *in vitro* and *in vivo* differentiation/mineralization potential. *J. Bone Miner. Res.* **14**, 893-903.

Wilson, C. J., Cleeg, R. E., Leavesley, D. I., Pearcy, M. J. (2005) Mediation of biomaterial-cell interactions by adsorbed proteins: a review. *Tissue Eng.* **11**, 1-18.

Xu, W., Doshi, A., Lei, M., Eck, M. J., Harrison, S. C. (1999) Crystal structure of c-Src reveal features of itsautoinhibitory mechanism. *EMBO J.* **18**, 188-197.

Yano, C. L., Marcondes, M. C. (2005) Cadmium chloride-induced oxidative stress in skeletal muscle cells *in vitro*. *Free Radic. Biol. Med.* **39**, 1378-1384.

Yashikawa, T. (2000) Bone reconstruction by cultured bone graft. *Mat. Sci. Eng. C.* **13,** 29-37.

Yellowley, C. E., Li, Z., Zhou, Z., Jacobs, C. R., Donahue, H. J. (2000) Functional gap junctions between osteocytic and osteoblastic cells. *J. Bone Miner. Res.* **15**, 209-217.

Yonezawa, N., Nishida, E., Iida, K., Yahara, I., Sakai, H. (1990) Inhibition of the interactions of cofilin, destrin and deoxyribonuclease I with actin by phoshoinositides. *J. Biol. Chem.* **265,** 8382-8386.

Zaildel-Bar, R., Itzkovitz, S., Maàyan, A., Iyengar, R., Geiger, B. (2007) Functional atlas of the integrin adhesome. *Nat. Cell Biol.* **9,** 858-867.

Zhang, Z. Y., Dixon, J. E. (1993) Active site labeling of the Yersinia protein tyrosine phosphatase: the determination of the pKa of the active site cysteine and the function of the conserved histidine 402. *Biochemistry*. **32**, 9340–9345.

Zhang, Z., Jimi, E., Bothwell, A. L. (2003) Receptor activator of NF-kappa B ligand stimulates recruitment of SHP-1 to the complex containing TNFR-associated factor 6 that regulates osteoclastogenesis. *J. Immunol.* **171**, 3620-3626.

Zheng, X. M., Resnick, R. J., Shalloway, D. (2000) A phosphotyrosine displacement mechanism for activation of Src by PTPa. *EMBO J.* **19**, 964-978.

Zimmermann, T. S., Lee, A. C., Akinc, A., Bramlage. B., Bumcrot. D., Fedoruk. M. N., Harborth, J., Heyes, J. A., Jeffs, L. B., John, M., Judge, A. D., Lam, K., McClintock, K., Nechev, L. V., Palmer, L. R., Racie, T., Röhl, I., Seiffert, S., Shanmugam, S., Sood, V., Soutschek, J., Toudjarska, I., Wheat. A. J., Yaworski, E., Zedalis, W., Koteliansky, V., Manoharan, M., Vornlocher, H. P., MacLachlan, I. (2006) RNAi-mediated gene silencing in non-human primates. *Nature*. **441**, 111-114.

Capítulo 7

Atividades não relacionadas ao projeto de tese (2005-2008)

PUBLICAÇÕES

1) Ferreira CV, Justo GZ, Souza AC, Queiroz KC, **Zambuzzi WF**, Aoyama H, Peppelenbosch MP (2006). Natural compounds as a source of protein tyrosine phosphatase inhibitors: application to the rational design of small-molecule derivatives. **Biochimie.** 88(12):1859-73.

2) de Souza Queiroz KC, **Zambuzzi WF**, Santos de Souza AC, da Silva RA, Machado D, Justo GZ, Carvalho HF, Peppelenbosch MP, Ferreira CV. (2007). A possible anti-proliferative and anti-metastatic effect of irradiated riboflavin in solid tumours. **Cancer Letters**, 258(1):126-34.

3) de Jesus MB, **Zambuzzi WF**, Sousa RRR, Areche C, Souza ACS, Aoyama H, Schmeda-Hirschmann G, Rodríguez JA, Brito ARMS, Peppelenbosch MP, den Hertog J, de Paula E, Ferreira CV (2008). Ferruginol suppresses survival signaling pathways in androgen-independent human prostate cancer cells. **Biochimie**, (*In Pres*, doi: 10.1016/j.biochi.2008.01.011).

