UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO: CIÊNCIAS EM GASTROENTEROLOGIA E HEPATOLOGIA

Efeito das drogas Dexametasona e Azatioprina na viabilidade, morfologia e comportamento migratório de células-tronco mesenquimais

NATÁLIA SCHNEIDER

DISSERTAÇÃO DE MESTRADO

Porto Alegre
2014

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO: CIÊNCIAS EM GASTROENTEROLOGIA E HEPATOLOGIA

Efeito das drogas Dexametasona e Azatioprina na viabilidade, morfologia e comportamento migratório de células-tronco mesenquimais

NATÁLIA SCHNEIDER

Orientadora: Profa. Dra. Ana Helena da Rosa Paz
Co-orientador: Prof. Dr. Marcelo Lazzaron Lamers
Dissertação apresentada ao Programa de Pós-Graduação: Ciências em Gastroenterologia e Hepatologia, Universidade Federal do Rio Grande do Sul, para obtenção de título de Mestre.

Porto Alegre
2014

CIP - Catalogação na Publicação

Schneider, Natália
Efeito das drogas Dexametasona e Azatioprina na viabilidade, morfologia e comportamento migratório de células-tronco mesenquimais / Natália Schneider. -- 2014.
112 f.

Orientadora: Ana Helena da Rosa Paz.
Coorientador: Marcelo Lazzaron Lamers.

Dissertação (Mestrado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Medicina, Programa de Pós-Graduação em Ciências em Gastroenterologia e Hepatologia, Porto Alegre, BR-RS, 2014.

1. Células-tronco mesenquimais. 2. Migração celular. 3. Doenças inflamatórias intestinais. 4. Azatioprina. 5. Dexametasona. I. Helena da Rosa Paz, Ana, orient. II. Lazzaron Lamers, Marcelo, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

Dedico essa dissertação à minha companheira
no amor e na vida, Ellen Nunes, pois teu apoio
e compreensão foram fundamentais nessa jornada.

AGRADECIMENTOS

Os meus sinceros agradecimentos:

À Profa. Dra. Ana Paz, por sua orientação não só neste trabalho, mas em todo o meu caminho desde a graduação. Por sempre, com muito carinho e paciência, acreditar no meu potencial e me incentivar a ir mais longe. Por ser uma orientadora presente, trabalhadora e sincera, mas além disso, por muitas vezes ser minha amiga, quase uma mãe, e parceira nos mais diversos momentos. Muito da minha evolução como pesquisadora e como pessoa devo a ti, a quem admiro e tenho como exemplo. Muito obrigada por tudo!

Ao Prof. Dr. Marcelo Lamers, por sua co-orientação neste trabalho, mesmo que à distância durante este último período. Por prontamente abrir as portas do seu laboratório ao nosso grupo e me orientar com muita paciência em todos os momentos. Aprendi muito contigo e espero continuar aprendendo, muito obrigada!

À Profa. Dra. Luíse Meurer, por todo o seu apoio, orientação, carinho e amizade, com os quais posso contar desde a minha época de iniciação científica. Também aos professores Dra. Sandra Vieira, Dr. Renato Fagundes e Dr. Sérgio Barros, não só pela incrível experiência que compartilhamos na Comissão Coordenadora do PPG, mas por todos os momentos que pudemos vivenciar e nos quais aprendo cada vez mais.

À Profa. Dra. Elizabeth Cirne-Lima e ao Prof. Dr. Eduardo Passos por nos acolherem em seu laboratório e por apoiarem nossos projetos. Às (aos) colegas do Laboratório de Embriologia e Diferenciação Celular pelo companheirismo durante toda essa jornada.

À minha colega e amiga MSc. Fabiany Gonçalves, por seu apoio, carinho e amizade desde a época da graduação, quando eu já ouvia falar do seu trabalho na pesquisa e não imaginava que um dia trabalharíamos juntas. Com muita alegria vim a fazer parte do teu trabalho mais tarde, onde aprendi e aprendo muito. Hoje posso te chamar de colega mais uma vez, e dizer que tenho uma grande admiração por ti. Obrigada por tudo Faby! Agradeço também ao Lucas Dreher, por suas habilidades artísticas e que com muito carinho ajudou a concretizar nossas ideias.

Às (aos) minhas/meus colegas Helena Mello, Fernanda Otesbelgue, Dra. Cristina Flores, Dr. William Lorenzi, Dra. Patrícia Lopez e Dr. Eduardo Chiela, pelo apoio e dedicação quando trabalhamos juntos, pela parceria e pela amizade que muito considero. Cada um de vocês foi importante em diferentes etapas deste processo e o resultado desse mestrado também é de vocês.

Aos colegas do Núcleo de Pesquisa Básica em Odontologia – UFRGS, Profa. Dra. Anna Fossati, Dra. Lisiane Bernardi, MSc. Alessandro Menna, MSc. Grasieli Ramos, Bibiana Matte, Leonardo Diel e Paloma de Campos, por me receberem em seu laboratório e sempre estarem dispostos a me auxiliar, até nos dias mais corridos para todos nós. À querida Profa. Dra. Fernanda Visioli por sempre deixar um abraço e uma palavra de carinho toda vez em que nos víamos.

Aos meus colegas do Centro de Pesquisa Experimental, Everaldo de Almeida, Flavinha Giustti, Jefferson da Silva, Jorge Lopes, Patrícia Koehler, Roger Pascoal, que não imaginam o quanto me ajudaram nos mais diversos momentos com sua pronta disposição, carinho e amizade.

À MSc. Bianca Pfaffenseller, por todas as horas que passamos em frente ao confocal tirando muitas fotos, por tudo o que me ensinaste e pela maravilhosa companhia que me fizeste durante todo esse período.

À MSc. Anelise Bergmann, por sua parceria em nossos projetos, por estar sempre disposta a me ensinar e por sua amizade.

Ao MSc. Álvaro Laureano por sua prontidão em nos ajudar com a citometria de fluxo no momento crucial de resposta aos revisores do paper deste trabalho.

À minha família, pelo suporte que me deram desde pequena para que eu chegasse até aqui, me incentivando nos estudos e a ir atrás dos meus objetivos. À minha namorada, Ellen Nunes, pelas noites de companhia enquanto trabalhava tanto em casa quanto no laboratório, pelas horas de ausência e finais de semana corridos, pela compreensão nos mais diversos momentos e pelo amor e carinho com os quais encarou esta jornada comigo. Aos amigos e colegas que me apoiaram e entenderam a minha falta, por cada palavra de incentivo, digo que valeu a pena!

“Aprender é a única coisa de que a
mente nunca se cansa, nunca tem
medo e nunca se arrepende.”

Leonardo da Vinci

SUMÁRIO

PARTE I

RESUMO 1
ABSTRACT 3
APRESENTAÇÃO 5
LISTA DE ABREVIATURAS 6
LISTA DE FIGURAS E TABELAS 8
1. INTRODUÇÃO E REVISÃO DA LITERATURA 9
1.1 Doenças Inflamatórias Intestinais 10
1.2 Tratamentos convencionais de Doenças Inflamatórias Intestinais 12
1.2.1 Azatioprina e Dexametasona 13
1.3 Células-Tronco Mesenquimais 14
1.3.1 Migração de células-tronco mesenquimais 17
1.4 Células-tronco mesenquimais em Doenças Inflamatórias Intestinais 19
1.5 Células-tronco mesenquimais e as drogas Azatioprina e Dexametasona 20
1.6 Protocolo de Migração Celular 21
JUSTIFICATIVA 23
QUESTÃO DE PESQUISA 23
HIPÓTESE 23
OBJETIVOS 24
PARTE II
CAPÍTULO 1: Analysis of mesenchymal stem cells migratory behavior by time-lapse microscopy assay 25
CAPÍTULO 2: Dexamethasone and azathioprine promote cytoskeleton changes and affect Mesenchymal Stem Cells migratory behavior 45
PARTE III
DISCUSSÃO 83
CONCLUSÕES 87
PERSPECTIVAS 88

REFERÊNCIAS BIBLIOGRÁFICAS.. 89
APÊNDICE.. 103

RESUMO

Glicocorticoides e outras drogas imunossupressoras são comumente utilizados para o tratamento de condições inflamatórias, como as Doenças Inflamatórias Intestinais (DIIs). Apesar dos avanços na terapia medicamentosa, a remissão da doença ainda é difícil de ser mantida. Devido às suas propriedades imunomodulatórias, as Células-Tronco Mesenquimais (MSCs – *Mesenchymal Stem Cells*) têm emergido como reguladoras da resposta imune, e sua viabilidade e propriedades migratórias são essenciais para o sucesso da terapia celular. Entretanto, pouco se conhece sobre os efeitos das drogas convencionalmente utilizadas no tratamento das DIIs no comportamento das MSCs. Portanto, o objetivo deste estudo foi avaliar a viabilidade, a morfometria nuclear, a polaridade celular, a distribuição da actina-F e da FAK (*Focal Adhesion Kinase*), e o comportamento migratório das MSCs na presença das drogas Azatioprina (AZA) e Dexametasona (DEXA). As células foram isoladas de membranas coriônicas humanas e caracterizadas pela diferenciação em adipócitos e osteócitos, bem como pela expressão de um painel de marcadores de superfície. As MSCs foram previamente tratadas com AZA ou DEXA por 24h ou 7d nas concentrações de 1μM ou 10μM, respectivamente. Ambas as drogas não afetaram a viabilidade celular analisada por MTT (3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide) e morfometria nuclear. Entretanto, a análise do índice de polaridade resultou em uma morfologia mais alongada após o tratamento com AZA, enquanto células mais arredondadas foram observadas na presença de DEXA. Os filamentos de actina foram marcados por Rodamina-Faloidina e sua análise mostrou que a AZA preservou parcialmente a formação de lamelipódios e aumentou a presença de fibras de estresse ventrais, enquanto que a DEXA inibiu a formação de lamelipódios, evidenciou uma maior presença de fibras de estresse ventrais e diminuiu a estabilidade das protrusões de membrana, observadas em vídeo. Através da análise de microscopia de série temporal, foi observado que as células sob o efeito da AZA por 7d migraram por maiores distâncias e tiveram um aumento em sua velocidade de migração (24,35%; $P < 0,05$; $n = 4$), ao passo que a DEXA diminuiu a velocidade migratória em 24h e 7d (-28,69% e -25,37%, respectivamente; $P < 0.05$; $n = 4$) e diminuiu a distância alcançada pelas células. Em conclusão, nossos dados sugerem que as drogas AZA e DEXA podem afetar diferentemente a morfologia e o comportamento migratório das MSCs,

possivelmente afetando o resultado da terapia celular. O protocolo de migração celular utilizado neste estudo foi estabelecido por nosso grupo de pesquisa, sendo que um artigo científico contendo todas as etapas do protocolo foi escrito para que outros laboratórios possam utilizá-lo de maneira simples e eficaz.

Palavras-chave: Células-Tronco Mesenquimais, Actina, Migração Celular, Azatioprina, Dexametasona, Doenças Inflamatórias Intestinais.

ABSTRACT

Glucocorticoids and other immunosuppressive drugs are commonly used to treat inflammatory disorders, such as Inflammatory Bowel Disease (IBD) and, despite few improvements, the remission of IBD is still difficult to maintain. Due to its immunomodulatory properties, Mesenchymal Stem Cells (MSCs) have emerged as regulators of immune response, and its viability and activation of migratory properties are essential for a successful cell therapy. However, little is known about the effects of immunosuppressant drugs used on IBD treatment on MSCs behavior. In this way, the aim of this study was to evaluate MSCs viability, nuclear morphometry, cell polarity, F-actin and FAK (Focal Adhesion Kinase) distribution and cell migration properties in the presence of the immunosuppressive drugs Azathioprine (AZA) or Dexamethasone (DEX). MSCs were isolated from human chorionic membranes and characterized through adipogenic and osteogenic differentiations, as well as a panel of surface markers. Cells were previously treated with AZA or DEX for 24 hrs or 7 days at 1μM and 10μM, respectively. Both drugs had no effects on cell viability analyzed through MTT (3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide) and nuclear morphometry. However, polarity index analysis showed that AZA treatment induced a more elongated cell shape while a greater presence of rounded cells was observed under DEX exposure. F-actin was stained by Rhodamine-Phalloidin and showed that AZA could partially preserve lamellipodia formation and increase the presence of ventral actin stress fibers, while DEX inhibited lamellipodia formation and increased the presence of ventral actin stress fibers while decreasing protrusion stability, observed in video. Through time-lapse microscopy, it was observed that after 7 days of treatment, AZA improved cell the spatial trajectory (ST) and increased migration speed (24.35%, $P < 0.05$, $n = 4$) while DEX impaired ST and migration speed after 24 hrs and 7 days treatment (-28.69% and -25.37%, respectively; $P < 0.05$, $n = 4$). In conclusion our data suggests these immunosuppressive drugs can differently affect MSCs morphology and migration capacity, possibly impacting the success of cell therapy. The migration protocol used in this study was successfully established by our group, leading to the writing of a protocol paper to facilitate the usage of this technique by other laboratories in a simple and efficient manner.

Keywords: Mesenchymal Stem Cells, Actin, Cell Migration, Azathioprine, Dexamethasone, Inflammatory Bowel Disease.

APRESENTAÇÃO

Esta dissertação está organizada em seções dispostas da seguinte maneira: Introdução e Revisão da Literatura, Justificativa, Questão de Pesquisa, Hipótese, Objetivos, Artigos Científicos, Discussão, Conclusões, Perspectivas, Referências Bibliográficas e Apêndice.

A **Introdução e Revisão da Literatura** mostram o embasamento teórico que nos levou a formular a proposta de trabalho. Os materiais, os métodos e os resultados, assim como as referências bibliográficas específicas, encontram-se no corpo dos artigos científicos denominados Capítulos 1 e 2. Esses trabalhos foram realizados no Laboratório de Embriologia e Diferenciação Celular e na Unidade de Análise Molecular e Proteínas do Centro de Pesquisa Experimental do Hospital de Clínicas de Porto Alegre, em colaboração com o Núcleo de Pesquisa Básica em Odontologia – Universidade Federal do Rio Grande do Sul, com auxílio financeiro CAPES e FIPE-HCPA.

A seção **Discussão** contém uma interpretação geral dos resultados obtidos nos artigos científicos; a seção **Conclusões** aborda as conclusões gerais obtidas na dissertação. A seção **Perspectivas** discute as possibilidades de desenvolvimento de projetos a partir dos resultados obtidos, dando continuidade a essa linha de pesquisa. A seção **Referências Bibliográficas** lista as referências utilizadas na Introdução e Discussão da dissertação. A seção **Apêndice** contém uma lista dos trabalhos realizados em co-autoria durante o período do mestrado.

LISTA DE ABREVIATURAS

DIIs – Doenças Inflamatórias Intestinais

MSCs – Células-Tronco Mesenquimais/*Mesenchymal Stem Cells*

UC – Colite Ulcerativa/*Ulcerative Colitis*

DC – Doença de Crohn

Th1 – Células T auxiliares/Type 1 *T helper cells*

IL-12 – Interleucina 12

INF-γ – Interferon γ

TNF-α – Fator de Necrose Tumoral α/*Tumor Necrosis Factor* α

Th2 – Células T auxiliares/Type 2 *T helper cells*

IL-5 – Interleucina 5

IL-13 – Interleucina 13

Th17 – Células T auxiliares/Type 17 *T helper cells*

IL-17 – Interleucina 17

IL-23 – Interleucina 23

AZA – Azatioprina

6-MP – 6-Mercaptopurina

6-TGN – Nucleotídeos 6-Tioguanina

TRAIL – Fator de Necrose Tumoral Relacionada ao Ligante Indutor de Apoptose/*Tumor necrosis factor-Related Apoptosis-Inducing Ligand*

TNFRS7 – membro 7 da Superfamília de Receptores do Fator de Necrose Tumoral/*Tumor Necrosis Factor Receptor Superfamily member 7*

MEK – Proteína Quinase ativada por Mitógeno/*Mitogen-activated protein Kinase*

NF-κβ – Fator Nuclear Kappa B/*Nuclear Factor Kappa B*

bcl-x_L – Linfoma de Células B Extra Grande/*B cell lymphoma-extra large*

HGPRT – Hipoxantina-Guanina-Fosforibosil-Transferase / *Hypoxanthine-Guanine-Phosphoribosyl-Transferase*

6-MeMP – 6-MetilMercaptopurina/*6-MethylMercaptopurine*

6-Me-Thio-IMP – S-Metil-Tioisina 5’monofosfato/*S-Methyl-Thioinosine 5'-Monophosphate*

TPMT – Tiopurina Metiltransferase/*Thiopurine Methyltransferase*

XO – Xantina Oxidase/*Xanthine Oxidase*

PDNS – Síntese de Novo de Purina/P*urine de Novo Synthesis*

DEXA – Dexametasona

Treg – Células T Regulatórias

CXCR4 – Receptor da Quimiocina C-X-C tipo 4/*C-X-C Chemokine Receptor type 4*

SDF-1 – Fator Derivado de Células Estromais-1/*Stromal Derived Factor*-1

CEs – Células Endoteliais

Células NK – Células exterminadoras naturais/*Natural Killer cells*

FAK – Quinase de Adesão Focal/*Focal Adhesion Kinase*

Rac-1 – Substrato C3 da Toxina Botulínica Relacionado a Ras 1/*Ras-related C3 botulinum toxin substrate 1*

ERM – Ezrina-Radixina-Moesina/*Ezrin-Radixin-Moesin*

iNOS – Óxido Nítrico Sintase induzível/*inducible Nitric Oxide Synthase*

IGF-1 – Fator de Crescimento semelhante à Insulina tipo 1/I*nsulin-like Growth Factor-1*

MTT - 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide

LISTA DE FIGURAS E TABELAS

INTRODUÇÃO

Figura 1: Doenças Inflamatórias Intestinais..11

Figura 2: Mecanismo de ação das tiopurinas..13

Figura 3: Efeitos imunomodulatórios das MSCs em células do sistema imune...........................16

Figura 4: Diferentes tipos de fibras de estresse em células mesenquimais.................................18

CAPÍTULO 1

Figura 1. Glass-bottomed dish preparation…..40

Figura 2. Cell culture..41

Figura 3. Microscope setup...42

Tabela 1. Comparison of *in vitro* cell migration techniques…………………………………..43

Tabela 2. Troubleshooting advice………………………………………………………………44

CAPÍTULO 2

Figura 1. MSCs characterization……………………………………………………………..76

Figura 2. MSCs viability and nuclear morphometry……………………………………………77

Figura 3. Cell Polarity Index……………………………………………………………………78

Figura 4. Actin cytoskeleton and FAK distribution……………………………………………..79

Figura 5. MSCs migration speed and spatial trajectory…………………………………………80

Figura Suplementar 1. Polarity index analysis…………………………………………………81

Figura Suplementar 2. Cytoskeleton morphology……………………………………………..82

1. INTRODUÇÃO E REVISÃO DA LITERATURA

As Doenças Inflamatórias Intestinais (DIIs) são doenças do trato gastrointestinal caracterizadas pela disfunção de células T e produção alterada de citocinas pró-inflamatórias. Esta inflamação é sustentada por alterações no sistema imunológico da mucosa intestinal (Bouma e Strober, 2003; Martínez-Montiel et al., 2014), tendo como componentes desencadeadores da patogênese a suscetibilidade genética, os fatores microbiais e ambientais, como dieta, depressão, estresse e a influência de radicais livres (Corridoni et al., 2014; Sobczak et al., 2014). O curso das doenças é caracterizado por crises que se alternam com períodos de remissão; sendo que uma minoria dos pacientes tem atividade contínua. A severidade das crises e a resposta ao tratamento variam, desde sintomas leves, sem manifestações sistêmicas, até risco de morte fulminante naqueles pacientes que não respondem ao tratamento e, portanto, são candidatos à intervenção cirúrgica (Cosnes et al., 2011). Apesar dos avanços nas terapias medicamentosas atuais, estas nem sempre são efetivas e novas terapêuticas que visem a imunomodulação e o reparo tecidual são necessárias (Duijvestein et al., 2010; Martínez-Montiel et al., 2014; Renna et al., 2014).

As Células-Tronco Mesenquimais (*Mesenchymal Stem Cells* – MSCs) têm surgido como uma opção para o tratamento de condições inflamatórias, pois apresentam grande plasticidade, multipotencialidade, atuam como reguladoras da resposta imune e têm a capacidade de migrar até o sítio de injúria (Forbes et al., 2014; Gonzalez-Rey et al., 2009; Le Blanc et al., 2003; Liang et al., 2011). As MSCs secretam uma variedade de citocinas que atuam na imunossupressão local, controlam a inflamação e auxiliam no reparo tecidual (Caplan e Dennis, 2006; Miller et al., 2008; Mokbel et al., 2011). Além disso, podem ser isoladas de diferentes órgãos e tecidos, incluindo medula óssea, músculo, tecido adiposo, e órgãos de origem materno-fetal (Hass et al., 2011; Indumathi et al., 2013; Meirelles et al., 2006).

Os tratamentos convencionais para as DIIs incluem drogas imunossupressoras, como as tiopurinas e os glicocorticoides. Entretanto pouco se sabe sobre a interação de MSCs e drogas imunossupressoras. Estudos anteriores analisaram a interação entre medicamentos e MSCs; no entanto, os resultados disponíveis na literatura não são conclusivos, uma vez que existem importantes divergências entre autores (Buron et al., 2009; Duijvestein et al., 2011; Hoogduijn et

al., 2008; Nichols et al., 2013; Nuzzi et al., 2012; Yun et al., 2011). Além disso, diferentes condições fisiológicas ou farmacológicas podem afetar a migração celular (Geiβler et al., 2012; Lamers et al., 2011), processo importante para a efetividade da terapia celular. Portanto, é essencial que seja estudada a interação entre MSCs e as drogas utilizadas como tratamento convencional das DIIs, na hipótese de serem utilizadas de maneira concomitante ou na proposta de transplante autólogo.

1.1 Doenças Inflamatórias Intestinais

As DIIs, como a Retocolite Ulcerativa (RCU) e a Doença de Crohn (DC), são caracterizadas por uma disfunção de linfócitos T da mucosa intestinal e alteração na produção e secreção de citocinas (Bouma e Strober, 2003), apresentando períodos de reincidência e remissão (Cosnes et al., 2011). Apesar de ser amplamente aceito que as DIIs resultem de uma resposta imune desregulada a fatores naturais em indivíduos geneticamente suscetíveis, a causa ainda não foi completamente elucidada (Corridoni et al., 2014). Nesse sentido, vários componentes estão sendo implicados na sua patogênese como suscetibilidade genética, fatores microbiais e ambientais, incluindo dieta, depressão, estresse, e a influência de radicais livres (Sobczak et al., 2014).

Inicialmente havia o consenso de que a DC estaria associada ao desequilíbrio de células T helper 1 (Th1), mediado pelas citocinas IL-12, INF-γ e TNF-α (fator de necrose tumoral α); enquanto que a RCU estaria associada ao desequilíbrio de células T helper 2 (Th2), mediado pelas citocinas IL-5 e IL-13 (Corridoni et al., 2014; Muzes et al., 2012). Porém, tem se visto recentemente que ambas as vias não são necessariamente exclusivas (Muzes et al., 2012). Além disso, células Th17 tem se mostrado importantes no quadro imunológico das DIIs, principalmente através das citocinas IL-17 e IL-23, ligadas à inflamação intestinal (Corridoni et al., 2014; Yen 2006).

A DC normalmente afeta o íleo e o cólon, mas pode atingir descontinuamente qualquer região do trato gastrointestinal (Figura 1A). Além disso, é caracterizada por inflamação transmural e pode ser associada à formação de granulomas, e desenvolvimento de estenoses e fístulas (Abraham et al., 2009; Corridoni et al., 2014; Sobczak et al., 2014). A doença torna-se sintomática quando existem lesões extensas, associadas a uma reação inflamatória sistêmica, ou

ainda quando há a presença de estenoses, abscessos ou fístulas (Cosnes et al., 2011). Dentre os sintomas estão: diarreia frequente, dor abdominal após as refeições, fadiga, perda de apetite, perda de peso, febre, estomatite, e fístulas ou fissuras perianais. Alguns pacientes ainda apresentam sangramento retal, artrite e eritema nodoso (Head e Jurenka, 2004).

A RCU envolve o reto e parte ou todo o cólon em um padrão contínuo e é caracterizada por uma inflamação limitada à mucosa, sem apresentação de fístulas ou granulomas (Figura 1B) (Abraham et al., 2009; Corridoni et al., 2014). A severidade da doença varia desde sintomas menores sem manifestações sistêmicas, até a necessidade de colectomia, muitas vezes chegando ao risco de morte fulminante quando não há resposta ao tratamento (Cosnes et al., 2011). Sintomas iniciais incluem o progressivo amolecimento das fezes, dor abdominal e diarreia. Com a progressão da doença, o paciente pode apresentar perda de peso, fadiga, perda de apetite, muco nas fezes, sangramento retal, febre e anemia (Head e Jurenka, 2003).

