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Liliana Sous Naasani

EXTRAÇÃO E CARACTERIZAÇÃO DE CÉLULAS TRONCO MESENQUIMAIS DE TECIDOS HUMANOS DE DESCARTE (PELE E LIMBO ESCLEROCORNEAL) E ANÁLISE DA SINALIZAÇÃO PURINÉRGICA

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Orientadora: Profa. Dra. Márcia Rosângela Wink

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RESUMO

A córnea é um tecido avascular transparente que proporciona proteção e apresenta propriedades de refração para a estrutura interna do olho. A pele humana não é apenas o maior órgão do corpo, mas também constitui-se em um dos mais complexos e importantes para a proteção contra agressões externas. Ambos tecidos atuam como uma barreira contra agentes externos, podendo ser físicos, químicos, patogênicos ou microbianos. Devido à alta taxa de pacientes com patologias da pele e córnea, como queimaduras, inflamação e cicatrização de feridas, reforça a importância de terapias alternativas para o tratamento dessas patologias. Tanto a córnea como a pele apresentam populações de células tronco mesenquimais (MSCs) com capacidade de auto-renovação, proliferação e potencial de diferenciação multi-linhagem e, por causa deste potencial, têm sido consideradas como uma fonte de células para o seu uso em medicina regenerativa, engenharia de tecidos e outras aplicações em dermatologia e oftalmologia, cujo mecanismo ainda está sob investigação. Tem-se demonstrado que os nucleotídeos extracelulares têm efeitos biológicos nas MSCs, alterando a proliferação, migração, diferenciação e apoptose. Portanto, mais informações sobre estes processos são cruciais para compreender a fisiologia destas células para o estabelecimento de futuras aplicações clínicas utilizando o seu potencial de diferenciação, mas sem efeitos secundários indesejáveis. Neste trabalho foram isoladas MSCs de tecido esclerocorneal e de pele humana, ambos considerados materiais de descarte, e foi demonstrado o seu potencial de diferenciação multi-linhagem e a expressão de marcadores mesenquimais. Foram avaliadas a expressão de ectoenzimas, a atividade enzimática de degradação de nucleotídeos extracelulares e o metabolismo de ATP extracelular em células mesenquimais isoladas da derme (D-MSCs) e do limbo esclerocorneal (L-MSCs). As D-MSCs e L-MSCs hidrolisaram baixos níveis de ATP extracelular e altos níveis de AMP, levando ao acúmulo de adenosina que pode regular a inflamação e o reparo tecidual. Além disso, demonstraram uma baixa atividade das nucleotídeo pirofosfatases/fosfodiesterases. Estas células expressaram mRNA para ENTPD1, 2, 3, 5 e 6, NPP1 e 2 o que corresponde com a atividade enzimática observada. Deste modo, considerando a degradação de ATP, as L-MSCs são muito semelhantes às D-MSCs. Assim, as células limbais podem ser outra fonte de MSCs a serem estudadas em modelos pré-clínicos e estudos potencialmente clínicos.

Palavras chave: Células mesenquimais, derme, limbo esclerocorneal, ATP, adenosina, ectoenzimas, regeneração tecidual.

ABSTRACT

Cornea is a transparent avascular tissue that provides protection and refractive properties for the internal structure of the eye; in turn, human skin is not only the largest organ in the body, but also one of the most complex and important for protection against external aggressions. Both tissues act as a barrier against external agents that can be physical, chemical, pathogenic or microbial usually. Due to the high rate of patients with skin and corneal pathologies such as burns, inflammation and wound healing, it reinforces the importance of alternative therapies for the treatment of these pathologies. Cornea and the skin have a populations of mesenchymal stem cells (MSCs) capable of self-renewal, proliferation and potential for multiline differentiation. Because of this potential, they have been considered as a source of cells to be used in regenerative medicine, tissue engineering and other applications in dermatology and ophthalmology, whose mechanisms are still under investigation. Extracellular nucleotides have been shown to have biological effects on MSCs, altering proliferation, migration, differentiation and apoptosis. Therefore, more information about o these processes would be crucial to understand the physiology of these cells for the establishment of future clinical applications using their differentiation potential, but without undesirable side effects. In this work, MSCs were isolated from sclerocorneal tissue and human skin, both considered as discard materials, and their potential for multilineage differentiation and the expression of mesenchymal markers were demonstrated. The expression of ectoenzymes, the enzymatic activity of degradation of extracellular nucleotides and extracellular ATP metabolism in dermal mesenchymal stem cells (D-MSCs) and limbal mesenchymal stem cell (L-MSCs) were evaluated. D-MSCs and L-MSCs hydrolyzed low levels of extracellular ATP and high levels of AMP, leading to accumulation of adenosine that can regulate inflammation and tissue repair. In addition, they demonstrated a low activity of the nucleotide pyrophosphatases/phosphodiesterases. These cells expressed mRNA for ENTPD1, 2, 3, 5 and 6, NPP1 and 2 which corresponds to the enzymatic activity observed. Thus, considering the degradation of ATP, L-MSCs are very similar to D-MSCs. Therefore, L-MSCs may be another source of MSCs to be studied in preclinical models and potentially clinical studies.

Key words: Mesenchymal cells, dermis, sclerocorneal limbus, ATP, adenosine, ectoenzymes, tissue regeneration.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS

ADP: Adenosina difosfato

ATP: Adenosina trifosfato

AMP: Adenosina monofosfato

CD73: ecto-5'-nucleotidases

D-MSCs: Células mesenquimais dérmicas

L-MSCs: Células mesenquimais do estroma limbal

MSCs: Células tronco mesenquimais

NDPs: Nucleotídeos difosfatados

NTPDases: Ectonucleósido trifosfato difosfohidrolases

NTPs: Nucleotídeos trifosfatados

NPPs: Nucleotídeo pirofosfatases/fosfodiesterases

p-Nph-5'-TMP: p-nitrofenil timidina 5 'monofosfato

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CAPITULO I

INTRODUÇÃO

1. A córnea humana

Um dos tecidos do corpo que atua como uma barreira física contra agentes externos físicos, químicos, patogênicos ou microbianos é a córnea (Campbell and Gubisch 1966). A córnea é um tecido avascular transparente que proporciona proteção e propriedades de refração para a estrutura interna do olho, permitindo a transmissão de luz para a retina, essencial para a acuidade (Meek and Leonard 1993). A transparência da córnea é essencial para a visão (Albon 2003). A falta de vasos sanguíneos e a estrutura regular composta por fibras de colágeno permitem a transparência deste tecido (Daniels et al. 2001).

A córnea é composta por cinco membranas conhecidas como o epitélio, a membrana de Bowman, o estroma, a membrana de Descemet e o endotélio, nomeados em ordem começando pela mais externa, onde só o epitélio, o estroma e o endotélio são membranas celulares (Derek W. DelMonte 2011) (Figura 1).

1.1. Epitélio

A membrana externa da córnea é caracterizada por um epitélio escamoso estratificado não queratinizado de 5-6 camadas de células epiteliais. O epitélio representa aproximadamente 10% da espessura da córnea e atua como uma barreira de defesa contra agentes externos (Pfister and Burstein 1977).

As células epiteliais da córnea tem uma vida de 7 a 10 dias, após esse período ocorre apoptose e dimensionamento, e isto permite a regeneração do epitélio da córnea, onde as células mais profundas substituem as células superficiais (Hanna, Bicknell, and O'Brien 1961).

Entre o epitélio e o estroma da córnea se encontra a membrana de Bowman, uma camada acelular de 15 mm de espessura, formada por fibras do colágeno do tipo I e tipo III, o que ajuda a manter a forma da córnea, mas não tem capacidade regenerativa (Derek W. DelMonte 2011).

1.2. Estroma

O estroma corresponde a 80% da espessura da córnea. Consiste em uma camada transparente, composta por colágeno tipo I e proteoglicanos produzidos pelos ceratócitos da córnea (Freegard 1997). Os ceratócitos são os principais tipos de células estromais com capacidade limitada de auto renovação e estão envolvidos na manutenção da matriz extracelular, ocupando cerca de 3% do volume do estroma (Byun et al. 2014).

O estroma e o endotélio são separados pela membrana de Descemet, que representa uma rede acelular formada por fibras de colágeno do tipo IV, laminina e fibronectina, produzidas por células endoteliais. Sua principal função é formar uma barreira resistente à perfuração da córnea (Araujo Costa et al. 2013).

1.3. Endotélio

O endotélio córneo é uma monocamada de células planas hexagonais unidas à membrana de Descemet por hemidesmossomas. Estas células expulsam o excesso de água para evitar o edema do tecido estromal e manter a transparência da córnea. Tanto a densidade celular e a topografia destas células são expostas a contínuas alterações ao longo da vida (Derek W. DelMonte 2011).

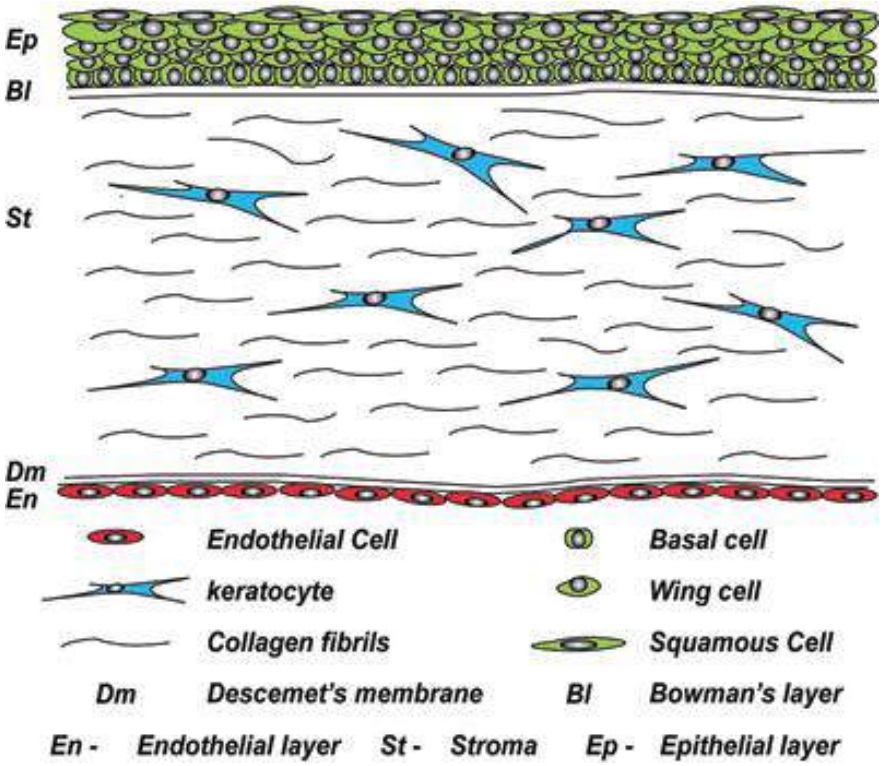

Figura 1. A córnea humana em corte transversal. Na superfície externa da córnea, há uma camada epitelial estratificada. A camada estromal média é conformada por ceratócitos rodeados por tecido conjuntivo denso. A camada final consiste em uma única camada de células endoteliais (Secker and Daniels 2009).

2. Limbo esclerocorneal

A córnea é capaz de se regenerar a partir da população de células tronco localizadas no limbo esclerocorneal, zona estreita entre a córnea e a esclera. Esta área foi descoberta pelo Dr. Tung-Tien Sun em 1986 (Schermer, Galvin, and Sun 1986). Uma das diferenças mais notáveis entre a córnea e o limbo é a presença de vasos sanguíneos que formam parte das paliçadas de Vogt, zonas vascularizadas e inervadas localizadas no limbo onde as células tronco epiteliais da córnea têm sido encontradas (Tseng 1989).

O limbo esclerocorneal é uma região onde há mudanças na estrutura da córnea. O epitélio forma cerca de 10-12 camadas celulares, ao contrário das cinco camadas observadas na córnea central, a membrana de Bowman desaparece e o estroma limbal perde a regularidade da estrutura formada pelas fibras de colágeno (Li et al. 2007; Takács et al. 2009).

Além de permitir a regeneração do epitélio da córnea pelas células tronco epiteliais, o limbo é caracterizado por apresentar respostas de hipersensibilidade, imunovigilância e função de barreira, impedindo que as células da conjuntiva não migrem até a córnea

(Forrester J. V., Dick A. D., McMenamin P. G., Roberts F. 2016). A resposta imunitária é produzida por células de Langerhans, envolvidas na ativação de linfócitos T supressores (Li et al. 2007) (Figura 2).

