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Potencial osteogênico *in vitro* e *in vivo* de células-tronco
mesenquimais de polpa dental e tecido adiposo

São Paulo

2012

Versão corrigida

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Potencial osteogênico *in vitro* e *in vivo* de células-tronco
mesenquimais de polpa dental e tecido adiposo

In vitro and *in vivo* osteogenic potential of mesenchymal stem cells from adipose
tissue and dental pulp

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Introdução

1.1 Medicina Regenerativa

O osso é um tecido conectivo com diversas funções no corpo, como: proteger estruturas internas, fornecer estabilidade e força ao esqueleto, abrigar a medula e atividade metabólica. O sistema esquelético é constituído por diferentes tecidos como o ósseo, cartilaginoso, nervoso, adiposo e hematopoiético, e a estrutura óssea é composta por uma matriz extracelular mineral e colágena, proteínas não colágenas, lipídios, e seus componentes possuem funções metabólicas, mecânicas e endócrinas (Rosen *et al.*, 2008).

Este importante tecido também possui a capacidade de auto-remodelação, auto-reparo e auto-regeneração, mas em alguns casos, dependendo do trauma e/ou doença, este mecanismo pode falhar, especialmente quando ocorrem grandes defeitos ou em caso de câncer e, na ocorrência destas circunstâncias, é necessária uma intervenção (Calvert *et al.*, 2003; Weinand, 2011).

Até o momento, a regeneração de tecidos ósseos tem ganhado muita importância no campo interdisciplinar da medicina regenerativa, estando presente na intersecção entre a engenharia, biologia e medicina. Podemos compreender o termo regeneração como o processo em que o tecido recupera sua condição fisiológica homeostática, recapitulando muitos aspectos da histogênese durante o desenvolvimento embrionário. Já quando ocorre uma falha no processo de

regeneração o tecido é progressivamente reconstituído por infiltrado adiposo ou fibrose (Seong *et al.*, 2010; Bonfanti *et al.*, 2012).

Nos Estados Unidos, no fim do século XX, cerca de 100.000 transplantes e 2.000.000 de implantes ósseos ou de materiais foram realizados. Estima-se que uma a cada cinco pessoas de até 65 anos irão receber um implante/órgão temporário ou permanente. Na Europa, cerca de um milhão de pacientes passam por cirurgias de reconstrução óssea anualmente. Este número tende a crescer devido ao envelhecimento da população e, nesta faixa de idade, as fraturas tendem a ocorrer com maior frequência em virtude do enfraquecimento ósseo ocasionado pela diminuição de cálcio e minerais essenciais para estrutura óssea (Nerem & Sambanis, 1995; Lysaght & Reyes, 2001; D' Aquino *et al.*, 2008; Gómez-Barrena *et al.*, 2011).

Pesquisa também desenvolvida nos Estados Unidos demonstrou que cerca de 500.000 a 600.000 enxertos ósseos foram realizados por ano no país (Greenwald, Boden *et al.*, 2001; Eppley *et al.*, 2005).

Dados referentes ao ano de 2001 da *U.S. Health Cost & Utilization Project* demonstraram que 33.000 procedimentos relacionados a traumas faciais foram realizados. Neste mesmo período, o setor privado de engenharia de tecidos gastou mais do que 600 milhões em pesquisa e desenvolvimento com 3.300 cientistas e suporte, com os investimentos excedendo 3.5 bilhões de dólares (Lysaght & Reyes, 2001).

Devido aos altos investimentos, e resultados ainda não totalmente satisfatórios, a engenharia de tecidos tem focado no desenvolvimento de novas tecnologias e estudos ao longo dos anos envolvendo a procura por terapias que aperfeiçoem os atuais procedimentos.

Os procedimentos utilizados para contornar as limitações ocasionadas por acidentes ou doenças envolvem principalmente o uso de dois tipos de transplantes: o transplante ósseo autólogo (enxerto ósseo é retirado do próprio paciente) e o transplante alogênico (enxerto ósseo é obtido de outro indivíduo imunologicamente compatível). Dentre estes, o mais utilizado atualmente e considerado o “padrão ouro”, é o transplante autólogo, o qual propicia três elementos essenciais para regeneração óssea: um substrato osteocondutivo, fatores osteoindutivos e células osteogênicas. Para melhor esclarecimento do jargão científico usado neste campo: “osteoindutivo” trata-se do processo em que a osteogênese é induzida e “osteocondutivo” pode ser definido como o processo que permite o crescimento ósseo em sua superfície ou através de seus poros, canais ou cânulas. Este tipo de transplante tem como principal característica a obtenção de tecido ósseo de regiões que não as danificadas (sítios secundários, geralmente da crista ilíaca) para ser implantado nas regiões afetadas pela perda óssea (sítio primário) (Wilson-Hench, 1987; Albrektsson & Johansson 2001; Glowacki & Mulliken, 1985; El-Ghannam, 2004).

Mesmo tratando-se da metodologia mais utilizada pelos cirurgiões e considerada o “padrão ouro”, cerca de 30% dos casos relacionados ao transplante ósseo autólogo apresentam taxas de insucesso (Jackson *et al.*, 1986; Scheller *et al.*, 2009).

Dentre as possíveis desvantagens apresentadas pelo transplante ósseo autólogo para a recuperação do tecido ósseo, temos:

- Quantidade limitante de tecido dos sítios doadores (sítio secundário);

- Dor, hematoma e morbidade no sítio doador, ocasionada pelo procedimento de obtenção do enxerto;
- Alta reabsorção óssea durante o processo de cicatrização;
- Alto custo e tempo extenso de recuperação (Abukawa *et al.*, 2006; Stevens *et al.*, 2008; Bhumiratana & Vunjak-Novakovic, 2012).

Com as complicações citadas associadas aos transplantes autólogos, uma alternativa seria o uso do transplante ósseo alogênico, que utiliza enxertos ósseos mantidos em bancos de ossos, provenientes de outros indivíduos, ou o xenotransplante/transplante heterólogo, o qual utiliza enxertos obtidos de animais. Mas estas abordagens também possuem limitações, incluindo incompatibilidade imunológica e podendo suscitar reações imunológicas e transmissão de doenças tanto virais quanto bacterianas (Buck *et al.*, 1989; Stevens *et al.*, 2008; Weinand *et al.*, 2011; Vo *et al.* 2012).

Como uma solução para os possíveis insucessos e desvantagens ocasionados pelos procedimentos de transplante ósseo autólogo, heterólogo e alogênico, temos como estratégia alternativa a bioengenharia de tecidos. Esta é uma área extremamente ampla com o objetivo de reconstrução, reparo e substituição do tecido perdido ou danificado, para a recuperação da arquitetura e função biológica do órgão afetado. (Badylak; Nerem, 2010; Glotzbach *et al.*, 2011).

A bioengenharia de tecidos compreende três principais frentes e estratégias (**Figura 1**), propostas primeiramente por Langer e Vacanti (1993), conhecidas como a tríade: Células progenitoras, biomateriais e sinais. O entendimento do funcionamento de cada um destes pilares é importante para a otimização de processos associados à reconstrução do tecido ósseo: as células progenitoras tem

papel fundamental para o processo de estabelecimento das funções do órgão, assim como na adequação de compatibilidade com o tecido alvo; os sinais utilizados na engenharia de tecidos ósseos podem ser fatores de crescimento, de diferenciação e proliferação que propiciarão o aceleração ou inibição de mecanismos pontuais no processo de formação óssea, como a osteoindução e angiogênese; e finalmente os biomateriais que terão a função de abrigar tanto estas substâncias, bem como permitir a adesão e nutrição das células que serão depositadas nestes (Langer & Vacanti, 1993; Nerem & Sambanis, 1995; Burg *et al.*, 2000; Abukawa *et al.*, 2006; Badylak & Nerem, 2010).

Figura 1. Tríade básica da Bioengenharia de Tecidos que compreendem: Biomateriais, células e substâncias/sinais e fatores. Figura adaptada de Glotzbach *et al.* 2011.

Para que ocorra o desenvolvimento de novas terapias na bioengenharia óssea é necessário tanto o estudo individual de cada um dos três elementos básicos da bioengenharia, como o estudo em conjunto para entender os mecanismos, falhas e adaptações que cada um deverá ter, para que através destes estudos haja um resultado satisfatório e adequado para cada doença (câncer, osteoporose), tipo

ósseo (osso longo, chato), e grau de acometimento (defeito de grandes ou pequenas dimensões).

Para a engenharia de tecidos, a previsão do potencial osteogênico de uma população celular anteriormente ao procedimento cirúrgico é de extrema relevância tanto do ponto de vista econômico, quanto do lado prático, porque tornará possível o isolamento da linhagem ou tipo celular que melhor se diferencie anteriormente ao procedimento. No presente trabalho optamos por abordar dois elementos da tríade básica da engenharia de tecidos ósseos (biomateriais e células) para estudar o **potencial inato** de diferenciação osteoblastogênica *in vitro* e potencial de indução osteogênica *in vivo* **sem indução de fatores externos**.

1.2 Biomateriais

O principal objetivo dos biomateriais na bioengenharia de tecidos e medicina regenerativa é prover uma superfície e área em que células possam aderir, crescer e formar o tecido e órgão desejado. Pode-se afirmar que, atualmente, existem três tipos principais de biomateriais utilizados na bioengenharia, estes são: as cerâmicas, os polímeros e os compostos (Abukawa *et al.*, 2006).

O desenvolvimento destes para substituição dos implantes ósseos autólogos proporciona vantagens significativas, tais como prevenção de cirurgias adicionais para a remoção de osso autólogo, diminuição do tempo cirúrgico, prevenção de transmissão de doenças e reação imunológica no caso de alotransplantes. Além destes fatores os biomateriais são úteis para aqueles pacientes que não possuem

uma quantidade de tecido ósseo suficiente para o transplante autólogo, ou tenha limitações devido a possíveis doenças ósseas (Legeros, 2002; Okuda *et al.*, 2007).

A facilidade e controle da síntese de materiais sintéticos que possuem parâmetros de produção otimizados têm como outras vantagens a oferta ilimitada, forma e características pré-determinadas relacionadas tanto à macro-estrutura (formato tridimensional, diâmetro dos poros), quanto à micro-estrutura (porosidade, interconectividade dos poros). Estas características e detalhes são pertinentes para obtenção do material ideal para cada tipo ósseo (Behraves, Yasko *et al.*, 1999; Lendlein & Langer, 2002; Gunatillake & Adhikari, 2003).

Podem ser considerados biomateriais ideais aqueles que possuem a mesma característica biomecânica análoga do osso autólogo, e podem ser completamente integrados no osso natural através do processo de remodelamento ósseo, em que a atividade osteoclástica e osteoblástica integrará o biomaterial ao osso (Hao *et al.*, 2010).

Para a bioengenharia de tecido ósseo um molde ideal promove a indução e a diferenciação de células progenitoras nele contidas ou circundantes (osteoidução), auxiliar na vascularização guiando a reconstrução óssea em local que não cicatrizaria naturalmente, e permitir a invasão de células do hospedeiro promovendo a integração ao osso do hospedeiro (osteocondução) (Burg *et al.*, 2000; Cavert *et al.*, 2003).

Devido à necessidade e ao avanço das tecnologias e pesquisas na área, já estão disponíveis diversas gerações de biomateriais. Dentre estes temos a primeira geração que tem como principal propriedade a proliferação e aderência celular (cola de fibrina e polímero de ácido poli-L-láctico), comercialmente disponíveis para uso clínico. A segunda geração consiste de uma mistura a partir da primeira

geração e novos materiais que promovem maior neoformação óssea. Como principais exemplos e representantes desta segunda geração temos o β -fosfato tricálcico (β -TCP), ácido poli-L-láctico e β -TCP/hidroxiapatita (HA). Os biomateriais “inteligentes” pertencem à terceira geração, e são compostos por materiais de primeira ou segunda geração tratados com fatores que estimulam o crescimento celular, regeneração de tecidos e facilitam a utilização de células progenitoras (Weinand *et al.*, 2011).

As cerâmicas de hidroxiapatita (HA), utilizadas há décadas em defeitos mandibulares para substituição óssea, apresentam estrutura molecular igual à fase mineral do osso (constituição óssea mineral possui aproximadamente 70% de hidroxiapatita), fazendo com que sejam extremamente osteocompatíveis (El-Ghannam, 2004; Rosso *et al.*, 2005).

O colágeno pode ser considerado um candidato ideal de biomaterial, por possuir o principal componente da matriz extracelular óssea. Esses materiais gelatinosos são na maioria colágenos desnaturados e disponíveis comercialmente como polímeros biodegradáveis. Sua principal característica e vantagem é sua aplicabilidade em diferentes composições e sua flexibilidade nas mudanças de composições e estruturas químicas (Sachlos *et al.*, 2003; Takahashi *et al.*, 2005).

As cerâmicas de fosfato de cálcio e os vidros bioativos foram introduzidos há mais de 30 anos como substitutos ósseos. Estes materiais são considerados bioativos, devido a sua característica de se ligar ao osso e promover a formação óssea. As cerâmicas de fosfato de cálcio mais utilizadas são as de tricálcio fosfato ($\text{Ca}_3 [\text{PO}_4]_2$), que possuem estrutura similar a fase mineral do osso, demonstram habilidade osteocondutivas, suscitam o crescimento de células, e deposição óssea em sua superfície (Ducheyne & Qiu, 1999; El-Ghannam *et al.*, 2004).

Estas cerâmicas possuem propriedades bioativas e osteocondutivas excelentes, sendo as formas mais usadas: o β -tricálcio fosfato [$\text{Ca}_3(\text{PO}_4)_2$, TCP] e a hidroxiapatita [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, HA]. O uso de cerâmicas de β -tricálcio fosfato é restrito em regeneração de pequenos defeitos ósseos devido à sua atividade osteoindutiva deficitária, mas sua grande vantagem está relacionada à suas características bio-reabsorvíveis (Anker *et al.*, 2005; Komaki *et al.*, 2006; Okuda *et al.*, 2007). Estes biomateriais cerâmicos permitem a diferenciação de células-tronco mesenquimais derivadas de medula óssea e contribuem com neoformação óssea *in vivo* (Matsushima *et al.*, 2009).

Outro tipo cerâmico importante, o tricálcio fosfato (TCP) é um material natural por sua composição química. Este já foi utilizado como substituto de enxerto ósseo nas cirurgias ortopédicas e cranio-maxilo-faciais devido a suas vantagens como: produção de acordo com o formato do defeito, propriedades similares ao tecido ósseo, promovendo a adesão de células e formação óssea *in vivo* (Abukawa *et al.*, 2006).

Sob condições fisiológicas, a HA não é completamente degradada e, após a regeneração do osso, ela pode permanecer no sítio em que foi colocada. Uma das tentativas de controle desta degradação *in vivo* é combinar a HA com materiais orgânicos como o colágeno e os co-polímeros, abordagem extensivamente utilizada na bioengenharia de tecidos ósseos (Daculsi *et al.*, 2003). Já o β -Tricálcio fosfato possui a vantagem de possuir uma biodegradação mais acelerada em relação à HA, no entanto este material é frágil quando comparado a HA (Takahashi *et al.*, 2005).

Com todas estas características e propriedades favoráveis, além de sua habilidade de se integrar ao tecido ósseo, os materiais constituídos por HA e TCP, ou a mistura de ambos com um aumento relativo da concentração de Tricálcio

fosfato, resulta em uma maior reabsorção do material pelo organismo após o procedimento. Com este conjunto agindo cada um com sua vantagem, estes materiais tornaram-se os mais estudados e utilizados no reparo de tecidos ósseos (Cancedda *et al.*, 2003).

Uma das maiores limitações no uso da engenharia de tecidos para reparo de fraturas ósseas tem sido a falta de células autólogas em abundância ou um tecido que possa também se diferenciar em matriz óssea sintetizada por osteoblastos, ou estimule o recrutamento de células-tronco para o local da fratura (Hicok *et al.*, 2004). Padronizando o biomaterial mais propício e que se adéque ao modelo que será utilizado, restará escolher o melhor tipo celular que possua características de diferenciação, proliferação e possivelmente seja uma fonte autóloga de células para que não ocorram respostas imunológicas. Não há grande quantidade de trabalhos que comparem os potenciais *in vitro* de células-tronco adultas humanas de diferentes fontes, e nem trabalhos que verifiquem se este potencial *in vitro* encontrado pode ser de alguma forma ditar o potencial *in vivo* destas células. Os resultados e correlação entre os potenciais osteogênicos *in vitro* e *in vivo* ainda são divergentes (Mendes *et al.*, 2004).

1.3 Células-Tronco Mesenquimais (MSCs)

Após a caracterização dos materiais que podem ser utilizados na bioengenharia e, constatada a necessidade da utilização de tipos celulares para aperfeiçoar e promover a osteoindução e construção do órgão desejado, uma importante e crucial tarefa é a escolha do tipo celular a ser utilizado em conjunto com os materiais, ou injetado sistemicamente para a neoformação óssea.

As células-tronco podem ser caracterizadas como o conjunto de células caracterizadas como aquelas que têm tanto a habilidade de auto-renovação, quanto habilidade de dar origem a células diferenciadas. Atualmente as células-tronco adultas são mais indicadas para propostas terapêuticas, pois são consideradas mais seguras para o transplante, com menor capacidade de formar tumores, diferentemente das células-tronco embrionárias (Gafni *et al.*, 2004).

As células-tronco mesenquimais (*Mesenchymal stem cells* – MSCs) podem ser extraídas de diferentes tecidos pós-natais com um protocolo específico para cada caso e suas principais vantagens são: uso autólogo, divisão moderada (sem riscos de formação de teratomas) e o seu comprometimento com tipos celulares específicos. Como desvantagens, este tipo celular pode não prover quantidades suficientes de células para o uso clínico terapêutico ou, o tempo de obtenção da quantidade necessária pode ser muito longa. Quando comparamos as desvantagens apresentadas por cada tipo celular, fica evidente que as questões éticas, reações imunológicas, possibilidade de integração viral e teratogênese ocasionadas pelas células-tronco embrionárias e células-tronco de pluripotência induzida, praticamente comprometem a utilização destas para uso clínico, e torna as células tronco adultas candidatas para terapias e ensaios clínicos (Robey, 2011).

Além das características apresentadas, as células-tronco adultas podem apresentar propriedades importantes como, por exemplo, a capacidade de regular resposta imune (imunomodulação) (Meirelles *et al.*, 2009); alguns autores acreditam que o benefício que este tipo celular pode proporcionar está relacionado com sua capacidade de agir como uma fonte de fatores tróficos. A propriedade de imunomodulação tornou as MSCs candidatas para o uso terapêutico em diversos tipos de doenças, inclusive as relacionadas a reações auto-imunes e imunológicas (Ghannam *et al.*, 2010). Outras propriedades de grande interesse para aplicações clínicas são a capacidade de *homing* (capacidade de migrar preferencialmente para sítios danificados) e também de *trafficking* para serem utilizadas na administração celular sistêmica para tratamento de inflamações (Ren *et al.*, 2012).

Em 2005 a *International Society for Cellular Therapy* (Vancouver, Canada) recomendou o uso do nome *multipotent mesenchymal stromal cell*, também abreviadas como as células-tronco mesenquimais: “MSCs”. Mas em 2011 a *International Society of Stem Cell Research* (Toronto, Canada) permaneceu adotando o termo células-tronco mesenquimais, também abreviadas com a sigla “MSCs” (Horwitz *et al.*, 2005).