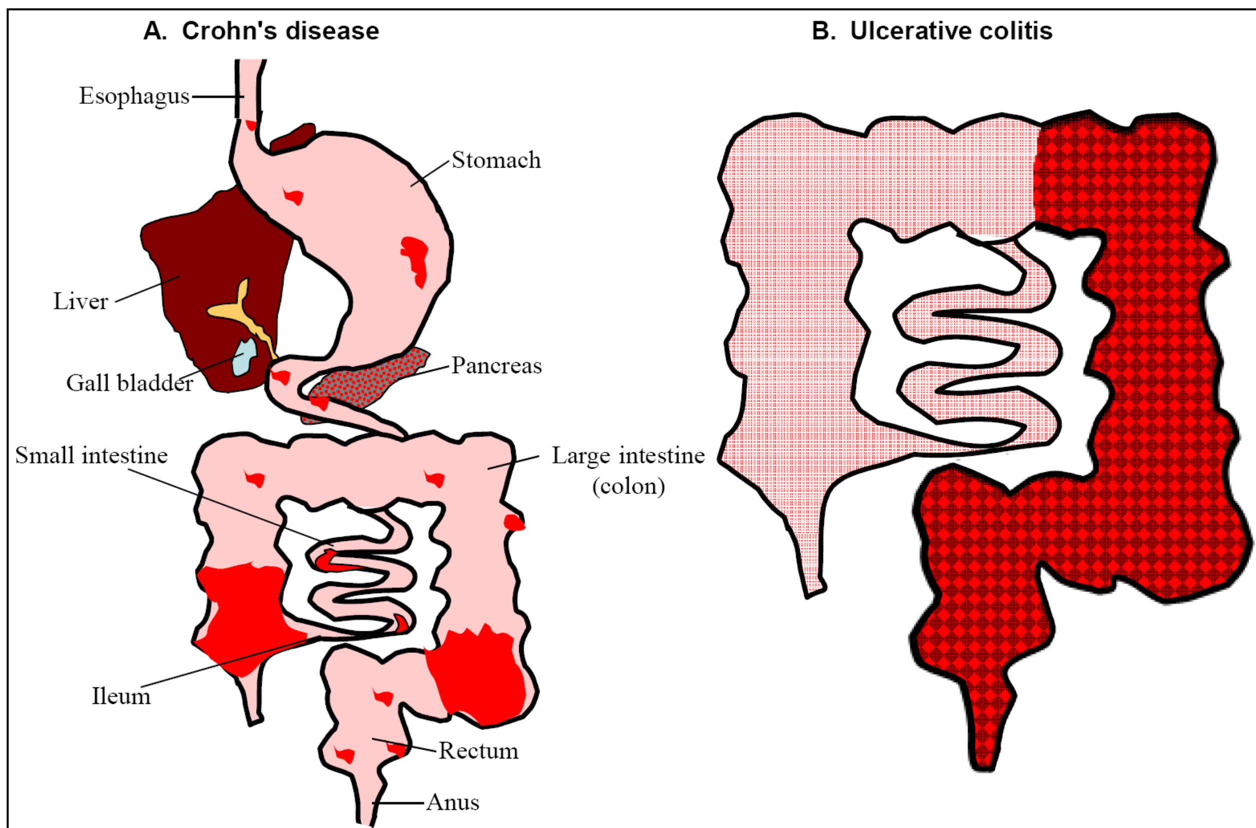


Figura 1. Doenças Inflamatórias Intestinais. (A) Doença de Crohn. A inflamação pode afetar todo o trato digestivo, da boca ao ânus. (B) Colite Ulcerativa. A inflamação é limitada ao cólon. Fonte: Singh et al, 2011.

A incidência e a prevalência das DIIs têm crescido ao redor do mundo, onde as maiores incidências são registradas na América do Norte e na Europa (Cosnes et al., 2011; Ponder e Long, 2013), e há um aumento em áreas antes consideradas de baixa incidência, como a Europa Oriental e a Ásia (Ng, 2014). Dados epidemiológicos ainda são escassos na América Central e do Sul, refletindo a baixa frequência ou a falta de registros sobre essas doenças (Cosnes et al., 2011).

1.2 Tratamentos convencionais de Doenças Inflamatórias Intestinais

Os correntes progressos no entendimento da fisiopatologia das DIIs permitiram avanços nos tratamentos disponíveis e nos objetivos terapêuticos. Substâncias como Aminossalicilatos, Corticosteróides, Tiopurinas, Metotrexato e Ciclosporina foram largamente utilizadas para induzir e manter a remissão da doença (Martínez-Montiel et al., 2014). Porém, a partir da identificação de que altos níveis da citocina pró-inflamatória TNF-α estão presentes na mucosa de pacientes com DIIs, as terapias biológicas anti-TNFα começaram a ser introduzidas, direcionando sua ação contra esta citocina (Sobczak et al., 2014). Inicialmente, o tratamento era centrado no controle dos sintomas; entretanto, a cicatrização da mucosa passou a ser um objetivo terapêutico, associada a um bom prognóstico, a um menor número de admissões hospitalares e de procedimentos cirúrgicos. Sendo assim, o tratamento convencional atualmente está focado na remissão profunda das DIIs, ou seja, no controle dos sintomas e da inflamação intestinal, na completa cicatrização das lesões da mucosa, na normalização dos índices de atividade sorológica (proteínas C-reativa e taxa de sedimentação de eritrócitos) e na restauração da função intestinal normal (Martínez-Montiel et al., 2014; Renna et al., 2014).

Os medicamentos hoje utilizados incluem, além daqueles convencionais, um arsenal cada vez mais diversificado de drogas biológicas (Sobczak et al., 2014). No entanto, a situação ainda está longe da ideal; por exemplo, 10% dos pacientes com DC não toleram ou não respondem primariamente a todas as drogas utilizadas para o seu tratamento. Entre os indivíduos que respondem à terapia anti-TNFα, um terço mostra a perda da resposta e requer otimização ou a mudança para outro agente biológico (Martínez-Montiel et al., 2014). A remissão da doença ainda é difícil de ser mantida e os pacientes sofrem com a baixa qualidade de vida devido à recidiva, à necessidade eventual de intervenção cirúrgica, a não rara apresentação de

manifestações extra-intestinais e os conhecidos e importantes efeitos colaterais das drogas (Duijvestein et al., 2010). Este quadro caracteriza a necessidade por novas terapias ou a otimização das mesmas, sendo a terapia com MSCs uma alternativa para a modulação do sistema imune e para reparação tecidual.

1.2.1 Azatioprina e Dexametasona

A Azatioprina (AZA) é uma droga imunossupressora análoga do nucleotídeo purina, pertencente ao grupo das tiopurinas. Após a sua ingestão oral, a AZA é rapidamente convertida em 6-mercaptopurina (6-MP), seu metabólito ativo, que em sequencia passa por uma complexa biotransformação em outros metabólitos ativos e inativos. O primeiro intermediário ativo, tioinosina monofosfato, é prontamente convertido em 6-TGN (nucleotídeos 6-tioguanina), o qual é considerado o principal efetor das tiopurinas. Seu efeito citotóxico e imunossupressor é atribuído à incorporação aos ácidos nucleicos das células, resultando em inibição da síntese de nucleotídeos e proteínas, e consequentemente inibição da proliferação de linfócitos (Figura 2) (Bär et al., 2013; Sahasranaman et al., 2008). O mecanismo de ação da AZA e da 6-MP inclui o aumento de apoptose (ou morte celular programada) de linfócitos T ativados, e o aumento de 6-TGN bloqueia a expressão de TRAIL, TNFRS7 e α4-integrina, provocando a diminuição da inflamação (Thomas et al., 2005; Sahasranaman et al., 2008). Também foi registrada sua ação através da inibição da ativação de Rac1 em células T, que tem como alvo os genes MEK, NF-κβ e bcl-x_L, levando à apoptose celular (Tiede et al., 2003).

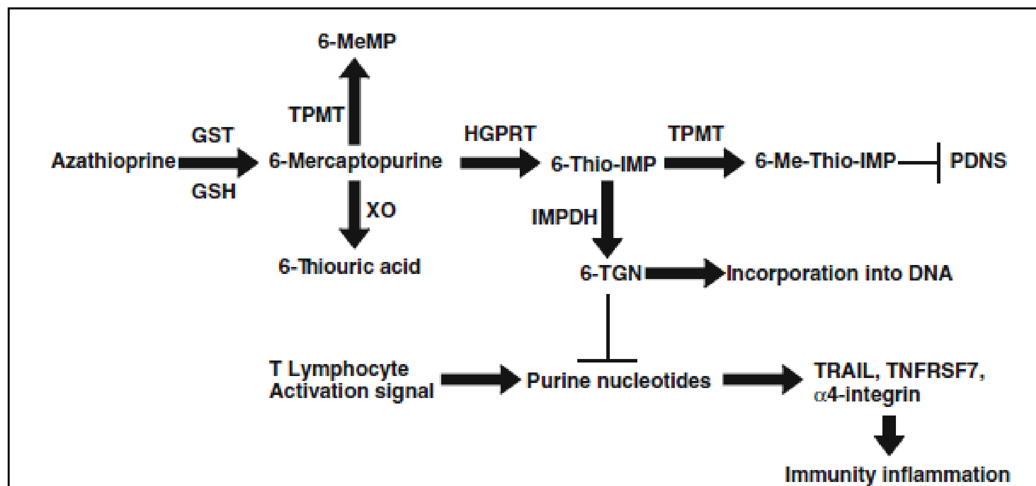


Figura 2. Mecanismo de ação das tiopurinas. *HGPRT* - Hypoxanthine-guanine-phosphoribosyl-transferase, *6-MeMP* - 6-methylmercaptopurine, *6-Me-Thio-IMP* - S-methyl-thioinosine 5′-monophosphate, *TPMT* - thiopurine methyltransferase, *XO* - xanthine oxidase, *PDNS* - purine de novo synthesis. Fonte: Sahasranaman et al, 2008.

A droga Dexametasona (DEXA) faz parte do grupo dos glicocorticoides, os quais possuem propriedades anti-inflamatórias e imunossupressoras; entretanto, seus mecanismos de atuação ainda não são totalmente esclarecidos (Wang et al., 2008). Glicocorticóides levam à expansão de células T regulatórias (Chen et al., 2006), interferem na migração de leucócitos pela inibição de proteínas de adesão (Wüst et al., 2008) e inibem a produção de citocinas pró-inflamatórias e quimiocinas (Almawi et al., 1996; Müller et al., 2013). Uma das ações conhecidas para os glicocorticoides, como a Dexametasona, é sua atuação sobre a ativação de NFκβ. Em seu estado inativo, NFκβ é transportado ao citoplasma pela proteína inibitória nomeada Iκβ. Quando Iκβ é inativado por sinais inflamatórios, o sinal de localização nuclear de NFκβ torna-se visível; dentro do núcleo, NFκβ se liga a sequencias do DNA chamadas de elementos NFκβ e estimula a transcrição de citocinas, quimiocinas, moléculas de adesão celular, fatores complementares e receptores para essas moléculas, estimulando a inflamação. Os glicocorticoides atuam induzindo a expressão de Iκβ, o qual inibe a transcrição dos elementos NFκβ e forma um heterodímero inativo com NFκβ, diminuindo a inflamação (Rhen e Cidlowski, 2005; Wang et al., 2008).

A DEXA é referenciada como uma droga utilizada no tratamento das DIIs (Duijvestein et al., 2010). Além disso, é facilmente diluída em meio de cultura para utilização em experimentos *in vitro* (D4902, Sigma) ao contrário de outros glicocorticoides, como Prednisona, Prednisolona ou Metilprednisolona.

1.3 Células-Tronco Mesenquimais

As MSCs têm demonstrado um grande potencial terapêutico para o tratamento das DIIs. Estas células apresentam grande plasticidade e multipotencialidade, podendo ser isoladas de diferentes órgãos e tecidos, incluindo medula óssea, músculo, tecido adiposo, e ainda órgãos de origem materno-fetal. (Hass et al., 2011; Indumathi et al., 2013; Meirelles et al., 2006). Quando nas condições ideais, as MSCs possuem a capacidade de diferenciação em tecidos como cartilagem, osso, tendões/ligamentos e músculo (Bielby et al., 2007; Caplan, 2005; Patel et al., 2013). Para que estas células possam ser identificadas como MSCs, elas devem expressar os marcadores de superfície CD105, CD73 e CD90, e não expressar CD45, CD34, CD14 ou CD11b, CD79α ou CD19 e HLA-DR. Além disso, as MSCs devem ser aderentes e ter a

capacidade de diferenciar-se em osteoblastos, adipócitos e condrócitos *in vitro* (Dominici et al., 2006).

O uso de tecidos materno-fetais tem sido cada vez mais aplicado para o isolamento de MSCs. Estudos têm utilizado tecidos placentários tanto de primeiro quanto terceiro trimestre de gestação, isolando MSCs das membranas amniótica e coriônica, e da camada decídua. Além disso, pode-se utilizar fontes como o cordão umbilical, o sangue de cordão umbilical e a geleia de Wharton (Hass et al., 2011). As MSCs derivadas destes tecidos têm sido caracterizadas quanto ao potencial de diferenciação, imunofenotipagem e morfologia, possuindo características semelhantes às MSCs derivadas de medula óssea. Além disso, os tecidos materno-fetais apresentam-se como uma fonte de fácil e pronto acesso, sem causar desconforto aos seus doadores e nem problemas éticos relacionados à sua coleta, uma vez que o material seria descartado (Hass et al., 2011; Indumathi et al., 2013).

Quando comparada a outras fontes, as MSCs derivadas de placenta apresentam benefícios relacionados às propriedades terapêuticas. Além de possuírem alguns marcadores característicos de células-tronco embrionárias (Yen et al., 2005), as MSCs derivadas de placenta demonstraram melhor taxa de proliferação (Barlow et al., 2008), superior capacidade de enxertia (Brooke et al., 2008) e propriedades imunossupressoras aumentadas (Li et al., 2014; Zhu et al., 2014). Estas células ainda possuem uma grande capacidade migratória tanto *in vitro* (Li et al., 2009) quanto *in vivo* (Kholodenko et al., 2012), e os resultados positivos em estudos experimentais levaram à administração de MSCs de tecido fetal em ensaios clínicos de fase I para tratamento de DC e UC (Liang et al., 2012; NCT01769755).

As MSCs atuam como reguladoras da resposta imune, justificando o interesse para sua utilização na terapia de doenças inflamatórias. Além disso, são conhecidas por apresentar baixa imunogenicidade, sendo capazes de “escapar” do reconhecimento por células T devido à baixa expressão de HLA de classe I e a ausência de HLA de classe II e de moléculas co-estimulatórias (Forbes et al., 2014; Gonzalez-Rey et al., 2009; Le Blanc et al., 2003). As MSCs secretam uma variedade de fatores solúveis que atuam no reparo tecidual, inibindo a fibrose, a apoptose e a transmigração de leucócitos. Também atuam estimulando a angiogênese, a proliferação e/ou diferenciação de células progenitoras intrínsecas do tecido lesado, e a proliferação de células T

regulatórias, atuando no controle da inflamação (Figura 3) (Caplan e Dennis, 2006; Ma et al., 2014; Miller et al., 2008; Mokbel et al., 2011; Yagi et al., 2010; Yi e Song, 2012).

Estas moléculas bioativas secretadas por MSCs podem atuar de forma direta ou indireta, promovendo a sinalização intracelular da célula alvo ou agindo sobre as células vizinhas para que as mesmas secretem agentes funcionalmente ativos (Caplan e Dennis, 2006). Entre os fatores solúveis liberados está a IDO, que atua inibindo a proliferação de linfócitos T pela diminuição do aminoácido essencial triptofano e pela indução de metabólitos reguladores do sistema imune (Ren et al., 2009). Também a IL-6, a qual auxilia na inibição da diferenciação de monócitos em células dendríticas (Djouad et al., 2007) e a IL-10, que atua inibindo a resposta de células T por alterações provocadas na função de células apresentadoras de antígenos (Beyth et al., 2005), além de inibir a diferenciação de células Th17 (Qu et al., 2012); e ainda a PGE2, que estimula a proliferação de células Treg (English et al., 2009) enquanto inibe a proliferação de células NK (Spaggiari et al., 2008) e a maturação de células dendríticas (Spaggiari et al., 2009).

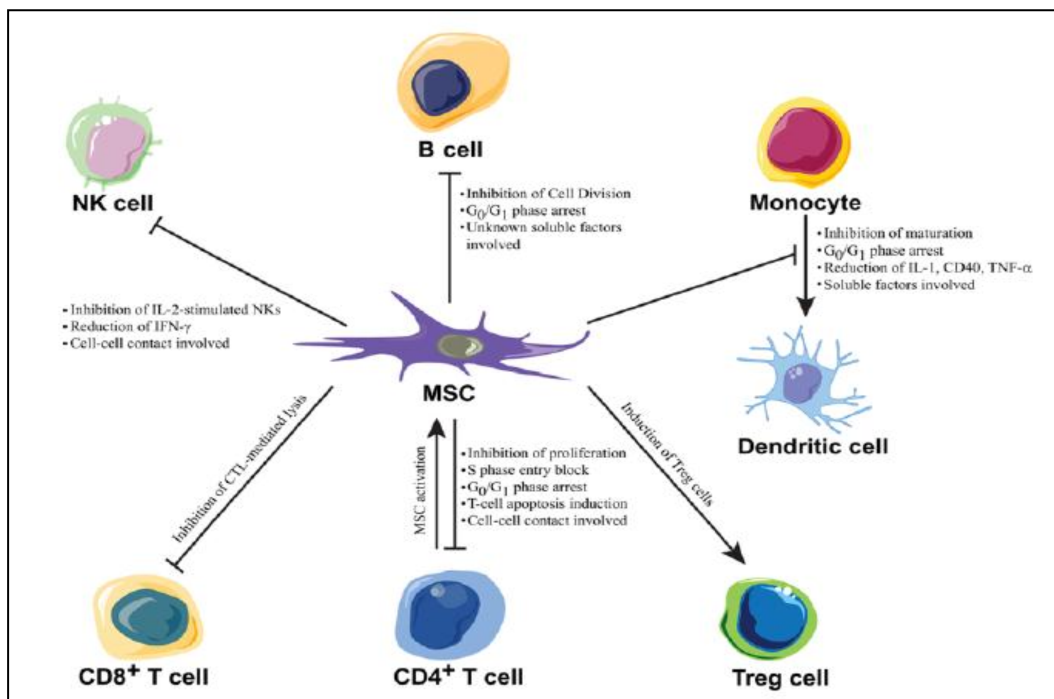


Figura 3. Efeitos imunomodulatórios das MSCs em células do sistema imune. Os efeitos imunomodulatórios das MSCs incluem a supressão da proliferação de linfócitos B e T, indução de células T regulatórias, inibição da maturação de monócitos em células dendríticas e inibição da função de células NK. Fonte: Yi e Song, 2012.

1.3.1 Migração de células-tronco mesenquimais

O processo migratório das MSCs é dependente da relação entre as quimiocinas liberadas por células do sítio inflamatório e os receptores presentes na membrana das MSCs. O recrutamento das MSCs ocorre por quimiotaxia, ou seja, a migração direcionada em resposta ao gradiente de quimioatratores (fatores de crescimento e quimiocinas). Um dos receptores mais estudados é o CXCR4, responsável pela quimioatração ao SDF-1 (*stromal derived factor*-1) (Sohni e Verfaillie, 2013; Marquez-Curtis e Janowska-Wieczorek, 2013). Em modelo experimental de DC, a administração sistêmica de MSCs derivadas de medula óssea superexpressando o receptor CXCR4 resultou em melhora clínica e histológica dos animais em comparação ao grupo controle (MSCs sem superexpressão de CXCR4), diminuindo a inflamação e mostrando a importância da adequada migração das MSCs também no contexto das DIIs (Liu et al., 2014).

Portanto, a terapia com MSCs necessita da migração das células até a inflamação ou local de injúria (processo também conhecido como *homing*) (Sohni e Verfaillie, 2013). A migração celular é um processo complexo que envolve o movimento do citoesqueleto de actina e a indução da polarização celular. Este movimento é caracterizado pela polimerização de actina e formação de adesões nascentes na parte anterior da célula, maturação das adesões celulares, contratilidade do corpo celular e a soltura na parte posterior da célula (Li e Gundersen, 2008; Pollard e Cooper, 2009; Swaney et al., 2010).

As fibras de estresse em células mesenquimais são compostas por agrupamentos de filamentos de actina, os quais são ancorados a adesões focais que as conectam a matriz extracelular (Tojkander et al., 2012). A associação das fibras de estresse com as adesões focais e a sua morfologia variam, podendo ser classificadas em fibras dorsais, ventrais e arcos transversos (Figura 4).

As fibras de estresse dorsais são ancoradas a adesões focais na porção distal da célula e atuam como uma plataforma para os outros tipos de fibras de estresse, assim como para ligá-las às adesões focais. Elas estão envolvidas na regulação das adesões frontais das células e na promoção da migração celular. Já os arcos transversais são agrupamentos curvos de filamentos de actina, não diretamente ligados às adesões focais, e que externam sua força contrátil através da conexão com as fibras dorsais. As fibras de estresse ventrais são ligadas às adesões focais em

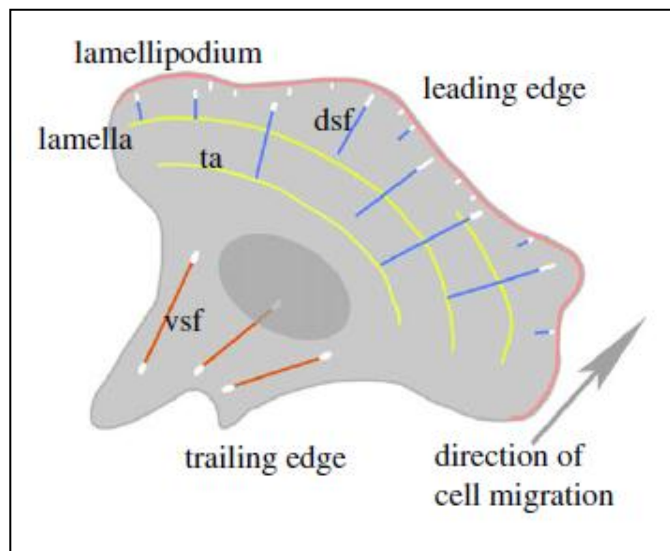


Figura 4. Diferentes tipos de fibras de estresse em células mesenquimais. Representação esquemática da rede de fibras de estresse de actina de células mesenquimais formadas por 3 diferentes tipos: dorsais (dsf – em azul), arcos transversais (ta – em amarelo) e ventrais (vsf – em vermelho). As adesões focais estão representadas pelos pequenos traços brancos. Fonte: Vallenius, 2013.

ambas as extremidades e estão envolvidas na regulação da polaridade celular. Elas são localizadas frequentemente na parte posterior da célula, onde podem promover a constrição celular e facilitar o movimento da célula (Tojkander et al., 2012; Vallenius, 2013).

A organização dos tipos de fibras de estresse é importante para o movimento migratório, assim como a sua interação com as adesões focais, pois estas asseguram uma comunicação entre a célula e a matriz extracelular durante os processos de adesão e migração. Nesse sentido, a FAK (*Focal Adhesion Kinase*) é um componente importante na modulação do movimento celular, pois quando ativada por diferentes estímulos pode influenciar o citoesqueleto, as adesões celulares e as protrusões de membrana (Mitra et al., 2005). Além disso, a migração celular depende da ativação de uma série de vias de sinalização que resultam na quebra da assimetria celular, onde uma célula mais alongada é correlacionada a uma migração mais efetiva e a presença de lamelipódios indica uma célula em movimento (Friedl e Alexander, 2011; Ridley et al., 2003; Tojkander et al., 2012; Vallenius, 2013).

Atualmente existem controvérsias envolvendo o mecanismo pelo qual as MSCs migram em direção ao tecido inflamado. Ao contrário da bem caracterizada cascata de adesão do *homing*

de leucócitos, há a falta de um claro mecanismo envolvendo a migração de MSCs. Ainda é incerto o exato posicionamento das MSCs após a sua infusão, o que torna mais difícil determinar se as células são capturadas entre os vasos ou se realizam a migração transendotelial (Karp e Leng Teo, 2009). Apesar de estudos terem visualizado as MSCs capturadas no pulmão após administração intravenosa (Parekkadan e Milwid, 2010; Schrepfer et al., 2007), outros têm encontrado MSCs administradas sistemicamente alcançando tecidos alvo de injúria, como o cérebro (Jackson et al., 2010; Kholodenko et al., 2012), a medula espinal (Kim et al., 2013), o coração (Zhang et al., 2007) e os rins (Zhuo et al., 2013) sugerindo que as MSCs têm a capacidade de migrar *in vivo* para o sítio de injúria após infusão sistêmica.

Nesse sentido, já foi demonstrado que as MSCs interagem com células endoteliais (CEs). Através de ensaio *in vitro* viu-se que as MSCs possuem a capacidade de rolamento sobre as CEs e que esse processo é seguido por firme adesão celular. Tal efeito foi aumentado quando as CEs foram previamente estimuladas com TNF-α. Além disso, os movimentos de rolamento e firme adesão foram observados *in vivo* (Rüster et al., 2006). Nosso grupo recentemente publicou um estudo sobre a administração de MSCs em modelo experimental de UC por diferentes vias, mostrando que a via intravenosa melhorou o quadro clínico e histológico dos animais quando comparada à via intraperitoneal (Gonçalves et al., 2014). Há evidências sobre os benefícios da aplicação local de MSCs (Kim et al., 2013; Charwat et al., 2008), porém, a administração intravenosa é uma opção minimamente invasiva que permite flexibilidade na terapia celular, como repetidas doses e o alcance de locais não disponíveis cirurgicamente.

1.4 Células-tronco mesenquimais em Doenças Inflamatórias Intestinais

A terapia celular tem sido utilizada como alternativa de tratamento para pacientes com DIIs devido às características imunomodulatórias e de reparo tecidual. Estudos experimentais em modelos animais têm demonstrado resultados satisfatórios quando MSCs são administradas como tratamento para as DIIs. Animais submetidos à terapia celular apresentam uma melhora clínica e histopatológica, como a diminuição da diarreia e inflamação, e o aumento da sobrevida (Gonzalez-Rey et al., 2009). Nesse sentido, Gonçalves et al., 2014 demonstraram que a administração de MSCs via intravenosa melhorou o quadro clínico e diminuiu a inflamação em modelo experimental de UC aguda, quando em comparação com a via intraperitoneal. Além

disso, Liang et al., 2011 demonstrou que MSCs derivadas de cordão umbilical foram capazes de migrar ao sítio de injúria e provocar uma melhora dos animais em modelo experimental de DC.

Ensaios clínicos têm sido conduzidos nesse sentido e apresentam resultados que, embora sejam promissores, ainda são controversos (Martínez-Montiel et al., 2014; Gazouli et al., 2014). Estudos de fase I com a aplicação de MSCs para o tratamento de DC demonstraram que as células são seguras e viáveis para uso clínico (Duijvestein et al., 2010; Ciccociopo et al., 2011); além disso estudos de fase II apresentaram resultados de melhora clínica dos pacientes com DIIs tratados com a terapia celular (Garcia-Olmo et al., 2009; Forbes et al., 2014). Também há estudos de fase III em andamento, como o que está sendo conduzido pela *Osiris Therapeutics Inc* (NCT00482092). Neste estudo, o tratamento com Prochymal (MSCs alogênicas derivadas de medula óssea; *Orphan Drug status*) foi aplicado em pacientes com DC ativa; apesar de uma pausa ter sido feita devido à alta resposta do grupo placebo, atualmente o estudo está em andamento e os resultados ainda não estão disponíveis (Martínez-Montiel et al., 2014). Além deste, outro estudo de fase III está em curso para o tratamento de DC fistulizante com a administração de MSCs derivadas de tecido adiposo; encontra-se na fase de recrutamento de pacientes (NCT01541579).

