PATRICIA ROMAGNOLLI

Adhesion and modulation of mouse embryonic stem cells hepatocyte progeny on mouse placental extracellular matrix

São Paulo
2018

PATRICIA ROMAGNOLLI

Adhesion and modulation of mouse embryonic stem cells hepatocyte progeny on mouse placental extracellular matrix

Thesis submitted to the Postgraduate Program in Anatomy of Domestic and Wild Animals of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences

Department:

Surgery

Area:

Anatomy of Domestic and Wild Animals

Advisor:

Prof. Maria Angelica Miglino, Ph.D.

São Paulo

2018

DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO

(Biblioteca Virginie Buff D'Ápice da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo)

T. 3622
FMVZ

Romagnolli, Patricia
Adhesion and modulation of mouse embryonic stem cells hepatocyte progeny on mouse placental extracellular matrix. / Patricia Romagnolli. -- 2018.
75 p. : il.

Título traduzido: Adesão e modulação da progênie hepatocitária de células-tronco embrionárias de camundongos sobre a matriz extracelular placentária de camundongos.

Tese (Doutorado) - Universidade de São Paulo. Faculdade de Medicina Veterinária e Zootecnia. Departamento de Cirurgia, São Paulo, 2017.

Programa de Pós-Graduação: Anatomia dos Animais Domésticos e Silvestres.

Área de concentração: Anatomia dos Animais Domésticos e Silvestres.

Orientador: Profa. Dra. Maria Angelica Miglino.

1. Scaffolds placentários de camundongos. 2. Recelularização. 3. Cultura rotativa 3D. 4. Células-tronco embrionárias. 5. Células hepatocyte-like. I. Título.

Ficha catalográfica elaborada por Denise Yamashita, CRB-8/8931.

CERTIFICADO

Certificamos que a proposta intitulada "Adesão e Modulação da Progênie Hepatocitária de Células-Tronco Embrionárias Murinas sobre a Matriz Extracelular Placentária Murina", protocolada sob o CEUA nº 5669271015, sob a responsabilidade de **Maria Angélica Miglino** *e equipe; Patricia Romagnolli; Joaquim Mansano Garcia* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 06/04/2017.

We certify that the proposal "Adhesion and Modulation of Hepatocyte Progenie from Murine Embryonic Stem Cells on Murine Placental Extracellular Matrix", utilizing 15 Isogenics mice (15 females), protocol number CEUA 5669271015, under the responsibility of **Maria Angélica Miglino** *and team; Patricia Romagnolli; Joaquim Mansano Garcia* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 04/06/2017.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 11/2015 a 07/2017 Área: Cirurgia

Origem:	Animais provenientes de doação espontânea						
Espécie:	Camundongos isogênicos	sexo:	Fêmeas	idade:	70 a 100 dias	N:	15
Linhagem:	C57BL/6			Peso:	150 a 300 g		

Resumo: Tecido hepático produzido scaffolds placentários tem potencial para se tornar um produto da engenharia tecidual diretamente utilizado como adjuvante na Medicina Regenerativa, pois a placenta, descartada após o parto, é uma fonte de material biológico, que quando eficientemente decelularizada propicia matriz extracelular adequada para recelularização. Hepatócitos são células que sobrevivem em sítios heterólogos por um período razoável, permitindo que seu implante auxilie as limitações provocadas pela impossibilidade de transplantes. Serão decelularizadas placentas de camundongos, para obtenção de scaffolds, os quais serão validados através de testes específicos, para seu estabelecimento como Biomaterial Ativo Placentário. Células-Tronco Embrionárias murinas (Linhagem H106) serão utilizadas para recelularização das BAPs, nas quais serão simulados os eventos de diferenciação hepática embrionária, até a produção de hepatócitos e, portanto, tecido hepático. Na Fase I (d0 a d6), para simular o sinal cripto-nodal e induzir a gastrulação, diferenciação da linha primitiva, mesoderma e endoderma primitivo, serão adicionados Activin-A e WNT3a. A fim de conduzir a diferenciação do endoderma definitivo à especificação em destino hepático (hepatoblastos), na Fase II (d6-d10) será feita adição de FGF2 e BMP4. A Fase III (d10-d14), destinada a proliferação celular e a primeira etapa de maturação dos hepatoblastos recém-formados, serão adicionados FGF8b, FGF1 e FGF4. Na Fase IV (d14 a d35), as células serão induzidas ao fenótipo hepatócito maduro através da adição de HGN como indutor hepatotrófico geral e Follistatina-288, que tratará da diferenciação hepatocitária em favor dos colonócitos. Serão adicionados insulina e dexametasona, para indução da expressão gênica específica de hepatócitos maduros.