4) Accorsi-Mendonça T, Paiva KB, **Zambuzzi WF**, Cestari TM, Lara VS, Sogayar MC, Taga R, Granjeiro JM (2008). Expression of matrix metalloproteinases-2 and -9 and RECK during alveolar bone regeneration in rat. **J Mol Histol**. 39(2):201-8.

5) de Fátima A, **Zambuzzi WF**, Modolo LV, Tarsitano CAB, Hyslop S, de Carvalho JE, Salgado I, Ferreira CV, Pilli RA. Cytotoxicity of Goniothalamin Enantiomers in Renal Cancer Cells: Involvement of Nitric Oxide and Apoptotic Pathways. (submetido para **Chemico-Biological Interactions**).

7) Sérgio Bertazzo, **Willian F. Zambuzzi**, Helder da Silva, Carmen V. Ferreira, Celso Bertran. Bioactivation of Alumina by Surface Modification: A Promising Alternative for Improving the Applicability of Alumina in Bone Repair. (Submetido para **Clinical Oral Implants Research**).

CO-ORIENTAÇÃO DE ALUNOS DE INICIAÇÃO CIENTÍFICA

1. Marylia Marqui Boff (2006-2007) **- Mecanismos Moleculares Disparados Pela Associação Riboflavina/Quimioterápicos Em Células De Câncer De Próstata-** (PIBIC-CNPq).

2. Mônica Ruzon (2006-2007) – **Investigação Bioquímica dos Efeitos Disparados pela Associação Resveratrol/Mitoxantrona no Tratamento de Células (PC-3).**

3. Rodolpho Cammarosano de Lima (2007-atual) - **Moduladores Estratégicos Da Invasibilidade de Células de Câncer De Próstata: Abordagem Química E Gênica** (FAPESP).

4. Luisa Leite Peres (2008-atual) – **Uso do silencimanto da LMWPTP (RNAi) para o estudo da fosforilação de Src quinase (Y_{416}-Src).**

ANEXOS

A tabela abaixo mostra as diferenças estatisticamente significante da fosforilação das seqüências consenso de peptídeos entre os grupos controle (2D) e o grupo onde as células foram cultivadas sobre o Matrigel.

Table 1. Significantly differentially phosphorylated consensus peptides

Spot	Protein Name	ID Protein	Sequence	Put. Kinase	*t*-Test	Fold
600	3-Phosphoinositide dependent protein kinase 1	NP_002604	TTSQLYDAVPI	c-Src	0,028	0,2
685	Acetyl-CoA carboxylase alpha	NP_942131	IRSSMSGLHLV	AMPK	0,035	1,3
668	Aralkylamine N-acetyltransferase	NP_001079	AQRRHTLPASE	PKA	0,012	0,3
128	ATP citrate lyase	NP_001087	STPAPSRTASF	GSk3	0,016	0,1
211	Beta glucuronidase	NP_000172	VVANGTGTQGQ	Beta-G1	0,036	-2,4
505	Beta-adrenergic receptor kinase1	NP_001610	PLVQRGSANGL	PKA	0,049	-3,0
749	Beta-catenin	NP_001895	HWQQQSYLDSG	CK2-beta	0,021	1,3
595	B-Myb	NP_002457	DNTPHTPTPFK	CDk2	0,013	-1,6
133	BRCA1	NP_009225	ASGLSSQSDIL	ATM	0,047	4,3
352	Calmodulin 1	NP_008819	KDGNGYISAAE	CKII	0,034	-2,6
637	cAMP-specific 3',5'-cyclic phosphodiesterase 4B	NP_002591	SQRRESFLYRS	Protein kinase A	0,022	-2,3
621	Caspase 9	NP_001220	RRRFSSLHFMV	ERK1 ERK2 MEK1 MAP2K2	0,014	1,2
657	Caspase 9	NP_001220	VLRPETPRPVD	AKT1	0,020	-0,1
701	CD45	NP_002829	SSDDDSDSEEP	CK2, alpha 1 CK 2, alpha 2	0,007	-2,9
652	CD5	NP_055022	SRLSAYPALEG	Lck Fyn	0,035	-0,1
810	CDC 25A	NP_001780	LLFAASPPPAS	CDC2	0,007	-2,0
554	CDC 25A	NP_001780	LKRSHSDSLDH	CDC2 PIM1	0,047	-2,3
167	Centromeric protein a	NP_001800	GPRRRSRKPEA	Aurora kinase B	0,041	15,2
145	c-Fos	NP_005243	PVVTATPSATA	MAP kinase	0,009	26,8
757	CK2, alpha 1	NP_001886	SVPTPSPLGPL	CK2, alpha 1	0,028	-2,2
441	Coagulation Factor III	NP_001984	SWKENSPLNVS	Protein Kinase C	0,023	-2,8
612	Cofilin 2	NP_068733	MASGVTVNDE	LIMK1	0,006	14,1
396	Colony stimulating factor 1 receptor	NP_005202	QGVDTYVEMRP	Colony stimulating factor 1 receptor	0,006	1,4
28	CRIP2	NP_001303	EERKASGPPKG	cGMP kinase I	0,043	-1,5
752	Crystallin, alpha B	NP_001876	FFPFHSPSRLF	nb	0,020	-4,5
323	c-Src	NP_005408	STEPQYQPGEN	Csk Fgr PTPase	0,040	2,3