1.5 Células-tronco mesenquimais e as drogas Azatioprina e Dexametasona

Os estudos sobre o efeito da AZA são principalmente focados em células do sistema imune, havendo poucos dados disponíveis sobre a ação da mesma em MSCs. Nesse sentido, trabalhos demonstraram que a AZA não altera a viabilidade, o fenótipo, a diferenciação, a proliferação, as características imunogênicas e a capacidade imunossupressora das MSCs (Duijvestein et al., 2011; Mancheño-Corvo et al., 2013). Entretanto, foi visto por outros autores a diminuição da proliferação e o aumento da apoptose de MSCs provocados por AZA (Huang et al., 2014). Além disso, Mancheño-Corvo et al., 2013 observou que AZA preservou a ação estimulatória das MSCs em células T regulatórias *in vitro* e protegeu as MSCs da lise provocada por células NK, o que sugere uma grande adição ao contexto da terapia celular.

O efeito da DEXA em MSCs também apresenta resultados diversos. Estudos demonstraram alterações provocadas por esta droga em relação à proliferação, à viabilidade, à morfologia celular e à imunossupressão (Nuzzi et al., 2012; Wyles et al., 2014; Wang et al.,

2012). Por outro lado, trabalhos mostraram que a DEXA não provocou nenhum efeito na expressão de antígenos e na proliferação das MSCs, e ainda protegeu as células da apoptose (Nuzzi et al., 2012; Wang et al., 2012; Song et al., 2009). Além disso, foi demonstrado em modelo animal que este glicocorticoide parece atuar anulando o efeito terapêutico das MSCs (Chen et al., 2014).

Atualmente, os resultados encontrados na avaliação da interação das MSCs com AZA ou DEXA são limitados e divergentes. Além disso, os estudos não avaliam a migração intrínseca e a morfologia por microscopia confocal, incluindo análise do citoesqueleto de actina e suas fibras de estresse, aspectos importantes para o sucesso da terapia celular.

1.6 Protocolo de Migração Celular

O estudo da base da migração celular tem sido de fundamental importância para o entendimento dos seus mecanismos, e nesse sentido, uma adequada técnica para avaliação dos seus aspectos é essencial. A migração intrínseca é o primeiro indicativo de como uma célula irá responder a um quimioatrator (Petrie et al., 2009; Swaney et al., 2010). A técnica de microscopia de série temporal fornece as imagens para a avaliação da migração celular, mas ainda é vista como de alto custo pelo equipamento e material requerido. Entretanto, é uma técnica que permite modificações e que já foi utilizada para diversos fins, como a filmagem do desenvolvimento larval (Chai et al., 2012), a medição da migração celular direcionada (Wu et al., 2014) , da meia-vida de proteínas (Geva-Zatorsky et al., 2012), e da expressão de genes em diferentes células (Young et al., 2011).

Neste trabalho, foi padronizada a técnica de microscopia de série temporal para MSCs, também com o objetivo de torná-la mais acessível. Outros protocolos comumente utilizados para a avaliação da migração celular incluem a técnica de *Transwell,* que consiste em uma membrana permeável por onde as células podem migrar de um compartimento ao outro em direção a um quimioatrator (Limame et al., 2012). Entretanto, não é possível a obtenção de imagens do movimento celular. Outro ensaio utilizado é o chamado *Wound Healing*, que consiste em um intervalo feito na monocamada celular com o objetivo de obter as imagens da migração celular em direção ao espaço livre (Liang et al., 2007). Apesar de fornecer imagens do comportamento

celular, muitas vezes a migração e a proliferação celular são confundidas, sendo difícil o seguimento individual das células.

O protocolo de microscopia de série temporal é adequado para a quantificação de medidas como a velocidade de migração e a trajetória espacial das células (Lamers et al., 2011), e a simplificação do método possibilita a utilização do protocolo com equipamentos mais modestos e materiais comumente utilizados nos laboratórios. Tais aspectos da migração intrínseca podem ser avaliados na presença de diversas drogas, como a interação entre imunossupressores e MSCs avaliada neste estudo.

JUSTIFICATIVA

Atualmente, o tratamento das DIIs é centrado no uso de medicamentos para a indução e manutenção da remissão da doença. Entretanto, a terapia convencional nem sempre é efetiva e muitas vezes apresenta efeitos colaterais, existindo a necessidade da busca por novos tratamentos. As MSCs têm sido cada vez mais utilizadas como terapia alternativa devido ao seu efeito imunomodulador e a sua atuação no reparo tecidual. Porém, estudos demonstrando a interação das MSCs com os medicamentos utilizados nas DIIs são limitados e divergentes, além de não aprofundarem-se nos aspectos da migração celular intrínseca e nos movimentos do citoesqueleto de actina. Considerando a importância destes aspectos, acreditamos que, para o sucesso da terapia celular em associação com a terapia medicamentosa, são necessários mais estudos para o esclarecimento do comportamento das MSCs frente aos medicamentos convencionais utilizados para as DIIs.

QUESTÃO DE PESQUISA

As drogas Azatioprina e Dexametasona modificam a viabilidade, a morfologia e o comportamento migratório das Células-Tronco Mesenquimais?

HIPÓTESE

Os medicamentos Azatioprina e Dexametasona podem alterar aspectos da viabilidade, da morfologia e do comportamento migratório das Células-Tronco Mesenquimais, podendo assim, alterar seu efeito terapêutico.

OBJETIVOS

Objetivo Geral

Avaliar a viabilidade, a morfologia e o comportamento migratório das células-tronco mesenquimais sob o efeito de Azatioprina e Dexametasona.

Objetivos Específicos

- Extrair, isolar e caracterizar células-tronco mesenquimais de membrana coriônica humana;
- Padronizar um protocolo de migração celular intrínseca para MSCs;
- Avaliar as MSCs sob o efeito de Azatioprina e Dexametasona quanto a:

  - Viabilidade celular e morfometria nuclear;
  - Polaridade celular;
  - Morfologia do citoesqueleto de actina;
  - Distribuição da FAK;
  - Velocidade e trajetória espacial da migração das MSCs.

CAPÍTULO 1

Analysis of mesenchymal stem cells migratory
behavior by time-lapse microscopy assay

Natália Schneider, Fabiany da Costa Gonçalves,
Marcelo Lazzaron Lamers, Ana Helena da Rosa Paz

Periódico: **Nature Protocols** (Qualis A1)

Status: **Em preparação**

Title: Analysis of mesenchymal stem cells migratory behavior by time-lapse microscopy assay

Authors:

Natália Schneider[a,b]

Email: natalia_@uol.com.br

Fabiany da Costa Gonçalves[a,b]

Email: fabygon@hotmail.com

Marcelo Lazzaron Lamers[c]

Email: marcelo.lamers@ufrgs.br

Ana Helena da Rosa Paz[a,b,c,*]

Email: anahpaz@gmail.com

a. Experimental Pathology Unit, Experimental Research Center, Hospital de Clínicas de Porto Alegre, Ramiro Barcelos 2350, CEP 90035-903 Porto Alegre, RS, Brazil.

b. Graduate Program in Gastroenterology and Hepatology Sciences, Universidade Federal do Rio Grande do Sul, Ramiro Barcelos 2400, CEP 90035-903 Porto Alegre, RS, Brazil.

c. Morphological Sciences Department, Health Basic Sciences Institute, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite 500, CEP 90050-170 Porto Alegre, RS, Brazil.

***Corresponding author:** Ana Helena da Rosa Paz, PhD, Morphological Sciences Department, Health Basic Sciences Institute, Universidade Federal do Rio Grande do Sul. Rua Sarmento Leite, 500 Sala 001D. CEP 90050-170, Porto Alegre, RS, Brazil.

anahpaz@gmail.com Telephone: +55-51-33083631 Fax: +55-51-33598761

Abstract

Cellular migratory behavior has been largely studied in the past few years, especially due to the growing interest in cellular therapy. In this way, studying the basis of mesenchymal stem cells (MSCs) homing becomes crucial for understanding their migratory process. Here we describe a protocol for monitoring cellular migration of mesenchymal stem cells through visualization and quantification of migratory activities. Cells are placed on a fibronectin-coated dish, stimulating cell migration for up to 20 h. In combination with microscopic imaging, this assay allows quantification of cell speed and spatial trajectory, also recording membrane movement and protrusions. The actual handling time for the assay takes 2-3 h in total. This protocol was developed to simplify imaging of cells providing options of materials easily reachable to most laboratories. It can be adapted to examine cell migration at a variety of adherent cells, including fibroblasts and MSCs.

Introduction

Overview of cellular migration

Cell migration has gained worldwide attention, especially after cellular therapies had become such a focus among treatments for several diseases. Studying the basis of cellular migration has been crucial for understanding the mechanisms in particular diseases and therapy outcomes. It is already known that cells must have a polarized morphology resulting from intracellular signaling to migrate properly. This process must follow a protrusion at the leading edge, efficient adhesion, contraction and detachment, concluding the motility cell cycle. In this way, directional cell migration involves both intrinsic cellular directionality and external regulation, and can be quantified regarding cell spatial trajectory (ST) and speed of migration. Cellular intrinsic migration is the first indicative of how a cell is going to respond in front of a chemoattractant, although it does not guarantee an outcome [1,2]. External factors can modify the basic mechanisms of cell motility [1], and here we provide a simple technique to begin understanding cellular behavior under different influences. Our protocol uses fibronectin as a known stimulator of cellular migration [3]. Besides not providing the cell a strict direction to follow, fibronectin is a proper matrix to assess intrinsic cell behavior under a range of experimental conditions.

Cell migration techniques

Commonly used cell migration techniques (Table 1) include the Transwell assay, which consists of a permeable membrane through where cells can migrate from one compartment to another. It usually involves a chemoattractant in the lower chamber, where cells are supposedly attracted to. However, it also can be used to assess intrinsic cellular behavior if both compartments contain fetal bovine serum (FBS) at the same percentage and no chemoattractant [4]. Despite widely used, is not possible to use this technique to image cellular migration behavior. In this way, an imaging assay frequently applied is the Wound healing assay (or Scratch assay), which consists of a gap in the cellular monolayer and the footage of cell movements to close it [5]. Although it provides imaging of cellular behavior, cell migration and proliferation is often misread in this technique. To provide the best technique to assess intrinsic cell migration imaging, we propose

the time-lapse microscopy technology, simplifying and demystifying this technique to be of broad reach to most laboratories.

Time-lapse microscopy

Time-lapse microscopy is often brought as a complicated and expensive technique, being out of reach to most of research laboratories. However, it is an excellent option to investigate cell behavior, expression and migration, while here we provide options to perform this technique with basic and low cost materials. Previous studies have applied this technique, with modifications, to image larval development [6], to measure directed cell migration [7] or the half-life dynamics of multiple proteins [8] and single-cell gene expression [9]. Our group has already tested high and low glucose concentrations on different fibroblastic cells, observing cellular behavior through this patronized technique [10]. Moreover, we are analyzing mesenchymal stem cell migration under influence of immunosuppressant drugs [11], observing that time-lapse microscopy is adequate to begin understanding cellular behavior in a range of conditions.

Experimental design

Before starting the procedure, some points should be considered. To obtain the best comparison between groups, the researcher should follow timing as strict as possible. It is highly important to start video shooting at the same point in all samples after step 8 of the protocol, so if any cellular changes appear on images, timing errors could be crossed out. Another important issue is the percentage of FBS that the cell type requires. Image testing should be done beforehand aiming to find the best medium fit for cells to migrate properly, so the shooting could be long enough to obtain good analyzing material. An option to this matter is a medium that does not require FBS complementation. Although a little more expensive, it is a valuable alternative. Moreover, the use of medium with phenol red is advised, along with a well vaseline-sealed petri dish, to avoid CO_2 exchange and maintain and control the ideal pH. This protocol can be performed to evaluate a serial of drugs and chemicals effects on intrinsic behavior of different cell types. Our proposal is to provide an inexpensive and simple technique to obtain good results regarding cell migration.

Limitations

This protocol has limitations when compared to other techniques. Differently from the Transwell assay, chemotaxis may not be evaluated. Also a chemical gradient of CO_2 exchange can not be controlled if your microscope is not equipped with stage incubator (which regulates temperature, CO_2 and humidity). However, our results demonstrated that Vaseline-sealed petri dishes associated to a temperature controlled-support promote adequate conditions to maintain MSCs for extended periods, as long as 20 h. Reagents described here are well established for mesenchymal stem cells, and adaptations should be made to obtain the best experimental fitting to your cell type.

MATERIALS

REAGENTS

CRITICAL All reagents must be sterile.

Dulbecco's Modified Eagle's Medium (DMEM; Gibco, cat. no. 31600-034)

Fetal Bovine Serum (FBS; Gibco, cat. no. 12657-029)

Trypsin-EDTA, 0.25% (wt/vol); divide it into 2 ml aliquots for single use and store them at -20 °C (Gibco, cat. no. 25200-072)

Penicillin Streptomycin Solution; divide it into 1 ml aliquots for single use and store them at -20 °C (PS; Gibco, cat. no. 15140-122)

Phosphate-Buffered Saline (PBS; Laborclin, cat. no. 590338)

Fibronectin (Sigma-Aldrich, cat. no.F0895-1MG)

Hydrochloridric Acid (HCl) ***CAUTION*** Hydrochloridric acid is a corrosive agent. Handle it with care.

Norland Optical Adhesive 68 (Ultraviolet curing glue, Norland, cat. no. 6801)

Solid Vaseline

Ethanol 96% ***CAUTION*** Ethanol 96% is a flammable liquid. Handle it with care.

EQUIPMENT

For cell culture

Disposables: 75-cm^2 flask (TPP), centrifuge tubes (15 ml, TPP), pipette tips (Vatten)

Pipetts (Pipetman) ***CRITICAL*** Pipettes and all other equipment for cell culture must be sterile.

CO_2 incubator set to 5% CO_2 and 37 °C (Thermo Fisher Scientific, cat. no. 3111)

Circular glass coverslips, 25 mm (see Equipment Setup; Fisherbrand, cat. no. 12-545-102)

Petri dishes, 35 mm (see Equipment Setup; Corning, cat. no. 430588)

Centrifuge (Eppendorf, cat. no. 5810-01418)

Water bath at 37°C ***CRITICAL*** Water bath must be sterile and maintained with sterile water, which should be periodically changed.

For cell migration

Inverted microscope (Axio Observer Z1, cat. no.491912-0003-000) equipped with a charge coupled device camera and with a temperature-controlled support for imaging (Temp Module S1, cat. no. 411860-9010-000)

Objective: x10 objective (Ecplan-Neofluar, x10 numerical aperture (NA): 0.3)

Image analysis software: Image J

REAGENT SETUP

Cell culture medium To 100 ml of DMEM low glucose w/ phenol red, add FBS (10% (vol/vol)) and PS (1% (vol/vol)). Store the medium at 4 °C for up to 1 month, and prewarm it to 37 °C in a water bath before use.

Fibronectin To 5 ml of PBS, add 10 µl of fibronectin 1 mg/ml (2 µg/ml final concentration). Use the working solution immediately after preparation.

EQUIPMENT SETUP

Circular glass coverslip preparation Submerge circular glass coverslips into hydrochloridric acid 10% solution (HCl (vol/vol) in Ethanol 96%) in an appropriate beaker, with the help of a coverslip porcelain support and tweezers. Keep the coverslips well separated during acid immersion and cover the beaker with parafilm. Let it incubate overnight at room temperature. After incubation, transfer the coverslips to a beaker filled with distilled water, assisted by a porcelain support and tweezers. Keep the coverslips submerged for 5 min and repeat the wash four more times. Leave the coverslips on the porcelain support in an incubator at 37 °C overnight. Dry-heat-sterilize the coverslips and store them in a sterilized container for future use for up to 6 months. Steps could be visualized in Figure 1. ***CAUTION*** Hydrochloridric acid is a corrosive agent. Handle it with care. ***CRITICAL*** Coverslip preparation needs to be performed over 3 d.

Glass-bottomed dish preparation Make a central hole in the 35mm petri dish, with the help of a drilling machine, not bigger than 20mm (smaller than the glass coverslips). Position the petri dish up-side-down and apply Norland Optical Adhesive 68 glue throughout the whole border. Place the circular coverslip, previously sterilized, closing the hole. Leave it under UV radiation during 2 h for the glue to go dry, and turn the petri dish up, leaving it under UV radiation for another 2 h, then close it with the lid. Steps could be visualized in Figure 1. ***CAUTION*** UV radiation is a hazard agent. Handle it with care.

Fibronectin coating Wash the glass-bottomed dish with PBS and coat with 1 ml of fibronectin working solution (2 µg/ml) only at the area of the glass coverslip (Figure 2). Incubate it at 4 °C overnight. Next day, wash the fibronectin coated area with PBS gently. It is advised to use the fibronectin glass-bottomed dish immediately, but it can be stored at 4 °C with PBS covering the fibronectin area up to 4 days. Prior to use, discard the PBS and wash the dish with warm culture medium. ***CRITICAL*** Always maintain fibronectin coated area covered either with PBS or culture medium, so it can not run dry. It is critical to keep the glass-bottomed dish sterile at all steps from the protocol.

PROCEDURE

Preparation of adherent cell culture *TIMING* ~ 1 week

1 Culture adherent cells in a 75-cm² flask in a humidified incubator at 37 °C with 5% CO_2.

2 Change the medium every 2-3 d by discarding the current medium and washing cells with 2 ml of prewarmed PBS (1x).

3 Discard the liquid and add 10 ml of prewarmed complete fresh medium (DMEM with 10% (v/v) FBS and 1% (v/v) PS).

4 Maintain the culture in a humidified incubator at 37 °C with 5% CO_2 until it reaches 80% of confluence. ***? TROUBLESHOOTING***

Adherent cells collection *TIMING* 2 h

5 After reaching 80% of confluence in culture, cells should be well attached and present a fibroblastic morphology. Detach cells by discarding current medium and washing cells with 2 ml of prewarmed PBS; discard the liquid and add 2 ml of trypsin-0.25% EDTA solution. Place the flask in the humidified incubator at 37 °C with 5% CO_2 for 2-3 min.

6 Transfer the suspension of cells and trypsin-0.25% EDTA to a 15-ml centrifuge tube, add 6 ml of complete medium and centrifuge at 200 x *g* for 5 min at 4 °C.

7 Discard the medium and resuspend the pellet in 1 ml of prewarmed complete medium. Place $2x10^4$ cells onto a thoroughly cleaned 20 mm fibronectin-coated glass-bottomed dish (2µg/ml) in a total of 2 ml. It is important to place cells only at the fibronectin-coated area.

8 Allow the cells to attach to the fibronectin-coated glass-bottomed dish for 1 h (Figure 2) in the humidified incubator at 37 °C with 5% CO_2. ***? TROUBLESHOOTING***

9 Add prewarmed complete medium to the fibronectin-coated glass-bottomed dish where the cells are placed, until it is completely filled.

10 Place solid vaseline on the border of the lid and close the dish to avoid any gas exchange and to maintain the medium pH while filming the cells.

Apparatus preparation for cell migration assay ***TIMING*** 30 min-1 h

11 Assemble the equipment for the migration assay as shown in Figure 3.

12 Set the dish support temperature to 37 °C to maintain the ideal medium temperature.

Time-lapse imaging ***TIMING*** ~ 20 h

13 Transfer the closed dish containing the cells to the prewarmed support of the microscope. Choose the best field with x10 objective considering the ideal of ~10 cells in the imaging field, and image without any shadows or bubbles.

14 For migration speed and ST measurement, use a x10 objective to obtain contrast phase time-lapse images at a 10 min intervals for up to 20 h. Membrane movement and protrusions can also be analyzed by taking pictures every 10 s for 50 min, using a x40 objective. ***? TROUBLESHOOTING***

Quantitative analysis ***TIMING*** 1 -2 h

15 After time-lapse image acquisition, plot the migratory paths of individual cells and determine the total distance randomly traveled by using the Image J software. Individual migration speed could be calculated considering distance traveled in the determined period and ST could be determined and plotted using SigmaPlot software. We recommend four independent experiments to allow appropriate statistical analysis.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

TIMING

Steps 1-4, cell culture: ~ 1 week

Steps 5-10, cell collection: ~ 2 h

Steps 11-12, preparation for migration assay: 30 min-1 h

Steps 13-14, time-lapse imaging: ~ 20 h

Step 15, quantitative analysis: 1-2 h

ANTECIPATED RESULTS

Our group has previously applied this technique to evaluate the effects of low glucose, high glucose or osmotic control in CHO.K1, NIH-3T3 fibroblasts or mouse embryonic fibroblasts migration. For image shooting, cells were kept in CCM1 medium, which does not require FBS complementation. Cellular speed and directionality were measured following imaging where images were captured every 10 min; also, kymografy analysis was performed after a 30 min video with captured images every 5 s. Data was compiled and analyzed over Image J [10].

In the same way, we used this protocol to assess the effects of Dexamethasone and Azathioprine on human chorionic mesenchymal stem cells. The images were taken every 10 min for a total of 20 h or every 10 s for a total of 50 min, and cells were kept in DMEM 10% FBS during the procedure. Cellular speed and ST were analyzed using Image J software [11].

Acknowledgments

The authors would like to thank Lucas Dreher for design assistance with the figures produced for this protocol paper.

REFERENCES

1. **Petrie RJ, Doyle AD, Yamada KM.** Random versus directionally persistent cell migration. *Nat Rev Mol Cell Biol.* 10(8):538-49 (2009).

2. **Swaney KF, Huang CH, Devreotes PN.** Eukaryotic chemotaxis: a network of signaling pathways controls motility, directional sensing, and polarity. *Annu Rev Biophys.* 39:265-89 (2010).

3. **Zou L, Cao S, Kang N, Huebert RC, Shah VH.** Fibronectin induces endothelial cell migration through β1 integrin and Src-dependent phosphorylation of fibroblast growth factor receptor-1 at tyrosines 653/654 and 766. *J Biol Chem.* 287(10):7190-202 (2012).

4. **Limame R, Wouters A, Pauwels B, Fransen E, Peeters M, Lardon F, De Weyer O, Pauwels P.** Comparative Analysis of Dynamic Cell Viability, Migration and Invasion Assessments by Novel Real-Time Technology and Classic Endpoint Assays. *Plos One*. 7(10): e46536 (2012).

5. **Liang CC, Park AY, Guan JL.** *In vitro* scratch assay: a convenient and inexpensive method for analysis of cell migration *in vitro*. *Nat Protoc.* 2(2):329-33 (2007).

6. **Chai Y, Li W, Feng G, Yang Y, Wang X, Ou G.** Live imaging of cellular dynamics during Caenorhabditis elegans postembryonic development. *Nat Protoc*. 7(12):2090-102 (2012).

7. **Wu HJ, Liu YJ, Li HQ, Chen C, Dou Y, Lou HF, Ho MS, Li MX, Gao Z, Duan S.** Analysis of microglial migration by a micropipette assay. *Nat Protoc*. 9(2):491-500 (2014).

8. **Geva-Zatorsky N, Issaeva I, Mayo A, Cohen A, Dekel E, Danon T, Cohen L, Liron Y, Alon U, Eden E.** Using bleach-chase to measure protein half-lives in living cells. Nat Protoc. 7(4):801-11 (2012).

9. **Young JW, Locke JC, Altinok A, Rosenfeld N, Bacarian T, Swain PS, Mjolsness E, Elowitz MB.** Measuring single-cell gene expression dynamics in bacteria using fluorescence time-lapse microscopy. *Nat Protoc*. 7(1):80-8 (2011).

10. **Lamers ML, Almeida ME, Vicente-Manzanares M, Horwitz AF, Santos MF.** High glucose-mediated oxidative stress impairs cell migration. *Plos One*. 6(8):e22865 (2011).

11. **Schneider N, Gonçalves FC, Pinto FO, Lopez PLC, Araújo AB, Pfaffenseller B, Passos EP, Cirne-Lima EO, Meurer L, Lamers ML, Paz AH.** Dexamethasone and Azathioprine promote cytoskeleton changes and affect Mesenchymal Stem Cells migratory behavior. *Plos One*. Submitted (2014).

FIGURE LEGENDS

Figure 1. Glass-bottomed dish preparation. Glass coverslips should be prepared by incubating them in HCl 10% ethanol overnight, following 5x washing in distilled water in the next day. Dry-heat sterilize the glass coverslips after they had gone dry and glue them on the 20mm hole petri dish. Expose each side of the dish to UV radiation for 2 h and the glass-bottomed dish is ready to be used.

Figure 2. Cell culture. Culture the cells until it reaches 80% of confluence and collect them. Place fibronectin (2µg/ml) in the glass portion of the glass-bottomed dish and then place the collected cells on the fibronectin coated area.

Figure 3. Microscope setup. (A) Photograph of migration assay through microscope imaging. **(B)** Controller of the temperature-controlled dish support. **(C)** Visualization of time-lapse images.

TABLE LEGENDS

Table 1. Comparison of *in vitro* cell migration techniques. Comparison of Time-Lapse Mycroscopy, Scratch Assay and Transwell Assay.

Table 2. Troubleshooting advice. Identification of problems, possible reasons and solutions.

FIGURE 1. Glass-bottomed dish preparation.