Local do experimento: Setor de Anatomia dos Animais Domésticos e Silvestres - Departamento de Cirurgia - FMVZ - USP

São Paulo, 24 de janeiro de 2018

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
Secretária Executiva
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

EVALUATION FORM

Author: ROMAGNOLLI, Patricia

Title: **Adhesion and modulation of mouse embryonic stem cells hepatocyte progeny on mouse placental extracellular matrix**

Thesis submitted to the Postgraduate Program in Anatomy of Domestic and Wild Animals of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences

Date: _____/_____/_____

Committee Members

Prof. _____

Institution:_____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution:_____ Decision: _____

Prof. _____

Institution:_____ Decision: _____

DEDICATION

To The Infinitude

“There are infinite numbers between 0 and 1. There's .1 and .12 and .112 and an infinite collection of others. Of course, there is a bigger infinite set of numbers between 0 and 2, or between 0 and a million. Some infinities are bigger than other infinities.”

John Green, The Fault in Our Stars

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my family, for the support given to my days away from home. I love you very much.

My gratitude to Professor Maria Angelica Miglino, for the opportunity of knowledge and evolution before science. Above all, for the friendship.

My sincere thanks to Professor Joaquim Mansano Garcia, for supplying the embryonic stem cells for this work, and for spending his time in benefit of my scientific training in cell culture.

I am very grateful to Professor Marcos Roberto Chiaratti, for granting biological samples for this research.

I warmly thank my dear friend Rodrigo da Silva Nunes Barreto, for his companionship dedication to our projects.

I would like to express my sincere gratitude to Raquel Castro, an angel who lent me her wings to rest, during the turbulence of my days.

I thank Professors and Technicians from Postgraduate Program in Anatomy of Domestic and Wild Animals of the School of Veterinary Medicine and Animal Science of the University of São Paulo.

I would like to thank São Paulo Research Foundation (FAPESP) (grant 14/50844-3), and National Council for Scientific and Technological Development (grant 467476/2014-4), for financial support.

“There is no time to wait for Darwinian evolution to make us more intelligent and better-natured”.

Stephen Hawking

RESUMO

ROMAGNOLLI, P. **Adesão e modulação da progênie hepatocitária de células-tronco embrionárias de camundongos sobre a matriz extracelular placentária de camundongos**. [**Adhesion and modulation of mouse embryonic stem cells hepatocyte progeny on mouse placental extracellular matrix**]. 2018. 75 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2018.