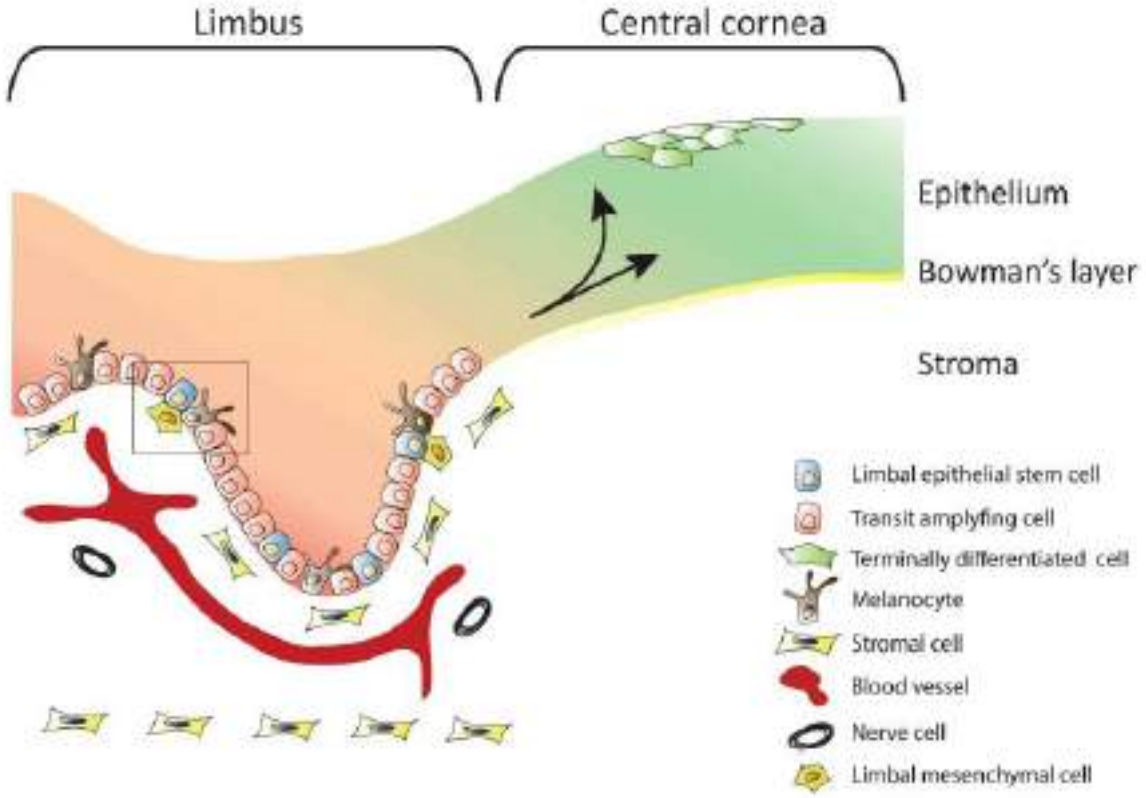

Figura 2. Esquema hipotético do limbo esclerocorneal. Células tronco epiteliais são encontradas na membrana basal do epitélio do limbo. Em este nível estão outros tipos de células epiteliais, tais como as células de amplificação transitória, melanócitos e as células de Langerhans. As células de amplificação transitória são diferenciadas em células de amplificação transitória tardia, localizadas na parte basal da córnea, em seguida são diferenciadas em células terminalmente diferenciadas. A membrana da lâmina basal, que separa o epitélio do estroma, tem vários componentes subjacentes como células mesenquimais (Dziasko and Daniels 2016).

3. A Pele humana

Um dos principais órgãos que também atua como barreira contra agressões externas físicas, químicas, imunológicas ou patogênicas é a pele, que, como a córnea, também precisa de uma rápida regeneração. Trata-se de uma associação de tecidos de várias origens (epitelial, conectivo, vascular, muscular e nervoso) organizados em camadas: a epiderme, a derme e a hipoderme. Embriologicamente, a epiderme é de origem ectodérmica, enquanto a derme e a hipoderme são de origem mesodermal (Kanitakis 2002) (Figura 3).

3.1. Epiderme

A camada mais superficial da pele, a epiderme, é composta por um epitélio escamoso estratificado constituído por vários tipos celulares (Suter et al. 1996). Os queratinócitos compreendem aproximadamente 90 a 95% das células da epiderme e são células epiteliais. Oriundas da ectoderme, sofrem um processo específico de diferenciação, resultando na produção de células anucleadas achatadas (corneócitos) que acabam por migrar até a superfície da pele. Ainda, cinco a dez por cento das células epidérmicas são compostas por células apresentadoras de antígenos (células de Langerhans), mecanorreceptores (células de Merkel) e células produtoras de pigmentos (melanócitos) (Taylor et al. 2000).

Como célula primária, os queratinócitos definem a estrutura e a organização da epiderme através da sua diferenciação terminal programada. Os queratinócitos estão dispostos em camadas sobrepostas, as quais são indicativas do estágio de diferenciação que a célula apresenta. A camada basal, adjacente à membrana basal, é a camada proliferativa e menos diferenciada de queratinócitos. Com o aumento da diferenciação, as células migram para a superfície da pele. Ao atingir a área superior da camada granular, sofrem um tipo de morte celular programada. Finalmente, as células já em escamas, são perdidas a partir da superfície da pele, completando a auto-renovação da epiderme (Ishida-Yamamoto and Iizuka 1998).

3.2. Derme

A derme é composta por diferentes tipos celulares e matriz extracelular , tais como elastina, glicosaminoglicanos e colágeno, principalmente tipo I e III, sendo esse último responsável por 98% da massa total de derme seca (Kanitakis 2002). A derme é altamente vascularizada, o que inclui a presença de células endoteliais contendo, também, unidades pilossebáceas e glândulas sudoríparas. Dentre as células estão presentes os fibroblastos, mastócitos, leucócitos infiltrantes e células mesenquimais (Menon 2002; Vapniarsky et al. 2015).

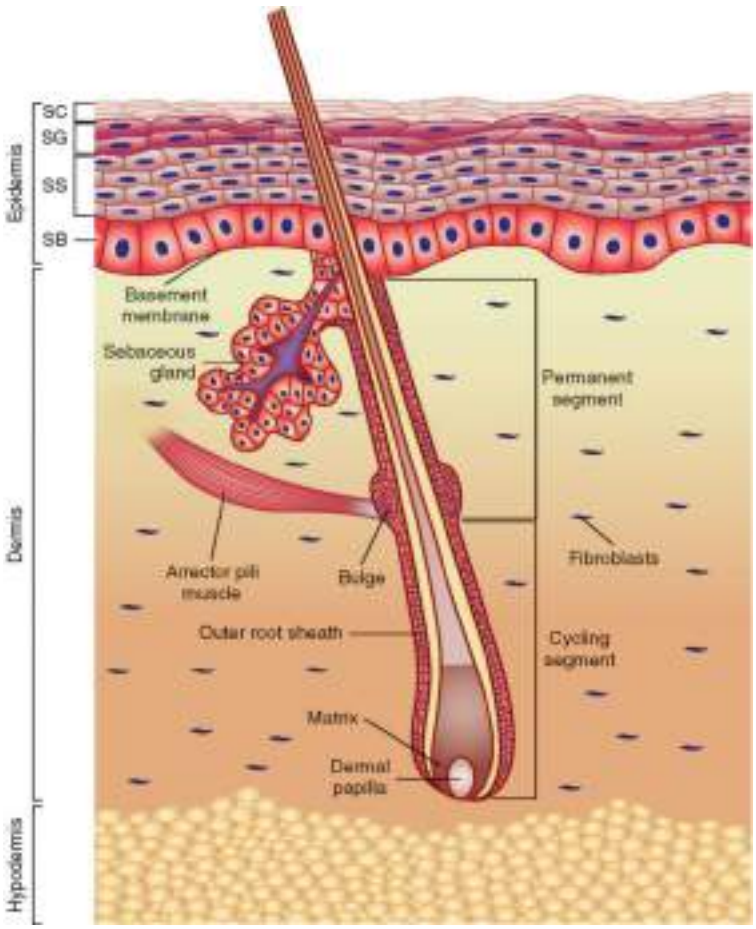

Figura 3. Anatomia da pele humana. A pele é composta de três camadas, começando com a camada mais externa: a epiderme, derme e hipoderme. A epiderme é um epitélio escamoso estratificado que se divide em quatro camadas: estrato córneo (SC), estrato granuloso (SG), estrato espinhoso (SS) e estrato basal (SB). A bainha externa da raiz do folículo piloso é contígua à camada epidérmica basal. Os nichos de células tronco incluem a camada epidérmica basal, a base da glândula sebácea, a protuberância do folículo piloso, as papilas dérmicas e a derme (Wong and Chang 2009).

4. Células tronco mesenquimais do estroma limbal

As células tronco mesenquimais (MSCs) são células não-hematopoiéticas com capacidade de auto-renovação, proliferação e potencial de diferenciação multilinhagem (Friedenstein, Chailakhjan, and Lalykina 1970; Pittenger 1999). Têm sido isoladas de diferentes tecidos adultos, incluindo medula óssea, tecido adiposo, gelatina de Wharton do cordão umbilical, placenta e pele e córnea (Dravida et al. 2005; Vapniarsky et al. 2015). As células mesenquimais do estroma limbal (L-MSCs), precursoras dos ceratócitos têm sido encontradas no estroma do limbo (Dravida et al. 2005; Yiqin Du et al. 2005). Estas células compartilham características com as células tronco mesenquimais da medula óssea, em relação à sua morfologia, marcadores específicos, alta taxa de proliferação e baixa imunogenicidade. Além disso, as células do estroma limbal fornecem um nicho, permitem a manutenção do fenótipo das células tronco limbais epiteliais e participam na angiogênese e regeneração do tecido estromal durante a cicatrização de feridas na córnea (Polisetty et al. 2008; Xie et al. 2011).

Tem sido demonstrado que as células mesenquimais do limbo são capazes de adquirir as funções de ceratócitos *in vivo* quando são introduzidas no estroma córneo de ratos, de modo que certos aspectos do microambiente do limbo favorecem a manutenção da potencialidade destas células (Du et al. 2009).

4.1. Células tronco mesenquimais da derme

As células mesenquimais dérmicas (D-MSCs) foram identificadas em diferentes nichos da derme (Vapniarsky et al. 2015). Diversos investigadores tem trabalhado no isolamento, caracterização e propagação *in vitro* destas células, demonstrando o seu potencial de diferenciação não só em células da linhagem mesodérmica (Crisan et al. 2008), mas também em células da linhagem endodérmica (Toma et al. 2005) e ectodérmica (Rodrigues et al. 2014).

Por causa deste potencial, têm sido consideradas como uma fonte de células para uso em medicina regenerativa, engenharia de tecidos e outras aplicações (Vapniarsky et al. 2015). As MSCs possuem muitas propriedades que aumentam seu potencial terapêutico em dermatologia, cujo mecanismo ainda está sob investigação.

Wu et al. 2007 sugeriram que as células mesenquimais isoladas de medula óssea podem promover a cicatrização de feridas através da diferenciação e liberação de fatores proangiogênicos em ratos, demostrando o potencial das células mesenquimais na regeneração de feridas na pele.

5. Abordagens alternativas para o tratamento de lesões na córnea e na pele

Diversas lesões ou patologias podem promover a deficiência das células epiteliais do limbo, fazendo com que a córnea não seja adequadamente regenerada (Daniels et al. 2001).

Após uma lesão ao nível do estroma, propõe-se que os ceratócitos estromais iniciam um processo de regeneração. Estes são ativados, perdem a quiescência e migram para a área da lesão, o que aumenta o tamanho e o número de organelas, iniciando assim a mudança de morfologia (West-Mays and Dwivedi 2006). Estes se diferenciam em fibroblastos, os quais participam no processo de cicatrização de feridas (Fini 1999). Ao contrário do tecido estromal saudável, a matriz extracelular no tecido cicatricial é desorganizada e opaca, resultando na perda da estrutura regular composta por fibras de colágeno e ao mesmo tempo

na deficiência da funcionalidade e da transparência da córnea (Ruberti and Zieske 2008) (Figura 4).

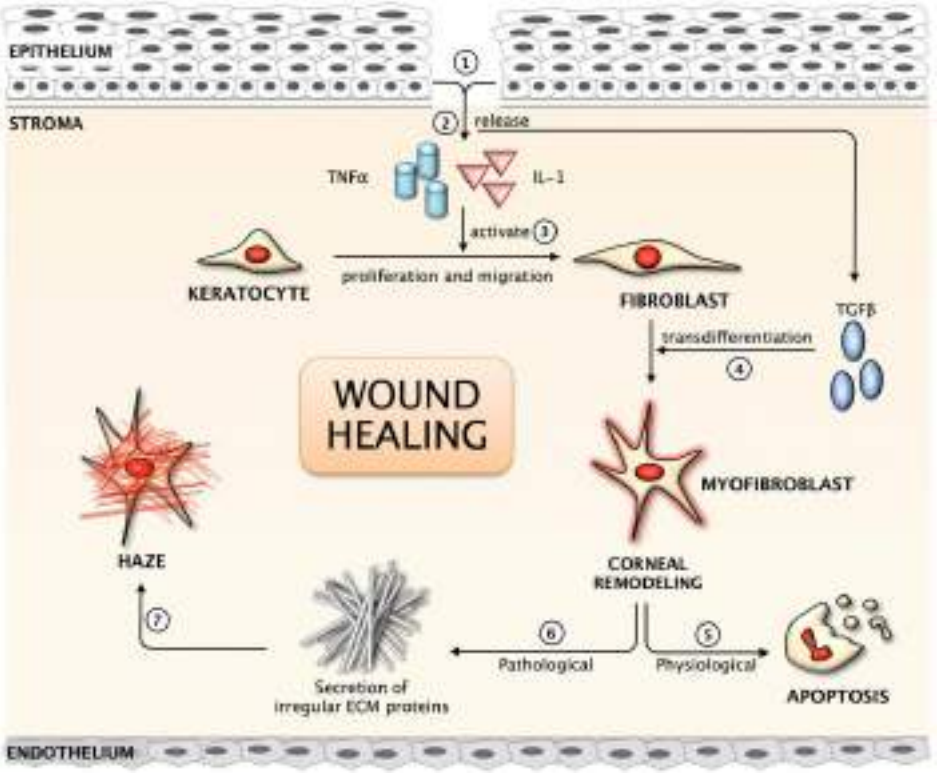

Figura 4. Representação esquemática do mecanismo de cicatrização da córnea. Após uma lesão na córnea, ocorre a ativação de queratócitos quiescentes e a trans-diferenciação de fibroblastos para miofibroblastos, o fenótipo de reparação; sob condições fisiológicas normais, os miofibroblastos sofrem apoptose após reparação da córnea; em condições patológicas, os miofibroblastos secretam matriz irregular resultando na perda da transparência da córnea (Chaurasia et al. 2015).

Em muitos casos, o transplante de córnea pode ser a única opção terapêutica para recuperar a anatomia da córnea. Isto requer a substituição da córnea lesionada por outra saudável, procedente de um doador cadáver. Entretanto, uma das principais limitações para este tratamento é que a quantidade de pacientes que necessitam deste tecido excede a disponibilidade de doadores.

Da mesma forma, a cicatrização óptima de uma ferida cutânea requer uma integração dos eventos biológicos e moleculares complexos de migração, proliferação celular e da deposição de matriz extracelular, angiogênese e remodelação. Nos casos em que são necessárias quantidades consideráveis de pele, a abordagem padrão-ouro é a de tomar enxertos que contêm toda a epiderme, mas apenas parte da derme. Estes são removidos de áreas saudáveis do corpo ou de pacientes cadáver (Martin 1997), mas o déficit de material

tem motivado o desenvolvimento de novas estratégias na área de engenharia de tecidos em busca de processos que favoreçam a regeneração da área lesionada e, portanto, permitam devolver a função tecidual (Carrier et al. 2009).