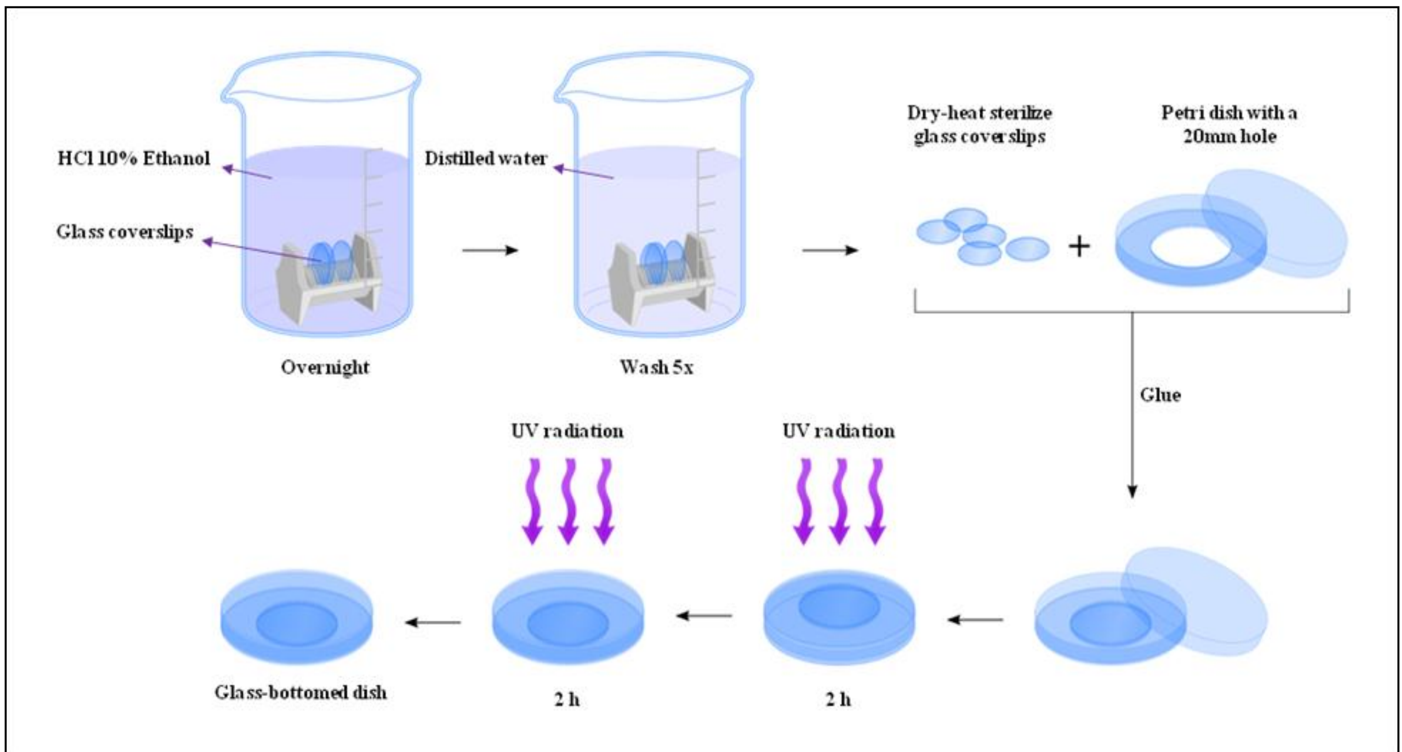


FIGURE 2. Cell culture.

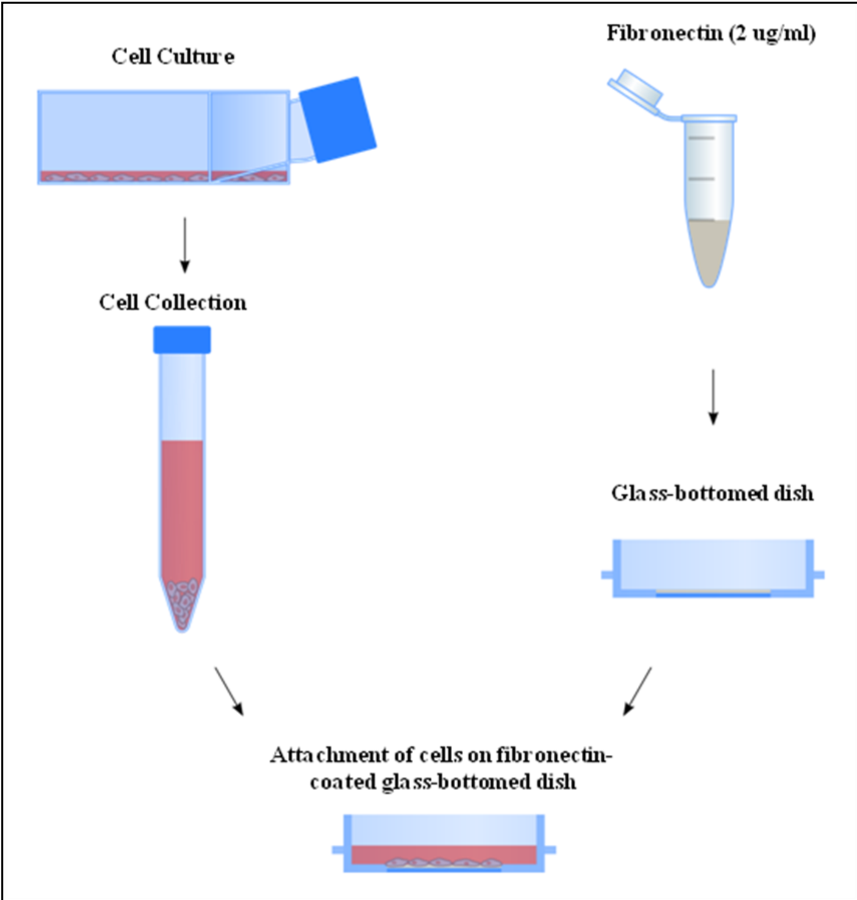


FIGURE 3.Microscope setup.

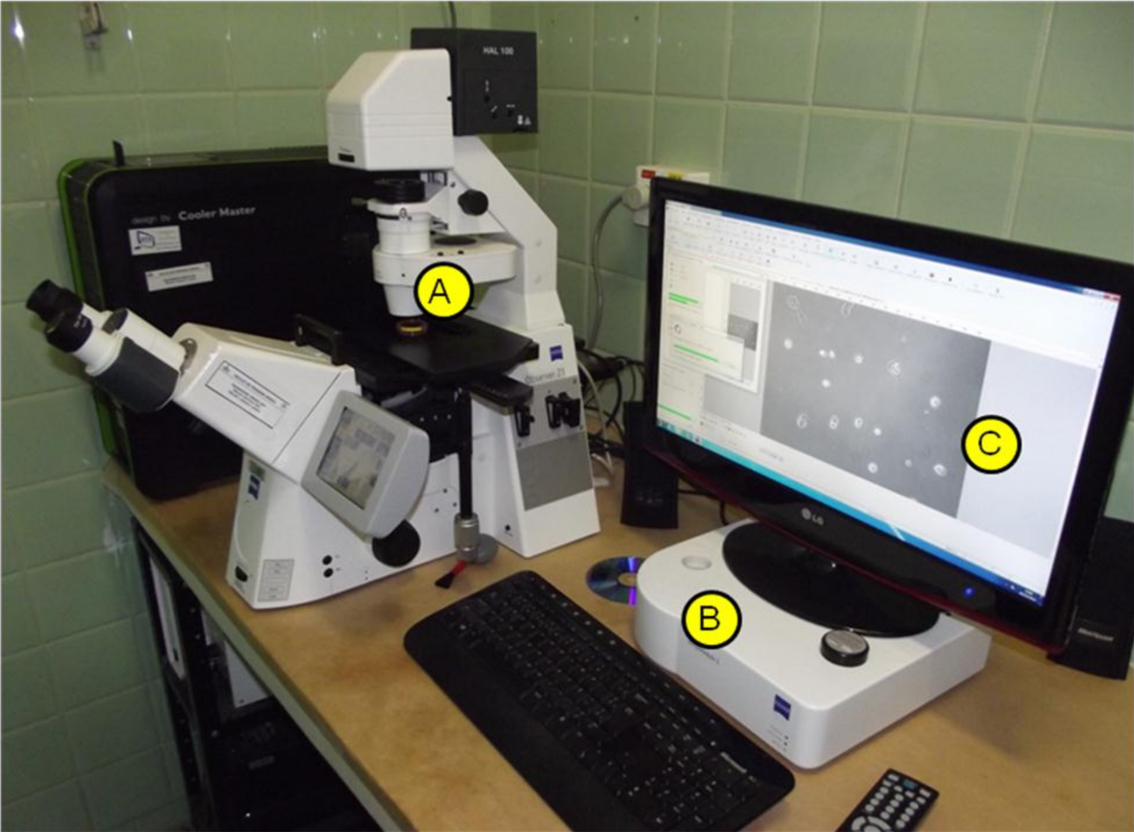


Table 1. Comparison of *in vitro* cell migration techniques.

	Time-lapse microscopy	**Scratch assay**	**Transwell assay**
Live imaging	Yes	Yes	No
Number of analyzed cells	Small	Small	Large
Precise individual tracking	Yes	No	No
Chemical gradient	No	No	Yes
Equipment set up	Self-made and easy to set up	Easy to set up	Transwell chambers commercially available

Table 2. Troubleshooting advice.

Step	Problem	Possible Reason	Solution
4	Poor cell attachment	Culture flasks surface are not adequate	Reach out for the fabricant
		Cell culture is contaminated	Use of antibiotics is always advised
	Slow cell growth	Insufficient supply of nutrients	Changing medium every 2-3 days is recommended
8	Poor cell attachment	Coated fibronectin is not adequate	Keep fibronectin glass-bottomed dish at 4 °C no longer than 4 days prior to use
		Cells were kept too long in trypsin-0.25% EDTA	Verify adequate incubation time with trypsin-0.25% EDTA according to cell type
14	Blurry image	The equipment was not able to sustain the settings overnight	Manually adjust focus in the equipment during the first 1-2 h of footage
		Bubbles were formed	The lid needs to be well closed with solid vaseline; focus on the center of the dish when choosing the best field and avoid the edges

CAPÍTULO 2

Dexamethasone and Azathioprine promote cytoskeleton
changes and affect Mesenchymal Stem Cells migratory behavior

Natália Schneider, Fabiany da Costa Gonçalves, Fernanda Otesbelgue Pinto, Patrícia Luciana da Costa Lopez, Anelise Bergmann Araújo, Bianca Pfaffenseller, Eduardo Pandolfi Passos, Elizabeth Obino Cirne-Lima, Luíse Meurer, Marcelo Lazzaron Lamers, Ana Helena da Rosa Paz

Periódico: **Plos One** (Qualis A1)

Status: **Submetido**

PLOS ONE

Dexamethasone and azathioprine promote cytoskeleton changes and affect Mesenchymal Stem Cells migratory behavior

--Manuscript Draft--

Manuscript Number:	PONE-D-14-30995R1
Article Type:	Research Article
Full Title:	Dexamethasone and azathioprine promote cytoskeleton changes and affect Mesenchymal Stem Cells migratory behavior
Short Title:	Immunosuppressants affect MSCs migratory behavior
Corresponding Author:	Ana Helena da Rosa Paz, Ph.D. Universidade Federal do Rio Grande do Sul Porto Alegre, Rio Grande do Sul BRAZIL
Keywords:	Mesenchymal Stem Cells; actin; cell migration; azathioprine; dexamethasone; Inflammatory Bowel Disease.
Abstract:	Glucocorticoids and immunosuppressive drugs are commonly used to treat inflammatory disorders, such as Inflammatory Bowel Disease (IBD) and, despite few improvements, the remission of IBD is still difficult to maintain. Due to its immunomodulation properties, Mesenchymal Stem Cells (MSCs) have emerged as regulators of immune response, and its viability and activation of migratory properties are essential for successful cell therapy. However, little is known about the effects of immunosuppressant drugs used on IBD treatment on MSCs behavior. The aim of this study was to evaluate MSC viability, nuclear morphometry, cell polarity, F-actin distribution and cell migration properties in the presence of the immunosuppressive drugs Azathioprine (AZA) or Dexamethasone (DEX). After initial characterization, MSCs were treated with DEX (10 μM) or AZA (1 μM) for 24 hrs or 7 days. Both drugs had no effects on cell viability or nuclear morphometry. However, AZA treatment induced a more elongated cell shape and increased the presence of ventral actin stress fibers, while DEX was associated to a more rounded cell shape with high presence of ventral actin stress fibers and a decrease on protrusion stability. After 7 days of treatment, AZA improved cell the spatial trajectory (ST) and increased migration speed (24.35%, $P < 0.05$, $n = 4$) while DEX impaired ST and migration speed after 24 hrs and 7 days treatment (-28.69% and -25.37%, respectively; $P < 0.05$, $n = 4$). In conclusion our data suggests these immunosuppressive drugs can differently affect MSCs morphology and migration capacity, possibly impacting the success of cell therapy.
Order of Authors:	Natália Schneider
	Fabiany da Costa Gonçalves
	Fernanda Otesbelgue Pinto
	Patrícia Luciana da Costa Lopez
	Anelise Bergmann Araújo
	Bianca Pfaffenseller
	Eduardo Pandolfi Passos
	Elizabeth Obino Cirne-Lima
	Luise Meurer
	Marcelo Lazzaron Lamers
	Ana Helena da Rosa Paz, Ph.D.

Title Page

Title: Dexamethasone and azathioprine promote cytoskeleton changes and affect Mesenchymal Stem Cells migratory behavior

Authorship:

Natália Schneider[a,b]

Email: natalia_@uol.com.br

Fabiany da Costa Gonçalves[a,b]

Email: fabygon@hotmail.com

Fernanda Otesbelgue Pinto[a]

Email: fernandaotesbelgue@hotmail.com

Patrícia Luciana da Costa Lopez[c]

Email: patilu17@gmail.com

Anelise Bergmann Araújo[c]

Email: anelise_araujo@yahoo.com.br

Bianca Pfaffenseller[c]

Email: bianca.pfaffenseller@gmail.com

Eduardo Pandolfi Passos[a]

Email: eduardo@segir.com.br

Elizabeth Obino Cirne-Lima[a]

Email: ecirnelima@gmail.com

Luíse Meurer[b]

Email: meurerrl@terra.com.br

Marcelo Lazzaron Lamers[d]

Email: marcelo.lamers@ufrgs.br

Ana Helena da Rosa Paz[a,b,d,*]

Email: anahpaz@gmail.com

a. Embryology and Cell Differentiation Laboratory, Experimental Research Center, Hospital de Clínicas de Porto Alegre, Ramiro Barcelos 2350, CEP 90035-903 Porto Alegre, RS, Brazil.

b. Graduate Program in Gastroenterology and Hepatology Sciences, Universidade Federal do Rio Grande do Sul, Ramiro Barcelos 2400, CEP 90035-903 Porto Alegre, RS, Brazil.

c. Experimental Research Center, Hospital de Clínicas de Porto Alegre, Ramiro Barcelos 2350, CEP 90035-903 Porto Alegre, RS, Brazil.

d. Morphological Sciences Department, Health Basic Sciences Institute, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite 500, CEP 90050-170 Porto Alegre, RS, Brazil.

***Corresponding author:** Ana Helena da Rosa Paz, PhD, Morphological Sciences Department, Health Basic Sciences Institute, Universidade Federal do Rio Grande do Sul. Rua Sarmento Leite, 500 Sala 001D. CEP 90050-170, Porto Alegre, RS, Brazil.
anahpaz@gmail.com Telephone: +55-51-33083631 Fax: +55-51-33598761

Authors contribution: NS, MLL and AHRP conceived and designed the study; NS, FCG, FOP, PLL, ABA, BP, MLL and AHRP performed the experiments; NS, MLL and AHRP analyzed the data; NS, FCG, MLL and AHRP contributed to the writing of the manuscript; EPP, EOC and LM contributed with reagents/materials/analysis tools; MLL and AHRP supervised the study design, execution, and analysis.

Abstract

Glucocorticoids and immunosuppressive drugs are commonly used to treat inflammatory disorders, such as Inflammatory Bowel Disease (IBD) and, despite few improvements, the remission of IBD is still difficult to maintain. Due to its immunomodulation properties, Mesenchymal Stem Cells (MSCs) have emerged as regulators of immune response, and its viability and activation of migratory properties are essential for successful cell therapy. However, little is known about the effects of immunosuppressant drugs used on IBD treatment on MSCs behavior. The aim of this study was to evaluate MSC viability, nuclear morphometry, cell polarity, F-actin distribution and cell migration properties in the presence of the immunosuppressive drugs Azathioprine (AZA) or Dexamethasone (DEX). After initial characterization, MSCs were treated with DEX (10 μM) or AZA (1 μM) for 24 hrs or 7 days. Both drugs had no effects on cell viability or nuclear morphometry. However, AZA treatment induced a more elongated cell shape and increased the presence of ventral actin stress fibers, while DEX was associated to a more rounded cell shape with high presence of ventral actin stress fibers and a decrease on protrusion stability. After 7 days of treatment, AZA improved cell spatial trajectory (ST) and increased migration speed (24.35%, $P < 0.05$, $n = 4$) while DEX impaired ST and migration speed after 24 hrs and 7 days treatment (-28.69% and -25.37%, respectively; $P < 0.05$, $n = 4$). In conclusion our data suggests these immunosuppressive drugs can differently affect MSCs morphology and migration capacity, possibly impacting the success of cell therapy.

Keywords: Mesenchymal Stem Cells, Actin, Cell Migration, Azathioprine, Dexamethasone, Inflammatory Bowel Disease.

Introduction

Inflammatory bowel disease (IBD) is a family of chronic inflammatory disorders of the gastrointestinal tract, that includes Crohn's Disease (CD) and Ulcerative Colitis (UC) and which is characterized by dysfunction of T cells and uncontrolled production of inflammatory cytokines [1]. Evidence supports that IBD results from the interaction between genetic, environmental and microbial factors, resulting in exaggerated and imbalanced mucosal immune response to the normal intestinal microflora. This inflammation is sustained by alteration of the mucosal barrier and immune system defects, which opens possibilities for new treatments targeting immunomodulation and tissue repair [1-3].

IBD patients usually suffer from a poor quality of life, multiple adverse effects and the disease remission often remains difficult to maintain. Despite improvements in current drug treatments, they are not entirely effective [1,4]. Furthermore, IBD has grown its appearance in pediatric patients, who present a history of multiple intestinal resections and immune modulating treatments with or without biological agents. Their long term response to treatment is uncertain, and that is one of the many reasons why there is a search for new therapies, putting mesenchymal stem cells as one of the best options to treat these inflammatory conditions [3].

Mesenchymal stem cells (MSCs) present great plasticity, multipotent capacity and have emerged as potent regulators of the immune response. These cells are known for having low immunogenicity, being able to escape recognition by T cells due to a low expression of HLA class I, and the lack of HLA class II and co-stimulatory molecules [5-7]. MSCs also secrete a variety of cytokines that act suppressing local immune system, controlling inflammation and assisting in tissue repair [8-10]. These cells can be isolated from different organs and tissues, including bone marrow, muscle, adipose tissue, and also feto-maternal organs. In addition, the usage of postnatal placental tissue has shown several benefits as a source of MSCs [11-13]. When compared to other sources, placental-derived MSCs have shown to possess a better proliferation rate [14], superior engraftment capacity [15], to share some of the same markers encountered in embryonic stem cells [16] and to present increased immunosuppressive properties [17,18]. These cells also possess a great migration capacity both *in vitro* [19] and *in vivo* [20].

These results lead to a successful administration of fetal-derived MSCs on a phase I study for the treatment of CD and UC [21].

Up to date, there are controversies involving the mechanisms by how MSCs migrate to inflamed tissues. MSCs homing is defined as the arrest of MSCs into the tissue vasculature followed by endothelial transmigration. Unlike the well-characterized adhesion cascade of leukocyte homing, there is a current absence of a clear mechanism for MSCs homing. The exact positioning of MCSs after infusion is unclear and makes it difficult to determine if cells have been arrested within the vessels (localization) or have gone through transendothelial migration (homing) [22]. Despite studies visualizing MSCs trapped into the lungs after intravenous infusion [23,24], several others have found MSCs systemically administered reaching the target injured tissue, such as brain [20,25], spinal cord [26], heart [27] and kidney [28]. These data suggest MSCs might have a homing capacity *in vivo* towards the injured site.

The use of cell therapies capable of local immunomodulation could be an alternative to improve current IBD outcome and different phase I-III clinical trials regarding IBD-treatments using Mesechymal Stem Cells (MSC) have resulted in promising and diverse outcomes [1,3,5,29-33].

MSCs therapeutic approaches still rely on adequate cell homing towards the inflammation or injured site. Cell migration is a complex process that involves the break of cell polarity with the formation of a cell front and rear, which is characterized by the polymerization of actin and formation of nascent adhesions at the cell front, maturation of cell adhesions, contractility of cell body and detachment of adhesions at the cell rear [34-36]. Actin polymerization is involved in the formation of cell protrusions and it depends of a fine regulation among several signaling and effector proteins [37] and the organization of the actin network is known to reflect different stages of cell migration [38,39]. The speed and the spatial trajectory of migrating cells are essential for MSCs homing process and it relies on an efficient regulation of cell polarity and actin dynamics, which might be impaired by different physiological or pharmacological condition [40,41].

IBD clinical treatments include immunosuppressive drugs, such as Azathioprine (AZA) and Dexamethasone (DEX). Their immunosuppressive action targets the inhibition of purine nucleotide synthesis and the synthesis and metabolism of RNA, or acts by controlling the

transcription of inflammatory genes, respectively [42,43]. Previous studies have analyzed immunosuppressive drugs interactions with MSCs, regarding cellular proliferation and functionality, including migratory chemotaxis capacity through transwell assay; however, while some studies conclude MSCs might be affected by these drugs, there are others who show otherwise [44-48]. Considering cell therapy as a strong alternative for the treatment of IBD, it is imperative to study the interaction of MSCs and drugs commonly used as conventional treatment. To address this matter, we examined human chorion-derived MSCs viability, nuclear morphometry, cell polarity, F-actin distribution and cell migration in the presence of Azathioprine or Dexamethasone at concentrations similar to observed in clinical treatments (1 μM and 10 μM, respectively) [1,45,49,50]. We observed early changes in cell polarity and cytoskeleton distribution (24 h) with both drugs and, after 7 d treatment, DEX impaired cell migration while AZA was able to increase cellular speed; both results have strong relevance to MSCs therapy outcome.

Materials and Methods

Ethics Statement

Ethical approval was given by the Research Ethics Committee of Hospital de Clínicas de Porto Alegre (GPPG12-0082).

Mesenchymal Stem Cells isolation and expansion

MSCs were isolated from human term chorionic membranes after written consent from women at delivery. The tissue was separated (3 x 2.5 cm) and thoroughly washed with phosphate-buffered saline (PBS); after, it was minced into small pieces and proteolytically digested with 1 mg/ml collagenase type I (Sigma, MO, USA) at 37 °C for 1 h 45 min and centrifuged at 500 x *g* for 10 min at room temperature. Cells were plated in 6 well plate (TPP, Trasadingen, CH) using Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, CA, USA) low glucose supplemented with 20% fetal bovine serum (FBS; Gibco, CA, USA), 1% 100 units/ml penicillin and 100 mg/ml streptomycin (PS; Gibco, CA, USA) - standard medium. Media was

replaced twice a week. For experiments, cells were used at passage 4–8 and results of each experimental condition were compared among cells at the same passage.

In vitro differentiation

In order to characterize MSCs in accordance with *The International Society for Cellular Therapy Statement* [51], two different experimental procedures were employed. Osteogenic differentiation was carried out using DMEM low glucose supplemented with 10% FBS, 1% PS, 0.1 μM dexamethasone, 10 mM β-glycerolphosphate (Sigma-Aldrich, MO, USA) and 50 μM ascorbic acid 2-phophaste (Sigma-Aldrich, MO, USA), with 5000 cells/well in 24-well plate for 21 d. Differentiation was further confirmed by Alizarin Red S (Sigma-Aldrich, MO, USA) staining [12].

Adipogenic differentiation was induced by culturing MSC for 21 d in DMEM low glucose supplemented with 10% FBS, 1% PS, 1 μM dexamethasone, 0.5 mM isobutyl methylxantine (Sigma-Aldrich, MO, USA), 10 μg/ml insulin and 200 μM indomethacin (Sigma-Aldrich, MO, USA). Adipogenic differentiation was confirmed by Oil Red O (Sigma-Aldrich, MO, USA) staining [12].

Flow cytometry analysis

In order to characterize the cell population according to surface molecular markers, immunophenotyping was performed. Cells were detached with Trypsin-0.25% EDTA (Gibco, CA, USA) and washed by centrifugation (300 x *g*, 5 min) with PBS. Antibodies (CD73, CD90, CD105, CD45, CD34, CD14, CD19 and HLA-DR) were added to the cells ($1x10^6$ cells) and incubated for 30 min at 4 °C following the manufacturer's protocol. Cells were then washed with PBS and analyzed by BD FACS-Calibur flow cytometer (Becton-Dickison, NJ, USA) using Cellquest and PAINT-A-GAIT software.

Inflammatory Bowel Disease drugs

Azathioprine and Dexamethasone (Sigma-Aldrich, MO, USA) were used in relevant clinical concentrations of 1 μM and 10 μM, respectively. AZA was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, MO, USA) and DEX in DMEM low glucose. The AZA and

DEX vehicle control groups were treated with complete standard medium supplemented with DMSO (0.5%) or DMEM low glucose, respectively. The range of concentrations of AZA and DEX used in the present study is comparable to those found in sera of patients with inflammatory conditions under these drugs treatments and are also commonly used for *in vitro* and *in vivo* studies [1,45,49,50].

Cell viability by MTT assay

MSCs were seeded in a 24-well plate at 5000 cells/well and cultivated in the presence of drugs for 24 h or 7 d. After that, MSCs were incubated with standard medium containing 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, MO, USA) (final concentration of 555,56 μg/ml) for 4 h at 37 °C. At the end of the experiment, medium was removed and 100 μl of DMSO were added. Absorbance was read at 570 nm in a 96-well plate using a microplate reader (Biochrom Anthos Zenyth 200rt microplate reader, Cambridge, UK) Experiments were performed in quadruplicates from four independent assays [45,52].