104	Cullin 5	NP_003469	MRKKISNAQLQ	Protein kinase A	0,023	2,8
403	Cyclin dependent kinase 2	NP_001789	PVRTYTHEVVT	nb	0,044	0,2
137	Cyclin dependent kinase 5	NP_004926	IGEGTYGTVFK	ABL	0,008	2,1
677	Cyclin dependent kinase inhibitor 2D	NP_001791	LKQGASPNVQD	nb	0,006	1,8
758	DNA topoisomerase II alpha	NP_001058	AEVLPSPRGQR	Proline directed kinase	0,045	-2,2
272	Docking protein 1	NP_001372	KEDPIYDEPEG	Insulin receptor	0,018	1,9
1020	Down syndrome critical region protein 1	NP_004405	ISPPASPPVGW	GSK-3	0,012	-1,7
550	E2F transcription factor 1	NP_005216	RLLDSSQIVII	ATM ATR	0,020	2,7
Spot	**Protein Name**	**ID Protein**	**Sequence**	**Put. Kinase**	***t*-Test**	**Fold**
468	EGF receptor	NP_005219	LPVPEYINQSV	EGF receptor	0,029	-2,0
926	EP4 receptor	NP_000949	ARIGGSRRERS	PKC	0,032	-1,8
817	Estrogen receptor, alpha	NP_000116	PPPQLSPFLQP	ERK2	0,046	4,4
258	FMS-related tyrosine kinase 3	NP_004110	DNEYFYVDFRE	FMS-related tyrosine kinase 3	0,011	2,9
841	GAB1	NP_002030	DKQVEYLDLDL	Insulin receptor EGF receptor	0,046	3,3
778	GAB1	NP_002030	HVSISYDIPPT	EGF receptor	0,028	-6,0
949	GAB2	NP_536739	RERKSSAPSHS	AKT1	0,006	-3,5
545	Glucocorticoid receptor	NP_000167	KETNESPWRSD	nb	0,044	2,2
959	Glycogen synthase 1	NP_002094	ASVPPSPSLSR	Glycogen synthase kinase 3 beta	0,033	-1,5
995	Growth hormone 1	NP_000506	KFDTNSHNDDA	nb	0,034	1,5
957	Guanine nucleotide binding protein, alpha Z polypeptide	NP_002064	HLRSESQRQRR	Protein kinase C	0,014	-3,2
539	Histone deacetylase 1	NP_004955	AEEEFSDSEEE	ck2	0,003	-4,9
227	HLA-B	NP_005505	SAQGSDVSLTA	nb	0,014	2,2
493	HOX B6	NP_061825	SASQLSAEEEE	Casein kinase II, be	0,014	9,0
484	Kinetochore associated 2	NP_006092	YPFALSKSSMY	Never in mitosis gene A-related kinase 2	0,012	-3,5
427	Lamin A/C	CAA27173	SSTPLSPTRIT	cdc2-kinase	0,046	1,5
970	Lck	AAH13200	IEDNEYTAREG	nb	0,001	2,0
59	Lck	AAH13200	IRESESTAGSF	PKC	0,016	-2,6
1024	LIG1	NP_000225	ARVLGSEGEEE	CK2, alpha 1	0,027	-2,2
971	MAPK12	NP_002960	SEMTGYVVTRW	MAP2K3	0,013	2,5
643	Metal regulatory transcription factor 1	NP_005946	KEVKRYQATFE	Tyrosine kinase	0,009	5,5
881	Nucleolar phosphoprotein p130	AAH01883	GEKRASSPFRR	Protein kinase A	0,000	-1,6
730	ORC1	NP_004144	FSEITSPSKRS	Cyclin dependent kinase 2	0,012	-2,1
209	p73	NP_005418	SASPYTPEHAA	CDC2 Cyclin dependent kinase 2 Cyclin E	0,040	-1,9