Para pacientes com queimaduras extensas, a expansão das células da pele que permitam uma função de barreira pode fazer a diferença entre a vida e a morte, e foi essa necessidade aguda que levou à iniciação da engenharia de tecidos na década de 1980 (MacNeil 2007).

Sabe-se que os compostos de purina e pirimidina desempenham um papel em muitos aspectos da fisiologia das células tronco mesenquimais, liberam e respondem aos receptores purinérgicos, alterando a proliferação, migração, diferenciação e apoptose (Glaser et al. 2012). Mais informações sobre estes processos seriam cruciais para o estabelecimento de futuras aplicações clínicas utilizando o potencial de diferenciação das MSCs sem efeitos colaterais indesejáveis, como por exemplo o risco de formação de tumores (Herberts et al 2011; Zhang et al. 2015).

6. Sistema purinérgico

A adenosina trifosfato (ATP) emergiu como uma das moléculas mais versáteis implicada em uma variedade de processos celulares, desde o fornecimento de energia até à sinalização célula-célula (Coppi et al. 2007). Danos celulares, morte celular aguda ou estímulos ambientais (estresse mecânico, osmolaridade e estresse oxidativo) desencadeiam a liberação de nucleotídeos das células. A lesão celular inicia a liberação de fatores de crescimento na ferida, bem como causa a saída de nucleotídeos os quais ativam os receptores purinérgicos (Gendaszewska-Darmach and Kucharska n.d.).

O papel dos nucleotídeos e nucleosídeos de purina como mensageiros extracelulares foi proposto pela primeira vez na década de 1970. Desde então, o progresso acelerado neste campo tem ajudado a determinar a participação desses compostos em processos bioquímicos e fisiológicos fundamentais em diferentes tecidos. O seu potencial terapêutico em uma ampla gama de enfermidades também está atraindo um interesse crescente (Burnstock, Knight, and Greig 2012).

Essa sinalização está envolvida no processo de regeneração e em demais processos terapêuticos de injúrias, logo, tem sido um importante objeto de estudo na comunidade científica. O ATP extracelular resulta na ativação de diferentes subtipos de receptores purinérgicos do tipo ionotrópicos, P2X (P2X1–7) e metabotrópicos, P2Y($P2Y_1$, $P2Y_2$, $P2Y_4$,

$P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14}$). Já a adenosina, produto de degradação do ATP ativa os receptores P1 para adenosina (A_1, A_{2A}, A_{2B}, e A_3) (Burnstock and Verkhratsky 2010).

A concentração de nucleotídeos e nucleosídeos é controlada com precisão pela família das NTPDases (nucleosídeo trifosfato difosfohidrolases), pela família de nucleotídeo pirofosfatases/fosfodiesterases (NPP) e pela ecto-5'-nucleotidase (CD73). Essas ectonucleotidases coordenam uma cascata de sinalização celular, essencial para o desenvolvimento e manutenção celular (Zimmermann 2001). O ATP, liberado por células danificadas e fisiologicamente em resposta a distúrbios mecânicos suaves, parece estar envolvido na cicatrização de feridas, reparação de tecidos e regeneração (Burnstock, Knight, and Greig 2012). Além do ATP, vários estudos têm proposto que o ADP também pode desempenhar um papel na cicatrização de feridas (Wang, Huang, and Heppel 1990).

A utilização dos agonistas dos receptores A_{2A} da adenosina demonstraram, em alguns estudos, um aumento na taxa de fechamento de feridas (Victor-Vega et al. 2002). Entretanto, em outros o tratamento tópico com adenosina promoveu a cicatrização de feridas via receptor A_{2B}, sugerindo que ambos os receptores A_{2A} e A_{2B} estão envolvidos na regulação da cicatrização de feridas. Outros grupos descobriram que o receptor A_{2A} da adenosina promove a produção de colágeno pelos fibroblastos dérmicos (Burnstock, Knight, and Greig 2012).

Do mesmo modo, as purinas desempenham um papel regulador importante na comunicação celular e no reparo de feridas na córnea (Sanderson et al. 2014). Durante a última década, o papel de vários nucleotídeos e seus receptores em processos oculares, incluindo a cicatrização da córnea (através do receptor $P2Y_2$), migração epitelial e organização do estroma (mediada pelo receptor P2X7) têm sido descrito em ratos (Mankus et al. 2012; Mayo et al. 2008) (Figura 5).

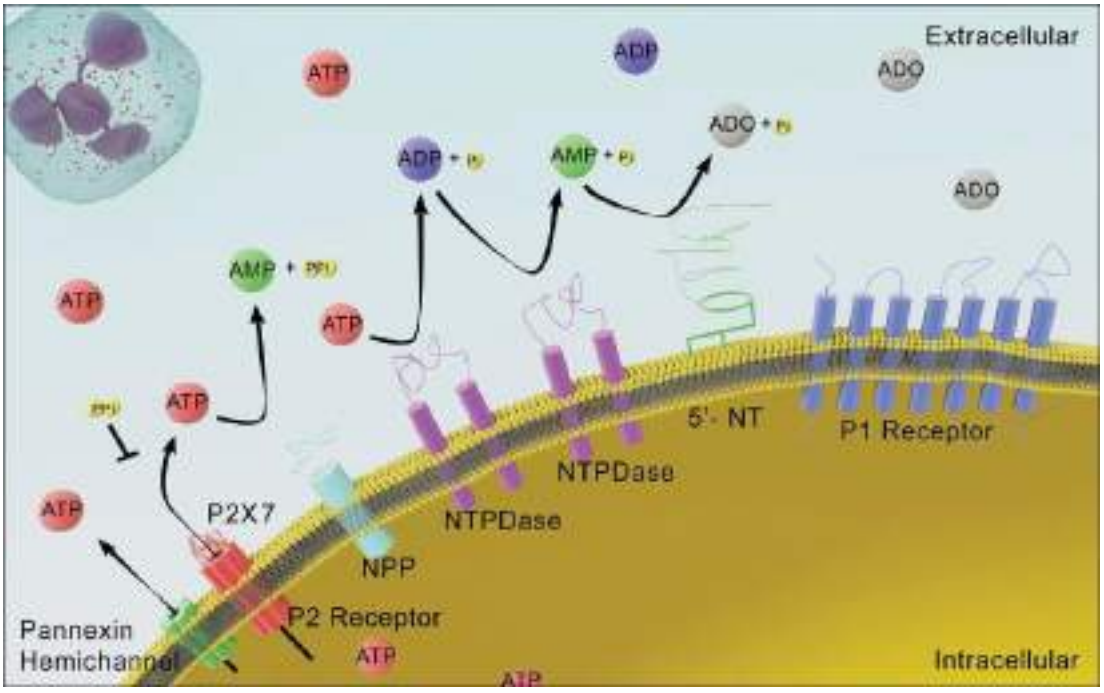

Figura 5. Representação esquemática da sinalização purinérgica. Os nucleotídeos são libertados das células por estimulação mecânica, liberação exocitótica ou lesão. O ATP extracelular pode ser o substrato para as enzimas que metabolizam nucleotídeos (ectonucleotidases). O ATP extracelular tem uma meia vida curta antes de ser degradado a adenosina. A adenosina pode ser convertida a inosina pela ação da adenosina deaminase ou por captação em células através de transportadores nucleosídicos. Quando são externalizados, a adenosina e seus derivados podem participar em processos fisiológicos e morte celular, portanto as purinas podem atuar como importantes mensageiros locais nos tecidos. Abreviaturas: ADO: Adenosina; 5'-NT: 5'-Nucleotidase; NPP: Nucleotide pirofosfatase/fosfodiesterase; NTPDases: Nucleosídeo trifosfato difosfohidrolase (Morandini, Savio, and Coutinho-Silva 2014).

6.1. Ectoenzimas

Várias famílias de enzimas estão envolvidas na sinalização purinergica: NTPDases, NPPs, fosfatase alcalina e ecto-5'-nucleotidase (Burnstock 2006).

A família das NTPDases é constituída por oito membros (NTPDase1-8) clonados e caracterizados, que diferem funcionalmente por hidrolisarem as purinas e pirimidinas com diferentes afinidades (Zimmermann 2001). As NTPDase1, 2, 3 e 8 são ligadas à membrana plasmática e hidrolisam os nucleosídeos trifosfatados (NTPs) e difosfatados (NDPs). As NTPDase5 e 6 apresentam uma localização intracelular e as NTPDase 4 e 7 são completamente intracelulares (Robson, Sévigny, and Zimmermann 2006; Zimmermann 2001). A NTPDase1 hidrolisa ATP e ADP, formando AMP e UTP para UDP, enquanto que a NTPDase2 hidrolisa ATP para ADP e a ecto-5'-nucleotidase hidrolisa o AMP para adenosina (Burnstock 2006). As NTPDase1, 2, 3 e 8 foram denominadas ecto-ATPDases

porque hidrolisam ATP e ADP. Durante a hidrólise de ATP para AMP pela NTPDase1 o ADP não é liberado como um intermediário, enquanto o aparecimento de ADP livre pode ser demonstrado pela hidrólise de ATP pelas NTPDase2,3 e 8 (Knowles 2011).

As ecto-fosfodiesterases hidrolisam ligações fosfodiester de nucleotídeos, resultando na produção de nucleosídeos monofosfato (Cristiana Stefan, Jansen, and Bollen 2005). As NPP1, NPP2 e NPP3 são capazes de hidrolisar vários nucleotídeos e são consideradas as enzimas importantes na via da sinalização purinérgica (C Stefan, Jansen, and Bollen 2006; Yegutkin 2008).

A ecto-5'-nucleotidase (CD73) considerada um importante marcador de superfície das MSCs é altamente expressa na membrana destas células. É uma glicoproteína ligada a membrana plasmática, sendo encontrada na maioria dos tecidos. Esta enzima é responsável por transformar o AMP em adenosina (Baldwin et al. 1999).

As ectoenzimas como elementos do sistema purinérgico são diversamente expressas em diferentes tipos de células e desempenham papéis diferentes nas células tronco. Existem vários estudos sobre os receptores purinérgicos em vários tipos de células tronco, mas há pouca informação sobre a expressão de membros da família das ecto-nucleotidases. Tem sido identificadas as NTPDases e a CD73 em MSCs isoladas de diferentes tecidos murinos (medula óssea, pulmão, veia cava, rim, pâncreas, baço, pele e tecido adiposo) (Iser et al. 2014). Recentemente, Rozsek et al. (2015) caracterizou o perfil de hidrólise de nucleotídeos extracelulares e a expressão das ectoenzimas em MSCs derivadas de cordão umbilical humano, demonstrando a importância do conhecimento sobre sinalização purinérgica na fisiologia das células tronco para aplicações terapêuticas (Roszek et al. 2015).

JUSTIFICATIVA

Tanto a córnea como a pele apresentam populações de células tronco mesenquimais com capacidade de auto-renovação, proliferação e potencial de diferenciação multi-linhagem e por causa deste potencial, têm sido consideradas como uma fonte de células para uso em medicina regenerativa e engenharia de tecidos. Considerando-se o número insuficiente de doadores e possibilidade de insucesso cirúrgico, são necessárias terapias alternativas para o tratamento de lesões na córnea e na pele. As purinas extracelulares (ATP, ADP, Adenosina) são moléculas extracelulares que regulam uma variedade de funções fisiológicas como proliferação, diferenciação e morte celular, tendo sido recentemente apontadas como importantes na fisiologia das células tronco mesenquimais. Assim, mais informações sobre estes processos são importantes para que se possa conhecer os aspectos da biologia celular destas células e então utilizá-las em modelos pré-clínicos e estudos potencialmente clínicos.

OBJETIVOS

Objetivo Geral

Padronizar o isolamento e o cultivo de células mesenquimais de pele e tecido esclerocorneal e investigar a sinalização purinérgica nestas células.

Objetivos Específicos

1. Padronizar o isolamento e o cultivo de células mesenquimais de materiais biológicos de descarte, como pele e tecido esclerocorneal humano.
2. Caracterizar fenotipicamente as culturas.
3. Avaliar o potencial de diferenciação das células mesenquimais isoladas.
4. Identificar o melhor gene normalizador e avaliar a expressão gênica dos componentes do sistema purinérgico.
5. Caracterizar a hidrólise dos nucleotídeos extracelulares ATP, ADP e AMP e p-nitrofenil timidina 5 'monofosfato (p-Nph-5'-TMP).
6. Avaliar o metabolismo do ATP extracelular e a formação de adenosina por HPLC.
7. Analisar a presença das ectoenzimas nas células mesenquimais extraídas do tecido esclerocorneal e da pele.

CAPITULO II

Extracellular nucleotide hydrolysis in Dermal and Limbal Mesenchymal Stem Cells: a source of adenosine production

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Extracellular Nucleotide Hydrolysis in Dermal and Limbal Mesenchymal Stem Cells: A Source of Adenosine Production

Liliana I. Sous Naasani, Cristiano Rodrigues, Rafael Paschoal de Campos, Liziane Raquel Beckenkamp, Isabele C. Iser, Ana Paula Santin Bertoni, and Márcia R. Wink*

Departamento de Ciências Básicas da Saúde e Laboratório de Biologia Celular, Universidade Federal de Ciências da Saúde de Porto Alegre–UFCSPA, Porto Alegre, RS, Brasil

ABSTRACT

Human Limbal (L-MSCs) and Dermal Mesenchymal Stem Cell (D-MSCs) possess many properties that increase their therapeutic potential in ophthalmology and dermatology. It is known that purinergic signaling plays a role in many aspects of mesenchymal stem cells physiology. They release and respond to purinergic ligands, altering proliferation, migration, differentiation, and apoptosis. Therefore, more information on these processes would be crucial for establishing future clinical applications using their differentiation potential, but without undesirable side effects. This study evaluated and compared the expression of ecto-nucleotidases, the enzymatic activity of degradation of extracellular nucleotides and the metabolism of extracellular ATP in D-MSCs and L-MSCs, isolated from discard tissues of human skin and sclerocorneal rims. The D-MSCs and L-MSCs showed a differentiation potential into osteogenic, adipogenic, and chondrogenic lineages and the expression of markers $CD105^-$, $CD44^+$, $CD14^-$, $CD34^-$, $CD45^-$, as expected. Both cells hydrolyzed low levels of extracellular ATP and high levels of AMP, leading to adenosine accumulation that can regulate inflammation and tissue repair. These cells expressed mRNA for *ENTPD*1, 2, 3, 5 and 6, and *CD73* that corresponded to the observed enzymatic activities. Thus, considering the degradation of ATP and adenosine production, limbal MSCs are very similar to dermal MSCs, indicating that from the aspect of extracellular nucleotide metabolism L-MSCs are very similar to the characterized D-MSCs. J. Cell. Biochem. 9999: 1–13, 2017. © 2017 Wiley Periodicals, Inc.