Nuclear Morphometric Analysis, Actin Cytoskeleton and Immuofluorescence

Cells were plated (5000 cells/well) in a 24-well plate, followed by treatments with drugs for 24 h and 7 days. In sequence, cells were detached with trypsin-0.25% EDTA, washed and plated on fibronectin-coated glass coverslips (2 μg/ml, Sigma-Aldrich, MO, USA) and allowed to attach overnight (ON) in an incubator (37 °C, 5% CO_2). MSCs were washed with PBS, fixed with 4% paraformaldehyde and sacarose 4% for 15 min at room temperature (RT), then washed (PBS) and the membrane was permeabilized with PBS 0.3% Tween 20 (Sigma-Aldrich, MO, USA) for 10 min at RT. Cells were washed (PBS), blocked with normal goat serum (1:10, 1 h, RT), incubated with FAK rabbit (Focal Adhesion Kinase; Cell Signaling) antibody ON at 4 °C, washed (PBS) and incubated with AlexaFluor 488 goat anti-rabbit IgG (Molecular Probes – Invitrogen, Oregon, USA) for 2 h, RT. After washing, actin staining was performed with Rhodamine-Phalloidin (1:100 in PBS, Molecular Probes - Invitrogen, Oregon, USA) for 1 h at 4°C and coverslips were mounted using Prolong (Sigma-Aldrich, MO, USA) containing dye (DAPI) for nuclear staining and sealed with nail polish.

Nuclear Morphometric Analysis (NMA) was performed as described by Filippi-Chiela et al [53] on Axio Observer Z1 microscope (Zeiss, Göttingen, Germany) with a charge coupled device camera (Axiocam mrn, Zeiss, Göttingen, Germany) using 10x objective (Ecplan-Neofluar 10x/0.3 aperture, Zeiss, Göttingen, Germany) and AxioVision Software (Zeiss, Göttingen, Germany). Briefly, DAPI stained cells were excited with mercury lamp and an excitation filter (EX G 365, EM BP445/50). Images were taken from a total of 100-300 nuclei, obtained from random fields, of 3-4 independent experiments. Images were analyzed using Image J Software, for the acquisition of nuclear area and the parameters of nuclear irregularity (Roundness, Aspect, Radius Ratio and Area/Box, which are grouped in an index, named nuclear irregularity index - NII). The plot of nuclear area per NII permits the separation of different nuclear populations and the inference of different cell death and growth inhibition mechanisms such as apoptosis (small and regular nuclei – SR nuclei), senescence (large and regular nuclei – LR nuclei) and irregular nuclei (I nuclei) [53,54].

Actin staining and FAK images were obtained in a confocal microscope (Leica TCS SP5 II, Wetzlar, Germany) using a 40x objective (HCX PL APO 40x/1.30 oil immersion). Rhodamine was excited using the 514 nm laser line of an Argon laser DAPI was excited with a 405 nm laser line of a 405 Diode laser and Alexa488 was excited with 488nm laser line of an Argon laser (Melles Griot, Albuquerque, NM). Fluorescence images were acquired using Leica LASAF Software (Leica, Wetzlar, Germany). In each experiment (n = 4) 10 images per condition were collected (total of 40 images) and the most representative samples of each set of experiments were selected.

Cell Polarity

For assessment of cell polarity, the polarity index was calculated as the length of the major migration axis (parallel to the direction of movement) divided by the length of the perpendicular axis that intersects the center of the cell nucleus (Supplementary Figure 1). It was analyzed a total of 80-100 cells in each condition from 4 independent experiments and the polarity index values distribution was analyzed through histogram [41].

Migration Assay

Cells were plated in a 6-well plate ($6x10^4$ cells/well), followed by treatments with drugs or vehicles media for 24 h or 7 d. After treatments, cells were detached with trypsin-0.25% EDTA, washed and $2x10^4$ cells were plated on fibronectin-coated glass-bottomed dishes (2 μg/ml) in DMEM low glucose 10% FBS with or without drugs for 1 h, and maintained in an incubator at 37 °C. For analysis of the migration properties, phase microscopy time-lapse images were captured for a period of 20 h at 10 min intervals (migration speed and spatial trajectory - ST) or 50 min at 10 s interval (protrusion activity) (Ecplan-Neofluar 10x/0.3 aperture objective) with a charge coupled device camera (Axiocam mrn, Zeiss, Göttingen, Germany) attached to an inverted microscope (Axio Observer Z1, Zeiss, Göttingen, Germany) using AxioVision Software (Zeiss, Göttingen, Germany). The values for assessment of migration speed and ST were carried out on Image J (*National Institute of Health*, MA, USA) software, and data was processed as previously described [41]. For ST analysis, it was performed a polar plot graph, which represents the spatial trajectory developed by each migratory cell, where the X and Y coordinates of each cell trajectory was normalized to start at a virtual (X=0 and Y=0) position. It was analyzed a total of 80-100 cells, from 4 independent experiments for each condition.

Statistics

Experimental data are presented as mean ± SEM. Statistical analysis were performed using GEE (Generalized Estimated Equations) for viability and ANOVA post-hoc Tukey HSD for migration speed in PASW Statistics (SPSS – version 18.0); ANOVA post-hoc Student-Newman-Keuls (SNK) for multiple comparisons was used for nuclear morphometric analysis in GraphPad INSTAT (GraphPad Software, San Diego, California, USA). Analyses were carried out and *P*-values less than 0.05 were considered significantly different.

Results

Isolation and characterization of chorion-derived MSCs

MSCs from human chorion membrane were isolated and characterized. To investigate the differential potential of MSCs, adipogenic and osteogenic differentiation was induced *in vitro*. A

clear potential for adipogenic differentiation was detected by oil red staining of lipid vacuoles (Fig. 1A), and osteogenic differentiation was confirmed as detected by alizarin red staining of calcium deposits (Fig. 1B). Cells expressed mesenchymal markers CD73 (99.5%), CD90 (99.8%) and CD105 (100%), but lacked CD45 (0.1%), CD34 (0.1%), CD14 (0.2%), CD19 (0.1%) and HLA-DR (0.1%) (Fig. 1C-J).

Cell viability and nuclear morphometry are not affected by IBD drugs

Alterations on cell viability could result in a diminished number of cells and a low effectiveness of cell therapy. In order to verify the effects of drugs on MSC viability, cells were incubated with immunosuppressive drugs for 24 h and 7 d and mitochondrial dehydrogenase activity was measured in the living cells by MTT assay [52]. Results showed no difference at the enzyme level under DEX and AZA treatments (Fig. 2A, $P > 0.05$, $n = 4$). Therefore, nuclear morphometry can indicate several cell fates such as early apoptosis and senescence, which may compromise MSCs quality for therapy purpose. In order to analyze nuclear morphometry, NMA was carried out and it was observed no difference in nuclear morphometry at all different drug treatments and time points (Fig. 2B, $P > 0.05$, $n = 4$). Altogether, our results from viability and NMA showed that DEX and AZA treatments are not toxic for MSCs.

IBD drugs change polarity profile of the cells

Cell polarity results from the internal organization of the cell and is a key step for induction of cell motility, where a more elongated morphology is usually associated to a better mesenchymal cell migratory performance [55,56]. The effects of the drugs on cell polarity at each time point (24 h and 7 d) were assessed through a polarity index (Fig. 3 and Supplementary Figure 1). AZA induced a slight increase of elongated shaped cells (11.7% and 12.2% with polarity index > 3; 24 h and 7 d, respectively) when compared with control group, while DEX treatment presented a higher presence of rounded shape cells (-42.8% and -22.4% with polarity index > 3; 24 h and 7 d, respectively). AZA and DEX vehicles had no effect on polarity index. This data suggest that dexamethasone could impair the migratory activity of MSCs.

IBD drugs alters MSCs actin organization

Actin filaments are responsible for changes of cell shape in response to stimuli, coordinating cellular protrusions and locomotion [35]. We observed morphological changes when drugs were added to culture; DEX induced a higher presence of stress fibers while AZA preserved some of the lamellipodia (Supplementary Figure 2). In mesenchymal cells, filamentous actin is observed as dorsal, transverse arcs or ventral stress fibers [39] that might reflect changes on cell adhesion as well as changes on migratory abilities of the cell. Control and vehicle-treated cells showed stable protrusions (Supplementary Movie 1) and most of migratory cells showed the presence of all stress fibers subtypes which was accompanied by the distribution of the cell adhesion marker Focal Adhesion Kinase (FAK) as small adhesions at cell cortex (Fig. 4A-L). After incubation with AZA for 24 h or 7 days it was still observed the presence of stable protrusions (Fig. 4M-R; Supplementary movie 2) but with a predominance of ventral fibers and large adhesions at the cell body. After 24h of DEX treatment, MSCs presented increased membrane activity that was followed by the presence of unstable protrusions after 7 days of treatment (Fig. 4S-X; Supplementary Movie 3). This phenotype was accompanied by a more intense presence of ventral actin stress fibers at the cell body after 7 days of DEX treatment, accompanied by adhesions mainly at the cell rear. This data suggest that immunosuppressive drugs may affect differently the actin dynamics of MSCs, with possible effects on its migratory activity.

Dexamethasone impairs MSCs migration

MSCs homing capacity towards inflammation and injured site is an essential process to a successful cell therapy. MSCs migration speed and spatial trajectory under 24 h or 7 d of immunosuppressants treatment was assessed through time-lapse analysis. The vehicle treatment had no influence on migration speed when compared to control group (Fig. 5A). The 24 h AZA treatment had small effects on cell migration, however it was observed a 24.35% increase after 7 d treatment ($P < 0.05$, $n = 4$). DEX treatment impaired cell speed after 24 h and after 7 d by -28.69% and -25.37%, respectively ($P < 0.05$, $n = 4$). The analysis of ST showed that the control group (Supplementary movie 4) presented a high number of migratory cells that were able to reach long distances from the starting migratory point and a few cells that did not explore the

field (Fig. 5B). The 7 d vehicles-treatment showed no changes on MSCs migration when compared to control group (Fig. 5E, H, Supplementary movie 5 and 6). AZA 24 h treatment induced no change of cells with a high ST (Fig. 5C, Supplementary movie 7), while the 7 d AZA treatment improved the number of cell that migrated long distances (Fig. 5D, Supplementary movie 8). DEX 24 h treatment showed cells with low ST (Figure 5F, Supplementary movie 9), and this effect was more pronounced after 7 d of treatment (Fig. 5G, Supplementary movie 10). Taken together, these results suggest that, after 7 d treatment, AZA might improve MSC migration, while DEX would impair MSCs migratory capacity.

Discussion

Current IBD available treatments rely on immunosuppressive drugs and glucocorticoids, but are not entirely effective and remission often remains difficult to maintain [1,2]. As a novel IBD therapeutic approach, MSCs have emerged as potent regulators of the immune response [5-7], but little is known about their interaction to immunosuppressive drugs. Besides controversies surrounding MSCs homing, it has been previously shown that these cells do interact with endothelial cells through coordinated rolling and adhesion [57]. Furthermore, our group has recently published a study about different routes of MSCs administration in experimental UC, and showed the intravenous via to ameliorate clinical and histological status of the animals [58]. There are evidences of the benefits from local delivery of MSCs in therapy [26,59], however, intravenously administration of MSCs is a convenient minimally invasive way that opens options for cell therapy as repeated doses and the reaching of sites not surgically available. Considering that confluence of cells in culture, MSCs passage and number of cells administered already modify MSCs homing capacity [22,60], it is imperative to better understand MSCs interactions with immunosuppressive drugs conventionally used for IBD.

We observed herein that the clinical concentrations of the IBD drugs Azathioprine and Dexamethasone did not substantially affect the viability of human-derived MSCs. Previous studies have tested different concentrations of DEX and AZA in order to register any changes in cell viability [45,48]. Corroborating with our data, it was postulated that both drugs have no influence on human MSC viability at a variable range of times. However, the glucocorticoid

DEX was shown to affect cell viability at high concentrations (up to 1 mg/ml) [45,48]. We used lower drugs concentrations in accordance to drug levels that would be found in the serum of IBD patient under conventional treatment, and to which MSCs would be exposed to. It is possible that the non-toxic effect of DEX observed in our study could be explained for its action reducing the cell density-related apoptosis on MSCs [61].

Besides no changes on cell viability, we found that DEX and AZA were able to alter cell shape and cell migratory behavior. Cell migration depends on the activation of several signaling pathways that result in the break of cell asymmetry, where a more elongated cell correlates to a more effective migration process [56,62]. Using a polarity index distribution, we demonstrated that 7 days of AZA treatment induced a more elongated cell shape, while DEX resulted in a more rounded morphology. The analysis of cytoskeleton organization, the distribution of FAK and the analysis of membrane dynamics using time-lapse corroborated with the polarity index, since control and AZA treated cells showed the presence of stable protrusions, while DEX-rounded shape cells showed a higher presence of ventral stress fibers and unstable protrusions. In this way, Chen et al. (2013) [63] demonstrated that DEX induced the presence of stress fibers on epithelial cells involving stomatin expression and F-actin rearrangements. It was also shown that endothelial cells treated with DEX presented higher levels of caveolin-1 protein, a regulatory protein of cell surface receptor also involved on the recycling of cell adhesions, as well as alteration on the activity of the GTPase Rac-1, that is involved in controlling protrusion dynamics [64]. All these mechanisms could be involved in the results herein observed.

The differences on cell polarity and actin organization induced by AZA and DEX might reflect in a differential effect of immunosuppressant drugs in the MSCs migratory ability. Changes on cell polarity and actin organization might reflect alterations on cell migratory properties [56,62], which is essential for MSC homing process. We observed that 7 days of AZA treatment resulted in significant increase of cell speed and spatial trajectory when compared to control group. Poppe et al. (2006) [65] showed that AZA suppressed T cells ability to create flexible regions in the plasma membrane and lamellipodia, concluding that AZA could block Ezrin-Radixin-Moesin (ERM) proteins dephosphorylation. Interestingly, in our work AZA treatment partially preserved these structures on MSCs. It is possible that these changes might be associated to adhesion signaling, since leukocytes might be considered as non-adherent cells.

However, it is still unknown how AZA acts on MSCs, once studies mainly focus on explaining the mechanisms affected on immune cells. Additionally, AZA could be positive to stem cell therapy since Mancheño-Corvo et al. (2013) [66] demonstrated an important role of this immunosuppressant as a protective drug towards MSCs due to a reduction in the activation and degranulation of Natural Killer cells, reducing cell mediated lysis of allogeneic MSCs. This study speculates that patients could benefit from concomitant treatment with AZA since the clearance of allogeneic MSCs might be delayed, creating an extended time window for these cells to perform their therapeutic effect. Along with our results of increased migratory speed, this immunosuppressant could be able to induce a faster and safer homing of MSCs towards the injury site, enabling a better cell therapy outcome.

Regarding DEX treatment we found that after 24 h there was a decrease on cell speed and spatial trajectory which was more evident after 7 d of treatment. These results might be associated to the higher presence of stress fibers observed on DEX treatment. Similar MSCs shape alterations were described by Geiβler et al. (2012) [40] who observed that chronological and *in vitro* aging promoted round shaped MSCs and diminished its migration potential, along with a decrease on the expression of genes associated to cytoskeleton organization. Another study demonstrated that DEX treatment induced a more rigid cell structure an impairment of T cells migration in a mechanism that involves the activation of the ERM complex, which is related to cytoskeleton rearrangements [67]. In addition to its effects on *in vitro* cell migration, a recently published paper demonstrated that DEX can abolish MSCs therapeutic effects *in vivo* in an animal model of liver inflammation by inhibition of MSCs nitric oxide synthase, recommending that concomitant treatment with steroids and MSCs should be avoided [68].

Despite the controversies involving migration of MSCs to damaged tissues, the homing process continues to be a key step for MSCs to participate in tissue repair and immunomodulation. Several factors are being identified as regulators of this process and studied in order to enhance cellular migration and improve cell therapy [69-71]. Also, studies have explored the interaction between MSCs migration and drugs, aiming to better understand this process and improve cell therapy. A recent study when testing the multi-tyrosine kinase inhibitor sorafenib on endometrial derived-MSC, demonstrated the increased migratory capacity of these cells could be reverted by this drug through inhibition of ezrin (an ERM component), an

important finding targeting eradication of endometriotic implants [72]. Xinaris et al. (2013) [73] demonstrated that MSC preconditioning with insulin-like growth factor-1 (IGF-1) resulted in the improvement of cell migration and the restoration of normal renal function in animal model, supporting the idea that improvement of MSC migration could increase therapeutically relevant effects.

Our study aimed to better understand the influence of conventional treatment of IBD in MSCs, focusing in the main key of a successful therapy. DEX decreased cell motility while AZA ameliorated cellular migration after a prolonged treatment, both results influenced by cellular morphology and cytoskeleton organization. Further studies are needed to better understanding of these processes; however, these findings provide substantial data to look more carefully into patients under DEX treatment ongoing MSCs therapy. In addition, AZA possibly opens new perspectives in the use of immunosuppressants concomitantly with MSC, enhancing the result of cell therapy in IBD.

Acknowledgments

The authors would like to thank Cristina Flores, MD, PhD (Hospital de Clínicas de Porto Alegre) for her support during this study and Álvaro Macedo Laureano, PhD (Graduate Program in Medical Sciences, UFRGS) for his assistance with flow cytometry analysis.

References

1. **Duijvestein M, Vos ACW, Roelofs H, *et al.*** (2010) Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. *Gut;* 59: 1662-1669. doi: 10.1136/gut.2010.215152
2. **Bouma G, Strober W.** (2003) The Immunological and Genetic Basis of Inflammatory Bowel Disease. *Nature Reviews, Immunology*; 3(7):521-33. doi:10.1038/nri1132
3. **Martínez-Montiel MP, Gómez-Gómez GJ, Flores AI.** (2014) Therapy with stem cells in inflammatory bowel disease. World J Gastroenterol 2014; 20(5): 1211-1227. doi: 10.3748/wjg.v20.i5.1211
4. **Anderson P, Souza-Moreira L, Morell M, *et al.*** (2013) Adipose-derived mesenchymal stromal cells induce immunomodulatory macrophages which protect from experimental colitis and sepsis. *Gut*; 62: 1131-1141. doi: 10.1136/gutjnl-2012-302152
5. **Forbes GM, Sturm MJ, Leong RW, *et al.*** (2014) A phase 2 study of allogeneic mesenchymal stromal cells for luminal Crohn's disease refractory to biologic therapy. *Clin Gastroenterol Hepatol;*12(1):64-71. doi: 10.1016/j.cgh.2013.06.021
6. **Gonzalez-Rey E, Anderson P, González MA, *et al.*** (2009) Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. *Gut;* 58:929–939. doi: 10.1136/gut.2008.168534
7. **Le Blanc K, Tammik C, Rosendahl K, *et al.*** (2003) HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol*; 31:890–896. doi:10.1016/S0301-472X(03)00110-3
8. **Caplan AI, Dennis JE.** (2006) Mesenchymal Stem Cells as Trophic Mediators. *Journal of Cellular Biochemistry*; 98:1076–1084. doi: 10.1002/jcb.20886
9. **Miller RJ, Banisadr G, Bhattacharyya BJ.** (2008) CXCR4 signaling in the regulation of stem cell migration and development. *Journal of Neuroimmunology*; 198 31–38. doi: 10.1016/j.jneuroim.2008.04.008
10. **Mokbel AN, El Tookhy OS, Shamaa AA, *et al.*** (2011) Homing and reparative effect of intra-articular injection of autologus mesenchymal stem cells in osteoarthritic animal model. *BMC Musculoskeletal Disorders*; 12:259. doi: 10.1186/1471-2474-12-259

11. **Hass R, Kasper C, Böhm S, Jacobs R.** (2011) Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. *Cell Commun Signal*; 14;9:12. doi: 10.1186/1478-811X-9-12
12. **Indumathi S, Harikrishnan R, Mishra R, *et al.*** (2013) Comparison of feto-maternal organ derived stem cells in facets of immunophenotype, proliferation and differentiation. *Tissue Cell*; 45(6):434-42. doi: 10.1016/j.tice.2013.07.007
13. **Meirelles LS, Chagastelles PC, Nardi NB.** (2006) Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *Journal of Cell Science*; 119: 2204-2213. doi: 10.1242/jcs.02932
14. **Barlow S, Brooke G, Chatterjee K, et al.** (2008) Comparison of human placenta- and bone marrow-derived multipotent mesenchymal stem cells. *Stem Cells Dev*; 17(6):1095-107. doi: 10.1089/scd.2007.0154
15. **Brooke G, Tong H, Levesque JP, Atkinson K.** (2008) Molecular trafficking mechanisms of multipotent mesenchymal stem cells derived from human bone marrow and placenta. *Stem Cells Dev*; 17(5):929-40. doi: 10.1089/scd.2007.0156
16. **Yen BL, Huang HI, Chien CC, *et al*.** (2005) Isolation of multipotent cells from human term placenta. *Stem Cells*; 23(1):3-9. doi: 10.1634/stemcells.2004–0098
17. **Li X, Bai J, Ji X, *et al.*** (2014) Comprehensive characterization of four different populations of human mesenchymal stem cells as regards their immune properties, proliferation and differentitation. *Int J Mol Med*. 34(3):695-704. doi: 10.3892/ijmm.2014.1821
18. **Zhu Y, Yang Y, Zhang Y, *et al.*** (2014) Placental mesenchymal stem cells of fetal and materni origins demonstrate different therapeutic potentials. *Stem Cell Res Ther*. 10;5(2):48. doi: 10.1186/scrt436
19. **Li G, Zhang XA, Wang H, *et al.*** (2009) Comparative proteomic analysis of mesechymal stem cells derived from human bone marrow, umbilical cord, and placenta: implication in migration. *Proteomics;* 9(1):20-30. doi: 10.1002/pmic.200701195
20. **Kholodenko IV, Yarygin KN, Gubsky LV, *et al.*** (2012) Intravenous xenotransplantation of human placental mesenchymal stem cells to rats: comparative

analysis of homing in rat brain in two models of experimental ischemic stroke. *Bull Exp Biol Med.* 154(1):118-23. doi: 10.1007/s10517-012-1890-6

21. **Liang J, Zhang H, Wang D, *et al.*** (2012) Allogeneic mesenchymal stem cell transplantation in seven patients with refractory inflammatory bowel disease. *Gut;* 61:468-469. doi: 10.1136/gutjnl-2011-300083
22. **Karp JM, Leng Teo GS.** (2009) Mesenchymal stem cell homing: the devil is in the details. *Cell Stem Cell.* 6;4(3):206-16. doi: 10.1016/j.stem.2009.02.001
23. **Parekkadan B, Milwid JM.** (2010) Mesenchymal stem cells as therapeutics. *Annu Rev Biomed Eng.* 15;12:87-117. doi: 10.1146/annurev-bioeng-070909-105309.
24. **Schrepfer S, Deuse T, Reichenspurner H, *et al.*** (2007) Stem cell transplantation: the lung barrier. *Transplant Proc.* 39(2):573-6.
25. **Jackson JS, Golding JP, Chapon C, *et al.*** (2010) Homing of stem cells to sites of inflammatory brain injury after intracerebral and intravenous administration: a longitudinal imaging study. *Stem Cell Res Ther.* 15;1(2):17. doi: 10.1186/scrt17.
26. **Kim JW, Ha KY, Molon JN, Kim YH.** (2013) Bone marrow-derived mesenchymal stem cell transplantation for chronic spinal cord injury in rats: comparative study between intralesional and intravenous transplantation. *Spine (Phila Pa 1976).* 1;38(17):E1065-74. doi: 10.1097/BRS.0b013e31829839fa.
27. **Zhang D, Fan GC, Zhou X, *et al.*** (2007) Over-expression of CXCR4 on mesenchymal stem cells augments myoangiogenesis in the infarcted myocardium. *J Mol Cell Cardiol.* 44(2):281-92. doi: 10.1016/j.yjmcc.2007.11.010
28. **Zhuo W, Liao L, Fu Y, *et al.*** (2013) Efficiency of endovenous versus arterial administration of mesenchymal stem cells for ischemia-reperfusion-induces renal dysfunction in rats. *Transplant Proc.* 45(2):503-10. Doi: 10.1016/j.transproceed.2012.07.162
29. A Randomized, Double-Blind, Placebo-Controlled Dose-Escalation Study to Determine the Safety and Efficacy of Intravenous Infusion of Human Placenta-Derived Cells (PDA001) for the Treatment of Crohn's Disease. ClinicalTrials, NCT01769755. Available from: http://clinical trials.gov/show/NCT01769755

30. **Gazouli M, Roubelakis MG, Theodoropoulos GE.** (2014) Stem cells ad potential targeted therapy for inflammatory bowel disease. *Inflamm Bowel Dis.* 20(5):952-5. doi: 10.1097/01.MIB.0000442922.85569.21.
31. **Ciccocioppo R, Bernardo ME, Sgarella A, *et al.*** (2011) Autologous bone marrow-derived mesenchymal stromal cells in the treatment of fistulizing Crohn's disease. *Gut.* 60(6):788-98. doi: 10.1136/gut.2010.214841.
32. **Garcia-Olmo D, Herreros D, Pascual I, *et al.*** (2009) Expanded adipose-derived stem cells for the treatment of complex perianal fistula: a phase II clinical trial. *Dis Colon Rectum.* 52(1):79-86. doi: 10.1007/DCR.0b013e3181973487.
33. **Osiris Therapeutics.** A phase III, multicenter, placebocontrolled, randomized, double-blind study to evaluate the safety and efficacy of Prochimal [tm] (ex vivo cultured adult human mesenchymal stem cells) intravenous infusion for induction of remission in subjects experiencing treatmentrefractory moderate-to-severe Crohn’s disease. ClinicalTrials, NCTO0482092. Available from: URL: http://www.clinicaltrials.gov/ct2/show/NCT00482092
34. **Li R, Gundersen GG.** (2008) Beyond polymer polarity: how the cytoskeleton builds a polarized cell. *Nat Rev Mol Cell Biol*; 9(11):860-73. doi: 10.1038/nrm2522
35. **Pollard TD, Cooper JA.** (2009) Actin, a central player in cell shape and movement. *Science*; 326(5957):1208-12. doi: 10.1126/science.1175862
36. **Swaney KF, Huang CH, Devreotes PN.** (2010) Eukaryotic chemotaxis: a network of signaling pathways controls motility, directional sensing, and polarity. *Annu Rev Biophys*; 39:265-89. doi: 10.1146/annurev.biophys.093008.131228
37. **Vicente-Manzanares M, Webb DJ, Horwitz AR.** (2005) Cell migration at a glance. *J Cell Sci.* 118(Pt 21):4917-9. doi: 10.1242/jcs.02662
38. **Tojkander S, Gateva G, Lappalainen P.** (2012) Actin stress fibers--assembly, dynamics and biological roles. *J Cell Sci.* 15;125(Pt 8):1855-64. doi: 10.1242/jcs.098087
39. **Vallenius T.** (2013) Actin stress fibre subtypes in mesenchymal-migrating cells. *Open Biol.* 19;3(6):130001. doi: 10.1098/rsob.130001
40. **Geißler S, Textor M, Kühnisch J, *et al.*** (2012) Functional Comparison of Chronological and In Vitro Aging: Differential Role of the Cytoskeleton and