Ultimamente têm ocorrido muitas críticas em relação à utilização do termo “células-tronco mesenquimais”, uma das principais ocorre porque a nomenclatura original se baseava na hipótese de que múltiplos tecidos além das linhagens osteogênicas poderiam ser gerados por células-tronco mesenquimais pós-natais, incluindo músculo esquelético, miocárdio, músculo liso, tendão entre outros. No entanto este potencial não foi comprovado em experimentos *in vivo*, e permanece controverso. Outra crítica está ligada à multipotência destas células que não é universalmente aceita porque há uma carência de padronização nos métodos para

isolamento, expansão e identificação destas células (Bianco *et al.*, 2008; Nombela-Arrieta *et al.*, 2011).

Definiremos células-tronco mesenquimais (MSCs), segundo a *International Society for Cellular Therapy position statement*, como todas as células extraídas de tecidos adultos que tenham como características: a multipotência (diferenciações condrogênica, adipogênica e osteogênica) verificada através de marcadores específicos para cada tipo celular; aderência ao plástico, e expressão específica de antígenos de superfície (Ag) positivos (>95%) para *cluster of differentiation* (CD): 13, 29, 44, 73, 90, 105 e 166, e negativos (<2%) 11, 11b, 14, 31, 34, 45 e 62E (Dominici *et al.*, 2006; Robey, 2011).

Com o refinamento de técnicas de extração e pesquisa foram identificadas uma enorme quantidade de fontes de onde podem ser isoladas células-tronco adultas, dentre elas alguns exemplos são: medula óssea (Friedenstein *et al.* 1966), sangue periférico (Zvaifler *et al.*, 2000), sangue de cordão umbilical (Erices *et al.*, 2000), tecidos fetais (Campagnoli *et al.*, 2001), membrana sinovial (De Bari *et al.*, 2001), tecido adiposo (Zuk *et al.*, 2001; Zuk *et al.*, 2002), líquido amniótico (In't anker *et al.*, 2003), cordão umbilical - *cord Wharton's jelly* (Sarugaser *et al.*, 2005), polpa dental adulta (Gronthos *et al.*, 2000) e de dente decíduo (Miura *et al.*, 2003). Recentemente nosso grupo de pesquisa (Centro de Estudos do Genoma Humano- Universidade de São Paulo) identificou duas novas fontes de células-tronco mesenquimais: de músculo orbicular do lábio (Bueno *et al.*, 2009) e de trompas de Falópio (Jazedje *et al.*, 2009).

As fontes de células progenitoras com potencial para aplicação na engenharia de tecido craniofacial que serão abordadas a seguir serão células-

tronco provenientes da medula óssea (**BMSCs**) por sua caracterização ter ocorrido há muito tempo, além dos diversos estudos, testes clínicos e utilização na medicina regenerativa ao longo dos anos, e provenientes da polpa dental adulta (**DPSCs**), dente decíduo (**SHED**) e de tecido adiposo (**ASCs**) por seu potencial uso e vantagens relacionadas à obtenção, osteogênese e proliferação.

Após verificar possíveis diferenças encontradas no ensaio *in vitro* e no modelo de duplo defeito crítico na calvária de Ratos Wistar, o próximo passo na nossa linha de pesquisa será investigar o motivo desta variação no potencial de diferenciação óssea de diferentes fontes de células-tronco. Uma abordagem para possivelmente esclarecer estas variações pode ser feita através do ensaio de *microarray* de expressão para observar o perfil de expressão gênica de células. Esta metodologia, permite observar e comparar a expressão de genes por diferentes tipos celulares em condições padronizadas, neste caso no processo de diferenciação osteogênica (Tamayo *et al.*, 1999; Kerr *et al.*, 2000; Young, 2000).

Um dos importantes itens quando à escolha do tipo celular a ser utilizado na engenharia de tecidos ósseos, é saber qual o potencial osteogênico destas células. Poucos trabalhos abordaram duas frentes (*in vitro* e *in vivo*), para verificar e comparar o potencial osteogênico de diferentes linhagens. Uma importante questão ainda não respondida nesta importante área de medicina regenerativa é se o potencial da uma linhagem no modelo *in vitro* poderia ser condizente, ou de alguma forma predizer o potencial *in vivo*.

Células-tronco provenientes de medula óssea (BMSCs)

As **BMSCs** (*Bone Marrow Mesenchymal Stem Cells*) foram primeiramente descritas por Friedenstein e colaboradores (1966), seu método de obtenção é extremamente doloroso, e expõem os pacientes a riscos de infecção, além da baixa frequência de MSCs que pode ser obtida no interior da medula (~ 1 a cada 10^5 células nucleadas). As BMSCs correspondem a uma população celular rara, que possui uma proporção inversa de quantidade de células de acordo com a idade do indivíduo. Este tipo celular também tem uma diminuição do potencial de diferenciação osteogênica após excessiva proliferação *in vitro* (Friedenstein *et al.*, 1966; Kadiyala *et al.*, 1997; Caplan, 2009; Seong *et al.*, 2010).

Estudos que utilizaram BMSCs em conjunto com biomateriais tridimensionais de hidroxiapatita associada à tricálcio-fosfato em defeitos críticos cranianos em ovelhas, coelhos, roedores e cães comprovaram formação óssea *in vivo* (Ohgushi *et al.*, 1989; Bruder *et al.*, 1998; Kon *et al.*, 2000; Arinzech *et al.*, 2003; Ge *et al.*, 2004).

A capacidade de formação óssea *in vivo* em reparo de modelos animais de defeitos ósseos críticos, foi constatado em diversos experimentos (Kadiyala *et al.*, 1997; Kon *et al.*, 2000; Petite *et al.*, 2000; Shang, Wang *et al.*, 2001; Bidic *et al.*, 2003; Weng *et al.*, 2006), além de sua associação com diferentes tipos e composições de biomateriais e fatores indutores (Wang *et al.*, 2010a; Burastrero *et al.*, 2008; Niemeyer *et al.*, 2010; Zong *et al.*, 2010) e nos transplantes de reparo ósseo humano (Horwitz *et al.*, 2001; Quarto *et al.*, 2001; Vacanti *et al.*, 2001; Jager *et al.*, 2009).

Por meio da análise minuciosa de colônias individuais de BMSCs notam-se diferenças quanto a taxas de proliferação e crescimento, tamanho, formato das células entre colônias clonais de células-tronco de medula óssea, além de haver diferenças relacionadas aos níveis de potencial de diferenciação e, capacidade de se diferenciar em osteoblastos, condrócitos e adipócitos. Comprovando desta forma que existem sub-populações dentro desta população heterogênea extraída de células-tronco mesenquimais de medula (Owen & Friedenstein, 1988; Kuznetsov *et al.*, 1997; Muraglia *et al.*, 2000; Derubeis & Cancedda, 2004).

Outros fatores que podem alterar as características das BMSCs inclusive o potencial osteogênico é a quantidade de passagens em que a cultura foi submetida e, a idade do paciente. Foram observadas diferenças evidentes nas taxas de crescimento e na atividade de fosfatase alcalina entre culturas de BMSCs com diferentes passagens e obtidas de doadores com idades diferentes (Phinney *et al.*, 1999; Mendes *et al.*, 2004).

Nakamura e colaboradores (2009) demonstraram que BMSCs em condições osteoindutivas, secretam osteocalcina para o meio de cultura, podendo ser um indicativo do potencial osteogênico de células-tronco proveniente de medula óssea. É importante ressaltar que este potencial é verificado apenas quando há indução de diferenciação *in vitro* das células (Nakamura *et al.*, 2009).

Mesmo com diversos experimentos utilizando este tipo celular na engenharia de tecidos ósseos, seus pontos negativos tornam-se um empecilho para sua utilização em larga escala para a regeneração de tecidos. A morbidade, dor, possíveis infecções e baixa quantidade de células obtidas criam obstáculos no seu efetivo uso para testes clínicos. Portanto a fonte celular ideal é aquela em que pode ser obtida através de um método pouco invasivo, sem riscos a saúde ou ocasione

morbidade ao paciente, esta população tenha um potencial mínimo de osteogêneses, ou de osteoindução e seja possível obter um número suficiente de células.

Células-tronco provenientes de tecidos Dentais

As células-tronco mesenquimais foram isoladas de diversos tecidos dentais. Primeiramente, Gronthos e colaboradores (2000) conseguiram isolar células da polpa dental de dentes adultos (*Dental Pulp Stem Cell – DPSC*) e, em 2003, Miura e colaboradores isolaram células de dentes decíduos (*Stem cells from Human Exfoliated Deciduous teeth - SHED*). Estas células possuem como características: obtenção extremamente fácil, alta eficiência na sua extração, interatividade com biomateriais na aplicação de engenharia de tecidos, expressão de marcadores de superfície similares a células tronco de medula óssea, células clonogênicas, de rápida proliferação (maior taxa proliferativa comparado as BMSCs), capacidade de diferenciação em uma variedade de tipos celulares como células neurais, osteoblastos, condrócitos, adipócitos e miócitos (Gronthos *et al.*, 2000; ; Kerkis *et al.*, 2006; Laino *et al.*, 2006; Lensch *et al.*, 2006; Mitsiadis *et al.*, 2007; Graziano *et al.*, 2008; Wang *et al.* 2010b). Após transplante *in vivo* em conjunto com um biomaterial cerâmico de hidroxiapatita e tricálcio fosfato, as SHEDs foram capazes de induzir formação óssea, além de também em outros ensaios gerar dentina, e sobreviver no cérebro de camundongos expressando marcadores neurais (Miura *et al.*, 2003).

Diversos experimentos e estratégias já foram feitos para verificar o potencial osteogênico de DPSCs. Laino e colaboradores (2005 e 2006) isolaram

uma sub-população SBP-DPSC (*Stromal bone producing cells*) positiva para os marcadores STRO-1, CD34 e c-kit, e negativo para CD45. Esta sub-população obtida representa cerca de 10% da população total. Demonstrou-se que elas apresentam intensa proliferação em cultura, longa durabilidade, e manutenção de sua multipotência durante muitas passagens (inclusive após congelamento). As SBP foram consideradas células pluripotentes, potencial comprovado através de diferenciação para células musculares, adipócitos, neurônios e osteoblastos. Quando implantadas *in vivo* as SBP-DPSCs formaram osso lamelar bem desenvolvido, com osteócitos presentes nas lamelas ósseas (Laino *et al.*, 2005; Laino *et al.*, 2006a; Laino *et al.*, 2006b; Papaccio *et al.*, 2006).

Outros ensaios *in vivo* desta sub-população de DPSCs utilizando biomateriais poliméricos, feitos de ácido poli-lático e carbonato de trimetileno colocados subcutaneamente em ratos Wistar formaram ossos adultos, inclusive com vasos tanto internamente quanto no exterior do tecido, demonstrando também uma capacidade de angiogênese além da osteoindução comprovada (D'Aquino *et al.*, 2007).

A maior parte dos trabalhos utilizando as SHED em modelos *in vivo* e *in vitro* abordaram somente questões de diferenciação comprovativas, ou seja, se estas células se diferenciam ou não. Deste modo, não promoveram uma comparação efetiva entre linhagens, metodologias, ou foram criados modelos que possam “prever” o potencial de células no modelo *in vivo* (Miura *et al.*, 2003; Kerkis *et al.*, 2006; Wang *et al.* 2010b) .

Em um ensaio clínico, DPSCs foram extraídas dos molares superiores de pacientes, e utilizadas em conjunto com um colágeno esponjoso para reparo de defeitos alveolares da mandíbula decorrentes do procedimento de extração de

terceiros molares (dente do siso). Como resultado após cerca de três meses do procedimento cirúrgico, as DPSCs autólogas em conjunto com um biomaterial, promoveram a completa restauração do defeito alveolar (D'Aquino *et al.*, 2009).

Em um ensaio *in vivo* SHED e BMSCs foram utilizadas para preencher um defeito crítico de 2.7 mm de diâmetro na calvária de camundongos *nude* (camundongos imunossuprimidos) em conjunto com um biomaterial particulado de cerâmica formado por hidroxiapatita e tricálcio fosfato. A quantidade de células utilizadas foi de 2×10^6 , após 6 meses de experimento foi possível constatar que as DPSCs foram capazes de induzir e formar osso *in vivo*, sendo desta forma uma fonte ideal para regeneração óssea (Seo *et al.*, 2008).

Células-tronco provenientes de tecido adiposo (ASCs)

A *International Fat Applied Technology Society* adotou o termo *Adipose-derived Stem Cells* (ASCs) para identificar a população celular multipotente e aderente ao plástico, isolada de tecido adiposo. Este tipo celular é obtido do material rotineiramente descartado após procedimentos em sua maioria estéticos como a lipoaspiração. O aumento no número de casos de obesidade, e de cirurgias plásticas para remoção do gordura tecidual, tem facilitado a obtenção de material para isolamento de ASCs (Gimble *et al.*, 2007; Witkowska-Zimny & Walenko, 2011).

Células-tronco provenientes de tecido adiposo (ASCs) possuem diversas vantagens, como: relativa facilidade de obtenção, mínimo desconforto para o paciente, pode ser feita principalmente em indivíduos adultos, e é possível obter caso necessário grande quantidade de células. Este procedimento pode ser

repetido quantas vezes forem necessárias, e as células extraídas podem ser cultivadas e expandidas *in vitro* para obtenção do número necessário de células (Zuk *et al.*, 2001; De Ugarte *et al.*, 2003; Cowan *et al.*, 2004). As ASCs têm sido utilizadas em experimentos *in vivo*, com aplicações potenciais para cirurgias plásticas e reconstrutivas devido a diversas vantagens como à sua maior taxa de proliferação durante expansão *in vivo* em relação a células BMSCs (Mao *et al.*, 2006).

Ao analisar os marcadores de superfície (imunofenótipo) das ASCs, estas apresentam uma expressão de marcadores similares às BMSCs. No entanto ainda há controversas em relação a dois marcadores STRO-1 e CD34. Alguns estudos demonstraram que as células multipotentes de tecido adiposo possuem uma marcação positiva para CD34, mas de acordo com as passagens em cultura *in vitro*, estas células podem de alguma forma ser selecionadas e, este marcador pode passar a ter uma expressão diminuída prejudicando desta maneira uma possível seleção das células multipotentes (Varma *et al.*, 2007).

Para as aplicações na bioengenharia e medicina regenerativa as ASCs possuem características importantes e criteriosas para serem candidatas no uso clínico como:

- I- Isolamento de grande quantidade do tecido adiposo;
- II- Ausência de morbidade ou dor para o paciente;
- III- Tecido acessível e procedimento minimamente invasivo quando comparado à obtenção de outros tipos de células-tronco mesenquimais como medula óssea, placenta, líquido amniótico e células do cordão umbilical (Witkowska-Zimny & Walenko, 2011).

Utilizando os métodos corretos, aproximadamente um bilhão de células pode ser isolado por litro de tecido adiposo, e com sua expansão *in vitro* de maneira mais robusta em relação às BMSCs, confere a esta população de células uma vantagem para sua aplicação na medicina regenerativa e engenharia de tecidos. (Glotzbach *et al.*, 2011).

Foram obtidos resultados positivos da associação de ASCs em conjunto com biomateriais, para testar o potencial osteogênico em diferentes situações (Dragoo *et al.*, 2003; Lee *et al.*, 2003; Cowan *et al.*, 2004; Hicok *et al.*, 2004; Dragoo *et al.*, 2005), incluindo a aplicação destas células com chips ósseos de crista ilíaca para a regeneração de defeitos calvariais em um paciente (Lendeckel *et al.*, 2004).

Estudos tem postulado diversas situações onde células-tronco provenientes de tecido adiposo (ASCs) podem ser usadas para reparo e regeneração de tecidos. Primeiramente, estas células implantadas em um tecido doente ou danificado secretariam citocinas e fatores de crescimento estimulando a recuperação de um modo parácrino. Além disso, as células ASCs poderiam também modular o “nicho de células-tronco” do hospedeiro através da estimulação do recrutamento de células-tronco endógenas para o local e da promoção de diferenciação destas células na linhagem requerida (Gimble *et al.*, 2007).

Como as células da medula óssea, as células ASCs são capazes de se diferenciar em múltiplas linhagens, inclusive podem formar estruturas osteóides *in vivo* (implantadas de forma subcutânea) quando associadas à biomateriais de hidroxiapatitas. Dessa forma, o uso destas células associadas a biomateriais para regeneração de tecido ósseo é promissor e ainda precisa ser melhor explorado (Hicok *et al.*, 2004).

Há estudos conflitantes onde o potencial osteogênico de ASCs mostrou-se deficitário, como no estudo de Dudas *et al.* (2006) onde ASCs foram associadas a moldes gelatinosos e implantadas em defeitos calvariais de coelhos. Portanto o uso destas células para regeneração de tecido ósseo é promissor e precisa ser melhor explorado. Deste modo, é de extrema importância a elucidação, estudo e identificação dos potenciais osteogênicos *in vivo* e *in vitro*, para que assim, seja possível filtrar e selecionar células para o uso clínico com potencial ideal de ossificação.

Alguns autores afirmam que é necessária e indispensável uma indução osteogênica para aperfeiçoar a habilidade osteogênica das células-tronco de tecido adiposo em modelos *in vivo* (Dudas *et al.* 2006; Hofmann *et al.*, 2007), mas outros acreditam que este processo de osteoindução aumenta o tempo de cultura, tornando o procedimento longo, e caro, tornando difícil sua utilização em aplicações clínicas (Li *et al.*, 2010). Mas mesmo com estas indefinições relacionadas a uma prévia indução osteogênica para obter um resultado e diferenciação melhor, as ASCs foram utilizadas com diferentes biomateriais e obtiveram sucesso no reparo ósseo de inúmeros modelos animais de defeito crítico ósseo (Yoon *et al.*, 2007; Di Bella *et al.*, 2008; Choi *et al.*, 2011).

As fontes de células-tronco mesenquimais disponíveis e de fácil acesso como as células-tronco de polpa dental e de tecido adiposo, possibilitaram o aumento de estudos e pesquisas de comportamento destes tipos celulares. Mesmo com o aumento de artigos e publicações, poucas linhas de pesquisa se concentraram na comparação entre os potenciais de diferenciação osteogênica entre as diferentes MSCs obtidas de tecidos humanos.

Objetivo Geral

O objetivo geral deste projeto é comparar o potencial osteogênico *in vitro* e *in vivo* de células-tronco mesenquimais de duas diferentes fontes: polpa de dente decíduo (SHED) e tecido adiposo (ASCs);

Objetivos específicos

- I) Comparar o potencial de diferenciação osteoblastogênica *in vitro* entre as linhagens de células-tronco mesenquimais de tecidos adiposo (hASCs) e as linhagens de polpa dental de dente decíduo (SHED);
- II) Promover a triagem do melhor biomaterial para ser utilizado no modelo *in vivo* já padronizado e estabelecido de duplo defeito ósseo na calvária de ratos Wistar;
- III) Testar e comparar o potencial de indução osteogênica das hASCs e SHED no modelo *in vivo*;
- IV) Analisar o perfil de expressão gênica durante o período de diferenciação osteoblastogênica *in vitro* inicial (0-6 dias) de hASCs, SHEDs e BMSCs;

Capítulo II

Human dental pulp stem cells display enhanced osteogenic potential when compared to adipose tissue-derived stem cells

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Abstract

Stem cells from human exfoliated deciduous teeth (SHED) and human adipose-derived stem cells (hASCs) are multipotent cells capable of undergoing osteogenesis *in vitro* and *in vivo*, and promising cell-source populations for bone tissue engineering given their easiness of isolation, expansion and differentiation. It is of great interest to understand which is the best cell type for osteogenic differentiation, thus the aim of this study is to compare the *in vitro* and the *in vivo* osteogenic differentiation potentials between SHED and ASCs. We isolated six stem cell populations from SHED (aged 7-12 years) and six from hASCs (from subjects aged 30-49 years) and cell culture was established.