KEY WORDS: LIMBAL MESENCHYMAL STEM CELLS; DERMAL MESENCHYMAL STEM CELLS; MSCs; PURINERGIC SIGNALING; NTPDases; CD73

Mesenchymal Stromal Cells (MSCs) are a population of non-hematopoietic stromal cells with a high proliferative, self-renewal and a multilineage differentiation potential [Friedenstein et al., 1970; Pittenger et al., 1999]. This cell population resides in various sites of adult, perinatal and fetal tissues where these cells have been isolated and characterized [Campagnoli et al., 2001]. According to the International Society for Cellular Therapy, to promote a uniform characterization of MSCs, they must have plastic-adherence when maintained in standard culture conditions, absence of expression of the surface molecules CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR and potential to differentiation in vitro into osteoblast, adipocytes, and chondroblast [Dominici et al., 2006].

Traditionally, MSCs were isolated from bone marrow [Friedenstein et al., 1970; Nemeth and Mezey, 2015], umbilical cord Wharton's jelly [Wang et al., 2004] and adipose tissue [Zuk et al., 2002], but other tissues such as the human skin and sclerocorneal limbus, were recently shown to be potential sources of these cells [Sellheyer and Krahl, 2010; Funderburgh et al., 2016]. Dermal stem cells were isolated and characterized from human dermal tissue, demonstrating multilineage differentiation potential [Toma et al., 2005]. These cells identified thus far, reside in a number of distinct niches. Among the first niches described are the hair follicle dermal papilla and the connective tissue dermal sheath [Jahoda et al., 2003]. In addition, cells residing in stroma of sweat gland

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*Correspondence to: Dra. Márcia Rosângela Win–Universidade Federal de Ciencias da Saúde de Porto Alegre–UFCSPA. Rua Sarmento Leite, 245, CEP 90050–170, Porto Alegre–RS, Brasil. E-mail: mwink@ufcspa.edu.br; marciawink@yahoo.com.br

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joined the list of multipotent stem cells of the dermis [Nagel et al., 2013].

In ocular surface, stromal stem cells were originally observed in the transitional zone between cornea and sclera, known as the sclerocorneal limbus. These cells localized to anterior stroma immediately subjacent to the epithelial basement membrane [Du et al., 2005]. Polisetty et al. [2008] showed that the stromal cells cultured from limbal explants share characteristics with mesenchymal stem cells of the bone marrow (BM-MSCs), in relation to its morphology, specific markers, high proliferation rate and low immunogenicity. In addition, the limbal stroma cells provide a niche allowing the maintenance of the phenotype of limbal epithelial cells. It has been recently shown that cells of the limbal stroma, in addition to establishing the niche for limbal epithelial stem cells (LESCs), participate in angiogenesis and regeneration of stromal tissue during corneal wounds healing [Xie et al., 2012].

MSCs possess many properties that increase their therapeutic potential in dermatology and ophthalmology. Recently, the purinergic signaling has emerged as a player in many aspects of mesenchymal stem cells physiology. MSCs release and respond to purinergic ligands, altering proliferation, migration, differentiation and apoptosis [Glaser et al., 2012]. More information on these processes would be crucial for establishing future clinical applications using their differentiation potential without undesirable side effects.

Nucleotides are among some of the oldest molecules used by living organisms for energy metabolism, storage of genetic information, transduction, and extracellular communication [Scarfì, 2014]. In fact, adenosine has been proposed to contribute to purinergic regulation in MSCs, through the activation of adenosine P1 receptors [Gharibi et al., 2011]. The extracellular hydrolysis cascade of nucleotides initiated by ATP hydrolysis produces ADP, AMP, and adenosine, resulting in the activation of different purinergic receptors (ionotropic P2X or metabotropic P2Y receptors for ATP/ADP and P1 selective for adenosine) [Burnstock, 1978]. The concentration of nucleotides and nucleosides is precisely controlled by the family of E-NTPDases (ectonucleoside triphosphate diphosphohydrolase), the nucleotide pyrophosphatase/phosphodiesterase (NPP) family and ecto-5′-nucleotidase (CD73). These ectonucleotidases coordinate a cellular signaling cascade essential for development and maintenance of MSCs [Robson et al., 2006].

So far, eight members of the NTPDase family (NTPDase1-8) that hydrolyze extracellular nucleotides with different affinities have been characterized. NTPDase1, 2, 3, and 8 are typical cell-surface located enzymes with two transmembrane regions and a large extracellular domain, which contains the catalytic site. NTPDases 4–7 have an intracellular localization, anchored to the membranes of organelles. NTPDase5 and 6 lack the C-terminal transmembrane domain and are also expressed in the plasma membrane or as secreted enzymes [Robson et al., 2006]. The ecto-5′-nucleotidase (CD73) is linked to the plasma membrane with its catalytic site exposed to the extracellular space and catalyzes a dephosphorylation of AMP to generate adenosine, being the major contributor to the pathway that completely hydrolyses from extracellular ATP to adenosine [Bavaresco et al., 2008].

Since MSCs are attractive cell populations for cell-based therapies, their potential for nucleotides hydrolysis is important to understand their physiology and for effective therapeutic applications. There are several studies on purinergic receptors in various types of stem cells, but there is little information about expression of members of ecto-nucleotidases families. Iser et al. [2014] identified the NTPDases and eNT/CD73 in MSCs isolated from different murine tissues (bone marrow, lung, vena cava, kidney, pancreas, spleen, skin, and adipose tissue). Recently, Roszek et al. [2015] characterized the ecto-nucleotides hydrolysis profile and ecto-enzymes expression in human umbilical cord-derived MSCs and chondrogenically induced MSCs, demonstrating the importance of knowledge about purinergic signaling in stem cells physiology for therapeutic applications.

Therefore, in order to contribute with the understanding of the importance of the purinergic signaling in mesenchymal cells from promising sources for therapy, this study evaluated and compared the expression of ecto-nucleotidades, the enzymatic activity of degradation of extracellular nucleotides and the metabolism of extracellular ATP in human dermal and limbal mesenchymal stem cells, isolated from discard tissues.

MATERIALS AND METHODS

ISOLATION AND CULTURE OF HUMAN LIMBAL AND DERMAL STROMAL CELLS

Discarded tissues of human skin (from abdominoplasty) and sclerocorneal rims (from cadaveric donors) were donated by the Skin Bank and Cornea Bank of Santa Casa de Misericórdia de Porto Alegre with the acceptance of Research Ethics committee on Human Beings (CEP) [Q5](N°. 54473316.5.0000.5345).

In both tissue types, we made a standardization of protocol published by Li et al. [1998]. Briefly, fragments of 4 mm^2 were washed, first with isopropyl alcohol/iodine (1:1), and then with phosphate buffer saline (PBS) and 2% penicillin/streptomycin (Sigma, Chemical Co. St. Louis, MO). Tissue fragments were incubated with 2 mg/ml dispase II (Sigma) with 1% penicillin/streptomycin overnight at 37°C to separate the epithelial membrane of the stroma. After removing the epithelial layer, the fragment of the stroma was seeded into 6-well microplates (Nest Biotech) to isolate the cells by migration. The growth medium used was a Dulbecco's Modified Eagle's Medium with low glucose (DMEM Low) (Sigma–Aldrich), supplemented by 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin, which was changed every 3 days. The cultures were maintained at 37°C, 5% CO2, 95% humidity to reach confluence. For each tissue, we work with three primary cultures obtained from different donors. When the primary cultures reached confluence, cells were trypsinized and expanded in 75 cm^2 culture flasks (Kasvi).

PHALLOIDIN STAINING

For F-actin staining, cells were seeded on coverslip and 24 h after culture were fixed with 4% paraformaldehyde solution in PBS for 30 min at 4°C. After each step, cells were washed with PBS. Then, they were incubated with permeabilization solution (0,1% Triton X-100

in PBS) for 1 h. F-actin was stained with FITC-Phalloidin (Sigma) and cells were also stained with 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) for the identification of nuclei. The incubation with Phalloidin and DAPI was for 40 min at 4°C in a dark room. Images were obtained with a fluorescence microscope (Olympus IX51/Olympus U-RFL-T).

ADIPOGENIC, OSTEOGENIC, AND CHONDROGENIC DIFFERENTIATION OF MSCS

For MSCs differentiation, the skin and limbal cells at 5 passage were seeded at a density of 1×10^4 cells/cm^2 into a 12-well plate (Nest Biotech). After 3 days in culture, the growth medium was changed by the StemPro® Adipocyte Differentiation Basal Medium, StemPro® Osteogenesis Differentiation Basal Medium and StemPro® Chondrocyte Differentiation, Basal Medium (Gibco) for adipogenic, osteogenic, and chondrogenic differentiation, respectively. The control cells were maintained with standard growth medium, that was changed every three days, until the specific periods of cultivation. After 14 days of adipogenic differentiation conditions, the culture was fixed with 4% formaldehyde solution for 30 min and staining with (1:3) Oil Red O on deionized water and the pre- and post-treatment was made with 60% (vol/vol) isopropanol. Otherwise, after 17 days in culture with chondrogenic medium, the synthesis of proteoglycans by chondrocytes was seen with 1% Alcian Blue staining, prepared in 0.1 N HCL. Finally, after 25 days under osteogenic differentiating conditions, the calcium deposited by osteocytes was staining with 2% Alizarin Red S solution (pH 4.2).

IMMUNOPHENOTYPING

The expression of cell surface markers was measured by a direct immunofluorescence staining. The cells were trypsinized and counted to incubate 1×10^5 cells for each antibody: FITC-anti-CD44, PE-anti-CD105, PE-anti-CD45, PE-anti-CD14, and FITC-anti-CD34 (Invitrogen™, Waltham, MA). To analyze the presence of the CD73 enzyme in the cells surface, 2×10^5 D-MSC and L-MSC were incubated in the presence of PE-anti-CD73 (BD Pharmingen™, San Diego, CA).

Analysis of cells was performed on a FACSCalibur™ flow cytometerequipped with 488 nm argon laser (Becton-Dickinson, San Diego, CA) with CellQuest software. At least 10,000 events were collected and data were evaluated using the Flowing Software 2.

NUCLEOTIDE HYDROLYSIS ON CELLS SURFACE

To evaluate the nucleotide hydrolysis on the MSCs surface, we made the enzymatic assay as already described [Wink et al., 2003]. Briefly, the 24-well microplates (Nest Biotech) containing confluents L-MSCs and D-MSCs, respectively, were washed three times with a phosphate-free incubation medium in the absence of nucleotides. For ATP and ADP hydrolysis, the reaction was started by the addition of 200 μl of the incubation medium containing 2 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes, pH 7.4 and nucleotides, 1 mM ATP and ADP, respectively, at 37°C. For AMP hydrolysis, the incubation medium was the same, except that the 2 mM $MgCl_2$ was used instead of $CaCl_2$, and the nucleotide was 1 mM AMP. The incubation time was chosen to assure the linearity of the reaction, 1 h for ATP and ATP and 30 min for AMP hydrolysis. To stop the reaction, an aliquot of the cell incubation medium was transferred to a tube containing trichloroacetic acid (TCA) to a final concentration of 5% (w/v). The inorganic phosphate (Pi) released was measured by the malachite green method [Chan et al., 1986], using KH2PO4 as a Pi standard. To determinate the spontaneous hydrolysis of nucleotides during the incubation, the medium was incubated with its respective nucleotide but without cells, to have net values of enzymatic activity. All samples were run in triplicate for each different sample. Specific activity was then expressed as nmol Pi released/min/mg of protein. To correct the released Pi by the cells, was determinate the mg of protein in each well. To this, after incubation, were added 100 μl of 1N NaOH and frozen at −20 °C. The protein was measured by the Comassie blue method, using bovine albumin as standard.

ASSAY OF ECTO–NUCLEOTIDE PYROPHOSPHATASE/ PHOSPHODIESTERASE (E–NPP) ACTIVITY

Phosphodiesterase activity was assessed using p −nitrophenyl thymidine 5′ monophosphate (p −Nph-5′ −TMP), an artificial substrate for E-NPPs [Buffon et al., 2010]. The cells were incubated with reaction medium containing 50 mM Tris–HCl buffer, 5 mM KCl, 120 mM NaCl, 0.5 mM CaCl2, 60 mM glucose, pH 7.4 and 8.9. The enzyme reaction was started by the addition of p-Nph-5′-TMP to a final concentration of 0.5 mM. After 90 min of incubation, the reaction was stopped by the addition of 200 μl 0.2 N NaOH and the samples were chilled on ice. Incubation time was chosen to ensure the linearity of the reaction. The amount of p-nitrophenol released from the substrate was measured at 400 nm. Enzyme activities were expressed as nmol p-nitrophenol release per minute per milligram of protein.