Pesquisas de diferentes campos ao redor do Mundo estão em busca de novas fontes tanto de biomateriais, quanto de potenciais hepatócitos, a fim de suprir testes de drogas, terapias celulares e transplante de células, como suporte terapêutico alternativo para doenças e lesões hepáticas. Placentas podem ser elegíveis como um novo modelo em Engenharia Tecidual em decorrência de sua rica matriz extracelular (ECM), e disponibilidade após o nascimento. Os scaffolds placentários foram produzidos por decelularização com SDS 0,01, 0,1 e 1% e Triton X-100 1%, os quais foram avaliados por meio da estrutura e composição. Posteriormente, os scaffolds placentários foram co-cultivados com fibroblastos embrionários de camundongos em um sistema rotativo tridimensional (3D). Os scaffolds placentários apresentaram uma MEC acelular bem conservada, contendo 9,42 ± 5,2 ng/dsDNA/mg/MEC. O fraco colágeno I nos nativos aparece claramente na MEC descelularizada, enquanto o colágeno III bem visível na placenta nativa estava ausente nos scaffolds. Esta observação interessante pode decorrido da solubilização das fibrilas de colágeno III, induzida pelo SDS durante a decelularização. A fibronectina foi bem observada nos scaffolds placentários, enquanto a laminina e o colágeno IV estiveram fortemente marcados. Recelularizados com fibroblastos por um sistema de cultura 3D, os scaffolds placentários mostraram potencial para repovoamento, com células aderidas ao longo de sua MEC acelular. Os scaffolds placentários foram então novamente recelularizados, visando agora a diferenciação de células tronco-embrionárias de camundongos em células hepáticas. Em um protocolo de 23 dias, foram simulados os grandes eventos do desenvolvimento embrionário do fígado, pela adição de fatores de crescimento. Como resultado, um alto índice de células aderiu, proliferou e migrou através das superfícies externa e interna dos scaffolds. A ausência de Oct4 e Nanog demostraram que o Activin A e o Wnt3a (d0-6) induziram o destino endoderma primitivo, e a marcação negativa para Foxa2 e Sox17 representaram a geração de

células endodermais definitivas pela diferenciação induzida por BMP4 e FGF2 (d6-10). Ainda, FGF1, FGF4 e FG8b (d10-14) induziram células do fenótipo hepatoblasto, que foram observadas positivas para os marcadores AFP e CK7. Finalmente, HGF e FS-288 (d14-23) induziram as células hepatocyte-like, positivas para os marcadores CK18 e Alb. The hepatocyte-like cells functional aspects were observed by glycogen storage. Though a heterogeneous cell hepatic lineage was confirmed, mouse placental scaffolds shown a useful model to support recellularization with simultaneous differentiation into hepatic fate simulating phases of embryonic development. Os aspectos funcionais das células hepatocyte-like foi observada pelo armazenamento de glicogênio. Embora uma linhagem hepática formada por células heterogêneas tenha sido confirmada, os scaffolds placentários de camundongos se mostraram um modelo útil para sustentar a recelularização com simultânea diferenciação em destino hepático, simulando fases do desenvolvimento embrionário.

Palavras-chave: scaffolds placentários de camundongos; recelularização; cultura rotativa 3D; células-tronco embrionárias; células *hepatocyte-like*

ABSTRACT

ROMAGNOLLI, P. **Adhesion and modulation of mouse embryonic stem cells hepatocyte progeny on mouse placental extracellular matrix**. [**Adesão e modulação da progênie hepatocitária de células-tronco embrionárias de camundongos sobre a matriz extracelular placentária de camundongos**]. 2018. 75 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2018.

Researches from different fields around the world are searching for both new sources of biomaterials and potential hepatocytes in order to supply drug tests, cell therapies, and cell transplantation as alternative therapeutic support to liver diseases and injuries. Placenta may be eligible as a new model in tissue engineering due to its rich extracellular matrix (ECM) and availability after birth. Placental scaffolds were produced by decellularization with 0.01, 0.1 and 1% SDS, and 1% Triton X-100 which were valued by means of structure and composition. Afterwards, placental scaffolds were co-cultured with mouse embryonic fibroblasts in a tridimensional (3D) rotating system. Placental scaffolds presented a well-preserved acellular ECM containing 9.42 ± 5.2 ng dsDNA per mg of ECM. Weak collagen I of the natives clearly appears in decellularized ECM while the collagen III, once well observed in native placenta, it was absent on scaffolds. This interesting observation may have been due to the solubilization SDS-induced of the collagen III fibrils during decellularization. Fibronectin was well-observed in placental scaffolds whereas laminin and collagen IV were strongly stained. Recellularized with fibroblasts by a 3D culture system, placental scaffolds showed potential for repopulation, with cells adhered throughout its acellular ECM. Placental scaffolds were then newly recellularized, aiming now for differentiation of mouse embryonic stem cells into hepatic cells. In a protocol of 23 days, it was simulated major events of liver embryonic development by adding growth factors. As result, a high index of cells adhered, proliferated and migrated throughout outer and inner scaffolds ECM surface. Absence of Oct4 and Nanog showed that Activin A and Wnt3a (d0-6) induced primitive endoderm fate, and negative label for Foxa2 and Sox17 representing BMP4 and FGF2 (d6-10) differentiation-induced generating definitive endoderm cells. Also, FGF1, FGF4 and FG8b (d10-14) induced hepatoblast phenotype cells, that were observed positive for AFP and CK7 markers. Finally, HGF and FS-288 (d14-23) induced to hepatocyte-like cells, positive for CK18 and Alb markers. The