After *in vitro* induction the populations were able to undergo osteogenic differentiation, as evidenced by alkaline phosphatase (9 days) and alizarin red S (14 and 21 days) stainings. Quantification of matrix mineralization after 21 days of osteogenic differentiation revealed an enhancement of 2.24-fold increase between SHED and hASCs differentiation.

To perform the *in vivo* experiment, we promoted a screening of six scaffolds to find out which would be best scaffold to our model, a calvarial critical-sized defect in Wistar non-immunosuppressed rats, with three different culture samples of SHED. After 45 days, CellCeram™ displayed the best *in vivo* bone neoformation, and was used to compare the *in vivo* osteogenic potentials between SHED and hASCs. Cells (10e6) were associated to 4.5 mm CellCeram™ discs, and control groups were performed transplanting the biomaterial free of cells. Bone healing was measured through histological hematoxylin/eosin staining 45 days after surgery. Newly formed bone was also evaluated by total bone island surface quantification through image analysis.

The association between SHED and CellCeram™ induced a mean of 7.24 times more bone formation when compared to the association between this same material and hASCs ($p < 0.0001$). The use of adult stem cells for bone regeneration is a robust therapeutic option, and calculate or predicts the osteogenic potential of the cell used are extremely important and necessary to future application, and translation to new strategies in bone tissue engineering.

Introduction

Bone tissue repair of large defects usually occurs spontaneously but delay or impairment of this process requires additional surgical intervention. About 90% of the grafting procedures aiming to regenerate or replace craniofacial bones involve the use of either autograft or allograft tissues, the former being considered the “golden standard” in reconstructive surgery [1, 2]. In spite of the usefulness and proved efficiency of these techniques to regenerate bony structures lost or damaged due to trauma, diseases or congenital malformations, there are several drawbacks related to them. Autografting has the restrictions of cost, limited supply of autologous bone, possibility of anatomical and structural incompatibility and morbidity of the donor site [3]. Allografting includes uncertainties concerning immunoreaction and disease transmission [1, 4]. Because of these disadvantages, surgeons and scientists have been seeking for alternative solutions.

Bone tissue engineering through the use of biomaterials associated with cells has the potential to overcome the problems stated above. A wide range of biomaterials with varying physicochemical and structural properties to embody biologically instructive characteristics were developed, but most of the empty scaffolds have largely failed to improve bone healing[5, 6]. Because of that, one of the main objectives in this field is to design delivery methods for osteogenic factors and stem cells/progenitor cells to repair or replace bone. Biomaterials can be combined with stem cells in cell-based therapies, where cells and materials are associated before the moment of the surgical intervention, with the purpose of delivering transplanted cells to guide the spatially complex process of tissue formation.

Mesenchymal stem cells (MSCs) isolated from most of the post-natal tissues, including several craniofacial tissues, have a well characterized potential to differentiate into all cellular lineages that constitute mesenchymal and connective tissues [9-11].

Dental pulp tissue is an interesting source of stem cells (DPSCs/SHED), a subtype of MSCs, used in bone bioengineering studies due to their multidifferentiation potential, noninvasive and efficient process of isolation,

immunomodulatory activity and similarity to osteoprogenitor cells [12-14]. Recently our group demonstrated their potential to aid in the regeneration of large calvarial defects in non-immunosuppressed rats when associated to a collagenous scaffold [15, 16]. Human adipose tissue is another source of MSCs with osteoprogenitor properties, which can be easily harvested in abundant quantities. It is suggested in the literature that adipose tissue-derived stem cells (ASCs) need minimal stimulation by exogenous cytokines to undergo osteogenesis and thus represent a promising option for skeletal tissue engineering trials [17, 18].

Although tissue engineering strategies using stem cells aim to ameliorate the prognosis of the grafted materials, there are several questions still to be addressed. The best materials to be used in different surgical sites and the best adult stem cells to be associated with these materials to improve and hasten the replacement of bone substitutes by newly formed bone tissue in an ideal fashion has to be determined [19, 20]. Clinical success of these strategies ultimately depends on an optimal cell source for bone reconstruction [21] and the majority of the studies so far has used either on *in vitro* or on *in vivo* approaches independently to access the properties of MSCs for bone [22-24]. Finally, the transition from the *in vitro* assays and the *in vivo* proof of principle using animal surgical models to the clinical application has been considerably lacking.

The aim of this study is to compare the differentiation potential of SHED and hASC into osteoblasts *in vitro* and the osteogenic potential of these 2 line cells *in vivo*.

Material and Methods

- Statement of financial interest

We certify that no financial support or benefits have been received by any co-author from any commercial source directly or indirectly related to the work reported in this article.

- Ethics statement

This study was approved by the Institute of Biosciences' Human Research Ethics Committee (permit number 104.120.09) and by the Institute of Biosciences' Animal Research Ethics Committee (permit number 037/2006) of the University of Sao Paulo. DPSCs populations were isolated from donated deciduous teeth obtained upon signed informed consent by the subjects' legal guardians. ASCs were isolated from human adipose tissue obtained from elective liposuction procedures. Wistar (NIS) rats were purchased from the Central Bioterium of the Institute of Biomedical Sciences / University of Sao Paulo. Animals were transferred to the Human Genome Center Bioterium and surgical procedures for stem cells transplantation were done within two weeks.

- Harvesting of dental pulp and adipose-tissue derived stem cells

Dental pulps were extracted from normal exfoliated deciduous teeth of six subjects (aged from 6 to 10 years). The donors were previously evaluated for systemic diseases and oral infections. Dental pulp fragments were accessed with a barbed nervbroach instrument, transferred to 60 X 15mm sterile Petri dishes and washed three times with sterile phosphate buffered saline solution (PBS solution; 0.01 M , pH = 7.5) supplemented with 100 U/ml penicillin and 100 g/ml

streptomycin. The fragments were then transferred to 6 wells culture plates (Corning, N.Y., USA) containing Dulbecco's modified Eagle's medium (DMEM) / Ham's F12 (1:1, Invitrogen, CA., USA) supplemented with 15% HyClone fetal bovine serum (FBS, HyClone, Utah, USA), 100 U/ml penicillin, 100 g/ml streptomycin, 2 mM L -glutamine, and 2 mM of nonessential amino acids.

Human adipose tissue was obtained from six subjects (aged from 24 to 68 years) undergoing elective liposuction procedure. The unprocessed lipoaspirate was washed with equivalent volume of PBS supplemented with 100 U/ml of penicillin and 100 g/ml of streptomycin and enzymatically dissociated with 0.075% collagenase type I (Sigma-Aldrich, St. Louis, USA). Enzyme activity was neutralized with DMEM-high glucose (DMEMHG-HG; Invitrogen, CA., USA) containing 10% FBS. Infranatant was centrifuged at 1,200g for 5 minutes to pellet the cells. Cells were then seeded in 6 wells culture plates (Corning, N.Y., USA) containing DMEM-HG supplemented with 10% FBS.

After 14 days cells were washed with 1XPBS, dissociated with a 0.25% trypsin solution (Invitrogen, CA., USA) and seeded at a 10^4 concentration in 25 cm² culture flasks (Corning, N.Y., USA). The cells were kept at 37°C in a 5% CO₂ incubator and maintained in semi-confluency in order to prevent differentiation. Passages were done every 4-5 days and medium was refreshed every 3 days. All experiments were performed between the third and the fifth subculture.

- Immunophenotyping

Immunophenotype characterization of the SHED and the hASCs populations in culture was done by flow cytometric analysis. In order to evaluate the cell-surface expression of specific marker proteins, cells were harvested with TrypLe (Invitrogen, CA, USA), washed with 1XPBS and incubated for 1 hour at 4°C with the following anti-human antibodies: CD29-PECy5, CD34PerCP, CD31-PE, CD45-FITC, CD90-R-PE, CD73-PE, CD105 (Becton, Dickinson and Company, NJ, EUA). Matched control samples were incubated with 1XPBS free of antibodies. Cell suspensions were washed with 1XPBS and 10.000 labeled cells were analyzed using a Guava EasyCyte flow cytometer running the Guava ExpressPlus software (Guava

Technologies Hayward, CA, USA). The immunophenotyping experiments were done in technical triplicates for each cell sample and for each antibody.

- ***In vitro* osteogenic differentiation**

Subconfluent cell populations of SHED and hASCs were treated with osteogenic medium containing DMEM-Low Glucose (Invitrogen, CA., USA) supplemented with 10% FBS (Invitrogen, CA., USA), 50 μ M ascorbate-2-phosphate, 10mM β -glycerophosphate, 0.1 μ M dexamethasone (Sigma-Aldrich, St. Louis, USA), 100 U/ml penicillin, 100 g/ml streptomycin (Invitrogen, CA., USA) for 21 days with media changes every three days. Alkaline phosphatase activity was assessed through a biochemical assay on the 9th day of *in vitro* osteogenic differentiation induction. In summary, cells were provided with phosphatase substrate (Sigma-Aldrich, St. Louis, USA) and the resulting p-nitrophenol was measured colorimetrically using a Multiskan EX ELISA plate reader (Thermo Scientific) at 405 nm. Calcium matrix production was analyzed after 21 days of *in vitro* osteogenesis by Alizarin Red-S staining. Briefly, cells were washed three times with 1XPBS, fixed with a 70% ethanol solution for 30 minutes at room temperature, washed three times with distilled water and stained with a 0.2% Alizarin Red-S solution (Sigma-Aldrich, St. Louis, USA) for 30 minutes at room temperature. Staining was removed with a solution of 20% methanol / 10% acetic acid and measured colorimetrically using a Multiskan EX ELISA plate reader (Thermo Scientific) at 450nm. As a negative control for both experiments we used the same cells but the cultures were performed in regular proliferation medium. Experiments were done in technical triplicates for each time point and for each cell line.

- **Biomaterials tested**

The materials tested are summarized on **Table 1**. Biocollagen® Gel (Bioteck®), Bio-Gen® (Bioteck®), BioGen® Mix Gel (Bioteck®) and CellCeram™ (Scaffdex Oy) are commercially available. HA/ β TCP-IPEN and β TCP-IPEN ceramics were

developed by the IPEN (Nuclear and Energy Research Institute) research Group of Ceramic Biomaterials. In this process, macroporous samples of calcium phosphate ceramic-based hydroxyapatite (HA) and β -tricalcium phosphate (β TCP) with porosity of about 70% were obtained using a consolidation methodology that comprises foaming and setting through protein action. These samples have been well characterized physico-chemically and preliminary *in vitro* experiments and *in vivo* studies with rabbits have shown promising results. Bio-Gen®, HA/ β TCP-IPEN and β TCP-IPEN were provided by the manufacturer as cubic blocks of different volumes. Discs with 4.5 mm of diameter were shaped using a trephine drill associated to a micromotor. CellCeram™ 4.5 mm discs were provided by the manufacturer.

Table 1

Material Type	Name	Characteristics
Calcium Phosphate Based	HA/ β TCP –IPEN	50 (wt%*) $\text{Ca}_3(\text{PO}_4)_2$ / 50 (wt%) $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ Porous/Synthetic
	β TCP –IPEN	100% $\text{Ca}_3(\text{PO}_4)_2$ Porous/Synthetic
	Cell Ceram™ (Scaffdex Oy)	60%(wt%) $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ 40% $\text{Ca}_3(\text{PO}_4)_2$ Porous/Synthetic
Collagen Type I Based	BioGen® Mix Gel (Bioteck®)	Mixture of cortical and cancellous bone in a water-based gel carrier
	Biocollagen® Gel (Bioteck®)	Collagen gel paste
	Biocollagen®(Bioteck®)	Collagen membrane
Decellularized Bone Matrix	Bio Gen® (Bioteck®)	Heterologous cancellous or cortical equine bone grafts obtained with enzymatic deantigenation method (Pagnutti et al., 2007)

* wt% - weight percent

- **Cell association with biomaterials**

To test the association of stem cells with the different materials to induce *in vivo* bone neoformation, 10^6 undifferentiated SHED were diluted in 40 μ L of proliferation medium and seeded onto the biomaterials with a 1cc, 28 gauge needle syringe (BD) in six 35 mm well plates each (Corning, Corning, NY). After 10 minutes the wells were filled with 2.5 mL of proliferation culture medium and the plates were kept in an incubator for 24 hours at 37°C in a 5% CO₂ atmosphere before transplantation. BioGen Mix Gels and Biocollagen Gels were sandwiched between two layers of Biocollagen membranes: one inserted between the duramater and the lower portion of the calvarial bone and the other inserted above the superior part of the calvarial bone. BioGen® Mix Gel and Biocollagen® Gel were added until complete fulfillment of the critical-sized defect and 10^6 undifferentiated hDPSC were diluted in 40 μ L of proliferation medium and seeded onto the biomaterials during surgery. To compare the *in vivo* bone formation potential of SHED and hASCs, CellCeram™ was selected to be used and the same cell association procedure described above was followed.

- **Surgical and transplantation procedures**

Two-months old non-immunosuppressed (NIS) male Wistar rats (body weights between 200-250 mg) were used to test the *in vivo* osteogenic differentiation of SHED associated with the different materials and for the comparison of the *in vivo* differentiation potential between SHED and hASCs. A total of 12 animals were used to test the different materials and each material was tested in biological duplicates. A total of 6 animals were used to compare the *in vivo* osteogenic potential of SHED and hASCs and experiments were performed in biological triplicates. Animals were kept in ventilated stants (Alesco, SP, Brazil) at a constant temperature of 22°C with a 12 hour light/day cycle and free access to drinking water and sterile food pellets.

Before surgery the animals were anesthetized with an intraperitoneal injection of a solution of ketamin hydrochloride (5%) and xylazine (2%) (0.5ml/100g of

body weight). A midline skin incision was done from the nasofrontal area to the external occipital area. Skin and periosteum were reflected laterally to fully expose the upper part of the calvaria. Two symmetrical full-thickness critical-sized defects were performed with a 4.5mm trephine drill attached to a micromotor and under constant irrigation with physiological solution to prevent overheating of the bone.

The underlining dura-mater and the flanking sutures were left undisturbed. Biomaterials associated to cells were always transplanted to the right side (experimental side) and the left side was used as a control (with the transplantation of a matched, same type material free of cells). The skin flaps were closed using nylon 4 sutures (Ethicon, SP, Brazil). Animals were euthanized 45 days after the surgical procedure using a CO₂ chamber.

- Histological preparations and *in vivo* bone formation quantification

The calvarial samples were dissected and fixed in 4% formaldehyde for 24 hours, decalcified in 5% formic acid for 48 hours, and paraffin-embedded. Slices of 5 µm were made using a microtome. The sections were then stained with hematoxylin and eosin and examined under a light microscope, Axiovert 200 (Carl Zeiss, Jena, Germany).

To verify and quantify the new bone formation we evaluated five transversal 5µm slices of the calvaria with 10 µm of distance between them for each animal. Ossification area of each defect was calculated through Axio Vision Carl Zeiss based on 10x amplified images obtained from Axio Observer A1 Carl Zeiss microscope (Carl Zeiss, Jena, Germany). Full images were reconstructed using the application Photomerge for Adobe Photoshop CS5® software and bone islands formation was quantified using Axiovision 4.7 software (Carl Zeiss, Jena, Germany). The percentage of the defect area that ossified at the experimental side was normalized by the percentage of the defect area that ossified at the control side, so that for each animal we obtained 5 ratio values.

-Immunohistochemistry

Human lamin expression was used to detect the distribution of human cells in the biomaterials 50 days after implantation. Briefly, for analysis of lamin A/C expression, serial 5µm sections were obtained from paraffin embedded tissue.

Slides were deparaffinized and rehydrated using xylene, ethanol at decreasing concentrations and water. Antigen retrieval was performed using heated sodium citrate (pH 6.0) and slides were treated with 3% (v/v) H₂O₂ in PBS (30 min) to block endogenous peroxidase activity prior to Vector MOM Immunodetection kit (Vector Labs, Burlingame, California, USA) application, accordingly to the manufacturer instructions. Mouse anti-lamina A/C (Millipore, Billerica, Massachusetts, USA) was diluted 1:100 in TBS – 0.3% (v/v) Tween 20 and incubated overnight (4°C). The sections were then incubated with biotin-conjugated secondary anti-mouse antibody (from MOM kit) for 1 h at room temperature and with streptavidin-peroxidase ABC complex (Vector Labs) (1 h at room temperature), according to the manufacturer's instructions. Peroxidase was visualized using 0.03% (w/v) 3,3'-diaminobenzidine in PBS with 0.03% (v/v) H₂O₂.

The sections were counterstained with methyl green. Negative controls included replacing the primary antibodies with the respective non-immune serum at similar concentrations or omitting the primary antibody step from the protocol.

A Nikon Eclipse E600 microscope was used for examining sections. Images were captured using a digital camera (Cool SNAP-Procf color; Roper Scientific, Trenton, New Jersey, USA) and Image Pro Plus software (Media Cybernetics, Silver Spring, Maryland, USA).

-Statistical analysis

Continuous variables were expressed by mean and standard deviation, and the groups were compared by Student's t-test. A p value < 0.05 was considered

statistically significant. The tests were done using the GraphPad InStat software (GraphPad).

Results

Immunophenotype characterization

Primary SHED and hASCs populations showed a spindle-shaped morphology after established in culture and during all passages. Cells did not exhibit any obvious significant cell death or morphological changes until the fifth passage. All cell populations stained negatively (< 4%) for endothelial (CD31) and hematopoietic (CD34, CD45) cell markers and positively (> 95%) for adhesion (CD29) and mesenchymal markers (CDs 73, 90 and 105) (**Figure 1**). These results allow us to attest the mesenchymal origin of these cells.

Figure 1. Bars show the mean percentage of staining with different immunophenotype markers for pooled data of 6 lineages of each cell source: SHED and hASCs. The error bars represent standard deviations for each biological and technical replicate.

***In vitro* osteogenic differentiation potential**

To verify the potential of SHED and hASCs to differentiate in osteoblasts *in vitro* and to compare this potential between these two MSCs from different niches, we assessed and quantified the osteogenic differentiation at 2 key points of the differentiation induction protocol. Under osteogenic medium treatment for 9 and 21 days all twelve samples (six SHED and six hASCs) were able to produce peak levels of alkaline phosphatase (ALP) and to secrete mineralized extracellular matrix, respectively. A 1.61-fold increase ($p < 0.05$) in ALP activity was displayed by SHED when compared to hASCs after 9 days of *in vitro* osteogenic differentiation and a 2.24-fold increase in the extracellular matrix calcium deposition was detected when SHED were compared to hASCs after 21 days of osteogenic differentiation ($p < 0.0001$) (**Figure 2, 3**).

Figure 2. Bars show means and standard deviations of technical replicates for pooled data of 6 lineages of each source in quantification of Alkaline Phosphatase. P-values are showed above each graph. Tests with p-values < 0.05 were considered to be statistically significant.