ANALYSIS OF EXTRACELLULAR ATP METABOLISM BY HPLC

Confluent cells seeded on 24-well microplates were incubated with 300 μl of incubation medium containing 100 μM of ATP at 37°C. Aliquots of the samples were collected at different times of incubation: 0, 10, 30, 60, 90, and 120 min and the reaction was stopped on ice. All samples were centrifuged at 400*g* in a refrigerated centrifuge at 4°C for 15 min and frozen at −80°C. The separation by HPLC was performed on a LC-20 system (Shimadzu, Kyoto, Japan), using a C18 column (5 μm, 150 × 4.6 mm) Shimadzu Shim-pack CLC (M) at a temperature of 32°C. Aliquots of 20 μl of each sample was injected in a flow of 1,2 ml/min during a 45 min run. The gradient was composed of a mobile phase consisting of a buffer A (60 mM KH_2PO_4, 5.0 mM $C_{16}H_{36}ClN$ at pH 5.9) and a buffer B (60 mM KH_2PO_4, 5.0 mM $C_{16}H_{36}ClN$ and 30% methanol, pH 5.9). Detection was analyzed at 254 nm and compared with the standards at their characteristic retention times.

RNA EXTRACTION, cDNA SYNTHESIS

Total RNA from MSCs cultures was isolated with TRI Reagent LS (Sigma)in accordance with the manufacturer's instructions. The cDNA species were synthesized with reverse transcriptase (SuperScript® III First-Strand Synthesis System Invitrogen, Carlsbad, CA) from up to 5 μg of total RNA, in a final volume of 25 μl, with random hexamers primers in accordance with the manufacturer's instructions.

TABLE I. Primer Sequences, Annealing Temperatures, and Fragment Size of Candidate Normalization Genes

Gene	Primer sequence	T (°C)	Fragment size (bp)
ACTβ F	5′-AAGACAGTGTTGTGGGTGTAGG-3′	60	126
ACTβ R	5′-TGGGATGGGGAGTCTGTTCA-3′		
B2M F	5′-AGATGAGTATGCCTGCCGTG-3′	60	120
B2M R	5′-TCATCCAATCCAAATGCGGC-3′		
GAPDH F	5′-GTCAAGGCTGAGAACGGGAA-3′	60	158
GAPDH R	5′-AAATGAGCCCCAGCCTTCTC-3′		
TBP F	5′-GCATCACTGTTTCTTGGCGT-3′	60	112
TBP R	5′-CGCTGGAACTCGTCTCACTA-3′		

Primer design based on this sequence. The database is the Ensemble database (http://www.ensembl.org). bp, base pairs; ACTβ, Beta-Actin; B2M; Beta-2-Microglobulin; GAPDH, Glyceraldehyde-3-phosphate Dehydrogenase; TBP, TATA box binding protein.

REVERSE-TRANSCRIPTION QUANTITATIVE PCR ANALYSIS

For the evaluation of RT-qPCR data four candidate normalization genes were tested: Beta-Actin (ACTβ), Beta-2-Microglobulin (B2M), Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH), and TATA box binding protein (TBP) (Table I). It was evaluated the expression of the genes *ENTPD1*, *ENTPD2*, *ENTPD3*, *ENTPD5*, *ENTPD6*, *ENTPD8*, *Ecto-5′NT/CD73*, *ENPP1*, *ENPP2*, *ENPP3*, and Alkaline phosphatase (*ALPL*) (Table II) for three n of each tissue type. Primers were designed using the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and analyzed using Integrated DNA Technologies Oligo Analyzer 3.1 software (http://www.idtdna.com/calc/analyzer/). RT-qPCR samples were prepared in a 12.5 ml final volume composed of 6.25 µl of SYBR® Select Master Mix (Applied Biosystems, Foster City, CA). 0.2 µl of primer pair solution (0.2 µM final concentration of each primer) 5.05 µl of water and 1 µl of diluted cDNA. qPCR was carried out in an Applied-BiosystemStepOnePlus™ Real-Time PCR cycler. Reaction settings were composed of an initial enzyme activation step of 20 s at 95°C, followed by 45 cycles of 3 s at 95°C and 30 s at 60°C for data acquisition. The melting curve analysis was performed in single cycle by heating samples from 65°C to 95°C at a ramp rate of 0.5 C/s with continuous fluorescent acquisition. To quantify mRNA expression levels, the relative standard curve method was used. cDNA standard curves were constructed using four serial twofold dilution points of a pool of cDNA samples. Relative gene expression was calculated from the quantity mean of target genes for each sample divided by the respective quantity mean of the reference gene.

STATISTICAL ANALYSIS

To evaluate the stability of the candidate reference genes in L-MSCs and D-MSCs, the NormFinder algorithm was used [Andersen et al., 2004]. In addition, the analysis of raw quantification cycle (Cq) values of each gene was used to evaluate their stability. The Mean Cq values, standard deviation (SD), coefficient of variation (CV), and maximum folds change (MFC, the ratio of the maximum and minimum values observed within the dataset) were calculated. Data were expressed as mean ± standard error of mean (SEM). Data were analyzed statistically by one-way ANOVA followed by Tukey post-hoc test for ATP, ADP and AMP hydrolysis and by Independent-samples *t*-test for relative mRNA expression using the statistical program IBM SPSS Statistics 24. Differences between mean values were considered significant when $P < 0.05$.

RESULTS

ISOLATION AND CULTURE OF HUMAN LIMBAL AND SKIN STROMAL CELLS

Cells with fusiform morphology were isolated from human skin and sclerocorneal samples from three donors of each tissue. Cells

TABLE II. Primer Sequences, Annealing Temperatures, and Fragment Size of Ectonucleotidases Genes

Gene	Primer sequence	T (°C)	Fragment size (bp)
ENTPDase3 F	5′-CCTCTCTCACGGAGACGGA-3′		
ENTPDase3 R	5′-TCTTGGAGCAAAATGTCTTCACG-3′	60	131
ENTPDase2 F	5′-CTCCTACTGCTGTGCGTCC-3′		
ENTPDase2 R	5′-TGTCGTTCTCCTTGTCTGCC-3′	60	133
ENTPDase3 F	5′-GCTAGTCGCCTTCTCCGAAT-3′		
ENTPDase3 R	5′-GCAAGACCACCAAGGCAATG-3′	60	136
ENTPDase5 F	5′-GGAGTGTCTTGGCTGAATCCT-3′		
ENTPDase5 R	5′-CACACCTGCAGAGGCAATTT-3′	60	150
ENTPDase6 F	5′-TCGCCTTTCCTTGTAGGGGA-3′		
ENTPDase6 R	5′-GCTCCCTCCTGGAGTTTTCA-3′	60	117
ENTPDase8 F	5′-GCGGACACAGAAGCGTCTAA-3′		
ENTPDase8 R	5′-CCAAGAAGACCTGCTCCTTCC-3′	60	120
Ecto-5′NT/CD73 F	5′-GAAGGCCTTTGAGCATAGCG-3′		
Ecto-5′NT/CD73 R	5′-CGACACTTGGTGCAAAGAACA-3′	60	144
ENPP1 F	5′-CCAAGGACCCCAACACCTATAAA-3′		
ENPP1 R	5′-AACTTCTTTGGCACAGCTTGG-3′	60	110
ENPP2 F	5′-GCTCGTTCCAGTCGTGTCA 3′		
ENPP2 R	5′-GGTCCAGGGGGAGTCTGATA-3′	60	146
ENPP3 F	5′-TGTGGATCAACAGTGGCTGG-3′		
ENPP3 R	5′-GGGTCCATGTGCCAGAAAGA-3′	60	118
Alkaline phosphatase F	5′-CGCTATCCTGGCTCCGTG-3′		
Alkaline phosphatase R	5′-AGAGATGCAATCGACGTGGG-3′	60	135

migrated at approximately 15 days of culture in both tissue types. After that, tissue fragments were removed to allow cells reached the confluence, which was about 25 days of cultivation (Fig. 1A–F). To obtain a purified culture, cells were extended to passage 4.

F-ACTIN STAINING IN L-MSCS AND D-MSCS

To observe the distribution of actin filaments in cells obtained from both tissue types, cells were stained with FITC-Phalloidin. Cells showed the filamentous actin stress fibers, characteristic of mesenchymal-migrating cells [Vallenius, 2013]. No morphological differences were observed between cells derived from skin and sclerocorneal limbus (Fig. 2A–H).

CHARACTERIZATION OF L-MSCS AND D-MSCS

To demonstrate the potential for multilineage differentiation of isolated cells from human skin and sclerocorneal tissue, cell cultures were induced with osteogenic, adipogenic, and chondrogenic media. After 14 days in adipogenic differentiation medium, cells showed the formation of intracellular lipid vacuoles, which were confirmed with Oil Red staining (Fig. 3A and B). The lipid vacuoles were not observed in the controls (data not shown). After 25 days under osteogenic differentiating conditions, it was observed a calcium-rich mineralized matrix as evidenced by Alizarin Red S staining (Fig. 3C and D). Similarly, both cells types demonstrate positive glycosaminoglycan production after 17 days in culture, as confirmed by Alcian blue staining (Fig. 3E and F). The analysis of surface markers indicated that the cell population isolated from skin and sclerocorneal limbus, have a very similar immunophenotype. The great part of the population of L-MSCs and D-MSCs was positive for mesenchymal markers (CD105 and CD44) and negative for hematopoietic markers (CD14, CD34, and CD45) [Onzi et al., 2016]Q6 (Fig. 3G and H). These results demonstrate that was possible to isolate a population of cells with potential for multilineage differentiation from both human tissues.

ECTO-NUCLEOTIDASES CHARACTERIZATION IN L-MSCS AND D-MSCS

After the evaluation of the time necessary to achieve the optimal conditions for kinetic assays with each cells and nucleotides (data not shown), the L-MSCs and D-MSCs were incubated with ATP and ADP for 1 h and with AMP for 30 min. The enzymatic assay showed a high hydrolysis rate for AMP and a low rate for ATP and ADP, without differences in the hydrolysis pattern between both cell types (Fig. 4A, Table III).

To investigate whether members of E-NPP family could contribute to nucleotide degradation in L-MSCs and D-MSCs, we investigated the hydrolysis of p −Nph-5′-TMP, the artificial substrate of NPPs, resulting in p-nitrophenol. The results showed that the higher p −Nph-50 −TMP hydrolysis occurs at alkaline pH (8.9), while the lower activity was observed at the physiological pH (7.4) (Fig. 4B), which is in accordance with previous data for E-NPPs [Rücker et al., 2007; Andrade et al., 2009]. At physiologic pH (7.4), we observed that the ATP, ADP, and AMP hydrolysis was approximately 200, 100, and 600 times higher, respectively, when compared to p-Nph-5′-TMP. These results evidenced that the participation of NPPs in the nucleotide degradation cascade on the surface of D-MSC and L-MSC is probably negligible in compassion to NTPDases and mainly, CD73.

Additionally, these cells were incubated with ATP at different time points, up to 120 min, and supernatants were analyzed by HPLC, confirming the low degradation of ATP and the generation of adenosine at the end of reaction. Additionally, we observed a slight increase in ATP concentration in the 90 or 120 min time points of the reaction (Fig. 4C and D).

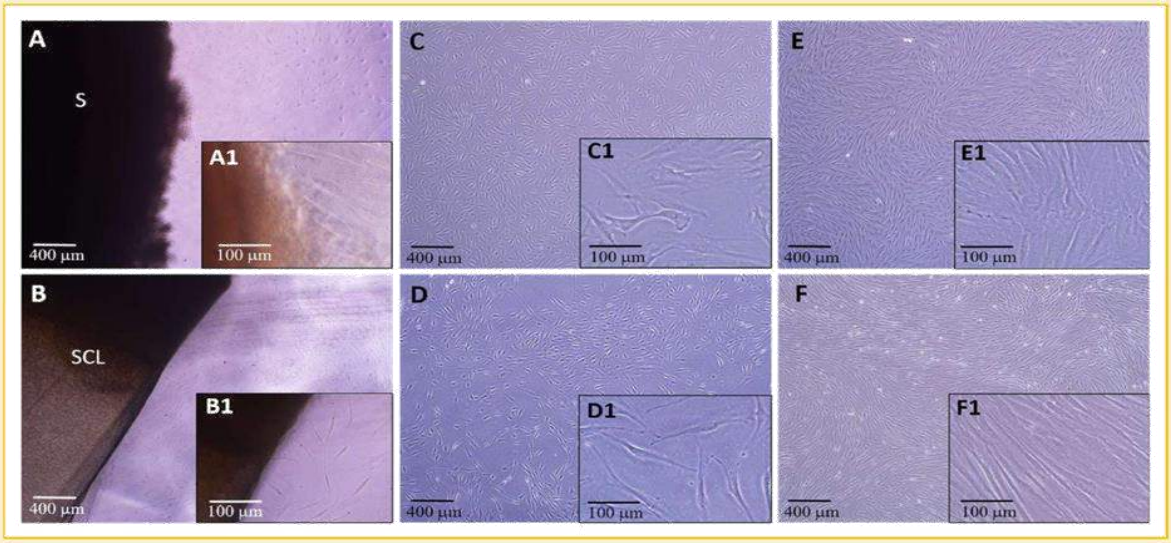

Fig. 1. Micrograph of MSCs isolated by migration. Cell migration from human skin tissue (A) and human sclerocorneal limbus (B) in 15 days of culture. Subconfluent culture of D-MSCs (C) and L-MSCs (D). Confluente primary culture of D-MSCs (E) and L-MSCs (F) in 25 days of culture. Higher magnification of images show the fusiform morphology of the cells. Fragment of human dermis (S), fragment of human sclerocorneal limbus (SCL). Phase contrast micrograph. Scale bars (A–F) 400 µm; (A1–F1) 100 µm.