hepatocyte-like cells functional aspects were observed by glycogen storage. Though a heterogeneous cell hepatic lineage was confirmed, mouse placental scaffolds shown a useful model to support recellularization with simultaneous differentiation into hepatic fate simulating phases of embryonic development.

Keywords: mouse placental scaffolds; recellularization; rotating 3D culture; embryonic stem cells; hepatocyte-like cells

LIST OF FIGURES

MOUSE PLACENTAL SCAFFOLDS: A THREE-DIMENSIONAL ENVIRONMENT MODEL FOR RECELLULARIZATION

MOUSE PLACENTAL SCAFFOLDS: A MODEL TO CULTURE HEPATOCYTE-LIKE CELLS INDUCED FROM MOUSE EMBRYONIC STEM CELLS IN A THREE-DIMENSIONAL DYNAMIC AND ROTATING SYSTEM

SUMMARY

1 INTRODUCTION

In the field of health sciences, Regenerative Medicine emerged with the goal of restoring cells, tissues, and organs in order to regenerate normal function (KATARI; PELOSO; ORLANDO, 2015; LONDONO; BADYLAK, 2014). Offering support to regeneration intents, Tissue Engineering deals with manufacturing of body parts ex vivo by seeding cells on and/or into a supporting scaffold (KATARI; PELOSO; ORLANDO, 2015).

Challenged to mimic what happens in nature (O'BRIEN, 2011), Tissue Engineering combines multidisciplinary elements (ALLAN, 1999; KEANE; BADYLAK, 2014) in order to produce functional replacements for damaged tissues and organs (DE KEMP et al., 2015; JANIK; MARZEC, 2015; KATARI; PELOSO; ORLANDO, 2015; RAHMANI DEL BAKHSHAYESH et al., 2017). Therefore, engineered tissues become an advantageous model system for biological and medical research for the purpose of getting more realistic answers to biological questions (FREED et al., 2006).

The 'material to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body' is defined by European Society for Biomaterials as 'biomaterial'. As part of the engineered tissues system, the term 'scaffold' refers to the biomaterial before cells addition (O'BRIEN, 2011) as well as tridimensional support for cell growth (KEANE; BADYLAK, 2014).

Scaffolds from biological sources became an alternative environment to the conventional implantation of organs and tissues (JANIK; MARZEC, 2015) in addition to cell attachment, maintenance, proliferation, and differentiation (BADYLAK; TAYLOR; UYGUN, 2011; KEANE; BADYLAK, 2014).

The constant flow state of biological scaffolds is suitable for cells functionalities in the presence of proper growth factors (RHEE, 2009), constituting an environment for long-term nutrients and oxygen diffusion (HOSSEINKHANI; MEHRABANI; KARIMFAR, 2014).