Figure 3. Bars show means and standard deviations of technical replicates for pooled data of 6 lineages of each source in quantification of Alizarin Red. P-values are shown above each graph. Tests with p-values < 0.05 were considered to be statistically significant.

In vivo screening of six biomaterials

Six different materials were screened aiming to select the best one to evaluate and compare the potential of induction of *in vivo* bone neoformation. We selected three samples of SHED to be used in this screening experiment.

Increased fibrous tissue infiltration and minimal bone regeneration processes was observed in control groups, where materials free of cells were transplanted (Figures 4 A, B and C). The same was true for experimental groups, where defects were filled with scaffolds loaded with SHED, corresponding to Bio Gen® (figure A), BioGen® Mix Gel and HA/βTCP- IPEN (figures 4 B) and βTCP-IPEN (figure 4 C). In these samples, a dense, almost avascular tissue is observed filling the pores of the material, suggesting that scarring, rather than osteogenesis, had occurred.

Concerning βTCP-IPEN, there was more connective tissue in the experimental side when compared to the control side, but it did not lead to formation of any center of new bone formation during the post-surgical recovery time of 45 days.

On the other hand, Biocollagen®Gel's and CellCeram™'s experimental groups clearly showed bone neogenesis. Biocollagen®Gel's experimental groups showed a single bony island formation at the implant surface, specifically at the site of association with SHED (Figure 4 B), and CellCeram™'s experimental groups presented a mature lamellar bone, with consistent and broad distribution of bony islands, including the central areas of the implants (Figures 4 C).

Bone neoformation induction of CellCeram™ and Biocollagen®Gel associated to SHED was assessed measuring total bone island surface areas by image analysis. The SHED associated to Biocollagen®Gel were able to induce a mean of 4.4 times more bone formation when compared to control sides and when these cells were associated to CellCeram™ they induced a mean increase of 8.4 times in the newly formed bone area. We thus selected CellCeram™, which displayed the best *in vivo* bone neoformation results, for the next experiments.

Figure 4 A, B and C: Histological hematoxylin-eosin analysis of *in vivo* bone formation in the rat calvarial critical defect 45 days post-surgery. Representative pictures of the materials tested with the experimental groups (materials associated with cells) and control groups (materials free of cells). Black or white arrows point to the different materials, red arrows indicate fibrous tissue infiltration and yellow arrows indicate bone island formation

Figure 4 B. Representative pictures of the materials tested with the experimental groups (materials associated with cells) and control groups (materials free of cells). Black or white arrows point to the different materials, red arrows indicate fibrous tissue infiltration and yellow arrows indicate bone island formation

β TCP - Ipen

CellCeram

Figure 4 C. Representative pictures of the materials tested with the experimental groups (materials associated with cells) and control groups (materials free of cells). Black or white arrows point to the different materials, red arrows indicate fibrous tissue infiltration and yellow arrows indicate bone island formation

Comparison between SHED and hASCs *in vivo* osteogenic potential

We then asked whether SHED also display enhanced osteogenic potential *in vivo* as compared to hASCs. Newly formed bone was also evaluated by total bone island surface quantification through image analysis. The association between SHED and CellCeram™ induced a mean of 7.24 times more bone formation when

compared to the association between this same material and hASCs ($p < 0.0001$)(Figure 5).

Figure 5. *In vivo* new bone formation ratio (ossification area/total defect area) of control group and three samples of each experimental groups, P-values are showed above each graph. Tests with p-values < 0.05 were considered to be statistically significant.

Human cells are detected in the biomaterials 50 days after implantation

Lamin A/C expression was used to identify human cells among host cells resident in the new formed tissues inside the biomaterials (figure 6). Cells of human origin are detected in the connective tissue deposited in the biomaterial's pores but not in the bone tissue of SHED and hASC loaded biomaterials. Bone cells (osteoblasts and osteocytes) do not express human-lamin A/C at 50 days post *in vivo* implantation. Our results suggest that human stem cells added to biomaterial have osteogenic potential *in vivo* due to the presence of bone tissue neo-formed in both groups, absent in the control.

Figure 6: Immunolocalization of human-laminA/C positive cells. Human cells differentiated from SHED and hASC are localized loose and dense connective tissue deposited inside the biomaterials 50 days after *in vivo* implantation in Wistar rat's calvaria (A and B). There are no human-lamin A/C positive cells in bone tissue localized inside the biomaterial (C and D). b: bone tissue; ct: connective tissue; black arrow: human-lamin A/C positive cells; white arrow: cell nuclei from host; bm: biomaterial; asterisc (*): blood vessel. Magnification: 40x.

Discussion

Almost all craniofacial structures are derived from mesenchymal cells [7, 8] and mesenchymal stem cells isolated from the bone marrow niche (BMSCs) have been thoroughly explored in tissue engineering approaches aiming to regenerate bone. Amongst craniofacial structures successfully regenerated with BMSCs are calvarial bone in mice [25], cranial defects of canines [26], mandibular critical sized defects in sheep [27] and maxillary sinus floor in humans [28]. Due to the scarcity of these cells and since bone marrow harvesting remains a painful and laborious procedure; there is a great interest in harvesting potential osteogenic cells from more convenient tissue sources, such as SHED from deciduous teeth or from teeth being extracted and hASCs obtained by liposuction.

Our *in vitro* assays showed a significant enhanced osteogenic potential associated to SHED as compared to hASCs. This is in accordance with extensive data in the literature, which shows multidifferentiation and osteogenic potentials associated to SHED and to hASCs [22-24].

To adequately translate *in vitro* findings to the clinical realm, strong *in vivo* data must be obtained to demonstrate and compare the osteogenic capacities of different MSC sources. Repair of craniofacial surgical defects is a well established model, mimicking the clinical reality, to investigate the efficacy of biomaterials to induce new bone formation [25, 29]. We therefore opted to screen six scaffolds of different constitution using only *in vivo* system.

The choice of the materials was based on their accessibility and on their similarity to the natural bone constitution: Bio-Gen® and BioGen® Mix Gel are based on heterogeneous bone substitutes of equine origin deantigenated through an enzymatic treatment, Biocollagen® Gel is based on a collagen type I gel paste and CellCeram™, HA/βTCP-IPEN and βTCP-IPEN are ceramics based on hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP). BioGen® Mix Gel and Biocollagen®Gel are moldable materials and Biocollagen® is a membrane based on type I collagen used to stabilize these two scaffolds in the surgical site.

Unlike the other bone graft substitutes tested, histological data and quantification of total bone surface formation showed that CellCeram™ and Biocollagen®Gel associated to SHED produced a significant increase in bone surface area when compared to control groups. We noticed, however, that a more homogenous induction was achieved when SHED were associated to CellCeram™ as compared to Biocollagen®Gel. We must point out that the distinctive performance of these scaffolds when associated to SHED was not due to a variation in cell sample since all SHED samples selected for this experiment showed uniform morphology, immunophenotype and *in vitro* osteogenic differentiation potential.

Finally, we selected CellCeram™ and compared the *in vivo* osteogenic potentials of SHED and hASCs using this same model. We found a mean enhancement of 7.24 times when SHED were associated to this scaffold, compared to the association of hASCs. We couldn't conclude that the *in vivo* evidences validated the *in vitro* data showing enhancement in the osteogenic potential of SHED. We need more experiments to perform this direct correlation between the

in vitro and the *in vivo* data of the osteogenic potential associated to these cells. We couldn't conclude by immunohistochemistry that bone cells formed after 50 days were from human origin, once initial time points were not evaluated.

Besides displaying multilineage differentiation potential, dental pulp and adipose tissue are both attractive sources of MSCs for bone tissue engineering for several reasons. ASCs can be easily and promptly isolated from human fat tissue, besides being readily available in large quantities. In addition, some studies have shown that ASCs have similar growth kinetics, senescence and osteogenic differentiation capacity when compared to BMSCs [18, 30]. It has also been reported that ASCs are able to acquire bone cell-like responsiveness and upregulate osteogenic genes in response to shear stress *in vitro*, which suggests that they are capable to respond to mechanical loads [31]. Scaffolds loaded with pre-differentiated ASCs cells show a more robust bone tissue regeneration when compared to scaffolds seeded with undifferentiated ASCs [32-35] but, in this study, we aimed to determine the true *in vivo* osteogenic potential of the cell niches, without the addition of morphogenetic factors, exogenous growth factors or the use of genetic manipulations, avoiding the drawbacks of these methodologies [36-39].

On the other hand, SHED can be isolated in a noninvasive and highly efficient fashion from deciduous teeth that are usually discarded without ethical concerns [12, 40]. They are considered to be analogous to bone cells since they show expression of osteogenic marker proteins as well as response to many growth factors for osteo/odontogenic differentiation [13, 41, 42] and also show increased immunosuppressive activity when compared to BMSCs, which could lead to potential advantages in clinical applications [14]. Last, DPSCs can be easily cryopreserved for long periods and retain their potential after preservation, which favors the establishment of cryobanks for adult tissue regeneration [43-45].

In the present manuscript we followed an experimental design to quantify and compare the osteogenic potential between SHED and hASCs. The use of adult stem cells for bone regeneration is a robust therapeutic option and our study reinforces that the understanding and the optimization of conditions to ideally match biomaterial scaffolds with stem cells harboring higher osteogenic potential will contribute to make stem cell-based strategies a better bid than the golden

standard autologous bone substitute for reconstruction and rehabilitation of craniofacial osseous defects.

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Capítulo III

Análise da expressão gênica de células-tronco mesenquimais na diferenciação osteoblastogênica inicial

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Abstract

Adult stem cells are the most frequently indicated for therapeutic approaches because they are considered safer regarding the ability of tumor formation and its autologous use. Several studies have demonstrated the osteogenic potential in vitro and in vivo of BMSCs, SHED and ASCs. It's important to know the potential of osteogenic differentiation of each cell type to be used in the bioengineering of bone tissue. In the previous chapter (Fanganiello & Ishiy et al. 2012) was able to verify that SHED showed greater osteogenic potential in relation to ASCs both in vitro and in vivo. The reason for this variation in potential between the cell types is still unclear. The aim of this new approach was to understand for what reasons DPSCs had better osteogenic potential both in vitro and in vivo, and mesenchymal stem cells derived from adipose tissue showed lower differentiation potential than DPSCs. The strategy used for this purpose was microarray expression assay. Two cell populations human exfoliated deciduous teeth (SHED), two populations of stem cells derived from adipose tissue (ASC) and two populations of stem cells from bone marrow (BMSCs) were selected for these experiment.

Three differentially expressed genes (DEGs) in common were found among the three cell types, and these genes (*APOD*, *METTL7A* and *FKBP5*) have been detected during osteogenic differentiation in several studies. Most of the genes related to pluripotency (*NANOG*, *SOX2*, *BST1*, *REX1* and *OCT4*) had higher expression by BMSCs, DPSCs with the greatest expression of *RUNX2* gene, and ASCs with lower expression among the cell types used. Among the top canonical pathways of all genes differentially expressed in all cell types used in the experiment, we highlight interleukin 17A, and LXR / RXR path (liver X receptor / retinoid X receptor).

Gene expression of cells in the basal state and during the process of osteogenic differentiation may be a promising approach for selecting osteogenesis markers. This approach will provide an understanding of the transcriptional pathways involved in the process of osteogenic stem cells from different niches, allowing the development of therapeutic approaches in bioengineering of bone tissue.

Introdução

Para propostas terapêuticas na área de medicina regenerativa e bioengenharia de tecidos, as células-tronco adultas (extraídas de diferentes tecidos pós-natais) são as mais indicadas atualmente por serem consideradas mais seguras no que tange a capacidade de formação de tumores e por seu uso autólogo [1,2]. Células-tronco mesenquimais (MSCs), além do seu multipotencial de diferenciação, apresentam propriedades importantes para uso na medicina regenerativa, como imunomodulação, capacidade de *homing* e *trafficking*, propriedades relacionadas a migração de células para o local lesionado [3-5].

Estudos já comprovaram o potencial osteogênico *in vitro* e *in vivo* de células-tronco de medula óssea - BMSCs [6-9], polpa dental – DPSCs/SHED [10-14] e de tecido adiposo – ASCs [15-18]. Para a escolha do tipo celular a ser utilizado na bioengenharia de tecidos ósseos é necessário saber qual o potencial de diferenciação osteogênica de cada tipo celular. No capítulo anterior (Fanganiello & Ishiy *et al.* 2012) foi possível verificar que as SHED apresentaram maior potencial osteogênico em relação às ASCs tanto no modelo *in vitro*, quanto no modelo *in vivo*. Mas os motivos desta variação de potencial entre as linhagens ainda não foi esclarecido.

Nesta abordagem do trabalho a principal questão é saber por quais motivos as SHED tanto *in vitro* quanto *in vivo* obtiveram melhor potencial osteogênico, e porque as células-tronco mesenquimais oriundas do tecido adiposo apresentaram uma diferenciação inferior às SHED. Uma estratégia que pode elucidar estas variações é o ensaio de *microarray* de expressão. Esta metodologia permite observar e comparar a expressão de genes por diferentes tipos celulares em condições padronizadas, neste caso no processo de diferenciação osteogênica [19-21].

Foram selecionadas para este experimento duas populações celulares provenientes de polpa de dente decíduo (SHED); duas populações de células-tronco provenientes de tecido adiposo (ASCs) e duas populações de células-tronco provenientes de medula óssea (BMSCs).

Focamos a análise de expressão gênica durante o período inicial de diferenciação osteogênica em três tempos de 0 a 6 dias (T0 dias, T4 dias e T6 dias), para observar se a diferença encontrada no potencial de diferenciação está relacionada com os diferentes níveis de expressão de genes nestes períodos escolhidos.

Material e Métodos

- Obtenção de material biológico e cultura

Para a coleta, isolamento e cultivo de células-tronco derivadas de tecido adiposo e polpa dental os procedimentos utilizados foram aqueles previamente padronizados por nosso grupo [22, 23].

Para diferenciação óssea das células-tronco, os protocolos foram conduzidos em triplicatas para cada uma das amostras (triplicatas técnicas). Para a diferenciação osteogênica as replicatas biológicas e técnicas, das células referidas acima foram tratadas com meio de proliferação (DMEM Low Glicose) suplementado com 50 μ M de ascorbate-2-phosphate, 10 mM de β -glicerofosfato (Sigma), e 0,1 μ M de dexametasona por 21 dias. As extrações de RNA foram realizadas em três tempos, primeiramente sem indução de diferenciação (T0), e após 4 e 6 dias de diferenciação osteoblastogênica *in vitro*.

- Experimento de microarray de expressão gênica

As análises da expressão das células que foram triadas para maior potencial de ossificação *in vitro* e *in vivo* foram realizadas por *microarrays* com o *chip Human Gene 1.0 ST (Affymetrix)*. Após três passagens em garrafas de 25 cm², as células contidas nas garrafas foram lisadas e o RNA total foi extraído de acordo com a técnica de extração de RNA isolado a partir de linfócitos com o uso de kit Nucleospin RNA II da Macherey-Nagel.

O RNA total foi diluído em água tratada com DEPC (dietil pirocarbonato) e a qualidade do RNA foi averiguada por meio de eletroforese (100 volts por 60 minutos) em gel de agarose (1,2 % de agarose, 2% de brometo de etídeo diluídos em tampão 1 X TBE). As bandas de RNA foram visualizadas com o uso de luz ultravioleta e o gel fotografado. Apenas amostras que apresentavam razão de intensidade de bandas de RNA ribossômico 28S:18S aproximadamente de 2:1 foram utilizadas.

De forma geral, o *GeneChip® Whole Transcript Sense Target Labeling* (Affymetrix) é designado para gerar DNAs senso amplificados e marcados com biotina, resultantes da expressão de todo o genoma. A partir de 100 ng do RNA total, o cDNA dupla fita é sintetizado com hexâmeros randômicos contendo uma cauda de T7, que age como uma seqüência promotora. O cDNA dupla-fita é subsequentemente utilizado como molde e amplificado pela polimerase T7 RNA (Affymetrix), produzindo muitas cópias de cRNA anti-senso. No segundo ciclo de síntese do cDNA, hexâmeros randômicos são utilizados como oligonucleotídeos iniciadores para a transcrição reversa do cRNA, resultando em DNA simple-fita. Durante o segundo ciclo da transcrição reversa, é também adicionado dUTP à reação. O DNA simples-fita é então tratado com uma combinação de DNA uracil glicosilase (UDG) e endonuclease apurínica/apiridínica 1 (APE 1), que reconhecem especificamente os resíduos de dUTP e quebram a fita de DNA. Em seguida, o DNA é marcado pela transferase deoxinucleotídeo terminal (TdT) com *DNA Labeling Reagent* (Affymetrix), que é covalentemente ligado à biotina. Este cDNA é hibridado por 17 horas ao *chip Human Gene 1.0 ST* com o auxílio de um forno de hibridação a 45 °C a 60 rpm. O processo de marcação por *Streptavidina* e de lavagem da lâmina ocorre na estação fluídica (*GeneChip® Fluidics Station 450*), comandada por um computador. Por fim, as lâminas são escanadas pelo *GeneChip® Scanner 3000 7G System* e um relatório de controle de qualidade é gerado pelo *Affymetrix® Expression Console™ Software*.

- Análise

Foi adotado o modelo de análise pareada, na qual foi possível comparar as amostras do mesmo indivíduo nos seguintes tempos: dia zero (Tempo 0), imediatamente antes da indução de diferenciação osteogênica *in vitro* e dias 4 e 6 (Tempo 4 e Tempo 6, respectivamente) pós-indução, para cada tipo celular. Utilizando este modelo, foi possível verificar quais eram os genes alterados nos diferentes tempos. A principal vantagem no uso deste modelo de análise pareada foi minimizar o efeito do indivíduo no processo de diferenciação.

Os dados foram normalizados com o método *Robust Multi-array Average* (RMA) do pacote Affy do projeto *Bioconductor* [24]. Em seguida foram adicionadas a cada arquivo de normalização as informações de anotação disponibilizadas pela ferramenta NETAFFX da Affymetrix (<http://www.affymetrix.com/>).

Após o processo de normalização, foi aplicado o filtro de dados $IQR < 0.5$ (*Interquartile Range*) para selecionar genes com maior variância, para serem utilizados como entrada no método de seleção de DEGs. Para a seleção dos DEGs adotamos o método *Significance Analysis of Microarrays* (SAM) *two-class paired*, com $p < 0.05$ corrigidos por FDR. Redes de interação e enriquecimento funcional dos DEGs foram obtidos por meio do software *Ingenuity Pathway Analysis* (*Ingenuity Systems*).

- Expressão de genes de pluripotência e marcadores osteogênicos.

Sem os filtros supracitados, foram analisados os dados de expressão de genes previamente relacionados à pluripotência (*NANOG*, *BST1*, *SOX2*, *REX1*, *C-MYC* e *OCT4*), e marcadores de osteogênese (*OMD*, *ALP*, *OSP*, *RUNX2*, *BSP* e *OSC*), para visualizar as possíveis variações desses durante os três tempos (T0, T4 e T6) e para comparação entre os diferentes tipos celulares. A comparação dos dados de expressão no período total foi feita através do teste-t de Student. Valores de $p < 0.05$ foram considerados estatisticamente significantes. Os testes foram feitos utilizando o *software* GraphPad InStat (GraphPad).