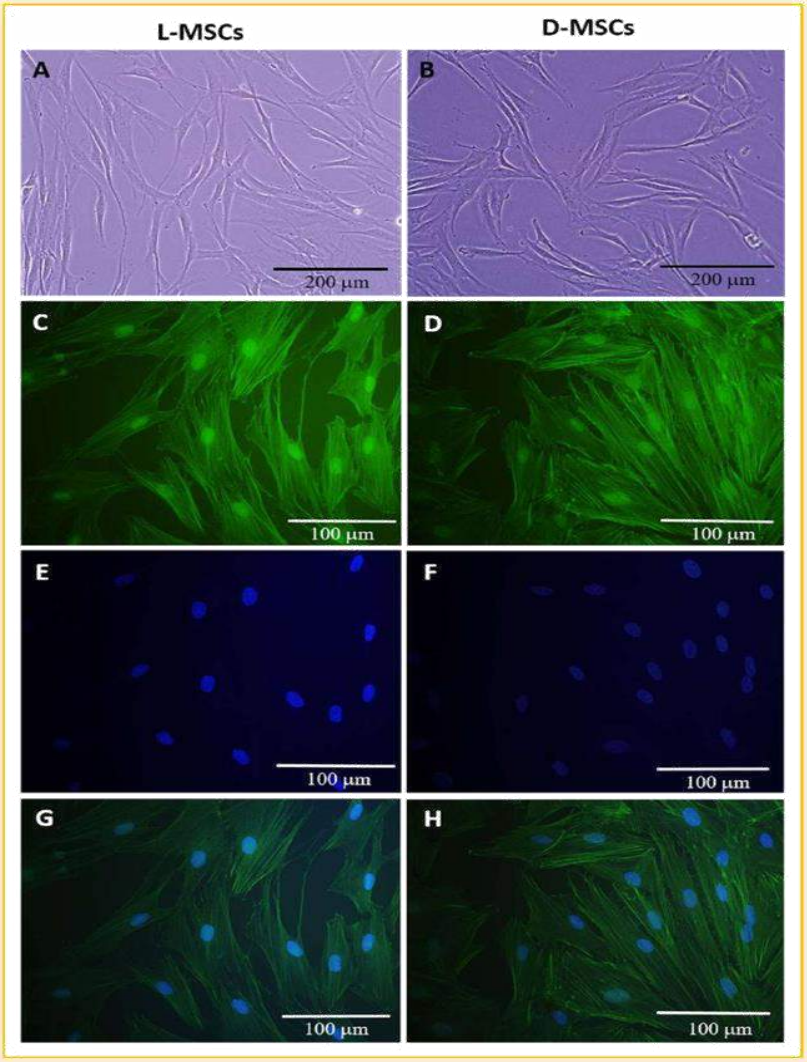

Fig. 2. Phalloidin staining of MSCs cultures. Fusiform morphology of L-MSCs (A) and D-MSCs (B). Actin filaments (Green) of L-MSCs (C) and D-MSCs (D). Nuclear staining with DAPI of L-MSCs (E) and D-MSCs (F). Overlapping micrographs of phalloidin and DAPI staining of L-MSCs (G) and D-MSCs (H). Phase contrast micrograph (A and B); fluorescence microscopy micrograph (C–H). Scale bars: (A–B) 200 µm; (C–H) 100 µm.

In view of the higher hydrolysis of AMP when compared to ADP and ATP, we decided investigate the expression of the CD73, one of the most important enzyme that catalyze AMP hydrolysis in cell membrane [Bavaresco et al., 2008]. The presence of CD73 marker on surface of cells was confirmed by flow cytometry. The positive cells percentage average was 51.05% ⊥ 5.07 and 82.22 ⊥ 0.54 for L-MSCs and D-MSCs, respectively (Fig. 4E and F).

ECTONUCLEOTIDASES GENE EXPRESSION IN L-MSCS AND D-MSCS

Four candidate genes were tested to find the best normalizing gene for analyze the relative amount of NTPDases mRNA. The TBP, B2M, and ACTβ show the efficiency, and a slope within the parameters of a good reaction (efficiency: 90–110%; slope: −3.10 to Q7 −3.58) and the R^2 was greater than 0.99, except for the gene ACTβ ($R^2 = 0.979$). Genes that exhibit the lowest CV and SD, as well as, MFC lower than 2 are considered the most stable genes (Table IV) [Zhu et al., 2013].

In our case, these parameters were not enough to choose the best normalizing gene, so we use the NormFinder to select the most stable candidate gene. NormFinder software ranks all reference gene candidates based on intra- and inter-group variations and combines both results into a stability value for each candidate gene [Zhong et al., 2011]. Q8 The most stable genes were

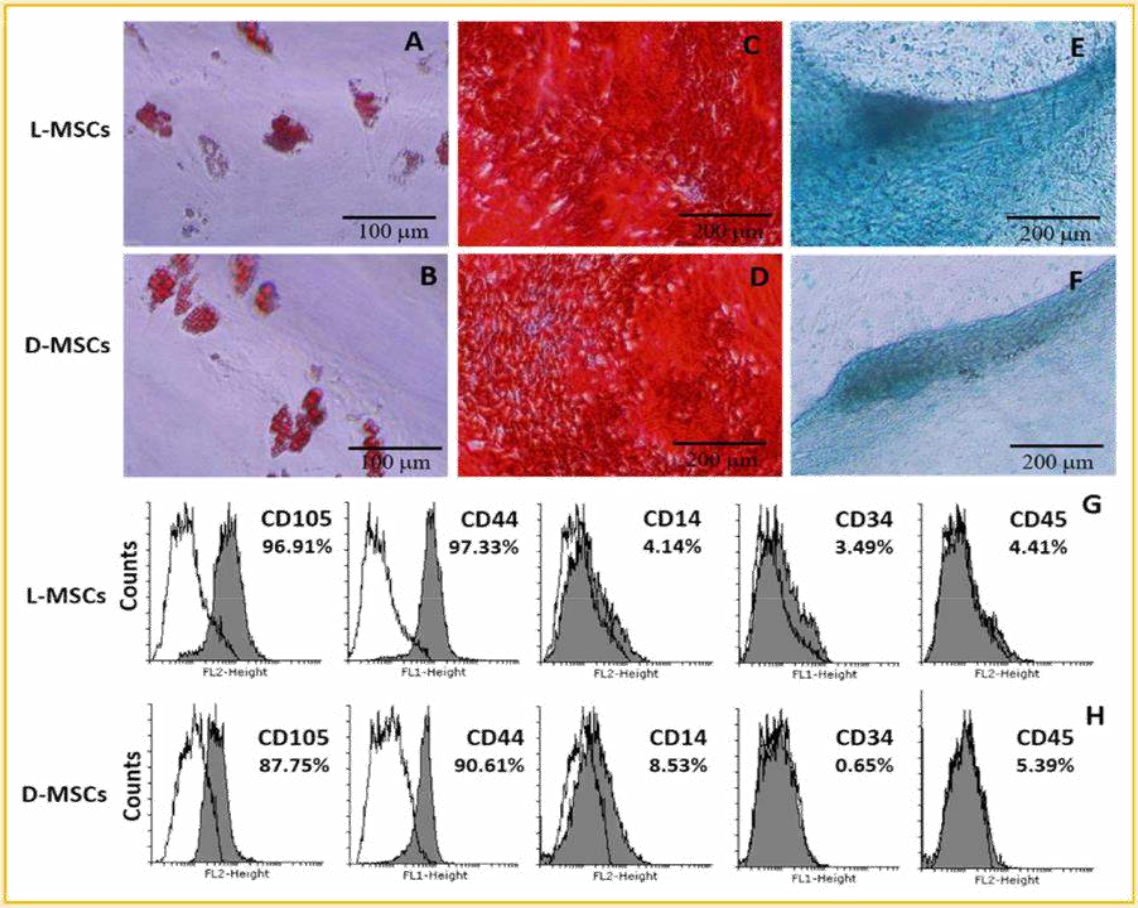

Fig. 3. D-MSCs and L-MSCs characterization. L-MSCs (A) and D-MSCs (B) differentiated into adipocytes showing the lipid vacuoles stained with Oil Red. L-MSCs (C) and D-MSCs (D) differentiated into osteoblasts showing the bone matrix stained by Alizarin Red. Alcian blue staining demonstrates positive glycosaminoglycan production in L-MSCs (E) and D-MSCs (F) cultures. Flow cytometry histograms show the expression (gray curves) of selected molecules ($CD105^+$, $CD44^+$, $CD14^-$, $CD34^-$, and $CD45^-$) by L-MSCs (G) and D-MCSc (H) populations compared with controls (white curves). Phase contrast micrograph. Scale bars: (A–B) 200 µm; (C–H) 100 µm.

TBP>ACTβ>B2M>GAPDH (Table V). All RT-qPCR assays produced a single peak of fluorescence in the melting curve (Figs. 5 and 6A). The quantification of mRNA expression levels showed that the L-MSCs and D-MSCs expressed the *ENTPD1*, *ENTPD2*, *ENTPD3*, *ENTPD5*, *ENTPD6*, *CD73*, *ENPP1*, and *ENPP2* in different levels. While the D-MSCs expressed higher mRNA levels of *ENTPD1* L-MSCs express higher levels of *ENTPD3* (Fig. 6B). In addition, the expression of *ENTPD8*, *ENPP3*, and *ALPL* was not observed in in L-MSCs and D-MSCs (Fig. 7B).[Q9]

DISCUSSION

Several clinical trials evaluate the use of MSCs in tissue engineering and cell-based therapies of various pathological conditions due to their potential for differentiation and tissue regeneration [Salem and Thiemermann, 2010].[Q10] Numerous studies have demonstrated the role of extracellular nucleotides in cellular biological processes. Recently, extracellular nucleotides have been included among the molecular signals produced by MSCs, demonstrating their importance in the maintenance, proliferation, and differentiation of these cells [Glaser et al., 2012; Scarfì, 2014].

The MSCs present purinergic receptors and ecto-enzymes that allow the control of nucleotide concentration [Iser et al., 2014]. The enzymatic hydrolysis of extracellular nucleotides allows the adjustment of signals, modulation of physiology, or specification of differentiation lineages [Roszek et al., 2015]. To evaluate the expression of ecto-nucleotidases and the degradation of extracellular nucleotides in human dermal and limbal mesenchymal stem cells, first, we isolate and characterize these cells from each discard tissues, showing their potential for multilineage differentiation. Branch et al. [2012] demonstrated that mesenchymal stem cells in the human corneal limbal stroma have the trilineage differentiation potential. Similarly, it has been demonstrated that Dermal MSCs have potential to differentiate on mesenchymal lineages [Toma et al., 2005; Nagel et al., 2013], which are in accordance with our results (Fig. 3A–F). Moreover, adherent skin-origin MSCs express markers specific for mesenchymal stem cell lineages CD73, CD90, and CD105, and are

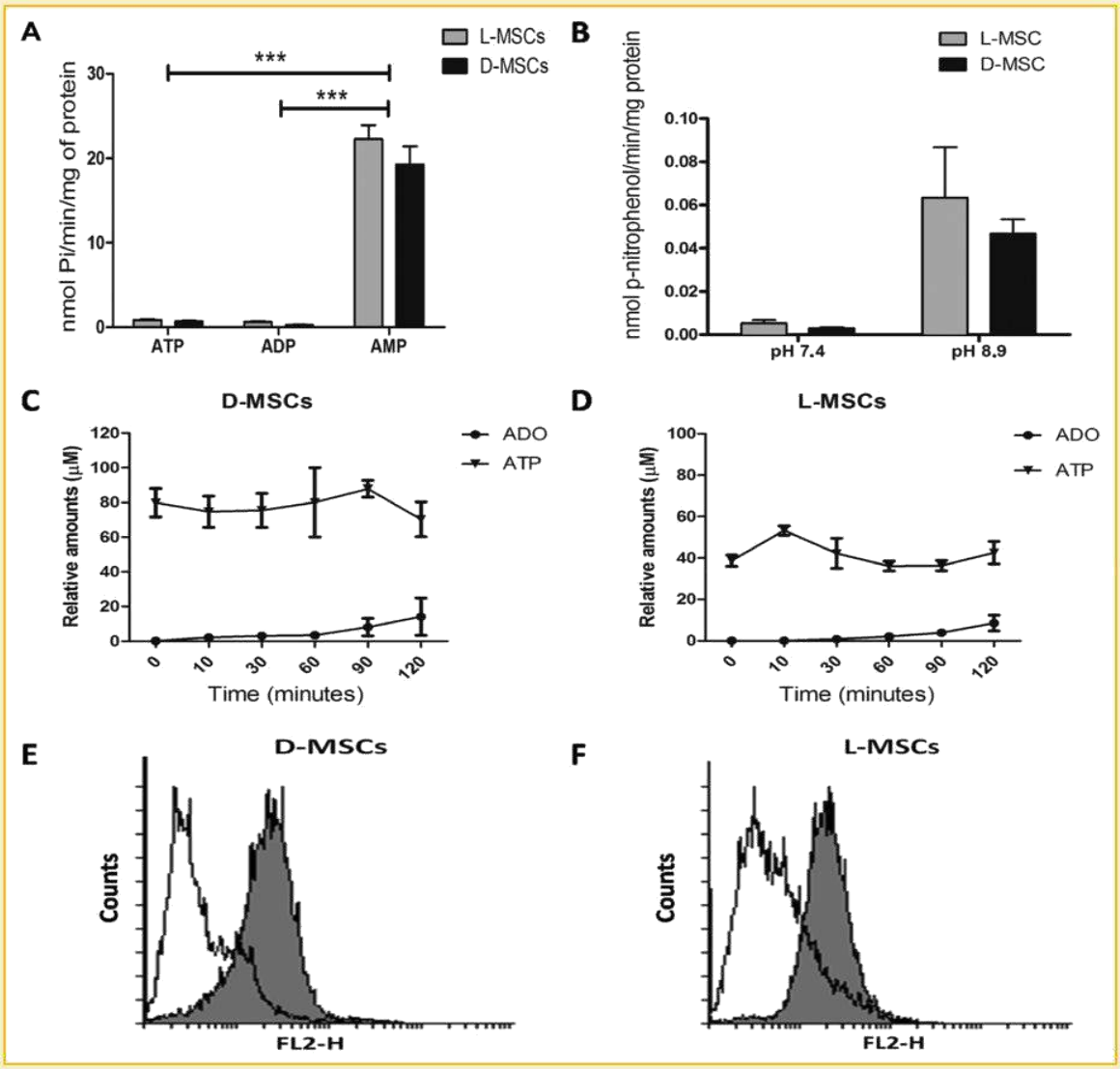

Fig. 4. Ectonucleotidase activities, metabolism of extracellular ATP and CD73 expression in D-MSCs and L-MSCs cultures. D-MSCs and L-MSCs cultures hydrolyzed AMP at significantly higher rates than ATP and ADP $P < 0.05$ (A). The results are expressed as specific activity (nmol Pi/min/mg of protein). The rate of P-Nph-5′-TMP hydrolysis by E-NPPs was very low in D-MSCs and L-MSCs (B).The metabolism of ATP and generation of adenosine (ADO) was determined after separation by HPLC (C and D). The low metabolism of ATP by both cell typeswas confirmed. However, all ATP degraded was converted in adenosine, which confirms the results of the enzymatic activity. The presence of CD73 enzyme on surface of cells was confirmed by flow cytometry (E and F). The positive cells percentage average was 51.05% + 5.07 and 82.22 + 0.54 for L-MSCs and D-MSCs, respectively. Bars represent means + SEM of three independents experiments performed in triplicate of each tissue. Data were analyzed statistically by one-way ANOVA followed by Tukey post-hoc test. * Different from AMP hydrolysis.