Biological scaffolds are produced by decellularization of allogeneic or xenogeneic organs and tissues (BADYLAK et al., 2012; SCHNEIDER et al., 2016;

TEODORI et al., 2014), i.e. skin, intestinal submucosa, urinary bladder, blood vessels, heart valves, and placenta, among others (BADYLAK; TAYLOR; UYGUN, 2011; REING et al., 2009). After removing original cell population, decellularization result must include preservation of the three-dimensional structure, architecture, and components of its extracellular matrix (FU et al., 2014), essential for further cell repopulation (BADYLAK; TAYLOR; UYGUN, 2011; DE KEMP et al., 2015).

Structure and composition of extracellular matrix are dynamic systems, continuously transformed by cell metabolism, mechanical demands, and prevailing conditions in the microenvironment (BADYLAK; TAYLOR; UYGUN, 2011). Proteins composition of extracellular matrix are adjuvant in cell behavior, including differentiation (BADYLAK; TAYLOR; UYGUN, 2011; DE KEMP et al., 2015; PARK et al., 2015) as observed in the signaling pathways of both embryonic stem cells, and their progenies (HUANG et al., 2015).

Therefore, the choice of a suitable scaffold will guide cells (DEBELS et al., 2015) during construction of tissues (XU et al., 2015) according to the purposes of Regenerative Medicine (REING et al., 2010).

During appropriate cell type search for culture system on intended tissue-engineered construction (BALDWIN et al., 2014), the advent of stem cell research had sparked a new direction in Regenerative Medicine (FRANCIPANE; LAGASSE, 2016) aiming therapies for injured organs and tissues (RAHMANI DEL BAKHSHAYESH et al., 2017).

Embryonic stem cells are derived from inner cell mass of blastocyst (BHARTIYA, 2015) which are cells with pluripotential nature (BISWAS; HUTCHINS, 2007), capable of self-renewal and differentiation into variety of cell lineages (BAHARVAND et al., 2006), and mature cell types (HOSSEINKHANI; MEHRABANI; KARIMFAR, 2014; YAMAMOTO et al., 2003).

One challenge in stem cell therapy is to control cell fate and phenotype (HUANG et al., 2015) once the environment contributes significantly to the regulation of stem cells (HUANG et al., 2015) as well as mechanical forces (FREED et al., 2006).

Since hepatocytes are one of the many cell types derived from embryonic stem cells, and yet do not proliferate sufficiently, or maintained their differentiated properties

in vitro (BAHARVAND et al., 2006), research across the world is very interested in stem cells differentiation into hepatocytes (YAMAMOTO et al., 2003) as a promising source of hepatocytes (WANG et al., 2012).

Early protocols for hepatocytes generation from embryonic stem cells included embryoid body formation as a result from differentiation of pluripotent cell into a mixed of cells from the three germ layers (SILLER et al., 2015). Achieving the embryoid body condition, hepatocytes were then differentiated by supplementing medium with specifics cytokines and growth factors for hepatic faith (WANG et al., 2012).

Recently, growth factors for stem cells differentiation into mesoderm and ectoderm faith were suppressed in favor of endoderm differentiation (SILLER et al., 2015) (BAHARVAND et al., 2006; SCHWARTZ et al., 2014). This is a significant advance in hepatic faith specification once endodermal differentiation subsequent hepatic cell lineage can be induced.

One step ahead, hepatocyte culture differentiation protocols became to be designed using cytokines, in order to simulate the original and natural stages of embryonic liver development (PAUWELYN et al., 2011; ROELANDT et al., 2010).

Based on the usage of cytokines to mimic hepatocyte embryonic development, human and mouse stem cells were cultured into four-steps protocol: 1) Activin-A and Wnt3a for induce primitive endoderm; 2) FGF2 and BMP4 to specify definitive endoderm; 3) FGF1, FGF4 and FGF8b for proliferation and first maturation of hepatoblasts; and 4) HGF to induce hepatocyte phenotype, and Follistatin-288 to favor hepatic at the expense cholangiocyte differentiation (PAUWELYN et al., 2011; ROELANDT et al., 2010).