Resultados

- Genes diferencialmente Expressos (DEGs)

Foram identificados nas ASCs onze DEGs no intervalo T0 x T4, vinte e quatro DEGs no T0 x T6 e três DEGs no intervalo T4 x T6. No período T0 x T4 e T0 x T6 foram encontrados oito DEGs em comum, e no total foram identificados trinta DEGs em todos os tempos das ASCs. Nas BMSCs foram constatados no total trinta e dois DEGs, sendo dezenove DEGs no intervalo T0 x T4, quatro DEGs no T0 x T6, e quatorze DEGs no intervalo T4 x T6. Ao cruzarmos os DEGs em busca de genes em comum para todos os tempos, foi obtido um gene em comum: *ABCA6*

Já nas SHED, foram observados em todos os tempos onze DEGs, distribuídos: cinco DEGs no intervalo T0 x T4, seis DEGs no T0 x T6 e nenhum no intervalo T4 x T6 (**Figura 1** e Tabelas I-III, **Material Suplementar**, página 72).

Figura 1. Quantidades de DEGs encontrados nos diferentes tempos dos três tipos celulares obtidos pela análise pareada com o método SAM.

Portanto, tivemos como números absolutos de genes diferencialmente expressos para cada tipo celular utilizado no experimento: trinta DEGs para as

ASCs, trinta e dois DEGs para as BMSCs e onze DEGs para as SHED (**Figura 2**). Quando cruzamos os resultados totais, buscando genes em comum entre os três tipos celulares, foram obtidos três genes no processo de diferenciação osteogênica. Estes foram: Apolipoproteína D (*APOD*), Metiltransferase tipo 7A (*METTL7A*) e *FK506 binding protein 5 (FKBP5)*.

ASCs (30 DEGs) x BMSCs (32 DEGs) x SHED (7 DEGs)

Figura 2. Diagrama de Euler-Venn em que a área de cada círculo é proporcional à quantidade de DEGs encontrados nos tipos celulares. Os números internos demonstram os genes em comum para cada grupo celular.

Ao caracterizarmos os DEGs únicos de cada tipo celular, ou seja, os genes diferencialmente expressos particulares de cada tipo celular que não se repetem em outros tipos celulares, foram observados dezenove DEGs particulares das ASCs, vinte e um DEGs para as BMSCs e um DEG para SHED. Este único gene particular das DPSCs no processo de diferenciação osteogênica inicial foi o *TIMP4*.

Através do *Ingenuity Pathway Analysis* (IPA) foram listadas as vias canônicas mais enriquecidas: *Role of IL-17A in Psoriasis* ($p=8,8E-04$), *LXR/RXR Activation* ($p=8,06E-03$) e *Role of IL-17A in arthritis* ($p=1,48E-02$). Dentre estas temos duas vias relacionadas com a interleucina-17A e, uma relacionada com ativação da LXR/RXR (receptor de ácido retinóico).

Plotando os dados de todos os tipos celulares utilizados no experimento no IPA, obtivemos uma lista de moléculas/genes superexpressos e sub-expressos que estão descritas na **Figura 3**. Pode-se destacar entre os superexpressos: *APOD* (Apolipoproteína D), *ABCA6* (*ATP-binding cassette ABC superfamily*), *OMD* (Osteomodulina) e *CPM* (carboxipeptidase M). Dentre a mais sub-expressas temos o gene *IGFBP5* (*insulin-like growth factor binding protein 5*), que está presente nesta análise global de genes dos três tipos celulares mas, ao analisarmos individualmente cada tipo celular, pode-se constatar que este gene sub-expresso é único das ASCs.

Top Molecules			
Fold Change up-regulated		Fold Change down-regulated	
Molecules	Exp. Value	Molecules	Exp. Value
<i>APOD</i>	↑5,435	<i>IGFBP5</i>	↓-4,032
<i>ABCA6</i>	↑5,082	<i>ACTC1</i>	↓-3,850
<i>OMD</i>	↑4,595	<i>SLC7A11</i>	↓-2,983
<i>CPM</i>	↑4,423	<i>RBM24</i>	↓-2,529
<i>SLC7A2</i>	↑4,191	<i>RNU5E-1</i>	↓-1,690
<i>FKBP5</i>	↑3,870	<i>ASA2</i>	↓-1,566
<i>CCL20</i>	↑3,341		
<i>TIMP4</i>	↑3,243		
<i>RERG</i>	↑3,224		
<i>FBN2</i> (includes EG:14119)	↑3,170		

Tabela 1. Lista dos genes superexpressos e sub-expressos durante o processo de diferenciação osteogênica inicial (0 – 6 dias) dos três tipos celulares ASCs, BMSCs e DPSCs com seus respectivos valores expressos em *fold change*.

Tendo em vista o maior potencial de diferenciação osteogênica das SHED em relação às ASCs, realizamos uma análise de expressão de genes sabidamente relacionados à pluripotência. Para a maior parte dos genes relacionados à pluripotência, há um padrão de expressão similar entre os tipos celulares estudados (**Figura 4**). No que concerne à expressão dos genes *NANOG*, *SOX2*, *BST1*, *OCT4* e *REX1*, as BMSCs apresentaram maior expressão, seguidas das SHED e com menor expressão as ASCs, nos três tempos testados.

As SHED e ASCs demonstraram um padrão de expressão mais similar para os genes *SOX2*, *REX1* e *NANOG*. É importante ressaltar que em nenhum destes genes relacionados à pluripotência as ASCs apresentaram expressão maior do que as BMSCs e SHED ($p < 0.05$).

Figura 4. Gráficos de expressão de genes relacionados à pluripotência, durante a diferenciação osteogênica.

A expressão de genes relacionados a marcadores osteogênicos (**Figura 5**) indicou um perfil aparentemente similar entre ASCs, BMSC e SHED para os marcadores osteomodulina (*OMD*) e Osteocalcina (*OSC*). Para a osteopontina (*OSP*) as BMSCs e ASCs apresentaram maior expressão em comparação às SHED ($p < 0.0001$) em todos os tempos. Além disso, nas BMSCs, a expressão do gene *RUNX2* mostrou-se similar as SHED ($p = 0,09$), e ambas apresentaram maior expressão em relação às ASCs ($p < 0.05$). As BMSCs obtiveram maior expressão ($p < 0.0001$) em todos os tempos de fosfatase alcalina (*ALP*) e sialoproteína óssea

(*BSP*) em relação às SHED e ASCs. Para estes marcadores, tanto as ASCs quanto as SHED apresentaram uma expressão similar e, menor do que as BMSCs.

Figura 5. Gráficos de expressão de genes relacionados a marcadores osteogênicos, durante a diferenciação osteogênica.

Quando observamos os gráficos de expressão de genes em comum (*APOD*, *METTL7A*, e *OMD*) dos três tipos celulares utilizados, verificamos um padrão similar de expressão nos três tempos.

Discussão e Conclusão

Três genes diferencialmente expressos em comum dentre os três tipos celulares foram encontrados, sendo que estes genes (*APOD*, *METTL7A* e *FKBP5*) já

foram detectados durante a diferenciação osteogênica em diversos estudos [25, 26]. Além disso, o gene *TIMP4* (Inibidor tecidual de metaloproteinase), que no nosso trabalho foi encontrado como diferencialmente expresso unicamente nas DPSCs, foi também encontrado por Baksh *et al.* (2004) [25]. Em Morsczeck *et al.*, 2009 [26], os autores observaram o perfil de expressão gênica de células do folículo dental durante o processo de diferenciação osteogênica e, dentre os DEGs apontados, temos o *TIMP4* e o *APOD* (Apolipoproteína D) em comum com nossos achados.

O papel efetivo do gene *TIMP4* especificamente no processo de diferenciação osteogênica ainda não foi estabelecido. Sabe-se que os inibidores de metaloproteinases (TIMPs) possuem um papel importante no processo de polarização, diferenciação, proliferação e participam na quebra de matriz extracelular através do balanço entre as TIMPs e MMPs (metaloproteinases de matriz) e, portanto, a expressão deste gene pode ser importante na diferenciação osteogênica [27-30].

Liu e colaboradores (2007) [31] propuseram, após análise de expressão gênica de ASCs e BMSCs, que os genes *OMD*, *FKBP5* e *APOD* têm forte contribuição no processo de diferenciação osteogênica onde, especificamente nas BMSCs, o gene *APOD* teve maior expressão do que nas ASCs. Em nossas análises o gene *OMD* foi encontrado nas ASCs e SHED, e os genes *FKBP5* e *APOD*, nos três tipos celulares.

Estes achados corroboram nossos resultados, nossas análises de expressão de genes relacionados à pluripotência indicam maior expressão da maior parte destes genes (*NANOG*, *SOX2*, *BST1*, *REX1* e *OCT4*) pelas BMSCs.

Ao analisarmos as vias canônicas mais enriquecidas (top *canonical pathways*) de todos os genes diferencialmente expressos em todos os tipos celulares utilizados no experimento, temos a via das interleucina 17A citada duas vezes na lista das vias canônicas mais enriquecidas. Esta via pode estar destacada devido à capacidade de imunomodulação já estabelecida das células-tronco mesenquimais [3-5]. Nesse contexto, Kocić *et al.* (2012) [32] relataram que a interleucina 17, através da ativação de ERK1 e 2, inibiu a diferenciação miogênica de células mioblásticas C2C12 promover a diferenciação osteogênica destas. Outra via canônica enriquecida interessante encontrada em nossa lista foi a via LXR/RXR (*liver X receptor/retinoid X receptor*), que está associada ao receptor de

ácido retinóico. Diversos estudos já comprovaram o efeito positivo do ácido retinóico individualmente ou em conjunto com *bone morphogenetic proteins* (BMPs) no processo de diferenciação osteogênica *in vitro* e *in vivo* [33-36]. Portanto, o enriquecimento das vias supracitadas na nossa análise indica que o processo de diferenciação osteogênica está condizente com a literatura e, portanto, a diferenciação inicial ocorreu de maneira correta.

No presente manuscrito estudamos a expressão gênica de células-tronco mesenquimais de polpa dental, de medula óssea e de tecido adiposo durante o processo inicial de diferenciação osteoblastogênica *in vitro*. Com os resultados obtidos, podemos concluir que o perfil de expressão de marcadores osteogênicos e de pluripotência no período estudado não são preditivos do potencial de diferenciação osteoblastogênica; genes de pluripotência estão menos expressos nas hASCs o que pode indicar maior comprometimento, menor plasticidade e consequentemente menor potencial osteoblastogênico *in vitro* e potencial de indução *in vivo* destas células em relação às BMSCs e SHED. A análise da expressão gênica de células em seu estado basal e durante o processo de diferenciação osteoblastogênica pode ser uma abordagem promissora para a seleção de marcadores de osteogênese. Esta abordagem proporcionará o entendimento das vias transcricionais associadas ao processo osteogênico em células-tronco de diferentes nichos. Este conhecimento é fundamental para o desenvolvimento de propostas terapêuticas na área de bioengenharia de tecidos ósseos.

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Material Suplementar

Tabela I. DEGs encontrados em todos os tempos das ASCs.

NM	Symbol	Entrez Gene Name
NM_005159	<i>ACTC1</i>	actin, alpha, cardiac muscle 1
NM_000668	<i>ADH1B</i>	alcohol dehydrogenase 1B (class I), beta polypeptide
NM_001647	<i>APOD</i>	apolipoprotein D
NM_019893	<i>ASAH2</i>	N-acylsphingosine amidohydrolase (non-lysosomal ceramidase) 2
NM_000064	<i>C3</i>	complement component 3
NM_005192	<i>CDKN3</i>	cyclin-dependent kinase inhibitor 3
NM_006587	<i>CORIN</i>	corin, serine peptidase
NM_001874	<i>CPM</i>	carboxypeptidase M
NM_002993	<i>CXCL6</i>	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)
NM_004820	<i>CYP7B1</i>	cytochrome P450, family 7, subfamily B, polypeptide 1
NM_006495	<i>EVI2B</i>	ecotropic viral integration site 2B
NM_001145775	<i>FKBP5</i>	FK506 binding protein 5
NM_000599	<i>IGFBP5</i>	insulin-like growth factor binding protein 5
NM_003637	<i>ITGA10</i>	integrin, alpha 10
NM_018640	<i>LMO3</i>	LIM domain only 3 (rhombotin-like 2)
NM_014033	<i>METTL7A</i>	methyltransferase like 7A
NM_001190839	<i>MGP</i>	matrix Gla protein
NM_176870	<i>MT1M</i>	metallothionein 1M
NM_001193582	<i>NRCAM</i>	neuronal cell adhesion molecule
NM_005014	<i>OMD</i>	osteomodulin
NM_001143942	<i>RBM24</i>	RNA binding motif protein 24
NM_032918	<i>RERG</i>	RAS-like, estrogen-regulated, growth inhibitor
NR_002754	<i>RNU5E-1</i>	RNA, U5E small nuclear 1
NM_015474	<i>SAMHD1</i>	SAM domain and HD domain 1
NM_005410	<i>SEPP1</i>	selenoprotein P, plasma, 1
NM_014331	<i>SLC7A11</i>	solute carrier family 7 (anionic amino acid transporter light chain, xc-system), member 11
NM_014752	<i>SPCS2</i>	signal peptidase complex subunit 2 homolog (<i>S. cerevisiae</i>)
NM_006006	<i>ZBTB16</i>	zinc finger and BTB domain containing 16
ENST0000036417 1	<i>RN5S242</i>	RNA, 5S ribosomal 242
NM_030754.2	<i>NCBI 6289 (SAA2)</i>	serum amyloid A2

Tabela II. DEGs encontrados em todos os tempos das BMSCs

NM	Symbol	Entrez Gene Name
NM_080284	<i>ABCA6</i>	ATP-binding cassette, sub-family A (ABC1), member 6
NM_000668	<i>ADH1B</i>	alcohol dehydrogenase 1B (class I), beta polypeptide
NM_003739	<i>AKR1C3</i>	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)
NM_001647	<i>APOD</i>	apolipoprotein D
NM_018154	<i>ASF1B</i>	ASF1 anti-silencing function 1 homolog B (<i>S. cerevisiae</i>)
NM_004591	<i>CCL20</i>	chemokine (C-C motif) ligand 20
NM_001874	<i>CPM</i>	carboxypeptidase M
NM_002993	<i>CXCL6</i>	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)
NM_134268	<i>CYGB</i>	cytoglobin
NM_004820	<i>CYP7B1</i>	cytochrome P450, family 7, subfamily B, polypeptide 1
NM_001017420	<i>ESCO2</i>	establishment of cohesion 1 homolog 2 (<i>S. cerevisiae</i>)
NM_006495	<i>EVI2B</i>	ecotropic viral integration site 2B
NM_001999	<i>FBN2</i> (includes EG:14119)	fibrillin 2
NM_203371	<i>FIBIN</i>	fin bud initiation factor homolog (zebrafish)
NM_001145775	<i>FKBP5</i>	FK506 binding protein 5
NM_001460	<i>FMO2</i>	flavin containing monooxygenase 2 (non-functional)
NM_001463	<i>FRZB</i>	frizzled-related protein
NM_018485	<i>GPR77</i>	G protein-coupled receptor 77
NM_016260	<i>IKZF2</i>	IKAROS family zinc finger 2 (Helios)
NM_003937	<i>KYNU</i>	Kynureninase
NM_014033	<i>METTL7A</i>	methyltransferase like 7 ^a
NM_015441	<i>OLFML2B</i>	olfactomedin-like 2B
NM_025179	<i>PLXNA2</i>	plexin A2
NM_000956	<i>PTGER2</i>	prostaglandin E receptor 2 (subtype EP2), 53kDa
NM_001161429	<i>RANBP3L</i>	RAN binding protein 3-like
NM_000331	<i>SAA1</i>	serum amyloid A1
NM_003046	<i>SLC7A2</i>	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2
NM_001080437	<i>SNED1</i>	sushi, nidogen and EGF-like domains 1
NM_024636	<i>STEAP4</i>	STEAP family member 4
NM_175861	<i>TMTC1</i>	transmembrane and tetratricopeptide repeat containing 1
NM_030754.2	<i>NCBI 6289</i> (SAA2)	serum amyloid A2
NM_001353.5	20-alpha (3-alpha)-hydroxysteroid dehydrogenase) (AKR1C1), mRNA	Homo sapiens aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1;

Tabela III. DEGs encontrados em todos os tempos das DPSCs

NM	Symbol	Entrez Gene Name
NM_080284	<i>ABCA6</i>	ATP-binding cassette, sub-family A (ABC1), member 6
NM_001647	<i>APOD</i>	apolipoprotein D
NM_001145775	<i>FKBP5</i>	FK506 binding protein 5
NM_014033	<i>METTL7A</i>	methyltransferase like 7A
NM_005014	<i>OMD</i>	osteomodulin
NM_003256	<i>TIMP4</i>	TIMP metalloproteinase inhibitor 4
NM_030754.2	<i>NCBI <u>6289</u> (SAA2)</i>	serum amyloid A2

Capítulo IV

Discussão geral e conclusões

Estudos já verificaram e comprovaram a capacidade de diferenciação osteogênica de MSCs, tanto *in vitro* quanto *in vivo*, mas a correlação destes dois modelos de abordagem, e a possibilidade de predição do potencial osteogênico ainda não foi totalmente esclarecida.

Primeiramente, verificamos que as SHED possuem maior potencial de diferenciação osteoblastogênica *in vitro*, quando comparadas às ASCs. A partir desta, fizemos uma triagem de biomateriais para verificar se o potencial *in vitro* poderia ser verificado no modelo *in vivo*. Criamos e estabelecemos um modelo capaz de comparar os potenciais de diferenciação óssea *in vivo* e provamos que SHED, que tiveram melhor diferenciação osteoblastogênica *in vitro*, promoveram no ensaio *in vivo* padronizado previamente por nosso grupo (De Mendonça *et al.*, 2008; Yeh *et al.*, 2011), maior indução de neoformação óssea pelo hospedeiro.

A variação do potencial osteogênico entre células-tronco mesenquimais de polpa dental de dente decíduo e de tecido adiposo humano não foi previamente explorada, mas muitas hipóteses já foram levantadas. Como as SHED utilizadas no presente manuscrito foram obtidas de dentes de leite de pacientes com faixa etária entre 7 e 12 anos, e as ASCs de adultos entre 30 e 49 anos, a idade em que as células foram obtidas pode influenciar na plasticidade e potencial de diferenciação delas. Caplan (2009) observou que células-tronco mesenquimais de medula óssea possuem uma proporção inversa de quantidade de células de acordo com a idade

do indivíduo, ou seja, com aumento da idade, menor a quantidade de células-tronco mesenquimais. Este resultado reforça a ideia de que a idade interfere diretamente no potencial de diferenciação osteogênica. Se estes achados puderem ser generalizados, as SHED obtidas de crianças (7-12 anos) iriam ter maior plasticidade de diferenciação celular em relação às ASCs. As ASCs apresentaram menor expressão dos genes relacionados à pluripotência, podendo ser um indicativo da menor plasticidade.

Alt e colaboradores (2012) também observaram a influência da idade em algumas respostas celulares das ASCs. Foram criados três grupos de ASCs obtidas de pacientes com diferentes idades (jovens, adultos e idosos), e foi constatado que o multipotencial diminuía com a idade, o envelhecimento reduzia a capacidade de proliferação celular e também contribuía para o aumento da senescência celular.