TABLE III. Specific Activities for the Hydrolysis of the ATP, ADP, AMP, and P-Nph-5′-TMP in D-MSCs and L-MSCs

	Specific activities (nmol Pi/min/mg of protein)			Specific activities (pnitrophenol/min/mg of protein)
Cell type	ATP	ADP	AMP	p-Nph-5′-TMP
D-MSCs	0.7388 ± 0.281	0.2818 = 0.1278	21.01 = 4.77	pH 7.4 = 0.003 ± 0.0005 pH 8.9 = 0.046 ± 0.0066
L-MSCs	0.8672 ± 0.201	0.6457 = 0.00676	22.27 = 2.84	pH 7.4 = 0.005 ± 0.0014 pH 8.9 = 0.063 ± 0.0230

Results are expressed as mean ± standard deviation.

TABLE IV. qPCR Parameters Providing the Standard Curve and Dispersion Data of Raw Cq Values for Each Primer Pair on Reference Genes for Each Cells

Symbol gene	Cells	Efficiency	R^2	Slope	Average	SD	CV%	MFC
TBP	D-MSCs	101.99	0.99	3.275	34.27	3.53	0.10	1.23
	L-MSCs	101.99	0.99	−3.275	27.26	0.62	0.02	1.04
GAPDH	D-MSCs	118.78	0.997	−2.941	23.99	2.52	0.10	1.20
	L-MSCs	118.78	0.997	−2.941	21.33	0.83	0.04	1.08
B2M	D-MSCs	104.87	0.991	−3.21	22.30	2.02	0.09	1.19
	L-MSCs	104.87	0.991	3.21	20.71	1.58	0.08	1.16
ACTβ	D-MSCs	102.59	0.979	3.261	28.86	1.45	0.05	1.11
	L-MSCs	102.59	0.979	−3.261	28.01	1.13	0.04	1.08

R^2, Correlation coefficient; SD, standard deviation; CV, coefficient of variation; and MFC, maximum fold change (the ratio of the maximum and minimum values).

TABLE V. Candidate Reference Genes for Normalization of RT-qPCR in L-MCSs and D-MSCs, According to Their Stability, as Calculated by NormFinder

Symbol gene	Stability value	Best gene
TBP	0.190	0.190
ACTβ	0.192	
B2M	0.408	
GAPDH	0.635	

negative for hematopoietic markers, including CD34, CD45, CD14, CD31, and HLA-DR [Klimczak and Kozlowska, 2016]. Markers associated with the phenotype of tissue-specific stromal cells have been identified. They are localized in the basal layer of epidermis and in the epithelium of adnexal structure of the skin [Klimczak and Kozlowska, 2016]. Shaharuddin et al. [2016] showed that limbal mesenchymal stromal cells expressed cell surface antigens associated with human MSC, while having no/low expression of negative hematopoietic lineage markers. Likewise, our result show that L-MSCs and D-MSCs express matrix receptors (CD44, CD105), but not/low hematopoietic lineage markers (CD34, CD45, CD14) (Fig. 3G and H).

The ACTβ and GAPDH genes have been widely used [Iser et al., 2015]. But Huggett et al. [2005] published that these genes are inappropriate due to their high variability in biological samples. Therefore, to evaluate the mRNA expression of ectonucleotidases in L-MSCs and D-MSCs, we first tested four candidate genes to find the

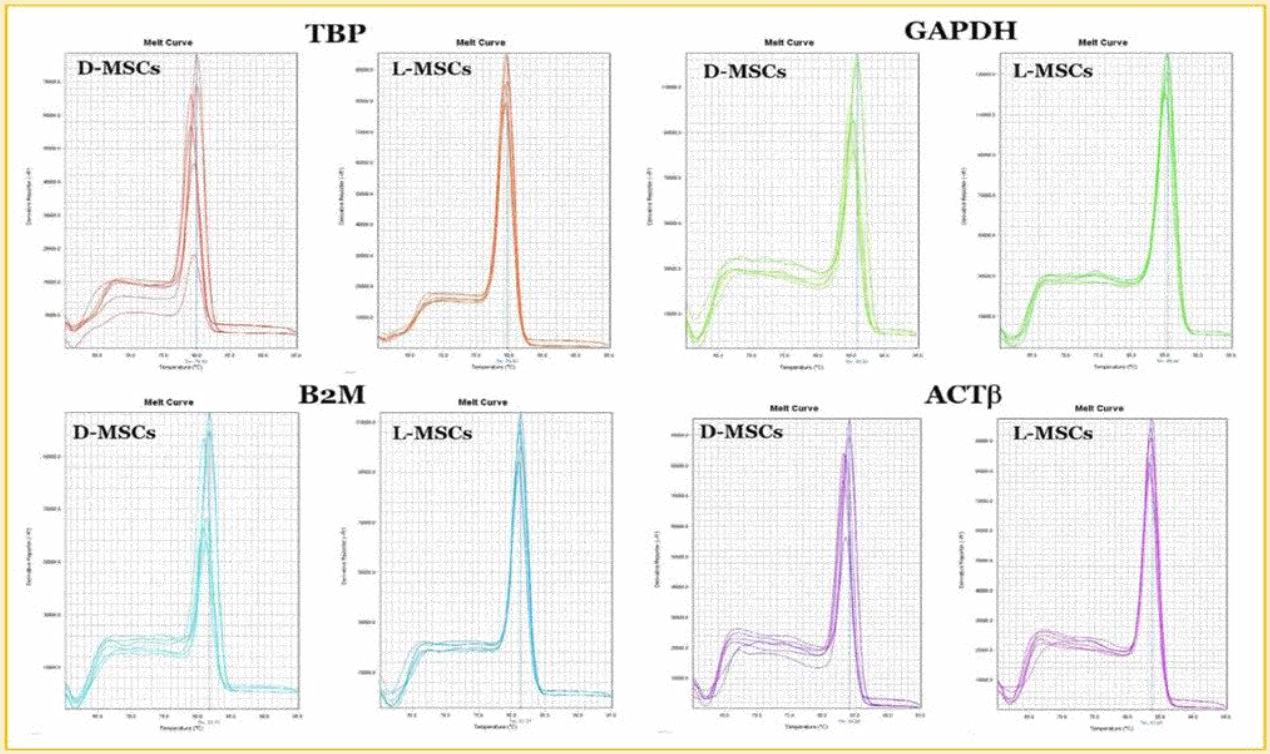

Fig. 5. Melting curves of the four reference genes for L-MSCs and D-MSCs. Melt curve peak chart collected using the StepOnePlusTM (Applied Biosystems).

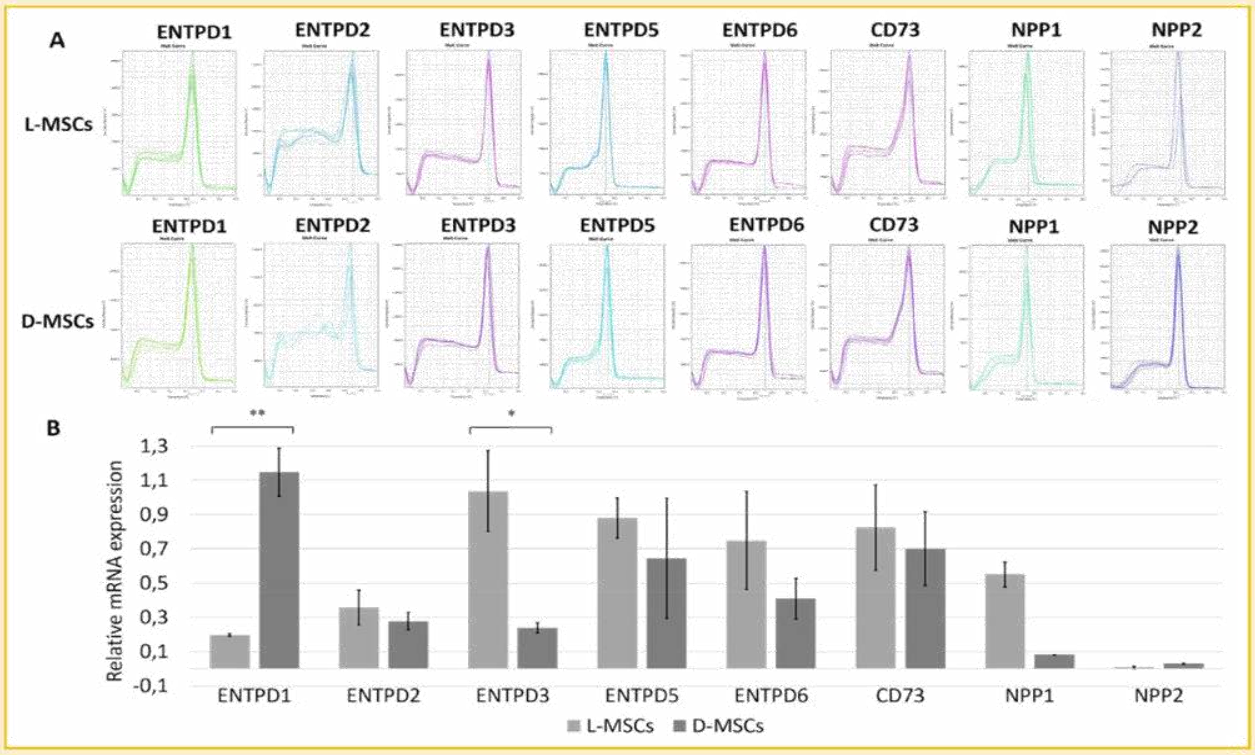

Fig. 6. Melting curves of the NTPDases genes (A) and quantification of ENTPD mRNA (B) expressed in L-MSCs and D-MSCs. The L-MSCs and D-MSCs expressed the enzymes *ENTPD1*, *ENTPD2*, *ENTPD3*, *ENTPD5*, *ENTPD6*, *ENPP1*, *ENPP2*, and CD73. The expression of *ENTPD8*, *ENPP3*, and *ALPL* was not observed. Bars represent mean ± SEM (n = 3). * *P*-value < 0.05 (*t*-test).

best normalizing gene. After analyzing the intra- and inter-group variations into a stability value for each candidate gene, we observed that TBP gene showed the best stability value (Table V).

Real-time PCR analysis showed that these cells, obtained from different donors, express mRNA of *ENTPD 1*, *2*, *3*, *5*, and *6*, plus *CD73*, what give them the capacity to promote the complete degradation of ATP until the adenosine. The D-MSCs expressed higher levels of *ENTPD1*, while the L-MSCs expressed *ENTPD3*, both NTPDase members that are capable to hydrolyze ATP and ADP to AMP (in ratios of 1:1 and 1:3, respectively). This can explain why both cell types showed a similarly ATPase activity. Nevertheless, the relation between the levels of expression of these enzymes does not imply their activity to degrade nucleotides, considering that they can be modulated by translation or post-translational regulation [Wink et al., 2000].

There is a growing interest in characterize the function of ectonucleotidases expressed on the surface of mesenchymal cells in recent years [Scarfì, 2014]. In the majority of cells, ATP acts as an antiproliferative and pro-inflammatory agent, so it should be efficiently hydrolyzed [Coppi et al., 2007]. However, in this study, when we incubated the L-MSCs and D-MSCs with ATP in various times and analyzed by HLPC, we observed a low degradation of ATP and the appearance adenosine at the end of reaction, but we observe a slight increase in ATP concentration in the last time points of the reaction (Fig. 4B and C). Interestingly, Roszek et al. [2015], when incubated the umbilical cord-derived human MSCs with ATP, also observed an increase in ATP concentration, after 3 h of incubation. These researchers argue that this effect might be due the release of intracellular ATP. In addition, that the low ATPase activity would be due the importance of ATP signaling in MSCs, an important signaling for their mobilization, anti-inflammatory action, differentiation, and cell regeneration. This explanation is in accordance with the work of Ferrari et al. [2011], which showed that MSCs are resistant to the cytotoxic effects of ATP and that cells stimulated with ATP has an increased in homing capacity and upregulation of genes involved in cell migration.