Mitochondria in Mesenchymal Stromal Cells. *PLoS One*; 7(12):e52700. doi: 10.1371/journal.pone.0052700

41. **Lamers ML, Almeida ME, Vicente-Manzanares M, *et al.*** (2011) High glucose-mediated oxidative stress impairs cell migration. *PLoS One*; 6(8):e22865. doi: 10.1371/journal.pone.0022865
42. **Liu L, Yuan S, Long Y, *et al.*** (2009) Immunomodulation of Rheum tanguticum polysaccharide (RTP) on the immunosuppressive effects of dexamethasone (DEX) on the treatment of colitis in rats induced by 2,4,6-trinitrobenzene sulfonic acid. *International Immunopharmacology;* 9:1568–1577. doi: 10.1016/j.intimp.2009.09.013
43. **Pham PT, Pham PC.** (2011) The impact of mycophenolate mofetil versus azathioprine as adjunctive therapy to cyclosporine on the rates of renal allograft loss due to glomerular disease recurrence. *Nephrol Dial Transplant;* 0: 1–7. doi: 10.1093/ndt/gfr731
44. **Buron F, Perrin H, Malcus C, *et al.*** (2009) Human Mesenchymal Stem Cells and Immunosuppressive Drug Interactions in Allogeneic Responses: An In Vitro Study Using Human Cells. *Transplantation Proceedings*; 41: 3347–3352. doi: 10.1016/j.transproceed.2009.08.030
45. **Duijvestein M, Molendijk I, Roelofs H, *et al.*** (2011) Mesenchymal stromal cell function is not affected by drugs used in the treatment of inflammatory bowel disease. *Cytotherapy*; 13: 1066–1073. doi: 10.3109/14653249.2011.597379
46. **Hoogduijn MJ, Crop MJ, Korevaar SS, *et al.*** (2008) Susceptibility of Human Mesenchymal Stem Cells to Tacrolimus, Mycophenolic Acid, and Rapamycin. *Transplantation*; 86: 1283–1291. doi: 10.1097/TP.0b013e31818aa536
47. **Nichols JE, Niles JA, Dewitt D, *et al.*** (2013) Neurogenic and neuro-protective potential of a novel subpopulation of peripheral blood-derived CD133+ ABCG2+CXCR4+ mesenchymal stem cells: development of autologous cell-based therapeutics for traumatic brain injury. *Stem Cell Res Ther;* 6;4(1):3. doi: 10.1186/scrt151
48. **Nuzzi R, Gunetti M, Rustichelli D, *et al.*** (2012) Effect of In Vitro Exposure of Corticosteroid Drugs, Conventionally Used in AMD Treatment, on Mesenchymal Stem Cells. *Stem Cells International*; 2012:946090. doi: 10.1155/2012/946090

49. Giamarellos-Bourboulis EJ, Dimopoulou I, Kotanidou A, *et al.* (2010) Ex-vivo effect of dexamethasone on cytokine production from whole blood of septic patients: Correlation with disease severity. *Cytokine*; 49(1):89-94. doi: 10.1016/j.cyto.2009.08.016

50. Schroll S, Sarlette A, Ahrens K, *et al.* (2005) Effects of azathioprine and its metabolites on repair mechanisms of the intestinal epithelium in vitro. *Regul Pept*; 131(1-3):1-11. doi10.1016/j.regpep.2005.03.001

51. Horwitz EM, Le Blanc K, Dominici M, *et al.* (2005) Clarification of the nomenclature for MSC. The International Society for Cellular Therapy position statement. *Cytotherapy*; 7: 393-395. doi: 10.1080/14653240500319234

52. Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods;* 16;65(1-2):55-63. doi:10.1016/0022-1759(83)90303-4

53. Filippi-Chiela EC, Oliveira MM, Jurkovski B, *et al.* (2012) Nuclear morphometric analysis (NMA): screening of senescence, apoptosis and nuclear irregularities. *PLoS One*; 7(8):e42522. doi: 10.1371/journal.pone.0042522

54. Lopez PL, Filippi-Chiela EC, Silva AO, *et al.* (2012) Sensitization of glioma cells by X-linked inhibitor of apoptosis protein knockdown. *Oncology*; 83(2):75-82. doi: 10.1159/000337978

55. Larsen M, Tremblay ML, Yamada KM. (2003) Phosphatases in cell-matrix adhesion and migration. *Nat Rev Mol Cell Biol;* 4(9):700-11. doi:10.1038/nrm1199

56. Ridley AJ, Schwartz MA, Burridge K, *et al.* (2003) Cell migration: integrating signals from front to back. *Science*; 302(5651):1704-9. doi: 10.1126/science.1092053

57. Rüster B, Göttig S, Ludwig RJ, *et al.* (2006) Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. *Blood;* 1;108(12):3938-44. doi: 10.1182/blood-2006-05-025098

58. Gonçalves FC, Schneider N, Pinto FO, *et al.* (2014) Intravenous versus intraperitoneal mesenchymal stem cells administration: What is the best route for treating experimental colitis? *World J Gastroenterol*; In press

59. **Charwat S, Gyöngyösi M, Lang I, *et al*.** (2008) Role of adult marrow stem cells in the repair of ischemic myocardium: current state of the art. *Exp Hematol.* 36(6):672-80. doi: 10.1016/j.exphem.2008.01.005

60. **Maijenburg MW, van der Schoot CE, Voermans C.** (2012) Mesenchymal stromal cell migration: possibilities to improve cellular therapy. *Stem Cells Dev.* 21(1):19-29. doi: 10.1089/scd.2011.0270

61. **Song IH, Caplan AI, Dennis JE.** (2009) Dexamethasone inhibition of confluence-induced apoptosis in human mesenchymal stem cells. *J Orthop Res.* 27(2):216-21. doi: 10.1002/jor.20726

62. **Friedl P, Alexander S.** (2011) Cancer invasion and the microenvironment: plasticity and reciprocity. *Cell.* 23;147(5):992-1009. doi: 10.1016/j.cell.2011.11.016.

63. **Chen JC, Cai HY, Wang Y, *et al.*** (2013) Up-regulation of stomatin expression by hypoxia and glucocorticoid stabilizes membrane-associated actin in alveolar epithelial cells. *J Cell Mol Med*; 17(7):863-72. doi: 10.1111/jcmm.12069

64. **Igarashi J, Hashimoto T, Shoji K, *et al.*** (2013) Dexamethasone induces caveolin-1 in vascular endotelial cells: implications for attenuated responses to VEGF. *Am J Physiol Cell Physiol*; 304(8):C790-800. doi: 10.1152/ajpcell.00268.2012

65. **Poppe D, Tiede I, Fritz G, *et al.*** (2006) Azathioprine suppresses ezrin-radixin-moesin-dependent T cell-APC conjugation through inhibition of Vav guanosine exchange activity on Rac proteins. *J Immunol*; 176(1):640-51. doi: 10.4049/jimmunol.176.1.640

66. **Mancheño-Corvo P, Franquesa M, de la Rosa O, *et al*.** (2013) Adipose mesenchymal stromal cell function is not affected by methotrexate and azathioprine. *Biores Open Access*; 2(6):431-9. doi: 10.1089/biores.2013.0040

67. **Müller N, Fisher HJ, Tischner D, *et al.*** (2013) Glucocorticoids indue effector T cell depolarization via ERM proteins, thereby impeding migration and APC conjugation. *J Immunol*; 190(8):4360-70. doi: 10.4049/jimmunol.1201520

68. **Chen X, Gan Y, Li W, *et al.*** (2014) The interaction between mesenchymal stem cells and steroids during inflammation. *Cell Death Dis*; 5e1009. doi: 10.1038/cddis.2013.537

69. **Li L, Jiang J.** (2011) Regulatory factors of mesenchymal stem cell migration into injured tissues and their signal transduction mechanisms. *Front Med*; 5(1):33-9. doi: 10.1007/s11684-011-0114-1

70. **Qiu Y, Marquez-Curtis LA, Janowska-Wieczorek A.** (2012) Mesenchymal stromal cells derived from umbilical cord migrate in response to complemente C1q. *Cytotherapy*; 14(3):285-95. doi: 10.3109/14653249.2011.651532

71. **Sohni A, Verfaillie CM.** (2013) Mesenchymal Stem Cells Migration Homing and Tracking. *Stem Cell Int*; 130763. doi:10.1155/2013/130763

72. **Moggio A, Pittatore G, Cassoni P, *et al.*** (2012) Sorafenib inhibits growth, migration, and angiogenic potential of ectopic endometrial mesenchymalstem cells derived from patients with endometriosis. *Fertil Steril*; 98(6):1521-30.e2. doi: 10.1016/j.fertnstert.2012.08.003

73. **Xinaris C, Morigi M, Benedetti V, *et al*.** (2013) A novel strategy to enhance mesenchymal stem cell migration capacity and promote tissue repair in an injury specific fashion. *Cell Transplant*; 22(3):423-36. doi: 10.3727/096368912X653246

Figure and Video Legends

Figure 1. MSCs characterization. Human-chorionic derived stem cells showed successful **(A)** adipogenic and **(B)** osteogenic differentiation potential, and presented positive signal for **(C-E)** CD73, CD90 and CD105, but lacked **(F-J)** CD45, CD34, CD14, CD19 and HLA- DR markers ($n = 4$).

Figure 2. MSCs viability and nuclear morphometry. MSCs were cultured for 24 h or 7 d under AZA 1 µM or DEX 10 µM treatments and accessed for cell viability through MTT **(A)** and nuclei irregularity index **(B)**. All groups and experiments were compared resulting in no difference between control and treatments.($n = 4$, $P > 0.05$) Time point in 2B is 7 days of treatment and 24 h time point is not shown. MTT assay was analyzed through GEE test and nuclear morphometry through ANOVA post-hoc SNK. Bar = 200 µm.

Figure 3. Cell Polarity Index. MSCs were cultured for 24 h or 7 d with or without AZA 1 µM or DEX 10 µM, and polarity index was measured according to Supplementary Figure 1. Images show the histogram of PI values obtained from all analyzed cells (80-100 cells, $n = 4$). DEX treatment resulted in a more rounded cell shape (PI < 3), while AZA showed a tendency to produce more elongated cells (PI > 3) when compared to control group.

Figure 4. Actin cytoskeleton and FAK distribution. MSCs were cultured for 24 h or 7 d with or without drugs, plated on fibronectin-coated dish overnight, fixed and stained for focal adhesion kinase (FAK) and actin analysis. FAK (green) and actin (magenta) co-localization (white) is demonstrated on merged images. Control cells showed small adhesions at the cell border (A and D) and actin stress fibers (B and E) that showed no variation in the presence of DMSO for 24 h (G-I) or 7 days (J-L). Cells treated with Azathioprine (1 µM) for 24 h showed small adhesions at the cell border (M) and the presence of ventral stress fibers (N), while 7 days of treatment did not change adhesions size (P) but actin distribution (Q) was similar to control. After 24 h or 7 days of Dexamethasone treatment there was an increase on adhesion size (S and

V) and ventral stress fibers (T, X), that was followed by an increase on membrane projections at 7 days (X). Bar = 40μm; representative images of *n* = 40 cells per condition.

Figure 5. MSC migration speed and spatial trajectory. MSCs were cultured for 24 h or 7 d with or without AZA 1 μM or DEX 10 μM, plated on fibronectin 2 μg/mL and imaged using time-lapse analysis. (A) DEX treatment induced a decrease on migration speed, while AZA was associated to a faster motility property. The Spatial Trajectory (ST) of each migratory cell was accessed through the use of X and Y values, which were normalized to start at a virtual migratory starting point (X=0 and Y=0) (B-H). Individual lines on polar plots represent the ST of each cell, while red lines represent cells that stayed close to starting point and blue lines represents more exploratory cells. DEX treated cells were associated to a lower ST, while AZA showed a tendency to induce a more exploratory phenotype. *$P < 0.05$, $n = 4$. MSCs migration speed was analyzed through ANOVA post-hoc Tukey HSD test and comparisons were made between all groups; the differences among the main groups are represented in the graphic.

Supplementary material legends

Supplementary Figure 1. Polarity index analysis. Polarity index was calculated as the length of the major migration axis parallel to the direction of movement (a) divided by the length of the perpendicular axis that intersects the center of the cell nucleus (b). (Bar = 100μm)

Supplementary Figure 2. **Cytoskeleton morphology.** MSCs were cultured for 24 h, 48 h or 7 d with or without drugs, plated on fibronectin-coated dish overnight, fixed and stained for actin analysis. Results showed control (A-C) and vehicle-treated (J-O) cells present lamellipodia (arrowheads) and a small amount of stress fibers in the cell body. After incubation with AZA for 24 h and 7 d (D-F) it was observed the presence of lamellipodia in some cells (arrowheads) and a few membrane projections (arrows). DEX treated cells showed a decrease on lamellipodia after 24 h (G) which was accompanied by an increase in the presence of thin membrane projections (H and I, arrows) and a more intense presence of actin stress fibers. (Bar = 100μm); nuclei staining = DAPI.

Supplementary Movie 1. Control. Movie shows the migratory activity of mesenchymal stem cells cultured with standard media in the presence (right cell) or absence (left cell) of DMSO (0.5%) for 7 days and plated under migration promotion conditions. Total time = 50 min, time interval = 10 s.

Supplementary Movie 2. Azathioprine. Movie shows the migratory activity of mesenchymal stem cells cultured with standard media in the presence of AZA (1 μM) for 24 h (left cell) or 7 days (right cell) and plated under migration promotion conditions. Total time = 50 min, time interval = 10 s.

Supplementary Movie 3. Dexamethasone. Movie shows the migratory activity of mesenchymal stem cells cultured with standard media in the presence of DEX (10 μM) for 24 h (left cell) or 7 days (right cell) and plated under migration promotion conditions. Total time = 50 min, time interval = 10 s.

Supplementary Movie 4. Control. Movie shows the migratory activity of mesenchymal stem cells cultured with standard media and plated under migration promotion conditions. This movie corresponds to Figure 5B. Total time = 20 h, time interval = 10 min.

Supplementary Movie 5. AZA Vehicle 7 d. Movie shows the migratory activity of mesenchymal stem cells cultured with AZA vehicle for 7 d and plated under migration promotion conditions. This movie corresponds to Figure 5E. Total time = 20 h, time interval = 10 min.

Supplementary Movie 6. DEX Vehicle 7 d. Movie shows the migratory activity of mesenchymal stem cells cultured with DEX vehicle for 7 d and plated under migration promotion conditions. This movie corresponds to Figure 5H. Total time = 20 h, time interval = 10 min.

Supplementary Movie 7. Azathioprine 24 h. Movie shows the migratory activity of mesenchymal stem cells cultured with AZA for 24 h and plated under migration promotion conditions. This movie corresponds to Figure 5C. Total time = 20 h, time interval = 10 min.

Supplementary Movie 8. Azathioprine 7 d. Movie shows the migratory activity of mesenchymal stem cells cultured with AZA for 7 d and plated under migration promotion conditions. This movie corresponds to Figure 5D. Total time = 20 h, time interval = 10 min.

Supplementary Movie 9. Dexamethasone 24 h. Movie shows the migratory activity of mesenchymal stem cells cultured with DEX for 24 h and plated under migration promotion conditions. This movie corresponds to Figure 5F. Total time = 20 h, time interval = 10 min.

Supplementary Movie 10. Dexamethasone 7 d. Movie shows the migratory activity of mesenchymal stem cells cultured with DEX for 7 d and plated under migration promotion conditions. This movie corresponds to Figure 5G. Total time = 20 h, time interval = 10 min.

FIGURE 1. MSCs characterization.

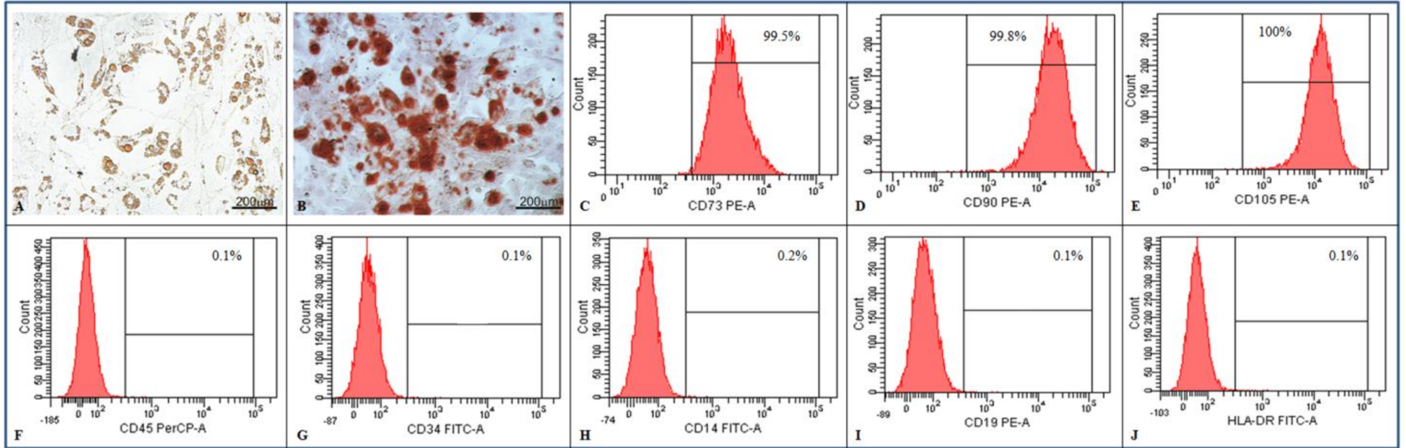


FIGURE 2. MSCs viability and nuclear morphometry.

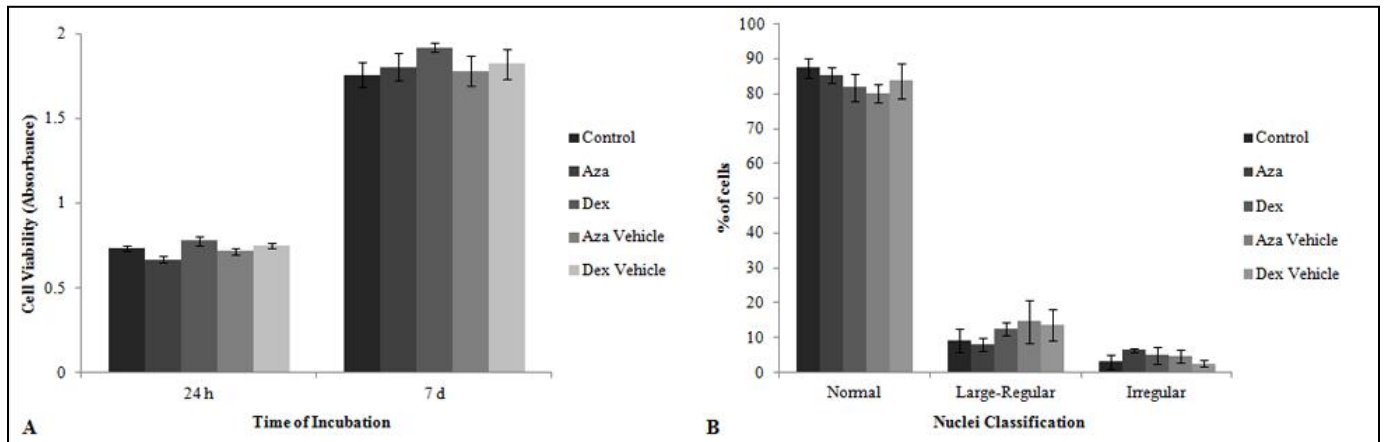


FIGURE 3. Cell Polarity Index.

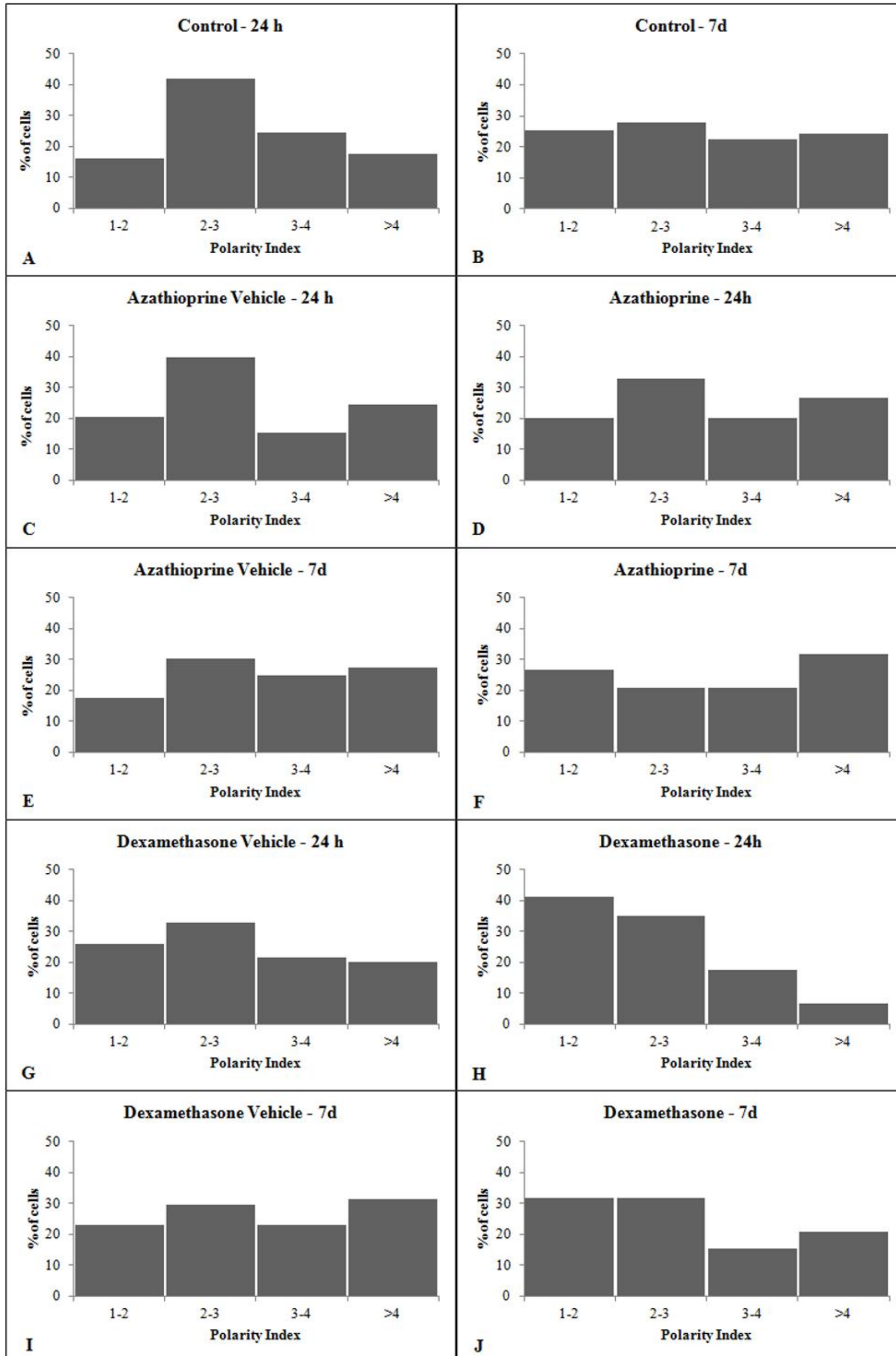


FIGURE 4. Actin cytoskeleton and FAK distribution.