Outra justificativa para a variação de potencial encontrado pode ser oriundo da origem das células-tronco mesenquimais obtidas. Como as SHED são originadas da crista neural, grupo de células com grande plasticidade e potencial, justificaria desta maneira, o maior potencial de neoformação óssea *in vitro* e *in vivo* observado (Graziano *et al.*, 2008).

Somado a isso, trabalhos já demonstraram que as ASCs têm necessidade de indução osteogênica para potencializar a sua diferenciação osteogênica em modelos *in vivo* (Dudas *et al.* 2006; Hofmann *et al.*, 2007), mas Gimble e colaboradores postularam que ASCs agiriam de maneira alternativa na regeneração, através da secreção de citocinas e fatores de crescimento modulando o “nicho de células-tronco” do hospedeiro de um modo parácrino (Gimble *et al.*, 2007)

Com o intuito de estudarmos os mecanismos, características e vias que levaram as SHED a apresentarem melhor potencial osteogênico nos dois ensaios em relação às ASCs, verificamos quais genes e vias poderiam proporcionar maior potencial das SHED. Para tal finalidade adotamos a análise de expressão gênica para comparação de DEGs durante o período de diferenciação osteoblastogênica *in vitro* inicial de ASCs (n=2), BMSCs (n=2) e SHED (n=2).

Três genes diferencialmente expressos em comum dentre os três tipos celulares já descritos durante o processo de diferenciação osteogênica foram observados *APOD*, *METTL7A* e *FKBP5* (Baksh *et al.*, 2004). As vias canônicas mais enriquecidas: do receptor de ácido retinóico e da via da interleucina 17A, são vias que possuem na literatura ligação com a diferenciação osteogênica, ou propriedade de imunomodulação das MSCs (Wan, *et al.* 2006, 2007; Meirelles *et al.* 2009; Zhang, *et al.* 2010; James *et al.* 2011; Kocić *et al.* 2012). Portanto esses achados corroboram e validam os resultados obtidos.

Após análise da expressão gênica de genes relacionados à pluripotência Tan *et al.* (2012) reportaram uma relação entre expressão do gene de pluripotência *Oct4* com uma maior capacidade de diferenciação (osteogênica, adipogênica, condrogênica e tenogênica) e proliferação em células-tronco isoladas do tendão quando comparadas com BMSCs. No nosso caso, a maior expressão destes genes tanto pelas BMSCs quanto para as SHED estão de acordo com o fenótipo encontrado nos experimentos *in vitro/in vivo* e com os dados da literatura em que as células-tronco provenientes de medula óssea e polpa dental possuem melhor potencial osteogênico em relação às ASCs. A menor expressão de genes de pluripotência pelas ASCs pode ser um indício de seu maior comprometimento

celular e, deste modo, conferir a estas um menor potencial de diferenciação osteogênica e plasticidade celular.

Através das análises e resultados obtidos no presente estudo podemos ressaltar:

1. SHEDs apresentaram maior potencial osteoblastogênico *in vitro* em comparação às hASCs
2. SHEDs promoveram maior indução inata (sem utilização de fatores exógenos) de neoformação óssea *in vivo* pelo hospedeiro em relação as hASCs.
3. O perfil de expressão de marcadores osteogênicos e de pluripotência no período estudado não são preditivos do potencial de diferenciação osteoblastogênica.
4. Genes de pluripotência estão menos expressos nas hASCs o que pode indicar maior comprometimento, menor plasticidade e consequentemente menor potencial osteoblastogênico *in vitro* e potencial de indução *in vivo* destas células em relação às BMSCs e SHEDs.
5. Foram identificados 3 DEGs em comum entre os tipos celulares que sofreram diferenciação osteoblastogênica inicial: *APOD*, *METTL7A* e *FKBP5*; Também identificamos 2 DEGs expressos somente pelas células com maior potencial osteoblastogênico (SHEDs e BMSCs): *TIMP4*, *ABCA6*

Resumo

Células-tronco humanas derivadas da polpa dental de dente decíduo (SHED) e células-tronco humanas derivadas de tecido adiposo (hASCs) são células multipotentes capazes de diferenciação osteogênica *in vitro* e *in vivo*, e promissoras fontes de células para a engenharia de tecido ósseo, dada a sua facilidade de expansão, isolamento e diferenciação. É de grande interesse compreender qual é o melhor tipo celular para diferenciação osteogênica, assim, o objetivo deste estudo foi comparar o potencial de diferenciação osteogênica *in vitro* e *in vivo* entre SHED e hASCs. Foram isoladas e estabelecidas seis populações de células-tronco de SHED (entre 7-12 anos) e seis da hASC (de indivíduos com idade entre 30-49 anos).

Após a indução *in vitro*, a diferenciação osteogênica foi comprovado através das colorações de fosfatase alcalina (9 dias) e vermelho de alizarina (14 e 21 dias). A quantificação da mineralização da matriz após 21 dias de diferenciação osteogênica revelou 2,24 mais ossificação das SHED em relação às hASCs.

Para realizar o experimento *in vivo*, foram triados seis biomateriais para verificar qual melhor biomaterial para o nosso modelo, defeito crítico em calvária de Ratos Wistar não imunossuprimidos, com três amostras de SHED. Após 45 dias, CellCeram™ exibiu a melhor neoformação óssea *in vivo*, e foi selecionado para comparar os potenciais osteogênicos *in vivo* entre SHED e hASCs. Células (10e6) foram associadas a discos de 4,5 mm CellCeram™, grupo controle foi realizado através do transplante do biomaterial livre de células. Neoformação óssea foi mensurada 45 dias após a cirurgia através da coloração histológica de hematoxilina / eosina. A formação óssea total foi quantificada através da análise de imagens de todas as ilhas de ossificação.

A associação entre SHED e CellCeram™ promoveu 7,24 vezes mais neoformação óssea quando comparado com a associação entre esse mesmo material e hASCs (p <0,0001). A utilização de células-tronco adultas para

regeneração óssea é uma ótima abordagem para uso terapêutico, e calcular ou prever o potencial osteogênico das células utilizadas é extremamente importante e necessário para futura aplicação em novas estratégias de bioengenharia de tecido ósseo.

Abstract

Stem cells from human exfoliated deciduous teeth (SHED) and human adipose-derived stem cells (AhSCs) are multipotent cells capable of undergoing osteogenesis in vitro and in vivo, and promising cell-source populations for bone tissue engineering given their easiness of isolation, expansion and differentiation. It is of great interest to understand which is the best cell type for osteogenic differentiation, thus the aim of this study is to compare the in vitro and the in vivo osteogenic differentiation potentials between DPSCs and ASCs. We isolated six stem cell populations from DPSCs (aged 7-12 years) and six from ASCs (from subjects aged 30-49 years) and cell culture was established.

After in vitro induction the populations were able to undergo osteogenic differentiation, as evidenced by alkaline phosphatase (9 days) and alizarin red S (14 and 21 days) stainings. Quantification of matrix mineralization after 21 days of osteogenic differentiation revealed an enhancement of 2.24-fold increase between hDPSCs and hASCs differentiation.

To perform the in vivo experiment, we promoted a screening of six scaffolds to find out which would be best scaffold to our model, a calvarial critical-sized defect in Wistar non-immunosuppressed rats, with three different culture samples of SHED. After 45 days, CellCeram™ displayed the best in vivo bone neoformation, and was used to compare the in vivo osteogenic potentials between hDPSCs and hASCs. Cells (10e6) were associated to 4.5 mm CellCeram™ discs, and control groups were performed transplanting the biomaterial free of cells. Bone healing was measured through histological hematoxylin/eosin staining 45 days after surgery. Newly formed bone was also evaluated by total bone island surface quantification through image analysis.

The association between SHED and CellCeram™ induced a mean of 7.24 times more bone formation when compared to the association between this same material and hASCs ($p < 0.0001$). The use of adult stem cells for bone regeneration

is a robust therapeutic option, and calculate or predicts the osteogenic potential of the cell used are extremely important and necessary to future application, and translation to new strategies in bone tissue engineering.

Capítulo VII

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Anexos

Lista de publicações

1) FGFR2 mutation confers a less drastic gain of function in mesenchymal stem cells than in fibroblasts

Yeh E., Atique R., **Ishiy F.A.A.**, Fanganiello R.D., Alonso N., Matushita H., Rocha K.M., Passos-Bueno M.R.

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2) Optimization of parameters for a more efficient use of adipose-derived stem cells in regenerative medicine therapies

Aguena M., Fanganiello R.D., Tissiani L.A.L., **Ishiy F.A.A.**, Atique, R. Alonso N., Passos-Bueno M.R.

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Resumos enviados para congressos:

1) Enhanced healing of rat calvarial critical size defects with beta-tricalcium phosphate discs associated with Dental Pulp and Adipose-derived stem cells

Fanganiello R.D., **Ishiy F.A.A.**, Ribeiro C., Bueno D.F., Bressiani A.H., Passos-Bueno M.R.

American Society of Bone and Mineral Research Conference, 15-19 de outubro de 2010, Toronto, Ontario, Canada

2) Scanning of biomaterial composition and microstructure for human adult stem cells association and optimization of large bone defects regeneration

Fanganiello R.D., **Ishiy F.A.A.**, Bueno D.F., Aguena M., Martins M.T., Ribeiro C., Bressiani A.H., Passos-Bueno M.R.

9th International Society of Stem Cell Research Annual Meeting,
15-18 de junho de 2011, Toronto, Ontario, Canada

3) Differences in osteogenic potential between human dental pulp stem cells and human adipose-derived stem cells in vitro and in vivo
Ishiy F.A.A., Fanganiello R.D., Agüena M., Larissa M.B., Martins M.T., Passos-Bueno M.R.
2011 Cold Spring Harbor Meetings and Conferences, Stem Cell Biology,
20-24 de setembro, Cold Spring Harbor, New York, EUA

Clinical Study

Optimization of Parameters for a More Efficient Use of Adipose-Derived Stem Cells in Regenerative Medicine Therapies

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Adipose tissue-derived stem cells (ASCs) association to fat in autologous lipotransfer is promising for a more effective soft tissue reconstruction, and optimization of protocols to isolate ASCs from lipoaspirate fat is much needed. We demonstrated that an increase in adipocyte differentiation is dependent on the number of ASCs. In a sample of 10 donors, we found a higher concentration of nucleated cells in the lower abdomen compared to flank ($P = 0.015$). In a sample of 6 donors we did not find differences in the cell yield obtained by manual or pump-assisted aspiration ($P = 0.56$). We suggest that the increase in the number of ASCs in the reinjected fat may enhance the efficiency of newly formed adipose tissue and that the anatomical region from which to harvest fat tissue needs to be considered to optimize the number of ASCs in the harvested tissue. Finally, pump-assisted aspiration can be used without any significant harm to the viability of cells.

1. Introduction

Fat transfer has been used over the past decades as an autologous dynamic filler in plastic surgery rehabilitation in several circumstances, such as in reconstruction of damaged adipose tissue due to burn injury, for craniofacial reconstruction due to congenital defects or trauma, in cancer or other tumors as well as for aesthetic reasons. Although this approach is considered successful, the need of several surgical interventions to reach the aimed result is not unusual. Therefore, the development of alternative protocols to achieve more effective reconstruction of soft tissues is of major interest. Nowadays, a current promise is to enhance adipose tissue survivability by the combination of fat transplantation and stem cell therapy, particularly with the use of adipose tissue-derived stem cells (ASCs), since stem cells potentially ameliorate neovascularization and partially halt inflammatory response [1, 2].

Adipose tissue-derived stem cells (ASCs) can be easily isolated from the stromal-vascular fraction (SVF) of human adipose tissue by simple surgical procedure, can be obtained

repeatedly, in large quantities and, in some cases, under local anesthesia, and are capable to undergo *in vitro* differentiation towards osteogenic, adipogenic, neurogenic, myogenic, and chondrogenic lineages when treated with specific factors [3, 4]. Multipotentiality of ASCs makes them interesting and promising candidates for mesodermal defect repair and disease management [5].

Regardless of using fat tissue as a sole fulfillment material or implemented with ASCs, there are several questions yet to be tackled in order to enhance its viability in tissue regeneration therapies. For instance, it is not well understood whether the percentage of ASCs in the reinjected fat tissue interferes in the efficiency of adipose tissue neogenesis. Moreover, the influence of the anatomical donor site in the number and type of mesenchymal cells is still uncertain: while some authors suggest that the subabdominal region is the most enriched site for mesenchymal stem cells (MSCs) [6, 7], others point out the hip as the best tissue to extract MSCs. Another important issue to be evaluated is the harvesting methodology, as some authors suggest that the

FIGURE 1: Oil Red-O staining for *in vitro* adipogenic differentiation of the mixed populations of ASCs and mature fibroblasts. Percentages indicate the proportion of ASCs. Pictures taken with 10X of magnification are showed in the upper line and pictures taken with 20X of magnification are showed in the lower line.

use of pump-assisted technologies decreases the viability of nucleated cells [8]. Considering that these factors might be controlled by the surgeon, their understanding will certainly have an impact in the final concentration of ASCs contained in the lipoaspirate [9].

In view of the questions listed above, the scopes of this work are to investigate if the proportion of ASCs influences the efficiency of the *in vitro* adipogenesis and to compare the quantity of nucleated cells in the SVF depending on the harvest area and finally, the harvesting methodology.

2. Materials and Methods

2.1. Ethics Statement. This study was approved by the Institute of Biosciences' Human Research Ethics Committee (permit number 095/2009-FR251136).

2.2. Lipoaspiration Surgeries. Lipoaspiration was done by a 10 mL syringe coupled to a cannula with a diameter of 2,5 mm, using the Coleman method [10] or by pump-assisted liposuction regulated at -350 mmHg using a cannula with a diameter of 3 mm. For pump versus manual lipoaspiration comparison experiments, 10 donors were submitted to sub-abdominal liposuction of right side with manual method and the sub-abdominal left side with pump method. For flank versus abdomen comparison experiments, 6 donors were submitted to liposuction in these distinct anatomical sites, with traditional pump suction lipoaspiration method. In order to minimize the differences between individuals, samples taken from the different locations and obtained using the different methods tested were paired in each series of subjects.

2.3. Adipose-Derived Stem Cells (ASCs) Isolation and Expansion. Adipose tissue from sub-abdominal and flank subcutaneous lipoaspirates were obtained by traditional pump suction from six healthy women undergoing cosmetic surgery procedures. In another instance, adipose tissue from sub-abdominal region of ten healthy women was isolated by

either pump-assisted liposuction, with controlled negative pressure, or manual lipoaspiration. The adipose tissue were washed extensively with sterile phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 pH 7.4, reagents from Sigma-Aldrich) to remove contaminating debris and red blood cells. Washed adipose tissue was treated with 0.075% collagenase (type IA; Sigma-Aldrich) in PBS for 40 min at 37°C with gentle agitation) as described previously [4]. The collagenase was inactivated with 2 volumes of HBSS (Invitrogen, CA, USA) and the infranatant was centrifuged for 5 min at 3000 g. Concerning the samples used to test whether there is an influence of harvesting pressure on cell concentration, the cellular pellet for seven samples obtained by pump-assisted liposuction and seven matched samples obtained by manual lipoaspiration were resuspended in DMEM F12 (Invitrogen, CA, USA) supplemented with 15% FBS (Hyclone), 1% non essential aminoacids (Invitrogen, CA, USA), 1% penicillin-streptomycin (Invitrogen, CA, USA) and seeded on conventional tissue culture flasks for immunophenotypic characterization.

2.4. Cell Count. Viable cells were counted using the trypan blue dye exclusion assay. A freshly prepared solution of 10 μL trypan blue at 0.05% (Sigma Aldrich) in distilled water was mixed with 10 μL of cellular suspension for 5 min., viable cells were counted in a Neubauer chamber using a light microscope (Nikon Eclipse TS100).

2.5. Immunophenotyping. Immunophenotype characterization of cell populations was done by flow cytometric analysis. For samples used in the comparison of cell yields depending on the anatomical region, we performed this characterization using the freshly isolated stromal-vascular fraction and each sample analyzed is a pool of the stromal-vascular fraction from five women donors. For samples used in the comparison of cell yields depending on the liposuction technique, the plated cell cultures were washed with PBS and digested

by trypsin solution (0,125% trypsin, 0,02% EDTA in PBS). The cells were incubated for 1 hour at 4°C with the following anti-human antibodies: CD29-PECy5, CD34PerCP, CD31-PE, CD45-FITC, CD90-R-PE, CD73-PE, CD105 (Becton, Dickinson and Company, NJ, EUA) and SH2, SH3, SH4 gently donated by professor Arnold Caplan (Case Western Reserve University). Matched control samples were incubated with PBS only. After a second wash with PBS, samples incubated with non-conjugated primary antibodies were incubated with anti-mouse-PE secondary antibody (Guava Technologies) for additional 15 min at 4°C. Cell suspensions were washed with PBS, fixed with 1% p-formaldehyde (Sigma-Aldrich) and 5.000 labeled cells were analyzed using a Guava EasyCyte flow cytometer running the Guava Express Plus software (Guava Technologies Hayward, CA, USA).

2.6. Preparation of Heterogeneous ASC and Mature Fibroblast Culture. A coronal suture periosteal fibroblast cell lineage and an adipose derived stem cell lineage were grown to 80–90% confluence in independent flasks. The cell lineages were treated with 0,125% trypsin, 0,02% EDTA (Invitrogen, CA, USA) and the suspended cells were mixed in different concentrations of these two cell lineages, as following: 5% ASC and 95% fibroblast; 25% ASC and 75% fibroblast; 50% ASC and 50% fibroblast; 75% ASC and 25% fibroblast and 100% ASC only. Cells were plated in a concentration of $10^4/\text{cm}^2$ for each experiment. These mixed cell lineages were seeded in 12 well plates and after 24 hour, the mixed cells were induced to adipogenic differentiation.

2.7. In Vitro Adipogenic Differentiation. To induce adipocyte differentiation, cells were cultured in adipogenic induction medium DMEM high glucose supplemented with 10% FBS (Gibco-Invitrogen, CA, USA), 1% penicillin-streptomycin (Invitrogen, CA, USA), $1\mu\text{M}$ dexamethasone (Sigma-Aldrich), $100\mu\text{M}$ indomethacin (Sigma-Aldrich), $500\mu\text{M}$ 3-isobutyl-1-methylxanthine (IBMX), and $10\mu\text{g/mL}$ insulin (Sigma-Aldrich) for 14 days.

2.8. Oil Red O Staining and Quantification. For Oil Red O staining, cells were cultured in 12-well culture plates and, for each experiment; four wells per condition were used. After adipogenic differentiation for 14 days, cells were washed with PBS, fixed in 4% formaldehyde for 1 hour, and then cells were washed with deionized water. After, the cells were washed with 60% isopropanol and the cell plate was dried at room temperature. The cells were stained with 0.6% (w/v) Oil Red O solution (60% isopropanol, 40% water) for 1 h at room temperature. Cells were then washed with deionized water three times to remove unbound dye and photographed. Stained Oil Red O was also eluted with 100% isopropanol (v/v) and quantified by measuring the optical absorbance at 500 nm. Oil Red O staining of undifferentiated cells grown in parallel culture served as the blank sample for this assay.