Many methods concerning to the differentiation of embryonic stem cells into hepatic faith were reported. Most of them directly on conventional two-dimensional culture dish (JONES et al., 2002) or culture plate (GABRIEL et al., 2012), and less in tridimensional culture, either by implantation into gel-form scaffolds (BAHARVAND et al., 2006) or even by seeding or perfusion on scaffolds (NAVARRO-TABLEROS et al., 2015). Recently, the simulated microgravity offered by a Rotary Cell Culture System is in use to produce tridimensional hepatospheroids (NELSON et al., 2010).

Microgravity enables presents for Tissue Engineering three-dimensional self-assembly of various cell types, where biological processes ensue without potential distortions resulting the gravitational force experienced on Earth (ALESHCHEVA et al., 2016). Simulated microgravity can be achieved by using a Rotating Wall Vessel bioreactor, a circular culture vessel rotating around its own central axis. The scaffold is suspended within the fluid-filled vessel with angular velocity of bioreactor leaving the scaffold in a state of ‘‘free fall’’. Rotating wall bioreactors have been used to cell culture, improving cell proliferation by up to five times when compared to static culture in different scaffolds, and still more efficiently distributing cells throughout the scaffolds (VARLEY; MARKAKI; BROOKS, 2017).

Faced with the causes, reasons, justifications, as well as the great effort and knowledge undertaken over the years, the advancement produced by Tissue Engineering is remarkable and surprisingly. However, the source for biomaterial production in large scale is still a restraining issue, therefore it is against this background that we seek to make our contribution. Nonetheless, the placentas are presented as biomaterial sources, rich in an extracellular matrix containing structure and composition compatibles with scaffolds production and, later biomaterials.

On the other hand, instead of other tissues, placentas are commonly discarded after birth (KAKABADZE et al., 2017). They are rich in extracellular matrix (DA ANUNCIAÇÃO et al., 2017), and they do not require invasive procedures to be harvested (LEONEL et al., 2018). Thus, according to the placental researchers in Tissue Engineering, human and cow decellularized placentas produces scaffolds with the natural microvascular structure for growing organs and tissues (BARRETO et al., 2017; KAKABADZE; KAKABADZE, 2015).

Mouse placenta has not been studied neither by scaffold or as biomaterials, even though it is being a recognized model of human placentation (CARTER, 2007) widely used with easy access (DA ANUNCIAÇÃO et al., 2017). Nevertheless, mouse haemochorial placentas are presented in a very complex arrangement (LEISER; KAUFMANN, 1994), structured in outer (deciduous), intermediate (junctional zone), and inner (labyrinth) layers (WATSON; CROSS, 2005). Labyrinth consists of a complex intercommunicating system where occurs the exchange between mother and fetus (COAN; FERGUSON-SMITH; BURTON, 2005). Within labyrinth, maternal blood

spaces are separated from fetal capillaries by the interhemal barrier (MESS; CARTER, 2009). There are three arranged layers of trophoblast between maternal and foetal blood as follows: the outer layer or cytotrophoblast; and the other two cast layers or syncytiotrophoblast (CARTER, 2008; WATSON; CROSS, 2005). The junctional zone is supplied only by maternal blood, and consists of three distinct cell types: spongioblasts, glycogen cells and trophoblastic cells (COAN; FERGUSON-SMITH; BURTON, 2005; TAKATA; FUJIKURA; SHIN, 1997). Decidua is formed during invasion of maternal tissues, when the fetal vessels are destroyed causing a decidual reaction and endometrial cells increasing in size (LEISER; KAUFMANN, 1994).

Composing the multidisciplinary constitution of Tissue Engineering, Basic Science Research encourages important scientific contributions and knowledge, fundamental for therapeutic and clinical applications in Regenerative Medicine.

The alignment of the present research with the main purposes of science and knowledge, designs the placentas, especially mouse, as a model for engineering three-dimensional biomaterials. Likewise, it also demonstrates the placental biomaterial potentialities to receive embryonic stem cells, and supporting its hepatic-specific inducted differentiation in a dynamic and microgravitational cell culture environment.