859	Phosphatidylinositol 3 kinase, catalytic subunit, delta	AAB53966	LAHNVSKDNRQ	PI3K, catalytic subunit, delta	0,042	-13,6
1007	Phospholipase C, beta 3	NP_000923	RKRHNSISEAK	PKG	0,043	-1,5
878	PPAR	NP_005027	AGDLESPLSEE	MAPK	0,043	5,1
1018	PPP1R9B	NP_115984	LPRASSLNENV	PKA	0,033	2,7
624	Protein kinase C, mu	NP_002733	IIGEKSFRRSV	Protein kinase C, mu	0,048	1,3
604	Protein phosphatase inhibitor 2	NP_006232	QEQESSGEEDS	Casein kinase 2	0,009	-1,7
466	Protein tyrosine phosphatase nonreceptor 7	NP_542155	IASVNTPREVT	Erk ERK2 MAPK14	0,002	4,6
819	Protein tyrosine phosphatase, receptor type, alpha	NP_002827	GSHSNSFRLSN	PKC delta	0,001	-2,3
934	Pyruvate dehydrogenase kinase, isoenzyme 1	NP_002601	ALSTDSIERLP	Pyruvate dehydrogenase kinase 1	0,013	-2,0
230	RAP1 GTPase activating protein 1	NP_002876	GSRRSSAIGIE	c-AMP dependent kinase	0,006	2,0
705	Regulator of G protein signaling 16	NP_002919	LKSPAYRDLAA	EGFR kinase	0,022	1,3
Spot	**Protein Name**	**ID Protein**	**Sequence**	**Put. Kinase**	***t*-Test**	**Fold**
475	Ribosomal phosphoprotein large P1	NP_000994	AKKEESEESDD	nb	0,016	-1,9
219	Ribosomal phosphoprotein large P2	NP_000995	EKKEESEESDD	nb	0,008	-3,3
678	Ribosomal protein S6 kinase alpha 3	NP_004577	KTPKDSPGIPP	EphB2	0,025	4,2
732	Ribosomal S6 kinase 1	NP_002944	EFTSRTPKDSP	ERK2	0,025	-1,4
382	Ryanodine receptor 2	NP_001026	RTRRISQTSQV	PKA	0,023	4,1
162	SMC1	NP_006297	QEEGSSQGEDS	ATM	0,030	1,6
482	Spermidine/spermine N	NP_002961	KRRGASDLSSE	CK2	0,010	2,8
232	SPIB transcription factor	Q01892	PPVPATPYEAF	ERK1	0,019	-2,1
834	STAT5B	NP_036580	PKDEVYSKYYT	c-Src	0,040	3,4
780	STAT6	AAA57193	KDGRGYVPATI	Interleukin 4	0,006	2,2
25	T-Cell acute lymphoblastic leukemia 2	NP_005412	NYQVPSPGPSH	nb	0,043	2,6
163	Telethonin	NP_003664	LRRSLSRSMSQ	Titin	0,020	4,5
151	TNF alpha	NP_000585	MSTESMIRD	Caesin Kinase	0,030	-1,7
740	Vesicle associated membrane protein 4	NP_003753	LLEDDSDEEED	CK2, alpha 1	0,030	7,7
832	Vesicular monoamine transporter 2	NP_003045	PIGEDEESESD	Casein kinase I Casein kinase II	0,046	1,8