2.9. Statistical Analysis. Continuous variables were either expressed individually or by mean and standard deviation and we used the nonparametric Wilcoxon signed-rank test for paired data in order to assess if the population mean

FIGURE 2: Linear regression showing significant correlation between the percentage of ASCs in the mixed cell population and the increase in Oil Red-O staining ($R^2 = 0.979$). Error bars represent standard deviation for quadruplicates of each sample.

rank differ. To assess the statistical significance of the correlation between pump-assisted and manual liposuction and between the percentage of ASCs in mixed cellular populations and adipogenic differentiation we used two-tailed Pearson's correlation test. Tests with P values < 0.05 were considered to be statistically significant. All the applied tests were done by the use of the GraphPad Prism 5 program.

3. Results

3.1. Influence of the Concentration of Adipose-Derived Stem Cells on the In Vitro Adipogenic Differentiation. To evaluate if there is an influence of the concentration of adipose-derived stem cells on the efficiency of the *in vitro* adipogenesis, we created five heterogeneous cell cultures composed of mixed subpopulations of ASCs and mature fibroblasts derived from cranial coronal suture, induced them to adipogenic differentiation and quantified the lipid vacuoles formation by Oil Red-O staining after 14 days (Figure 1). Applying a linear regression model, as showed in Figure 2, there is a significant correlation between the percentage of ASCs and the increase in Oil Red-O staining ($R^2 = 0.979$).

3.2. Influence of Donor Site on Cell Concentration. In order to determine if the number and proportion of mesenchymal cells varies according to the donor site of the adipose tissue, we evaluated cells from SVF from six female patients with mean age of 37 years (range, 26–51 years) submitted to liposuction at the lower abdomen and at the flank following medical recommendations. We observed a significantly higher concentration of nucleated cells in fat from the lower abdomen when compared to fat from the flank ($P = 0.015$; Figures 3(a) and 3(b)).

As showed in Figure 4, we observed a higher proportion of cells positive for mesenchymal (79.7–84.7%) and adhesion cell markers (CD29; 96%) in samples isolated from the abdomen as compared to the samples obtained from the flank (24.36–28.40% and 50.48%, resp.). Furthermore, samples

FIGURE 3: Bar graphs showing (a) cell quantity ($\times 10^6$) per volume of fat tissue (mL) obtained from abdomen or flank from six individuals and (b) Mean and standard deviation from data showed in graph "a". The Wilcoxon signed-rank test indicates significant difference between the samples ($P < 0.05$).

from the flank had a more prominent subpopulation of cells of hematopoietic (53.75%) and endothelial (21.56%) origin when compared to the ones from the abdomen (29.38 and 15.8%, resp.).

3.3. Influence of Harvesting Methodology on Cell Concentration after 1 Passage. A group formed by 10 patients, with mean age of 49 (range, 22–73 years) and mean body mass index of 24.88 (range, 22–29.6), submitted to sub-abdominal liposuction according to medical recommendation, was used to evaluate if there is an influence of the harvesting method on the yield of nucleated cells in the SVF. Liposuction was performed applying 2 commonly used harvesting methods: manual aspiration using a syringe and pump-assisted aspiration, with a controlled pressure of -350 mmHg. After 1 passage in culture, cells aspirated manually and obtained by pump-assisted aspiration showed positive staining ($>89\%$) for mesenchymal (SH2, 3 and 4) and adhesion (CD29)

markers and negative staining ($<4\%$) for hematopoietic (CD45) and endothelial (CD31) markers. Fold change differences in the amount of cells obtained by pump-assisted method were calculated compared to manual aspiration and are represented in Figure 5(a). Applying a linear regression model (Figure 5(b)), there is no statistically significant difference on the cell yield obtained using these 2 methods.

4. Discussion

In autologous fat transplantation with large volume transfer, fat survival rather than fibrosis is desired. Other expected characteristics, which are usually highly variable, are the clinical longevity of fat graft and the maintenance of volume of the transplanted fat. To amend these variables, the combination of stem cell therapy with fat transfer, supplementing fat grafts with adipose-derived stem cells, has been reported as a method of autologous tissue transfer termed cell-assisted lipotransfer [9, 11, 12] and is a promise for the rehabilitation of several patients.

Using an *in vitro* model of admixed heterogeneous cell populations we found a positive correlation between the percentage of ASCs and the increase in the *in vitro* adipocyte differentiation. Thus, we suggest that the increase in the number of ASCs in the reinjected fat tissue may enhance the efficiency of newly formed adipose tissue. These results would thus support the idea that enrichment of adipose tissue with mesenchymal stem cells can influence the rehabilitation process of patients submitted to autologous fat graft [5].

Therefore, establishing the ideal parameters to optimize the number of viable mesenchymal cells, such as settling preferred donor sites of lipoaspiration from which to isolate the ASCs and determining if the method of lipoaspiration interferes in the quantity and quality of the mesenchymal cells will certainly contribute to a more successful use of fat transplantation enriched with mesenchymal cells.

The finding of a significantly higher concentration ($P < 0.05$) of nucleated cells in the lower abdomen when compared to flank together with the observation of an enriched subpopulation of cells from mesenchymal origin in samples from the lower abdomen suggests that SVF from the lower abdomen might be a better source of mesenchymal stem cells than adipose tissue isolated from the flank. Our findings are consistent with the work of Padoin et al. [7], where they found a significantly higher concentration of nucleated cells in the SVF obtained from the lower abdomen when compared to upper abdomen, inner thigh, trochanteric region, knee, and flank. Jurgens et al. [6] also suggested, after CFU assays, that the abdomen is preferable to the hip/thigh region for harvesting mesenchymal stem cells.

If we take into account the number and type of mesenchymal cells, it thus seems that the adipose tissue from the lower abdomen is better than those from the flank. However, further studies are necessary in order to elucidate the functional effect on tissue regeneration as we do not know if the higher proportion of hematopoietic and endothelial cells contained in the flank might actually facilitate neovascularization, which is critical for the success of the surgery procedure.

FIGURE 4: Immunophenotype characterization of fresh human stromal vascular fraction isolated from adipose tissue. The donors were submitted to liposuction in 2 different sites (abdomen and flank), and each sample analyzed is a pool of the stromal-vascular fraction from five women donors. Histogram for each sample (areas displayed in black) comparing cell number and fluorescence intensity with matched negative controls (areas displayed in grey). The percentage of positively stained cells is showed above each histogram.

FIGURE 5: (a) bar graph depicting the $\log_2$ of the fold changes between the quantity of cells obtained by pump-assisted method compared to the amount of cells obtained by manual aspiration for ten individuals. (b) linear regression model showing no statistical difference in cell number depending on the liposuction method used. The difference in the slopes of the linear regression of experimental data (black line) is not significant ($P = 0.56$) compared to the theoretical line (dotted line, constructed assuming there is no difference between the data).

We did not find significant differences in SVF cell yields comparing manual liposuction using a syringe or pump-assisted liposuction. Indeed, Fraser et al. [13] also tested these two approaches of fat tissue aspiration and concluded that the frequency of clonogenic cells was not impacted by the harvesting methodology. Although these data suggest no influence of these methodologies in the SVF nucleated cell quantity, a study by Mojallal et al. [8] concluded that liposuction at a controlled pressure of -350 mmHg gives a greater cell yield when compared to power-assisted aspiration with a negative pressure of 700 mmHg and syringe aspiration. However, these results should be considered with caution, as they performed this test using only 3 patients.

In this paper, we demonstrate that even though there is no difference on the nucleated cell yield obtained by manual aspiration using a syringe or with pump-assisted aspiration with a pressure of -350 mmHg, the anatomical region from which to harvest fat tissue needs to be considered as a means to optimize the total number of nucleated cells in the SVF and, consequently, the quantity of adipose-derived stem and progenitor cells. For the foreseeable future, implementations to the cell-assisted lipotransfer will lead to improve fat-grafting outcomes for restoration of tissues for either aesthetic or reconstructive purposes. Further, our results support the hypothesis that enrichment of adipose tissue with mesenchymal stem cells might improve the regeneration process following a cell-assisted lipotransfer.

Authors' Contributions

M. Agüena and R. D. Fanganiello contributed equally to this work.

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FGFR2 Mutation Confers a Less Drastic Gain of Function in Mesenchymal Stem Cells Than in Fibroblasts

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Abstract Gain-of-function mutations in *FGFR2* cause Apert syndrome (AS), a disease characterized by craniosynostosis and limb bone defects both due to abnormalities in bone differentiation and remodeling. Although the periosteum is an important cell source for bone remodeling, its role in craniosynostosis remains poorly characterized. We hypothesized that periosteal mesenchymal stem cells (MSCs) and fibroblasts from AS patients have abnormal cell phenotypes that contribute to the recurrent fusion of the coronal sutures. MSCs and fibroblasts were obtained from the periosteum of 3 AS patients (S252W) and 3 control individuals (WT). We evaluated the proliferation, migration, and osteogenic differentiation of these cells. Interestingly, S252W mutation had opposite effects on different cell types: S252W MSCs proliferated less than WT MSCs, while S252W fibroblasts proliferated more than WT fibroblasts. Under restrictive media conditions, only S252W fibroblasts showed enhanced migration. The presence of S252W mutation increased in vitro and in vivo osteogenic differentiation in both studied cell types, though the difference compared to WT cells was more pronounced in S252W fibroblasts. This osteogenic

differentiation was reversed through inhibition of JNK. We demonstrated that S252W fibroblasts can induce osteogenic differentiation in periosteal MSCs but not in MSCs from another tissue. MSCs and fibroblasts responded differently to the pathogenic effects of the *FGFR2*^{S252W} mutation. We propose that cells from the periosteum have a more important role in the premature fusion of cranial sutures than previously thought and that molecules in JNK pathway are strong candidates for the treatment of AS patients.

Keywords Mesenchymal stem cells · Fibroblasts · *FGFR2* · Apert syndrome · Osteogenesis

Introduction

Apert Syndrome (AS), the most severe form of craniosynostosis [1], is characterized by premature fusion of the coronal sutures, severe syndactyly of the hands and feet and by a range of skeletal abnormalities, including progressive joint limitation [2–4]. This autosomal dominant syndrome is caused by gain-of-function mutations in the *FGFR2* gene. The most prevalent mutation, accounting for approximately 65% of all AS cases, is S252W. *FGFR2*, by means of alternative mRNA splicing, can be transcribed into an epithelial and a mesenchymal isoform. Both isoforms are tyrosine kinase receptors that bind to a specific subset of fibroblast growth factors (FGFs) to induce a variety of cell functions, such as cell migration, proliferation, and differentiation [5]. In the presence of the S252W mutation, *FGFR2* shows enhanced ligand-binding affinity to FGF2 and loses isoform-ligand specificity for most of the other ligands [6]. This mutation affects both the epithelial and the mesenchymal *FGFR2* isoforms. Although most of the clinical features of AS arise as a consequence of signaling disturbance during

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embryonic development, FGFR2^{S252W} also interferes in post-natal organism homeostasis. Surgical opening of the coronal sutures is a mandatory procedure for AS patients. However, the excessive and repetitive closure of these sutures after the procedure (resynostosis) requires multiple interventions from birth until adulthood [1].

It has been postulated that FGFR2^{S252W} enhances cell proliferation, which would account for the higher ossification rate at the sutures [7]. However, we still do not know if this enhanced proliferation is observed in all cell types. Most studies have focused on murine osteoblasts harboring the AS mutation, but both increased [8] and decreased [9] proliferation have been observed. Studies on the effect of FGFR2^{S252W} on osteogenic potential have also produced contradictory results [8–10]. Few studies have investigated the functional effect of FGFR2^{S252W} in human cells, which are considerably different from murine cells in regard to cell signaling [11–16]. We have conducted a preliminary study in which we suggested that S252W fibroblasts have an increased osteogenic potential [17], a finding that we sought to replicate using in vivo models. On the other hand, there are no studies on human AS mesenchymal stem cells (MSC), the possible precursors of osteoblasts. The premature suture fusion and the resynostosis process after surgical interventions are not likely the result of alterations in one particular cell type, such as osteoblasts, but the result of perturbations in signaling and in interactions between different cell types and tissues of the cranial suture complex [18].

The cranial suture complex comprises the overlying periosteum of the calvaria, the osteogenic fronts of the bone plates, the intervening mesenchyme, and the underlying dura mater. This complex allows skull deformation during birth, expansion during brain growth and regulates the balance between proliferation and differentiation of osteogenic precursors [18]. Several studies have indicated dura mater as a crucial tissue that regulates suture patency, so it is assumed that signaling by this tissue is deregulated in AS patients, contributing to premature embryonic synostosis as well as adult resynostosis [1, 19]. Remarkably, the removal of the periosteum diminishes calcification of cranial defects in animal models [20, 21], which highlights the importance of the periosteum in cranial bone formation. The periosteum contributes not only to normal bone growth, but also to bone healing and regeneration [22, 23]. This highly cellular tissue contains multipotent MSC, fibroblasts, differentiated osteogenic progenitor cells and osteoblasts [24, 25], and acts as a major source of skeletal stem cells/progenitors during bone repair [26]. It is thus possible that periosteal cells also have a major contribution to the premature suture fusion in AS patients; however, their role in this process is still poorly characterized. In addition, it is still unknown how the different cell types from the calvarial

periosteum interact and what is the functional effect of the FGFR2^{S252W} mutation in these cells.

JNK (c-Jun N-terminal kinases) has been reported as crucial for the final stage of differentiation in pre-osteoblasts and pluripotent cells [27–30], and is considered a critical regulator of important osteogenic differentiation markers [30, 31]. In addition, two negative regulators of JNK activity of the same family are associated with craniosynostosis: DUSP6 and DUSP2. The loss of *Dusp6* leads to coronal craniosynostosis in mice [32], and we have previously reported that *DUSP2* was one of the most significant differentially expressed genes in AS periosteal cells [17]. Therefore, JNK is an interesting candidate for the altered osteogenic potential of S252W cells.

In view of the above, we conducted this study to access the effect of the FGFR2^{S252W} mutation in fibroblasts and MSCs derived from the coronal periosteum. We examined these cells' proliferative capacity, motility, and osteogenic potential and also evaluated whether there is a functional interaction between them. Finally, we have evaluated the role of JNK in the increased osteogenic potential of S252W fibroblasts.

Materials and Methods

Subjects

Coronal suture periosteal fibroblasts and MSCs from three unrelated AS patients and from three age- and sex-matched control subjects were obtained as previously described [17]. The presence of the c.934 G>C (p.S252W) mutation was confirmed by direct DNA sequencing and expression of the mesenchyme-specific isoform of FGFR2 in the primary fibroblasts was examined by Western Blot and RT-PCR [17].

Cell Culture

Periosteum harvested from AS patients or control individuals were split in half for fibroblast and MSC extraction. Primary periosteal fibroblasts derived from periosteal flaps were grown in fibroblast growth medium (DMEM High-Glucose, 20% fetal bovine serum [FBS; GIBCO] and 100 U/mL penicillin and 100 µg/mL streptomycin [1% Penicillin Streptomycin; GIBCO]). Cells were passaged at near confluency with trypsin-EDTA. MSC cultures were obtained from finely minced periosteum after 30 min of trypsin incubation and grown in MSC growth medium (DMEM-F12 [Invitrogen] supplemented with 15% FBS [GIBCO] and 1% Penicillin Streptomycin [GIBCO]). All cells were cultured in a humidified incubator at 37°C and 5% CO₂. All tests were performed between the third and the fifth subcultures.

For each of the 12 cell lines (three S252W fibroblasts, three WT fibroblasts, three S252W MSCs and three WT MSCs), we performed experiments in technical triplicates. For all the experiments, we used all twelve cell lines for each condition, the exceptions are indicated by an “n” value. Thus we tried to ensure that the results we obtained were representative of the biological variance seen in human patients.

Immunophenotyping

To analyze cell-surface expression of typical protein markers, adherent cells were incubated with the following anti-human primary antibodies: CD29-PECy5, CD31-phycoerythrin (PE), CD45-fluorescein isothiocyanate (FITC), CD90-R-PE, CD117-PE, and SH3 (Becton Dickinson). A total of 5,000 labeled cells were analyzed using a Guava EasyCyte flow Cytometer running Guava ExpressPlus software (Guava Technologies).

Cell Proliferation Analysis

A density of 4,000 cells/cm² was plated to each well of a 12-well flat bottom plate in fibroblast growth medium. After 24 h, when total cell adhesion was verified, the fibroblasts were serum-starved for 24 h and MSCs for 48 h. At the initial time point (0 h), we changed the starvation medium (fibroblast growth medium or MSC growth medium without FBS) for the respective cell growth medium or starvation medium supplemented with 0.5%, 10% and 20% FBS. At the indicated times, the cells were trypsinized and counted using Guava EasyCyte Flow Cytometer (Guava Technologies). The experiment was done in triplicates for each time point and cell line.

In Vitro Wound Healing Assay

We plated the cells (3×10^5) on 12-well culture plates (Corning) in the respective cell growth medium. Upon reaching 100% confluence, the fibroblasts were serum-starved for another 24 h and MSCs for 48 h. After starvation, a single wound was created in the center of the cell monolayer by gentle removal of the attached cells with a sterile plastic pipette tip. The cell layer was then scratched with a P-200 pipette tip, the debris was removed by washing with PBS (Phosphate Buffered Saline) and we added fibroblast or MSC growth medium. Photographs of the wound adjacent to reference lines scraped on the bottom of the plate were taken using an Axio Observer microscope under 5× field (Zeiss) at 0 h and 12 h after the wound was done. We used the ImageJ software [33] and Adobe Photoshop CS3 (Adobe) to analyze and calculate the number of cells that moved into the wound. The experiment was done in triplicates for each treatment and each cell line.

In Vitro Osteogenic Differentiation

To induce osteogenic differentiation, periosteal fibroblasts and MSCs from three AS patients and from three controls were plated in 24-well plates (5×10^3 cells/cm²) and cultured for 3 weeks in osteogenic medium (DMEM Low-Glucose, 0.5% FBS [GIBCO], 0.1 mM dexamethasone (Sigma-Aldrich Corp., St. Louis, MO), 50 mM ascorbate-2-phosphate (Sigma-Aldrich), 10 mM β-glycerophosphate (Sigma-Aldrich), and 1% Penicillin Streptomycin [GIBCO]). For the co-culture assay, the cells were plated at the same concentration onto 12-mm transwell inserts of 12-well plates, 0.4 μm pore size (Corning Costar). Media changes occurred every three to 4 days. As an internal control of the experiment, the same cells were maintained throughout the 21 days of differentiation in regular growth medium.

Alkaline phosphatase activity was assessed on the 9th day of differentiation through a biochemical assay. The cells were provided with phosphatase substrate (Sigma-Aldrich) and the re-sulting p-nitrophenol was measured colorimetrically by the use of a Multiskan EX ELISA plate reader (Thermo Scientific) at 405 nm.

After 14 and 21 days, calcified matrix production was analyzed by alizarin red staining and quantification was done as previously described [34].

In Vivo Osteogenic Differentiation

A 4.5 mm in diameter ceramic scaffold (60% hydroxyapatite and 40% of β-tricalcium phosphate; Cellceram ScaffoldTM) was moistened with osteogenic medium and mixed with 10^6 human fibroblasts or MSCs. The cells attached to the scaffold were pre-differentiated in osteogenic medium and incubated at 37°C in 5% CO₂ for 5 days.