The results presented in this article indicate that the rate of AMP hydrolysis is higher in L-MSCs and D-MSCs, when compared with the hydrolysis of ATP and ADP, leading to adenosine formation. In addition, the presence of the enzyme CD73 was confirmed through the expression of the CD73 marker, by flow cytometry, in both cell types. Adenosine through the ligation of adenosine receptors has an essential role in regulating inflammation and tissue repair. Evans et al. [2006], evidenced for the first time, the presence of the adenosine receptor and its function on the surface of MSCs, demonstrating the formation of extracellular adenosine. Katebi et al. [2009], showed that adenosine signaling affects proliferation and

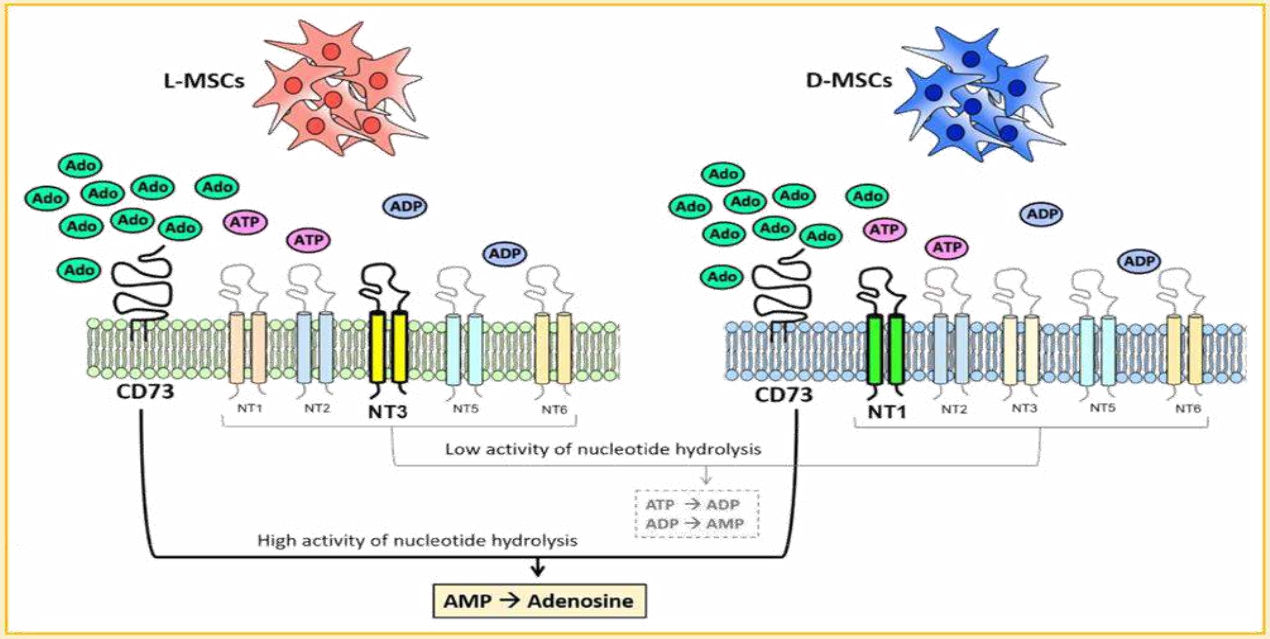

Fig. 7. Schematic representation of nucleotide hydrolysis profile in human limbal (L-MSCs) and dermal mesenchymal stem cell (D-MSCs). In our molecular analysis, L-MSCs and D-MSCs express CD73 and NTPDases (NT) 1,2,3,5, and 6. Interestingly, comparing both cell types, L-MSC expresses higher levels of NT3 mRNA, while D-MSCs expresses higher levels of NT1. However, in our enzymatic activity analysis, we demonstrated that in the cells analyzed, the ATP and ADP degradation is low when compared to AMP hydrolysis. Here, we hypothesized that although there are differences in mRNA expression of NTPDases between these MSCs, probably their ability of nucleotide degradation is of less important in these cells in relation to AMP hydrolysis. Moreover, it is important to consider that mRNAs could undergo post-transcriptional modifications or other mechanisms that could alter the production of proteins within cells. At the end, as result of the high hydrolytic activity of the CD73 enzyme, there will be an abundant production of adenosine, which in turn, can exert many roles in the extracellular space interacting with other cells.

development of BMMSCs, and by targeting the A2AR receptor, could increase the proliferation of MSCs. Additionally, studies in vitro and in vivo evaluated the contribution of adenosine signaling in MSC differentiation [Gharibi et al., 2011].

Furthermore, MSCs possess immunoregulatory functions and can attenuate various types of immune responses [Parekkadan et al., 2008]. The regenerative effects depend of their ability to regulate inflammation and tissue homeostasis, by secreting a number of immunosuppressive factors, such as cytokines and growth factors. Thus, inhibiting inflammatory responses and facilitating the proliferation and differentiation of cells in tissues [Cavaliere et al., 2015]. The immunomodulatory activity has also been related to the CD39/CD73 cascade that actively produces extracellular adenosine, which has paracrine and immunosuppressive effects in these cells [Chen et al., 2013]. The anti-inflammatory effects are produced by blocking the proliferation of T-lymphocytes through the A2A subtype and the addition of A2A antagonists or CD39 inhibitors significantly counteracts this effect [Lee et al., 2014]. The importance of these findings is shown by the result obtained with the A2A-receptor agonist, polydeoxyribonucleotide (PDRN) that nearly doubled the rate of healing of diabetic foot ulcers, when compared with placebo [Squadrito et al., 2014]. Chen et al. [2016] investigated the role of the CD73/adenosine pathway in immune modulation by MSCs using a mouse model of experimental autoimmune uveitis. They demonstrate that this pathway is involved in the immunomodulatory function of MSCs in autoimmune responses. This confirms the essential role of extracellular adenosine in the physiology of MSCs, stimulating its properties to immunomodulatory and pro-healing, suggesting a possible use of this nucleoside or its agonists in cellular therapies.

In conclusion, we showed that considering the degradation of ATP and adenosine production, limbal MSCs are very similar to dermal MSCs, indicating that from the aspect of extracelular nucleotide metabolism, L-MSCs are very similar to the characterized D-MSCs. Thus, limbal cells can be another source of MSCs to be studied in pre-clinical models and potentially clinical studies.

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CAPITULO III

DISCUSSÃO

Devido ao potencial de diferenciação e capacidade imunomoduladora, as MSCs têm sido consideradas como uma fonte potencial de células para seu uso em bioengenharia de tecidos e terapias celulares (Salem and Thiemermann 2010). Neste trabalho foi possível isolar células mesenquimais de materiais que geralmente são descartados nos centros hospitalares. Estima-se que a necessidade anual de transplantes de córnea no Brasil seja de 17.168 transplantes (Bonfadini et al. 2014). Após o transplante de córnea, o tecido circundante (limbo esclerocorneal) é descartado, a partir do qual é possível isolar, com técnicas de cultivo celular, as células tronco que participam na regeneração da córnea (Dravida et al. 2005). Do mesmo modo, após uma cirurgia de abdominoplastia, o excesso de pele retirada também é considerada material de descarte, e da mesma forma é possível isolar células tronco não só mesenquimais dérmicas, mas também queratinócitos, melanócitos e células mesenquimais adiposas.

Neste trabalho as células mesenquimais foram isoladas por migração, após uma digestão enzimática que permitiu retirar o epitélio das amostras. Em aproximadamente duas semanas em cultura, foi possível observar as células migrando a partir dos fragmentos de tecido, as quais alcançaram confluência em 25 dias de cultura. As células isoladas de cada tecido mostraram seu potencial de diferenciação em linhagens osteogênicas, adipogênicas e condrogênicas. Além disso, apresentaram a expressão de marcadores de superfície mesenquimais (CD105 e CD44), mas não hematopoiéticos (CD14, CD34, CD45), confirmando a sua identidade tronco, conforme descrito na literatura (Branch et al. 2012; Toma et al. 2005).

Nos últimos anos têm aumentado o interesse em desvendar as funções das ectonucleotidases expressas na superfície das células mesenquimais. Isso porque os nucleotídeos e seus produtos de hidrólise podem exercer uma diversidade de efeitos nessas células (Roszek et al. 2015; Scarfì 2014). Neste estudo foi caraterizada a expressão genica das ectoenzimas, demostrando-se que as L-MSCs e D-MSCs expressam mRNA das ENTPDases 1, 2, 3, 5, 6, CD73, ENPP1 e 2, o que lhes dá a capacidade de promover a degradação de ATP até a adenosina. No entanto, foi observado que estas células degradaram

baixos níveis de ATP e ADP em comparação com o AMP. Também foi avaliada a atividade fosfodiesterase das NPP usando-se como substrato artificial o p-nitrofenil timidina 5 'monofosfato (p-Nph-5'-TMP). Nossos resultados evidenciaram uma baixa atividade enzimática das NPPs em L-MSCs e D-MSCs, o que indica que estas enzimas apresentam pouca participação na degradação de ATP nessas células.

A baixa atividade ATPasica poderia ser pela importância que o ATP tem na sinalização das MSCs, modulando a sua mobilização, diferenciação e regeneração celular. Esta explicação está de acordo com o trabalho de Ferrari Davide et al. 2011 que mostrou que MSCs são resistentes aos efeitos citotóxicos do ATP. No entanto, a alta hidrolise de AMP, possivelmente pela atividade da ecto-5'-nucleotidase (CD73), expressa na superfície das L-MSCs e D-MSCs, poderia estar relacionada com o papel que tem a adenosina na atenuação da inflamação e cicatrização de tecidos (Scarfì 2014). Também tem se demostrado que a adenosina tem efeitos na proliferação e diferenciação das MSCs (Gharibi et al. 2011; Katebi, Soleimani, and Cronstein 2009).

As MSCs têm propriedades imunomoduladoras (Parekkadan, Tilles, and Yarmush 2008) e foi investigado o efeito da via de sinalização de CD73/adenosina em modelo animal de uveíte autoimune experimental, demostrando que esta via está envolvida na função imunomoduladora das MSCs em respostas autoimunes. Isto confirma o papel essencial da adenosina extracelular na fisiologia das MSCs, estimulando as suas propriedades imunomoduladoras e pró-cicatrizantes, sugerindo um possível uso deste nucleosídeo ou dos seus agonistas em terapias celulares(Chen X, Shao H, Zhi Y, Xiao Q, Su C, Dong L, Liu X, Li X 2016).

Tanto o ATP como a adenosina têm demostrado papéis pleiotrópicos que afetam tanto as propriedades imunomoduladoras as MSCs como seu comprometimento com a diferenciação nas linhagens. O ATP tem sido mais associado à inibição da proliferação, propriedades proinflamatórias e de migração celular. Por outro lado, a adenosina tem sido associada a uma atividade autócrina, protetora bem como a uma atividade imunossupressora parácrina, contrariando a estimulação de ATP.

Portanto, o conhecimento do papel dos nucleotídeos e nucleosídeos é essencial para a compreensão da fisiologia das MSCs que, num futuro próximo, permitirá definir o envolvimento destas células na reparação de tecidos.

CONCLUSÃO GERAL

Nesse estudo padronizamos o isolamento de células mesenquimais do limbo esclerocorneal (L-MSCs) e da derme (D-MSCs), ambos considerados tecidos de descarte, as quais demonstraram o potencial de diferenciação multilinhagem. Ambos os tipos celulares, L-MSCs e D-MSCs, expressaram mRNA para as E-NTPD1, 2, 3, 5, 6, CD73, E-NPP1 e 2, e hidrolisam baixos níveis de p-Nph-5'-TMP, ATP e ADP em comparação com AMP.

CONCLUSÕES ESPECÍFICAS

1. Foi padronizado o isolamento e cultivo de células tronco mesenquimais a partir de materiais de descarte humano, como pele e tecido esclerocorneal. As células iniciaram a migração desde os tecidos em aproximadamente 15 dias de cultivo e alcançaram a confluência aos 25 dias de cultivo.
2. As células isoladas mostraram a morfologia fusiforme em cultura e as populações de L-MSCs e D-MSCs foram positivas para marcadores de células mesenquimais (CD105 e CD44) e negativas para marcadores hematopoiéticos (CD14, CD34, CD45).
3. As L-MSCs e D-MSCs mostraram o potencial de diferenciação multilinhagem ao se diferenciar nas linhagens osteogênica, adipogênica e condrogênica.
4. O melhor gene normalizador para avaliar a expressão gênica nas L-MSCs e D-MSCs foi o TBP. Estas células expressam mRNA para as *E-NTPD1, 2, 3, 5, 6, CD73, E-NPP1 e 2* e não foi observada a expressão da *E-NTPD8, E-NPP3* e fosfatase alcalina em ambos tipos celulares.
5. A baixa hidrólise do substrato artificial das NPPs, p-Nph-5'-TMP, mostra que essas enzimas não possuem uma participação na hidrólise do ATP.
6. As L-MSCs e D-MSCs hidrolisam níveis baixos de ATP e ADP extracelular e altos níveis de AMP, que levam a formação de adenosina.
7. As L-MSCs e D-MSCs expressam a enzima CD73 na superfície celular.

PERSPECTIVAS

Este trabalho proporciona várias perspectivas de investigação para um maior conhecimento destas MSCs, de forma que possam ser consideradas em estudos potencialmente clínicos, como:

1. Investigar a expressão dos receptores purinérgicos nas células para complementar sua caracterização.
2. Avaliar o papel do ATP e da adenosina na biologia das MSCs.
3. Isolar demais tipos celulares (quetarinócitos, melanócitos) e desenvolver um modelo tridimensional em conjunto com as células mesenquimais para estudar o funcionamento *in vivo* das células e o papel dos componentes da sinalização purinérgica nas lesões.

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ANEXOS

Parecer de aprovação do Comitê de Ética

IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE - ISCMPA

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise da sinalização purinérgica em queratinócitos e melanócitos humanos isolados de materiais de descarte.

Pesquisador: Marcia Rosangela Wink

Área Temática:

Versão: 1

CAAE: 47513315.0.0000.5335

Instituição Proponente: ISCMPA

Patrocinador Principal: MINISTERIO DA CIENCIA, TECNOLOGIA E INOVACAO

DADOS DO PARECER

Número do Parecer: 1.209.474

Apresentação do Projeto:

Trata-se de uma pesquisa de análises bioquímicas e de biologia molecular, onde serão recolhidos materiais de descarte das cirurgias plásticas de redução abdnominal e córneas impróprias para transplante por deformidades ou tecido de sobra das córneas após o transplante.

Trata-se de uma pesquisa de análises bioquímicas e de biologia molecular

Resumo:

A Pele não é apenas o maior órgão do corpo, e também um dos mais complexos e importantes para a proteção contra agressões externas, de forma semelhante, a córnea é um tecido avascular transparente que proporciona proteção e propriedades de refração da estrutura interna do olho. Ambos tecidos atuam como uma barreira contra agentes externos podem ser físicos, químicos, patogênicos ou microbianos geralmente. Devido à alta taxa de pacientes com patologias da pele e córnea como queimaduras, inflamação, cicatrização de feridas e câncer de pele, reforça a importância de terapias alternativas para o tratamento dessas patologias, Nucleotídeos e nucleosídeos de purinas extracelulares têm efeitos biológicos em uma variedade de tipos de células e tecidos. A sinalização purinérgica está envolvida em patologias de pele e da córnea e seu

Endereço: R. Profª Annes Dias,285 Hosp.Dom Vicente Scherer
Bairro: 6º andar - Centro **CEP:** 90.020-090
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3214-8571 **Fax:** (51)3214-8571 **E-mail:** cep@santacasa.tche.br