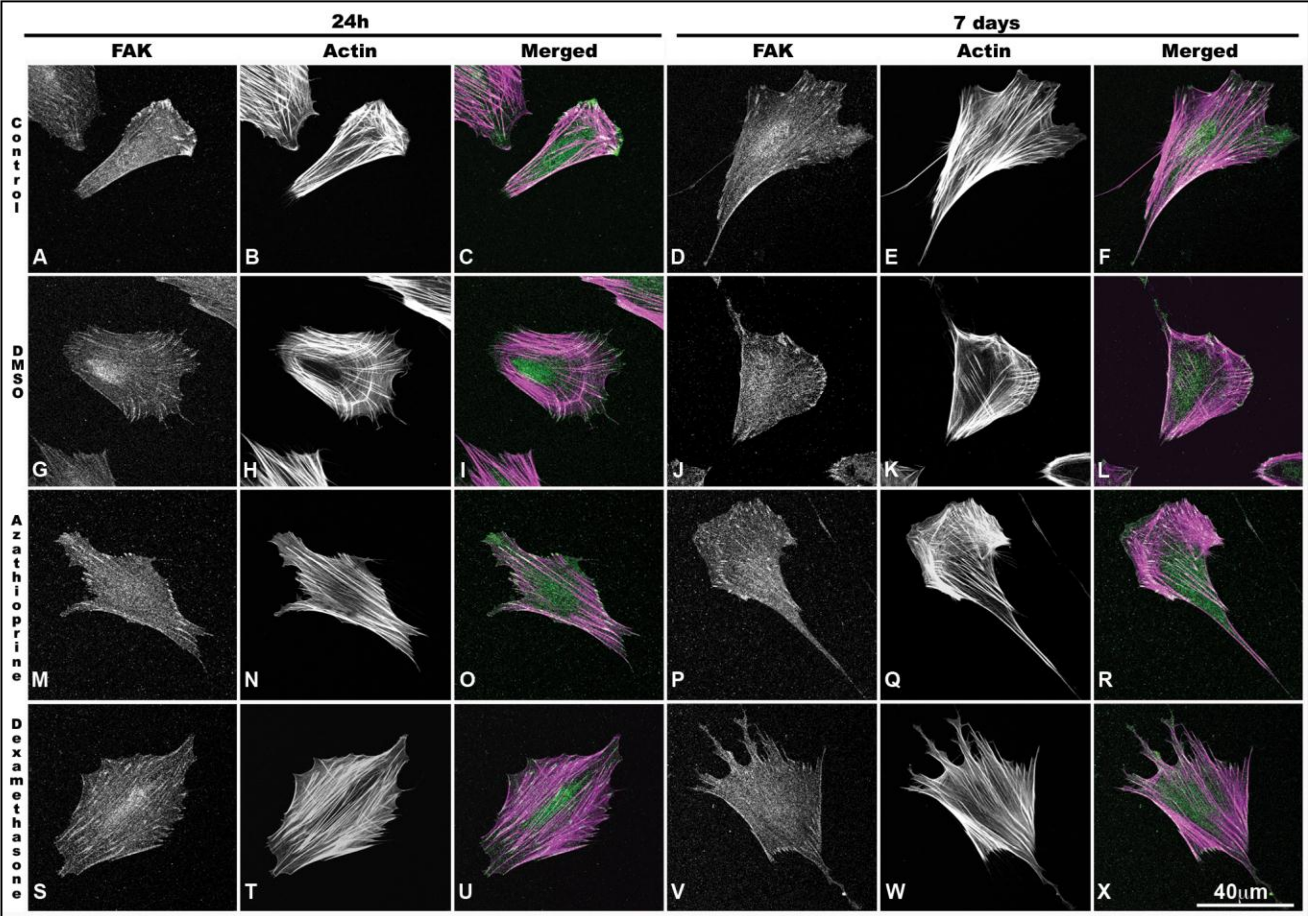
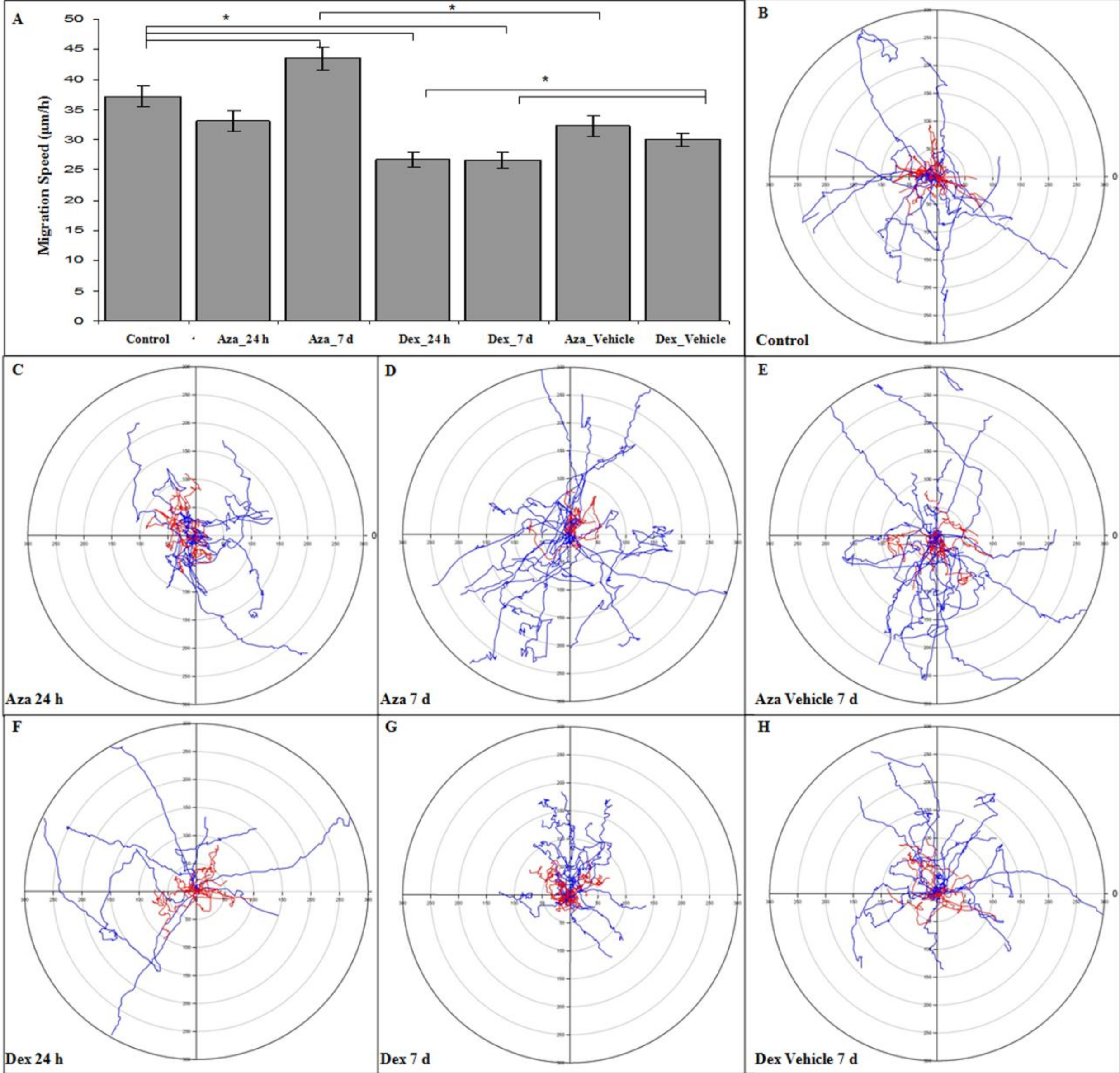
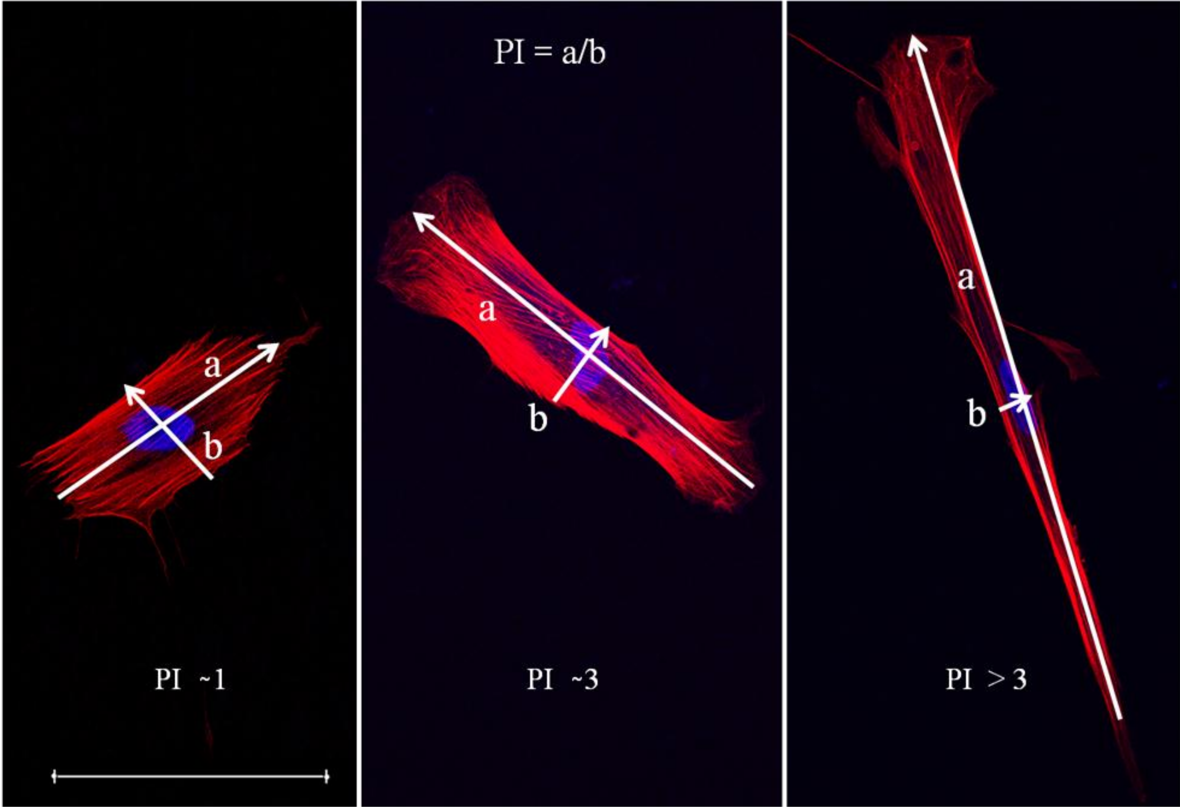


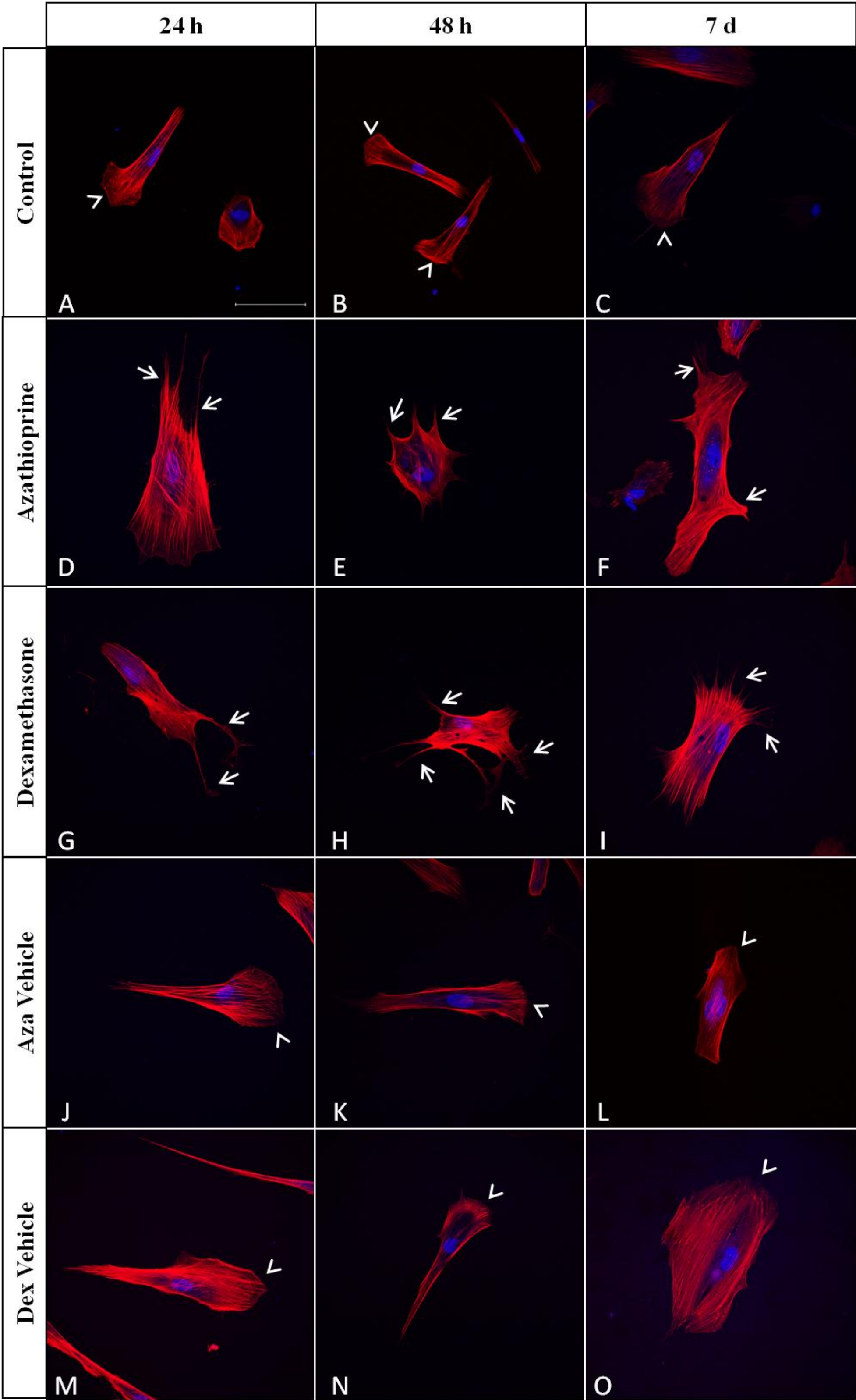
FIGURE 5. MSC migration speed and spatial trajectory.



Supplementary Figure 1. Polarity index analysis.



Supplementary Figure 2. Cytoskeleton morphology.



DISCUSSÃO

Os tratamentos para os dois principais representantes clínicos das DIIs incluem imunossupressores e glicocorticoides, e apesar dos avanços alcançados nesta área, a remissão da doença ainda é difícil de ser mantida (Bouma e Strober, 2003; Duijvestein et al., 2010). Como uma alternativa para o tratamento, as MSCs têm sido estudadas por suas propriedades imunomoduladoras e de reparação tecidual (Forbes et al., 2014; Gonzales-Rey et al., 2009; Le Blanc et al., 2003). Entretanto, pouco é conhecido sobre a sua interação com as drogas convencionais, incluindo os efeitos sobre o citoesqueleto e a migração celular. Além disso, a utilização da terapia combinada poderia potencializar o efeito terapêutico das MSCs, sendo que eventualmente, doses mais baixas dos medicamentos poderiam ser utilizadas.

Os resultados obtidos neste estudo demonstraram que as concentrações terapêuticas de AZA e DEXA não são capazes de afetar a viabilidade das MSCs. Esses dados estão de acordo com outros estudos que observaram que ambas as drogas não tiveram influência na viabilidade celular em diferentes tempos de tratamento (Duijvestein et al., 2011; Nuzzi et al., 2012). Entretanto, foi demonstrado que a DEXA pode afetar a viabilidade das MSCs e a AZA pode promover aumento da apoptose quando utilizadas em altas concentrações (1 mg/ml e 0,20-0,30 mg/ml, respectivamente) (Nuzzi et al., 2012; Huang et al., 2014). Nós utilizamos concentrações menores, procurando mimetizar aquelas encontradas no soro de pacientes em condições inflamatórias.

Apesar de não haver mudanças na viabilidade das MSCs, observamos que a AZA e a DEXA foram capazes de alterar a morfologia celular e o comportamento migratório. Utilizando a distribuição do índice de polaridade, demonstramos que o tratamento com a AZA induziu o alongamento da célula, indicando uma possível melhora no desempenho migratório; enquanto que a DEXA promoveu uma morfologia celular arredondada, indicando um perfil de diminuída migração celular. A análise do citoesqueleto de actina, da distribuição da FAK e da dinâmica da membrana das MSCs por microscopia de série temporal corroborou com o índice de polaridade. Células dos grupos controle e AZA apresentaram protrusões de membrana estáveis, enquanto que as células tratadas com DEXA demonstraram uma maior presença de fibras de estresse ventrais e protrusões instáveis. Tal efeito da DEXA também foi observado no trabalho de Chen

et al., 2013, onde a presença da droga induziu um aumento das fibras de estresse em células epiteliais, envolvendo a expressão da proteína estomatina e o rearranjo dos filamentos de actina. Além disso, outro estudo demonstrou que o tratamento com DEXA em células endoteliais provocou a diminuição da migração celular e da ativação da proteína Rac-1, envolvida no controle da dinâmica de protrusão de membrana. Este efeito foi observado pelo aumento dos níveis de caveolina-1, uma proteína regulatória de receptores de membrana envolvida na reciclagem de adesões celulares (Igarashi, 2013; Boscher e Nabi 2012). Esses estudos demonstram o potencial efeito de DEXA em alterações do citoesqueleto e da migração celular, que vão de encontro aos resultados observados em nosso trabalho.

Sabendo que modificações na polaridade celular e na organização do citoesqueleto de actina podem alterar a migração celular (Friedl e Alexander, 2011; Ridley et al., 2003), as mudanças provocadas por AZA e DEXA podem refletir em diferentes comportamentos migratórios. Neste trabalho, foi padronizada a técnica de microscopia de série temporal para avaliação da capacidade migratória das MSCs. A técnica de microscopia de série temporal já foi descrita por outros autores para a migração randômica, partindo de uma técnica semelhante à de *Wound Healing* (Jain et al., 2012). Entretanto, em nosso protocolo, propomos a análise dos movimentos desde a adesão celular, além de apresentarmos uma alternativa para a ausência da câmara de CO_2, passível para até 20 h de filmagem, e para a construção de material pelo próprio pesquisador, como uma opção mais acessível.

Utilizando o protocolo desenvolvido, observamos que o tratamento prolongado com a AZA resultou no aumento da velocidade de migração e no aumento da capacidade das MSCs em alcançarem longas distâncias, comparando-se ao grupo controle. Poppe et al., 2006 demonstrou que a AZA suprimiu a habilidade das células T de criar regiões flexíveis na membrana plasmática e de formar lamelipódios, concluindo que a AZA poderia bloquear a defosforilação das proteínas ERM (*Ezrin-Radixin-Moesin*) e suprimir a conjugação com células apresentadoras de antígenos. Interessantemente, em nosso trabalho, o tratamento com a AZA resultou na preservação parcial dos lamelipódios e da presença da FAK nessas estruturas. É possível que estas mudanças estejam associadas às vias de sinalização de adesão celular, já que leucócitos podem ser considerados como células não aderentes. Além disso, a presença da FAK aumentada em ambas as extremidades das fibras ventrais das MSCs pode indicar uma maior contratilidade

celular e consequente potencialização dos movimentos migratórios. Os estudos disponíveis com a AZA focam principalmente nos mecanismos das células do sistema imune, e nesse sentido, ainda não está claro qual o mecanismo de atuação da droga em outros tipos celulares como as MSCs. Entretanto, o trabalho de Mancheño-Corvo et al., 2013 demonstrou o importante papel deste imunossupressor como uma droga protetora das MSCs, devido à redução da ativação e degranulação das células NK, resultando na redução da lise das MSCs alogênicas. Este estudo ainda especula que os pacientes poderiam ser beneficiados pelo uso concomitante de AZA e terapia celular com MSCs, partindo do princípio de que a reação às MSCs alogênicas seria retardada e as células teriam mais tempo para agir terapeuticamente. Juntamente com nossos resultados do aumento da velocidade celular, a AZA poderia auxiliar no efeito terapêutico das MSCs.

Em relação à DEXA, nossos resultados mostraram que após 24h de tratamento houve uma diminuição da velocidade de migração e as células alcançaram distâncias menores em sua maioria, efeitos ainda mais evidentes após 7 dias de tratamento. Estes resultados podem estar associados ao aumento da presença de fibras de estresse ventrais, à redução da presença de lamelipódios e à disposição da FAK, a qual se mostrou distribuída em todo o corpo celular e ausente nas projeções de membrana, levando à instabilidade das mesmas e à baixa motilidade celular. Resultados similares foram descritos por Geiβler et al., 2012, que observou que o envelhecimento *in vitro* e cronológico das MSCs promove o arredondamento celular e um menor potencial migratório, além da diminuição da expressão de genes relacionados à organização do citoesqueleto. Outro artigo demonstrou que o tratamento com DEXA foi capaz de induzir maior rigidez celular e a inibição da capacidade de migração de células T, mecanismo que envolve a ativação do complexo ERM, relacionado ao rearranjo do citoesqueleto (Müller et al., 2013). Por outro lado, Yun et al., 2011 observou que a DEXA poderia aumentar o potencial migratório das MSCs quando em concentrações menores (entre 10^{-8} e 10^{-6} M) do que a por nós utilizada. Seus dados demonstraram que este efeito foi associado ao aumento da expressão de FAK e β1-integrina. Porém, quando a concentração correspondente à utilizada em nosso trabalho (10µM) foi aplicada, não houve diferença no comportamento migratório. Apesar de mais estudos serem necessários para o esclarecimento destas diferenças, nossos resultados vão ao encontro do estudo recentemente publicado por Chen et al., 2014. Neste trabalho, os autores demonstraram que o

tratamento com DEXA pode anular o efeito terapêutico das MSCs, em modelo animal de inflamação hepática¸ através da inibição da iNOS em MSCs, recomendando que o uso concomitante de MSCs e esteroides seja evitado.

Apesar das controvérsias envolvendo a migração das MSCs, o *homing* para sítios de injúria continua a ser muito importante para a atuação destas células no reparo tecidual e na imunomodulação. Vários fatores reguladores deste processo estão sendo identificados e estudados para a melhora da migração e da terapia celular (Li e Jiang, 2011; Qiu et al., 2012; Sohni e Verfaillie, 2013). Grupos de pesquisa têm explorado a interação entre a migração das MSCs e drogas utilizadas nos tratamentos convencionais de diversas doenças. Moggio et al., 2012 testaram o efeito do inibidor multi-tirosina quinase Sorafenib em MSCs derivadas do endométrio e demonstrou que a capacidade migratória aumentada destas células pôde ser revertida pela droga através da inibição da Ezrina (componente do grupo ERM), um importante achado para a erradicação dos implantes endometrióticos. Somado a isso, Xinaris et al., 2013 demonstrou que o pré-condicionamento das MSCs com IGF-1 (*insulin-like growth factor-1*) resultou na melhora da migração celular e na restauração da função renal em modelo animal, apoiando a ideia de que a potencialização da capacidade migratória das MSCs pode auxiliar no seu efeito terapêutico.

Nosso estudo teve como objetivo o melhor entendimento da influência do tratamento convencional das DIIs sobre as MSCs, focando no principal aspecto para uma terapia bem sucedida. A DEXA provocou uma diminuição na motilidade celular, enquanto a AZA potencializou a migração celular após o tratamento prolongado. Ambos os resultados foram influenciados pela morfologia celular e pela organização do citoesqueleto. Acreditamos que mais estudos são necessários para o melhor entendimento destes processos, entretanto, nossos achados fornecem dados importantes para o uso concomitante de DEXA e MSCs. Ao passo que AZA abre novas possibilidades para a terapia combinada, podendo melhorar o resultado da terapia celular nas DIIs.

CONCLUSÕES

Os resultados obtidos nesse trabalho nos permitem concluir que:

1. Foi possível isolar, expandir e caracterizar células-tronco mesenquimais de membrana coriônica humana de acordo com as normas da Sociedade Internacional de Terapia Celular;
2. O protocolo de migração celular por microscopia de série temporal foi desenvolvido e padronizado para avaliação das MSCs, e foi descrito em um artigo científico a ser submetido em uma revista de protocolos;
3. A viabilidade das MSCs não foi afetada pelas drogas Azatioprina e Dexametasona em concentrações terapêuticas de 1μM e 10μM, respectivamente;
4. O índice de polaridade indicou um perfil mais alongado das MSCs quando na presença de Azatioprina, principalmente em 7 dias, ao passo que indicou um perfil mais arredondado nas MSCs quando na presença de Dexametasona;
5. A avaliação do citoesqueleto de actina por microscopia confocal revelou um aumento da presença de fibras de estresse ventrais no tratamento com AZA e a preservação parcial de lamelipódios. Entretanto, a DEXA resultou em aumento ainda maior de fibras de estresse ventrais e perda de lamelipódios;
6. O tratamento com AZA preservou a distribuição da FAK nas projeções de membrana, bem como apresentou um aumento da mesma em ambas as extremidades das fibras de estresse ventrais, indicando aumento da contratilidade celular. A DEXA resultou na distribuição da FAK por todo o corpo celular e ausência da mesma nas projeções de membrana, indicando uma redução da motilidade celular;
7. A velocidade de migração das MSCs foi aumentada na presença de AZA após o tratamento prolongado, o que permitiu às células alcançarem maiores distâncias pela melhora da trajetória espacial. Entretanto, a DEXA promoveu uma diminuição na velocidade de migração em 24h e 7d e provocou uma piora na trajetória espacial das células, resultando em uma atividade migratória de curtas distâncias.

PERSPECTIVAS

Como continuação desse trabalho, pretende-se seguir com os seguintes objetivos:

- Avaliar a atividade migratória em direção ao quimioatrator SDF-1 por ensaio de *transwell* sob a influência de Dexametasona e Azatioprina;
- Avaliar o secretoma das MSCs na presença de Dexametasone e Azatioprina;
- Avaliar o ciclo celular das MSCs sob o efeitos destas drogas imunossupressoras;
- Avaliar a viabilidade, a morfologia e o comportamento migratório das MSCs sob a influência de outras drogas utilizadas para o tratamento das DIIs, como Ciclosporina e Infliximab.

REFERÊNCIAS BIBLIOGRÁFICAS

Abraham C and Cho JH. (2009) Inflammatory Bowel Disease. N Engl J Med. 361(21): 2066-2078.

Almawi WY, Beyhum HN, Rahme AA, Rieder MJ. (1996) Regulation of cytokine and cytokine receptor expression by glucocorticoids. J Leukoc Biol. 60(5):563-72.

Bär F, Sina C, Fellermann K. (2013) Thiopurines in inflammatory bowel disease revisited. World J Gastroenterol. 19(11):1699-706.

Barlow S, Brooke G, Chatterjee K, Price G, Pelekanos R, Rossetti T, Doody M, Venter D, Pain S, Gilshenan K, Atkinson K. (2008) Comparison of human placenta- and bone marrow-derived multipotent mesenchymal stem cells. Stem Cells Dev. 17(6):1095-107.

Beyth S, Borovsky Z, Mevorach D, Liebergall M, Gazit Z, Aslan H, Galun E, Rachmilewitz J. (2005) Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. Blood. 105(5):2214-9.

Bielby R, Jones E and McGonagle D. (2007) The role of mesenchymal stem cells in maintenance and repair of bone. Injury. 38 Suppl 1: S26-32.

Boscher C and Nabu IR. (2012) Caveolin-1: role in cell signaling. Adv Exp Med Biol. 729:29-50.

Bouma G and Strober W. (2003) The Immunological and Genetic Basis of Inflammatory Bowel Disease. Nature Reviews, Immunology. 3(7):521-33.

Brooke G, Tong H, Levesque JP, Atkinson K. (2008) Molecular trafficking mechanisms of multipotent mesenchymal stem cells derived from human bone marrow and placenta. Stem Cells Dev. 17(5):929-40.