For the in vivo differentiation we used 8 non-immunosuppressed (NIS) Wistar rats (all males, aged 2 months, weighing a maximum of 200 g) as previously described by our group and approved by the ethical committee of our Institute [35]. We used a trephine bur of 4.5 mm diameter to obtain two cranial critical defects which were made in the parietal region, lateral to the sagittal suture, where two scaffolds were implanted per animal, one side being filled by biomaterial alone (left defect) and the other by the biomaterial associated with cells (right defect). The animals were kept in ventilated racks with standard conditions of temperature and lighting (22°C, 12 h light cycling per day) with free access to food and water. Four weeks after surgery, the rats were sacrificed in a CO₂ chamber. The calvaria was removed and fixed in 10% formalin for 24 h and then decalcified in 5% formic acid for 48 h and embedded in paraffin. Slices of 5 μm were obtained and stained with hematoxylin and eosin.

We analyzed three transversal 4 μm slices of the calvaria with 10 μm of distance of each animal. Ossification area of each defect was calculated through Axio Vision Carl Zeiss based on 10x amplified images obtained from Axio Observer.A1 Carl Zeiss microscope. The percentage of the defect area that ossified at the right side was normalized by the percentage of the defect area that ossified at the left side, so that for each animal we obtained 3 ratio values.

JNK Inhibitor Treatment

To test for the role of JNK in the enhanced osteogenic potential of S252W fibroblasts, we used the reversible ATP-competitive JNK inhibitor SP600125 (Sigma-Aldrich). Stock solutions of at least 20 mM were made using 100% dimethyl sulfoxide.

First, we evaluated the levels of p-JNK to confirm the inhibition of JNK activity by SP600125, since the inhibitor has been described to downregulate the autophosphorylation activity of JNK [36]. Total protein extracts were prepared using Phosphosafe extraction reagent (EMD Biosciences) according to the protocol provided by the manufacturer. The levels of P-JNK were assessed by western blotting. The antibodies used were anti-p-JNK (Thr183/Tyr185) Rabbit mAb (Cell Signaling), anti-SAPK/JNK Rabbit mAb (Cell signaling), anti-Rabbit IgG-HRP (Cell Signaling) and Anti-B-actin-HRP (AbCam) (Supplementary figure 1).

During osteogenic differentiation, media were supplemented with 2 μM and 4 μM of the inhibitor, which corresponds to IC50 and twice the IC50 respectively [37].

Statistical Analysis

Continuous variables were expressed by mean and standard deviation, and the groups were compared by Student's *t*-test. A *p* value < 0.05 was considered statistically significant. The tests were performed using the GraphPad InStat software (GraphPad).

Results

Characterization of the Immunophenotype

In order to certify that the results we obtained were representative of the biological variance seen in human patients, we performed all experiments in fibroblast or MSC cultures from three unrelated AS patients (S252W cells) and from three control individuals (WT cells). Each cell culture was studied in three technical replicates.

All cell cultures were adherent and with a fibroblast-like appearance. We performed flow cytometry experiments with different markers in order to characterize the immunophenotype of fibroblast and MSC cultures (Table 1). S252W and WT cells were highly positive for mesenchymal cell markers (>85%) and negative for hematopoietic and endothelial cell markers. These results confirm that these cells are of mesenchymal origin.

Cell Proliferation and Cell Migration

In order to determine the effect of the FGFR2^{S252W} mutation in periosteal cells, we first accessed whether the presence of the mutation altered the proliferation rates of S252W fibroblasts and S252W MSCs. The S252W mutation increased cell proliferation in fibroblasts at all times of culture (24 h: *p* < 0.001, 48 h: *p* < 0.001; 72 h: *p* < 0.001) (Fig. 1a) and in different culture conditions (0.5% FBS medium: *p* = 0.014; 10% FBS medium: *p* = 0.04; and 20% FBS medium: *p* < 0.001) (Fig. 1c). On the other hand, in MSCs, the mutation decreased cell proliferation after 72 h in MSC growth medium (72 h: *p* = 0.002) (Fig. 1b) and in enriched medium (20% FBS medium: *p* = 0.004) (Fig. 1d).

We next evaluated the effect of FGFR2^{S252W} on cell migration. The S252W mutation increased cell migration in fibroblasts only in restrictive medium condition (0.5% FBS medium: *p* < 0.001) (Fig. 1e), but had no effect in MSCs (Fig. 1f).

Table 1 Percentage of positive cells for mesenchymal (SH3, CD90, CD29), hematopoietic (CD117 and CD45) and endothelial (CD31) cell lines antibodies

	MSCs		Fibroblasts	
	S252W (n=2)	WT (n=2)	S252W (n=4)	WT (n=4)
SH3	96.32–99.78%	94.7–98.68%	88.92–98.45%	93.92–97.87%
CD90	94.92–95.52%	94.02–98.7%	89.12–99.7%	89.46–98.94%
CD29	98.48–99.12%	88.1–98.98%	85.14–99.02%	70–99.25%
CD31	<5%	<5%	<5%	<5%
CD117	<5%	<5%	<5%	<5%
CD45	<5%	<5%	<5%	<5%

Fig. 1 **a** Comparative analysis of the proliferation of WT (from 3 individuals) and S252W (from 3 patients) fibroblasts and **b** WT (from 3 individuals) and S252W (from 3 patients) MSCs. Each point indicates the average for each time and each condition and the error bars represent the standard deviation for the biological replicate (same S252W or WT cell type culture results summed). Analysis of **c** WT (from 3 individuals) and S252W (from 3 patients) fibroblasts and **d** WT (from 3 individuals) and S252W (from 3 patients) MSCs when grown in culture medium with different FBS concentration. Each point indicates the average of each medium after 48 h and the error bars represent the standard deviation for the biological replicate. **e** Wound healing assay of S252W ($n=3$) and WT ($n=3$) fibroblasts and **f** and S252W ($n=3$) and WT ($n=3$) MSCs in high FBS and low FBS growth medium. The bars represent the average number of cells that migrated toward the wound after 12 h for each condition and error bars represent the standard deviation for the biological replicate (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$)

In Vitro Osteogenic Differentiation

Next, we assessed the effects of the S252W mutation on osteogenic differentiation. We performed experiments at key points of our in vitro differentiation protocol in order to evaluate whether the mutation plays a more prominent part at a specific stage of osteogenic differentiation.

The ninth day of differentiation is ideal to access the levels of alkaline phosphatase (ALP) in cultures, as in this period there is a peak production of the enzyme. ALP provides the phosphate needed for the production of cellular matrix calcium. S252W fibroblasts showed 6-fold increase in ALP activity in comparison to WT fibroblasts ($p<0.001$) (Fig. 2a), while S252W MSCs had 3-fold increase in comparison to WT MSCs ($p<0.001$) (Fig. 2b).

After 2 weeks in osteogenic medium, we analyzed initial calcium deposition in the extracellular matrix (ECM)

through alizarin red staining. S252W fibroblasts showed 2.7-fold increase in ECM calcium in comparison to WT fibroblasts ($p<0.001$) (Fig. 2c), while S252W MSCs had 1.5-fold increase in comparison to WT MSCs ($p=0.016$) (Fig. 2d).

Finally, we evaluated concentration of ECM calcium at the end of differentiation (21st day). S252W fibroblasts showed a 1.7-fold increase in ECM calcium in comparison to WT fibroblasts ($p=0.002$) (Fig. 2e), while S252W MSCs had a 1.5-fold increase in comparison to WT MSCs ($p<0.001$) (Fig. 2f).

In Vivo Osteogenic Differentiation

To validate these data in vivo, we performed the bilateral cranial critical defect experiment using Wistar NIS rats as previously described by our group [35]. At the right-side

defect we introduced either S252W or WT cells associated with biomaterial, and at the left-side defect we introduced biomaterial only as an internal control of each animal's osteoregeneration. Four weeks after the surgery, the right-side:left-side ossification ratio was 4.9 in S252W fibroblasts and 1.9 compared to WT fibroblasts (2.6-fold higher; $p=0.036$) (Fig. 2g). Likewise, this ratio was 11.8 in S252W MSCs and 2.6 in WT MSCs (4.5-fold higher; $p=0.001$) (Fig. 2h).

Interactions between Periosteal MSCs and Fibroblasts During Osteogenic Differentiation

As shown above, we observed *in vitro* and *in vivo* that the cellular phenotype alterations due to S252W mutation seemed more drastic in fibroblasts than in MSCs, and that S252W fibroblasts are particularly more prone to osteogenic differentiation. These data thus raised the question whether S252W fibroblasts could induce osteogenic differentiation in other cells.

Therefore, in order to test the hypothesis that a cell population with the S252W mutation alters normal signaling in adjacent cells, we used a co-culture system to simulate the *in vivo* anatomic link between the fibroblasts and MSCs in the periosteum, allowing the paracrine signaling without physical cell interaction. S252W fibroblasts induced 30% more differentiation of periosteal MSCs, whether WT ($n=3$) or S252W ($n=2$), both by ALP assay (WT MSCs: $p<0.001$; S252W MSCs: $p=0.037$) and alizarin red staining (vs. WT MSCs: $p=0.007$; vs. S252W MSCs: $p=0.016$) (Fig. 3a and c). Interestingly, S252W fibroblasts did not induce osteogenic differentiation of MSC from another tissue, such as dental pulp stem cells (DPSC) (Fig. 3a and c). Further, S252W MSCs and WT MSCs exhibit no influence on the osteogenic differentiation of S252W fibroblasts (Fig. 3b and d).

Potential Molecule Involved in Altered Fibroblast Osteogenic Potential

To assess whether JNK plays a role in the increased osteogenic potential of S252W fibroblasts, we treated S252W fibroblasts with 2 μM (IC₅₀) [37] and 4 μM (twice IC₅₀) of SP600125, a JNK phosphorylation inhibitor, during osteogenic differentiation. We observed a lower ALP activity as we increased the concentration of SP600125 (untreated vs. +2 μM SP600125: $p=0.025$; +2 μM SP600125 vs. +4 μM SP600125: $p=0.014$). This effect was also observed by alizarin red staining (untreated vs. +2 μM SP600125: $p=0.006$; untreated vs. +4 μM SP600125: $p=0.003$) (Fig. 4a and b). At the maximal inhibition of JNK (4 μM), ALP activity of S252W fibroblast and WT fibroblasts were equivalent. Therefore,

Fig. 2 S252W and WT fibroblasts and MSCs (all conditions: $n=3$) in response to osteogenic medium during different phases of osteogenic differentiation. **a, b** Analysis of alkaline phosphatase activity on the 9th day of osteogenic differentiation; **c, d** alizarin red staining quantification at the 14th day of differentiation; **e, f** alizarin red staining quantification at the 21st day of osteogenic differentiation. The columns represent the absorbance at wavelength indicated for each condition and error bars represent the standard deviation for the biological replicate. **g** Percentage of ossification area of calvarial defects with WT or S252W fibroblasts in rats 4 weeks after surgery. **h** Percentage of ossification area of calvarial defects with WT or S252W MSCs in rats 4 weeks after surgery (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$)

inhibition of JNK activity rescued the increased osteogenic potential of S252W fibroblasts.

Discussion

The only treatment available nowadays for AS patients is surgical intervention, which consists of artificial reconstruction of the coronal sutures [38]. Nevertheless, post-surgical ossification of the sutures (resynostosis) is frequent and any surgical treatment for craniosynostosis during childhood is considered a procedure to delay but not to prevent synostosis [1]. To understand the cause and prevent resynostosis of the sutures so that they remain open during the growth phase of the individual, it is important to identify the main molecules and mechanisms involved in this process. This knowledge can contribute to a better management of the affected child, enhancing their life quality. Bone regeneration studies have pointed to the periosteum as a significant contributor to the ossification process [22, 23], not only through molecular signaling, but also by providing osteoprogenitor cells [26]. Despite the periosteum's possible contribution to premature suture ossification in AS and other craniosynostosis, the mechanism by which FGFR2 gain-of-function mutations achieve this effect is not clear. Unbalanced FGF signaling can accelerate proliferation, migration, and differentiation in osteoprogenitor cells from the periosteum and contribute to resynostosis, though this process continues to be poorly understood, particularly in AS patients. Study of cells derived from the periosteum of the AS patients can help to elucidate these questions, as we can compare the effect of the mutation in different cell types from the same niche. This could be of particular relevance considering that cell signaling can be different between mice and humans, and that the range of phenotypic effects of a mutation is not always the same in both [13–16]. Here we successfully established fibroblasts and MSCs cell cultures from the periosteum of both AS and control individuals. In accordance to the literature [38], we were not able to distinguish these two cell types based on morphology and positive staining for mesenchymal cell markers. However, the

Fig. 3 Effects of interaction between periosteal MSCs and fibroblasts. **a** Analysis of alkaline phosphatase activity on the 9th day and **c** alizarin red staining at the 21st day of osteogenic differentiation of S252W MSCs ($n=2$), WT MSCs ($n=2$) and WT DPSCs ($n=1$) co-cultured with fibroblasts with or without the mutation in the presence of osteogenic medium. **b** Analysis of alkaline phosphatase activity on the 9th day and **d** alizarin red staining at the 21st day of osteogenic differentiation of S252W fibroblast ($n=1$) co-cultured with MSCs with or without the mutation in the presence of osteogenic medium. The columns represent the absorbance at wavelength indicated for each condition and error bars represent the standard deviation for the biological replicate (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$)

positive bone differentiation seen in WT MSCs but not in WT fibroblasts confirms that our protocols allow for the establishment of two distinct cell populations, as we have shown in our previous reports [17, 39].

The S252W mutation enhanced cell proliferation in fibroblasts, even in critical culture conditions, such as 0.5% FBS medium, but it had a negative effect on MSCs in 20% FBS culture environment. Literature data regarding the presence of FGFR2^{S252W} and cell proliferation are controversial. Some studies that investigated human periosteal fibroblasts [17], murine osteoblasts [8], murine stem cells [40], or

calvarial cells during embryonic sutures formation in murine AS models [41, 42] point to the FGFR2^{S252W} mutation as responsible for increasing cell proliferation. Interestingly, the murine MSCs with FGFR3 mutation proliferate less than the wild type cells [43]. These comparative analyses are complex, as the experiments have not only included different cell types but also cells from different species and from different niches. Therefore, although we cannot rule out differences in the protocols used, our results suggest that the effect of the FGFR2^{S252W} mutation on cell proliferation might depend both on the tissue, niche of origin, and cell type under

Fig. 4 Effects of JNK inhibition in periosteal S252W ($n=2$) and WT ($n=2$) fibroblasts. **a** Analysis of alkaline phosphatase activity on the 9th day of osteogenic differentiation. **b** Alizarin red staining quantification at the 21st day of osteogenic differentiation. The columns represent the absorbance at wavelength indicated for each condition and error bars represent the standard deviation for the biological replicate (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$)

Fig. 5 In the periosteum (pink) overlying the suture (brown), fibroblasts (green cells) and MSCs (blue cells) have similar cell growth and cell migration rates. However, fibroblasts exhibit low while MSCs show higher osteogenic potential (left). The $FGFR2^{S252W}$ mutation has a positive effect on both proliferation and migration of fibroblasts, and a negative effect on MSC proliferation. It has no consequence on MSCs migration. Both cell types have enhanced osteogenic differentiation and S252W fibroblasts show positive influence on MSCs differentiation. Inhibition of JNK phosphorylation by SP600125 can null the effect of the mutation over osteogenic differentiation of fibroblasts

analysis, which would explain at least in part the controversy in the literature.

Through the wound healing assay, we have shown that $FGFR2^{S252W}$ has an effect on the migratory property of fibroblasts, but not of MSCs. This effect on the fibroblasts, however, was dependent on the availability of FBS, a culture medium supplement that provides not only growth factors, but also cellular growth inhibitors [44]. It was previously reported that $FGFR2^{S252W}$ has enhanced affinity to different FGFs [45], therefore, the altered S252W cell proliferation and cell migration in response to different FBS concentrations suggests that FBS contains growth stimulating FGFs to which S252W fibroblasts are more sensitive to, and FGFs that in high concentration inhibit migration of S252W fibroblasts. It is possible that during embryonic and even post-natal development, atypical cell responses are dependent on which FGF is available at that period.

Regarding the osteogenic potential, we found that $FGFR2^{S252W}$ induces increased osteogenic differentiation in both cell types under study, especially at the early stages of the *in vitro* process. We were able to confirm these results *in vivo* using non-immunosuppressed rats and human cells, as an increased ossification rate was observed for both fibroblasts and MSCs harboring the $FGFR2^{S252W}$ mutation as compared to corresponding WT cells. These results thus confirm our preliminary work on S252W fibroblasts [13] which showed that the $FGFR2^{S252W}$ mutation confers a new function to these cells. In addition, our results for MSCs, the osteoblast precursors, are in agreement with most of the literature, ranging from studies with murine stem cells to human pre-osteoblasts transfected with $FGFR2^{S252W}$, which showed that the $FGFR2^{S252W}$ mutation enhances osteogenic potential [8, 9, 40, 41, 46].

A dynamic system based on integrated signals between stem cells and cells from their surrounding niche, such as fibroblasts, is necessary to maintain proper tissue physiology [47]. Thus, we judged it necessary to evaluate the effect of S252W fibroblasts on the MSCs, the expected osteoblast precursors. Interestingly, our co-culture assay showed that the presence of S252W fibroblasts promotes osteogenic differentiation of both S252W and WT periosteal MSCs. In addition, S252W fibroblasts did not induce osteogenic differentiation in DPSC, which suggests that the cells we used must still harbor regulatory network programs that are specific to the tissue from which they are extracted and that are not erased in cell culture.

The increased osteogenic potential of S252W fibroblasts seems to be mediated by JNK, since JNK inhibition reverted this phenotype. Shukla et al. demonstrated that ERK (extracellular-signal-regulated kinase) is directly involved in the craniosynostosis in a $Fgfr2^{+/S252W}$ mouse [48]. Furthermore, ERK indirectly increases JNK activity [49]. Our findings thus suggest that ERK-JNK pathway is also disturbed in human AS patients and that a key molecule involved in craniosynostosis lies downstream to ERK and JNK. Altogether, we propose that identifying this key molecule might have a better therapeutic potential in the surgical treatment of AS patients than ERK and JNK inhibitors, as inhibition of either molecule leads to severe side effects as both are involved in important signaling pathway in the whole organism. We cannot rule out the possibility that other MAPKs, such as p38, downstream to FGFR activation might be responsible for the increased osteogenic potential in the S252W fibroblasts, as these molecules have been shown to have enhance activation in Apert mouse models [41, 43, 48, 50–54].

Based on our results, we propose that the FGFR2^{S252W} mutation confers a most pronounced gain-of-function in fibroblast cells. Of the cell phenotypes evaluated, the most strikingly altered one is their increased osteogenic potential. This represents an acquired new function for fibroblasts, apparently mediated by JNK pathway. It has been suggested that the premature suture fusion in S252W cells is a result of excessive cell proliferation [7]. In the present study, FGFR2^{S252W} mutation leads to increased proliferation, migration, and osteogenic potential of both fibroblasts and MSCs. Therefore, the premature ossification process might result from a more complex mechanism than only alteration of the proliferative capacity. Further, we show that fibroblasts enhance the osteogenic potential of MSCs of the same niche. These results allow us to suggest that periosteum cells might contribute to premature suture fusion in these patients (Fig. 5), a characteristic that has previously been attributed to dura mater [49]. To better understand the molecular mechanisms underlying our findings, it is crucial to identify molecules secreted by S252W fibroblasts that may contribute to intensify osteogenic differentiation in other cells and whether they are related to the JNK pathway. These molecules could lead to the identification of candidate drugs that could ameliorate the surgical prognosis of AS patients.

Disclosures The authors indicate no potential conflicts of interest.

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