Buron F, Perrin H, Malcus C, Héquet O, Thaunat O, Kholopp-Sarda M-N, Moulin FT, Morelon E. (2009) Human Mesenchymal Stem Cells and Immunosuppressive Drug

Interactions in Allogeneic Responses: An In Vitro Study Using Human Cells. Transplantation Proceedings. 41: 3347–3352.

Caplan AI. (2005) Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. Tissue Eng 11: 1198-1211.

Caplan AI and Dennis JE. (2006) Mesenchymal Stem Cells as Trophic Mediators. Journal of Cellular Biochemistry. 98:1076–1084.

Chai Y, Li W, Feng G, Yang Y, Wang X, Ou G. (2012) Live imaging of cellular dynamics during Caenorhabditis elegans postembryonic development. Nat Protoc. 7(12):2090-102.

Charwat S, Gyóngyösi M, Lang I, Graf S, Beran G, Hemetsberger R, Nyolczas N, Sochor H, Glogar D. (2008) Role of adult marrow stem cells in the repair of ischemic myocardium: current state of the art. Exp Hematol. 36(6):672-80.

Chen X, Oppenheim JJ, Winkler-Pickett RT, Ortaldo JR, Howard OM. (2006) Glucocorticoid amplifies IL-2-dependent expansion of functional FoxP3(+)CD4(+)CD25(+) T regulatory cells *in vivo* and enhances their capacity to suppress EAE. Eur J Immunol. 36(8):2139-49.

Chen JC, Cai HY, Wang Y, Ma YY, Song LN, Yin LJ, Cao DM, Diao F, Li YD, Lu J. (2013) Up-regulation of stomatin expression by hypoxia and glucocorticoid stabilizes membrane-associated actin in alveolar epithelial cells. J Cell Mol Med. 17(7):863-72.

Chen X, Gan Y, Li W, Su J, Zhang Y, Haung Y, Roberts AI, Han Y, Li J, Wang Y, Shi Y. (2014) The interaction between mesenchymal stem cells and steroids during inflammation. Cell Death Dis. 5e1009.

Ciccocioppo R, Bernardo ME, Sgarella A, Maccario R, Avanzini MA, Ubezio C, Minelli A, Alvisi C, Vanoli A, Calliada F, Dionigi P, Perotti C, Locatelli F, Corazza GR. (2011) Autologous bone marrow-derived mesenchymal stromal cells in the treatment of fistulizing Crohn’s disease. Gut. 60(6):788-98.

Corridoni D, Arseneau KO, Cominelli F. (2014) Inflammatory bowel disease. Immunol. Lett. 161(2):231-5.

Cosnes J, Gower-Rosseau C, Seksik P, Cortot A. (2011) Epidemiology and natural history of inflammatory bowel diseases. Gastroenterology. 140(6):1785-94.

Djouad F, Charbonnier LM, Bouffi C, Louis-Plence P, Bony C, Apparailly F, Cantos C, Jorgensen C, Nöel D. (2007) Mesenchymal stem cells inhibit the differentiation of dendritic cells through an interleukin-6-dependent mechanism. Stem Cells. 25(8):2025-32.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 8(4):315-7.

Duijvestein M, Vos ACW, Roelofs H, Wildenberg ME, Wendrich BB, Verspaget HW, Kooy-Winkelaar EMC, Koning F, Zwaginga JJ, Fidder HH, Verhaar AP, Fibbe WE, van den Brink GR, Hommes DW. (2010) Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. Gut. 59: 1662-1669.

Duijvestein M, Molendijk I, Roelofs H, Vos ACW, Verhhar AP, Reinders MEJ, Fibbe WE, Verspaget HW, van den Brink GR, Wildenberg ME, Hommes DW. (2011) Mesenchymal stromal cell function is not affected by drugs used in the treatment of inflammatory bowel disease. Cytotherapy. 13: 1066–1073.

English K, Ryan JM, Tobin L, Murphy MJ, Barry FP, Mahon BP. (2009) Cell contact, prostaglandin E(2) and transforming growth factor beta 1 play non-redundant roles in human mesenchymal stem cell induction of CD4+CD25(High) forkhead boxP3+ regulatory T cells. Clin Exp Immunol. 156(1):149-60.

Forbes GM, Sturm MJ, Leong RW, Sparrow MP, Segarajasingam D, Cummis AG, Philips M, Herrmann RP. (2014) A phase 2 study of allogeneic mesenchymal stromal cells

for luminal Crohn's disease refractory to biologic therapy. Clin Gastroenterol Hepatol. 12(1):64-71.

Friedl P and Alexander S. (2011) Cancer invasion and the microenvironment: plasticity and reciprocity. Cell. 23;147(5):992-1009.

Garcia-Olmo D, Herreros D, Pascual I, Pascual JA, Del-Valle E, Zorrilla J, De-La-Quintana P, Garcia-Arranz M, Pascual M. (2009) Expanded adipose-derived stem cells for the treatment of complex perianal fistula: a phase II clinical Trial. Dis Colon Rectum. 52(1):79-86.

Gazouli M, Roubelakis MG, Theodoropoulos GE. (2014) Stem cells as potential targeted therapy for inflammatory bowel disease. Inflamm Bowel Dis. 20(5):952-5.

Geißler S, Textor M, Kühnisch J, Könnig D, Klein O, Ode A, Pfitzner T, Adjaye J, Kasper G, Duda GN. (2012) Functional Comparison of Chronological and In Vitro Aging: Differential Role of the Cytoskeleton and Mitochondria in Mesenchymal Stromal Cells. Plos One. 7(12):e52700.

Geva-Zatorsky N, Issaeva I, Mayo A, Cohen A, Dekel E, Danon T, Cohen L, Liron Y, Alon U, Eden E. (2012) Using bleach-chase to measure protein half-lives in living cells. Nat Protoc. 7(4):801-11.

Gonçalves FC, Schneider N, Pinto FO, Meyer F, Visioli F, Pfaffenseller B, Passos EP, Cirne-Lime EO, Meurer L, Paz AHR. (2014) Intravenous versus intraperitoneal mesenchymal stem cells administration: What is the best route for treating experimental colitis? World J Gastroenterol. In press.

Gonzalez-Rey E, Anderson P, González MA, Rico L, Büscher D, Delgado M. (2009) Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. Gut. 58:929–939.

Hass R, Kasper C, Böhm S, Jacobs R. (2011) Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. Cell Commun Signal. 14;9:12.

Head KA and Jurenka JS. (2003) Inflammatory bowel disease Part 1: ulcerative colitis – pathophysiology and conventional and alternative treatment options. Altern Med Rev. 8(3)247-83.

Head K and Jurenka JS. (2004) Inflammatory bowel disease. Part II: Crohn's disease – pathophysiology and conventional treatment and alternative treatment options. Altern Med Rev. 9(4):360-401.

Hoogduijn MJ, Crop MJ, Korevaar SS, Peeters AM, Ejiken M, Maat LP, Balk AH, Weimar W, Baan CC. (2008) Susceptibility of Human Mesenchymal Stem Cells to Tacrolimus, Mycophenolic Acid, and Rapamycin. Transplantation. 86: 1283–1291.

Huang HR, Zan H, Lin Y, Zhong YQ. (2014) Effects of azathioprine and infliximab on mesenchymal stem cells derived from bone marrow of rats *in vitro*. Mol Med Rep. 9(3):1005-12.

Indumathi S, Harikrishnan R, Mishra R, Rajkumar JS, Padmapriya V, Lissa RP, Dhanasekaran M. (2013) Comparison of feto-maternal organ derived stem cells in facets of immunophenotype, proliferation and differentiation. Tissue Cell. 45(6):434-42.

Igarashi J, Hashimoto T, Shoji K, Yoneda K, Tsukamoto I, Moriue T, Kubota Y, Kosaka H. (2013) Dexamethasone induces caveolin-1 in vascular endotelial cells: implications for attenuated responses to VEGF. Am J Physiol Cell Physiol. 304(8):C790-800.

Jackson JS, Gonding JP, Chapon C, Jones WA, Bhakoo KK. (2010) Homing of stem cells to sites of inflammatory brain injury after intracerebral and intravenous administration: a longitudinal imaging study. Stem Cell Res Ther. 1(2):17.

Jain P, Worthylake RA, Alahari SK. (2012) Quantitative analysis of random migration of cells using time-lapse video microscopy. J Vis Exp. (63):e3585

Karp JM and Leng Teo GS (2009) Mesenchymal stem cell homing: the devil is in the details. Cell Stem Cell. 4(3):206-16.

Kholodenko IV, Yarygin KN, Gubsky LV, Konieva AA, Tairova RT, Povarova OV, Kholodenko RV, Burunova VV, Yarygin VN, Skyortsova VI. (2012) Intravenous xenotransplantation of human placental mesenchymal stem cells to rats: comparative analysis of homing in rat brain in two models of experimental ischemic stroke. Bull Exp Biol Med. 154(1):118-23.

Kim JW, Ha KY, Molon JN, Kim YH. (2013) Bone marrow-derived mesenchymal stem cell transplantation for chronic spinal cord injury in rats: comparative study between intralesional and intravenous transplantation. Spine (Phila Pa 1976). 38(17):E1065-74.

Lamers ML, Almeida ME, Vicente-Manzanares M, Horwitz AF, Santos MF. (2011) High glucose-mediated oxidative stress impairs cell migration. Plos One. 6(8):e22865.

Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringdén O. (2003) HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. Exp Hematol. 31:890–896.

Li G, Zhang XA, Wang H, Meng CL, Chan CY, Yew DT, Tsang KS, Li K, Tsai SN, Ngai SM, Han ZC, Lin MC, He ML, Kung HF. (2009) Comparative proteomic analysis of mesechymal stem cells derived from human bone marrow, umbilical cord, and placenta: implication in migration. Proteomics. 9(1):20-30.

Li L and Li Jiang. (2011) Regulatory factors of mesenchymal stem cell migration into injured tissues and their signal transduction mechanisms. Front Med. 5(1):33-9.

Li R and Gundersen GG. (2008) Beyond polymer polarity: how the cytoskeleton builds a polarized cell. Nat Rev Mol Cell Biol. 9(11):860-73.

Li X, Bai J, Ji X, Li R, Xuan Y, Wang Y. (2014) Comprehensive characterization of four different populations of human mesenchymal stem cells as regards their immune properties, proliferation and differentiation. Int J Mol Med. 34(3):695-704.

Liang CC, Park AY, Guan JL. (2007) *In vitro* scratch assay: a convenient and inexpensive method for analysis of cell migration *in vitro*. Nat Protoc. 2(2):329-33.

Liang J, Zhang H, Wang D, Feng X, Wang H, Hua B, Liu B, Sun L. (2012) Allogeneic mesenchymal stem cell transplantation in seven patients with refractory inflammatory bowel disease. Gut. 61:468-469.

Liang L, Dong C, Chen X, Fang Z, Xu J, Liu M, Zhang X, Gu DS, Wang D, Du W, Zhu D, Han ZC. (2011) Human umbilical cord mesenchymal stem cells ameliorate mice trinitrobenzene sulfonic acid (TNBS)-induced colitis. Cell Transplant. 20(9):1395-408.

Liu X, Zuo D, Fan H, Tang Q, Shou Z, Cao D, Zou Z. (2014) Over-expression of CXCR4 on mesenchymal stem cells protect against experimental colitis via immunomodulatory functions in impaired tissue. J Mol Histol. 45(2):181-93.

Limame R, Wouters A, Pauwels B, Fransen E, Peeters M, Lardon F, De Weyer O, Pauwels P. (2012) Comparative Analysis of Dynamic Cell Viability, Migration and Invasion Assessments by Novel Real-Time Technology and Classic Endpoint Assays. Plos One. 7(10): e46536.

Ma S, Xie N, Li W, Yuan B, Shi Y, Wang Y. (2014) Immunobiology of mesenchymal stem cells. Cell Death Differ. 21(2):216-25.

Mancheño-Corvo P, Franquesa M, de la Rosa O, Ramírez C, García-Benzaquén L, Fernández V, Menta R, Beraza A, Dalemans W, Hoogduijn MJ, Lombardo E. (2013) Adipose mesenchymal stromal cell function is not affected by methotrexate and azathioprine. Biores Open Access. 2(6):431-9.

Marquez-Curtis LA and Janowska-Wieczorek A. (2013) Enhancing the migration ability of mesenchymal stromal cells by targeting the SDF-1/CXCR4 axis. Biomed Res Int. 2013:561098.

Martínez-Montiel MP, Gómez-Gómez GJ, Flores AI. (2014) Therapy with stem cells in inflammatory bowel disease. World J Gastroenterol. 20(5): 1211-1227.

Meirelles LS, Chagastelles PC, Nardi NB. (2006) Mesenchymal stem cells reside in virtually all post-natal organs and tissues. Journal of Cell Science. 119: 2204-2213.

Miller RJ, Banisadr G, Bhattacharyya BJ. (2008) CXCR4 signaling in the regulation of stem cell migration and development. Journal of Neuroimmunology. 198 31–38.

Mitra SK, Hanson DA, Schlaepfer DD. (2005) Focal adhesion kinase: in command and control of cell motility. 6(1):56-68.

Moggio A, Pittatore G, Cassoni P, Marchino GL, Revelli A, Bussolati B. (2012) Sorafenib inhibits growth, migration, and angiogenic potential of ectopic endometrial mesenchymal stem cells derived from patients with endometriosis. Fertil Steril. 98(6):1521-30.e2.

Mokbel AN, El Tookhy OS, Shamaa AA, Rashed LA, Sabry D, El Sayed AM. (2011) Homing and reparative effect of intra-articular injection of autologus mesenchymal stem cells in osteoarthritic animal model. BMC Musculoskeletal Disorders. 12:259.

Müller N, Fisher HJ, Tischner D, van den Brandt J, Reichardt HM. (2013) Glucocorticoids indue effector T cell depolarization via ERM proteins, thereby impeding migration and APC conjugation. J Immunol. 190(8):4360-70.

Muzes G, Molnár B, Tulassay Z, Sipos F. (2012) Changes of the cytokine profile in inflammatory bowel diseases. World J Gastroenterol. 18(41):5848-61.

NCT01541579, Clinical Trials. Adipose derived mesenchymal stem cells for induction of remission in perianal fistulizing Crohn’s Disease (ADMIRE-CD). Available from: http://clinicaltrials.gov/show/NCT01541579

NCT01769755, ClinicalTrials. A Randomized, Double-Blind, Placebo-Controlled Dose-Escalation Study to Determine the Safety and Efficacy of Intravenous Infusion of Human Placenta-Derived Cells (PDA001) for the Treatment of Crohn's Disease. Available from: http://clinical trials.gov/show/NCT01769755

NCTO0482092, ClinicalTrials. A phase III, multicenter, placebocontrolled, randomized, double-blind study to evaluate the safety and efficacy of Prochimal [tm] (ex vivo cultured adult human mesenchymal stem cells) intravenous infusion for induction of remission in subjects experiencing treatmentrefractory moderate-to-severe Crohn's disease. Osiris Therapeutics. Available from: http://www.clinicaltrials.gov/ct2/show/NCT00482092

Ng SC. (2014) Epidemiology of inflammatory bowel disease: focus on Asia. Best Pract Res Clin Gastroenterol. 28(3):363-72.

Nichols JE, Niles JA, DeWitt D, Prough D, Parsley M, Vega S, Cantu A, Lee E, Cortiella J. (2013) Neurogenic and neuro-protective potential of a novel subpopulation of peripheral blood-derived CD133+ ABCG2+CXCR4+ mesenchymal stem cells: development of autologous cell-based therapeutics for traumatic brain injury. Stem Cell Res Ther. 6;4(1):3.

Nuzzi R, Gunetti M, Rustichelli D, Roagna B, Bardelli FF, Fagioli F, Ferrero I. (2012) Effect of In Vitro Exposure of Corticosteroid Drugs, Conventionally Used in AMD Treatment, on Mesenchymal Stem Cells. Stem Cells International; 2012:946090.

Parekkadan B and Milwid JM (2010) Mesenchymal stem cells as therapeutics. Annu Rev Biomed Eng. 15;12:87-117.

Patel DM, Shah J, Srivastava AS. (2013) Therapeutic potential of mesenchymal stem cells in regenerative medicine. Stem Cells Int. 2013:496218.

Petrie RJ, Doyle AD, Yamada KM. (2009) Random versus directionally persistent cell migration. Nat Rev Mol Cell Biol. 10(8):538-49.

Pollard TD and Cooper JA (2009) Actin, a central player in cell shape and movement. Science. 326(5957):1208-12

Ponder A and Long MD. (2013) A clinical review of recent findings in the epidemiology of inflammatory bowel disease. Clin Epidemiol. 5:237-47.

Poppe D, Tiede I, Fritz G, Becker C, Bartsch B, Wirtz S, Strand D, Tanaka S, Galle PR, Bustelo XR, Neurath MF. (2006) Azathioprine suppresses ezrin-radixin-moesin-dependent T cell-APC conjugation through inhibition of Vav guanosine exchange activity on Rac proteins. J Immunol. 176(1):640-51.

Qiu Y, Marquez-Curtis LA, Janowska-Wieczorek A. (2012) Mesenchymal stromal cells derived from umbilical cord migrate in response to complement C1q. Cytotherapy. 14(3):285-95.

Qu X, Liu X, Cheng K, Yang R, Zhao RC. (2012) Mesenchymal stem cells inhibit Th17 cell differentiation by IL-10 secretion. Exp Hematol. 40(9):761-70.

Ren G, Su J, Zhang L, Zhao X, Ling W, L'huillie A, Zhang J, Lu Y, Roberts AI, Ji W, Zhang H, Rabson AB, Shi Y. (2009) Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression. Stem Cells. 27(8):1954-62.

Renna S, Cottone M, Orlando A. (2014) Optimization of the treatment with immunosuppressants and biologics in inflammatory bowel disease. World J Gastroenterol. 20(29):9675-90.

Rhen T and Cidlowski JA. (2005) Antiinflammatory action of glucocorticoids – new mechanisms for old drugs. N Engl J Med. 353(16):1711-23.

Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR. (2003) Cell migration: integrating signals from front to back. Science. 302(5651):1704-9.

Rüster B, Göttig S, Ludwig RJ, Bistrian R, Müller S, Seifried E, Gille J, Henschler R. (2006) Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. Blood. 1;108(12):3938-44.

Sahasranaman S, Howard D, Roy S. (2008) Clinical pharmacology and pharmacogenetics of thiopurines. Eur J Clin Pharmacol. 64(8):753-67.

Schrepfer S, Deuse T, Reichenspurner H, Fischbein MP, Robbins RC, Pelletier MP. (2007) Stem cell transplantation: the lung barrier. Transplant Proc. 39(2):573-6.

Sobczak M, Fabisiak A, Murawska N, Wesolowska E, Wierzbicka P, Wlazlowski M, Wójcikowska M, Zatorski H, Zwolinska M, Fichna J. (2014) Current overview of extrinsic and intrinsic factors in etiology and progression of inflammatory bowel diseases. Pharmacol Rep. 66(5):766-75.

Sohni A and Verfaillie CM. (2013) Mesenchymal Stem Cells Migration Homing and Tracking. Stem Cell Int. 130763.

Song IH, Caplan AI, Dennis JE. (2009) Dexamethasone inhibition of confluence-induced apoptosis in human mesenchymal stem cells. J Orthop Res. 27(2):216-21.

Spaggiari GM, Capobianco A, Abdelrazik H, Becchetti F, Mingari MC, Moretta L. (2008) Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. Blood. 111(3):1327-33.

Spaggiari GM, Abdelrazik H, Becchetti F, Moretta L. (2009) MSCs inhibit monocyte-derived DC maturation and function by selectively interfering with the generation of immature DCs: central role of MSC-derived protaglandin E2. Blood. 113(26):6576-83.

Swaney KF, Huang CH, Devreotes PN. (2010) Eukaryotic chemotaxis: a network of signaling pathways controls motility, directional sensing, and polarity. Annu Rev Biophys. 39:265-89.

Thomas CW, Myhre GM, Tschumper R, Sreekumar R, Jelinek D, McKean DJ, Lipsky JJ, Sandborn WJ, Egan LJ. (2005) Selective inhibition of inflammatory gene expression in activated T lymphocytes: a mechanism of immunesuppression by thiopurines. J Pharmacol Exp Ther. 312(2):537-45.

Tiede I, Fritz G, Strand S, Poppe D, Dvorsky R, Strand D, Lehr HA, Wirtz S, Becker C, Atreya R, Mudter J, Hildner K, Bartsch B, Holtmann M, Blumberg R, Walczak H, Iven

H, Galle PR, Ahmadian MR, Neurath MF. (2003) CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes. J Clin Invest. 111(8):1133-45.

Tojkander S, Gateva G, Lappalainen P. (2012) Actin stress fibers--assembly, dynamics and biological roles. J Cell Sci. 15;125(Pt 8):1855-64.

Vallenius T. (2013) Actin stress fibre subtypes in mesenchymal-migrating cells. Open Biol. 19;3(6):130001.

Wang H, Pang B, Li Y, Zhu D, Pang T, Liu Y. (2012) Dexamethasone has variable effects on mesenchymal stromal cells. Cytotherapy. 14(4):423-30.

Wang X, Nelin LD, Kuhlman JR, Meng X, Welty SE, Liu Y. (2008). The role of MAP kinase phosphatase-1 in the protective mechanism of dexamethasone against endotoxemia. Life Sci. 83 (19-20):671-80.

Wu HJ, Liu YJ, Li HQ, Chen C, Dou Y, Lou HF, Ho MS, Li MX, Gao Z, Duan S. (2014) Analysis of microglial migration by a micropipette assay. Nat Protoc. 9(2):491-500.

Wüst S, van den Brandt J, Tischner D, Kleiman A, Tuckermann JP, Gold R, Lühder F, Reichardt HM. (2008) Peripheral T cells are the therapeutic targets of glucocorticoids in experimental autoimmune encephalomyelitis. J Immunol. 180(12):8434-43.

Wyles CC, Houdek MT, Wyles SP, Wagner ER, Behfar A, Sierra RJ. (2014) Differential cytotoxicity of corticosteroids on human mesenchymal stem cells. Clin Orthop Relat Res. Epub ahead of print.

Xinaris C, Morigi M, Benedetti V, Imberti B, Fabricio AS, Squarcina E, Benigni A, Gagliardini E, Remuzzi G. (2013) A novel strategy to enhance mesenchymal stem cell migration capacity and promote tissue repair in an injury specific fashion. Cell Transplant. 22(3):423-36.

Yagi H, Soto-Gutierrez A, Parekkadan B, Kitagawa Y, Tompkins RG, Kobayashi N, Yarmush ML. (2010) Mesenchymal stem cells: Mechanisms of immunomodulation and homing. Cell Transplant. 19(6):667-79.

Yen BL, Huang HI, Chien CC, Jui HY, Ko BS, Yao M, Shun CT, Yen ML, Lee MC, Chen YC. (2005) Isolation of multipotent cells from human term placenta. Stem Cells. 23(1):3-9.

Yen D, Cheung J, Scheerens H, Poulet F, McClanahan T, McKenzie B, Kleinschek MA, Owyang A, Mattson J, Blumenschein W, Murphy E, Sathe M, Cua DJ, Kastelein RA, Rennick D. (2006) IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. J Clin Invest. 116(5):1310-6.

Yi T and Song SU. (2012) Immunomodulatory properties of mesenchymal stem cells and their therapeutic applications. Arch Pharm Res. 35(2):213-21.

Yun SP, Ryu JM, Han HJ. (2011) Involvement of β1-integrin via PIP complex and FAK/paxilin in dexamethasone-induced human mesenchymal stem cells migration. J Cell Physiol. 226(3):683-92.

Young JW, Locke JC, Altinok A, Rosenfeld N, Bacarian T, Swain PS, Mjolsness E, Elowitz MB. (2011) Measuring single-cell gene expression dynamics in bacteria using fluorescence time-lapse microscopy. Nat Protoc. 7(1):80-8.

Zhang D, Fan GC, Zhou X, Zhao T, Pasha Z, Xu M, Zhu Y, Ashraf M, Wang Y. (2007) Over-expression of CXCR4 on mesenchymal stem cells augments myoangiogenesis in the infarcted myocardium. J Mol Cell Cardiol. 44(2):281-92.

Zhu Y, Yang Y, Zhang Y, Hao G, Liu T, Wang L, Yang T, Wang Q, Zhang G, Wei J, Li Y. (2014) Placental mesenchymal stem cells of fetal and maternal origins demonstrate different therapeutic potentials. Stem Cell Res Ther. 10;5(2):48.

Zhuo W, Liao L, Fu Y, Xu T, Wu W, Yang S, Tan J. (2013) Efficiency of endovenous versus arterial administration of mesenchymal stem cells for ischemia-reperfusion-induced renal dysfunction in rats. Transplant Proc. 45(2):503-10.

APÊNDICE

Co-autoria de trabalhos realizados durante o mestrado:

1. **Gonçalves FC, Schneider N, Mello HF, Passos EP, Meurer L, Cirne-Lima EO, Paz AH.** (2013) Characterization of acute murine dextran sodium sulfate (DSS) colitis: severity of inflammation is dependent on the DSS molecular weight and concentration. Acta Scientiae Veterianariae. 41:1142
2. **Gonçalves FC, Schneider N, Pinto FO, Meyer F, Visioli F, Pfaffenseller B, Passos EP, Cirne-Lime EO, Meurer L, Paz AH.** (2014) Intravenous versus intraperitoneal mesenchymal stem cells administration: What is the best route for treating experimental colitis? World J Gastroenterol. In press.
3. **Gonçalves FC, Grings M, Schneider N, Pinto FO, Garcez TNA, Passos EP, Cirne-Lima EO, Meurer L, Leipnitz G, Paz AH.** (2014) Mesenchymal stem cells acts as antioxidant and elevates reduced glutathione levels in a murine model DSS-induced colitis. Molecular Genetics and Metabolism. Manuscrito em preparação.
4. **Lorenzi W, Gonçalves FC, Schneider N, Silva EF, Paz AH, Saueressig MG.** (2014) Repetead systemic administration of adipose stem cells reduces tracheal obliteration in a murine model of bronchiolitis obliterans. European Journal of Cardio-Thoracic Surgery. Paper submetido.