2 MOUSE PLACENTAL SCAFFOLDS: A THREE-DIMENSIONAL ENVIRONMENT MODEL FOR RECELLULARIZATION

Patricia Romagnolli, Rodrigo da Silva Nunes Barreto, Paula Fratini, Andrea Maria Mess; Maria Angelica Miglino

University of Sao Paulo

Correspondence: Maria Angelica Miglino, University of Sao Paulo, Av. Prof. Orlando Marques de Paiva, 87, University City, Butantã, CEP: 05508-270, Sao Paulo, Brazil. Email: miglino@usp.br

ABSTRACT

The crescent field of tissue engineering demands new biomaterial sources. Due to the rich extracellular matrix and availability of the material, the placenta could be eligible as an alternative source. Placental mouse scaffolds were produced by decellularization with 0.01, 0.1 and 1% SDS, and 1% Triton X-100. They were evaluated by means of structure and composition to be considered as a biomaterial. Afterwards, they were co-cultured in a rotatory system with mouse embryonic fibroblasts. It was possible to provide cell-free ECM, presenting both structure and composition well-preserved containing 9.42 ± 5.2 ng dsDNA per mg of ECM. Proteoglycans were widespread throughout all ECM in the scaffolds without cell nuclei and other cell remnants. Collagen I, weak in natives, clearly appears in ECM after recellularization. On the contrary, collagen III well observed in native placental tissue, was absent on scaffolds. This interesting observation may have been found due to the solubilization SDS-induced of the collagen III fibrils during decellularization. Fibronectin was well-observed in placental scaffolds whereas laminin and collagen IV were strong expressed. Placental scaffolds potential for repopulation was confirmed after recellularization in a 3D dynamic suspension culture, using a Rotary Cell Culture System (RCCS). Mouse embryonic fibroblasts were seeded, resulting in massive scaffold repopulation with cell-cell interactions and cell-matrix adhesion maintaining their natural morphology, though. The small size whole placental scaffolds provided a 3D environment for recellularizations, as a useful tool for tissue engineering and cellular biology purposes.

Keywords: tissue engineering; biomaterial; whole placental scaffolds; extracellular matrix; recellularization

1 INTRODUCTION

In tissue engineering biomaterials and cells are produced with the objective of tissues and organs regeneration (KATARI; PELOSO; ORLANDO, 2015), by fundamentals of engineering and biology combination for further applications (KEANE; BADYLAK, 2014).

Biomaterials constituting of three-dimensional (3D) biological microenvironments, are produced by the Tissue Engineering technique of decellularization, removing cells from several organs with minor disruption of their extracellular matrix (ECM) structure and composition (FU et al., 2014a; KEANE; SWINEHART; BADYLAK, 2015; SYED et al., 2014).

Decellularized ECM in the resulting suitable scaffolds is configured as a complex 3D structure with functional protein and glycosaminoglycan composition (HREBIKOVA; DIAZ; MOKRY, 2015). Under this condition, bioactive ECM inner scaffolds are potential for cell repopulation and establishment of functional tissues (DE KEMP et al., 2015; KEANE; SWINEHART; BADYLAK, 2015). Thus, scaffolds with minor DNA fragments (<50 ng of dsDNA per mg of ECM) (CRAPO; GILBERT; BADYLAK, 2011; SYED et al., 2014) are suitable for pre-clinical and clinical studies (XU et al., 2015), avoiding antigen gene expression, and resulting in less immunological rejection after transplantation (METHE et al., 2014).

Different techniques combining physical, chemical and enzymatic agents are used for decellularization (GILBERT; SELLARO; BADYLAK, 2006). Detergents, such as sodium dodecyl sulfate (SDS) and octylphenol ethoxylate (Triton X-100) are chemical agents commonly applied for decellularization (FAULK et al., 2014), combined with orbital agitation as mechanical supporter (CRAPO; GILBERT; BADYLAK, 2011).

SDS is an ionic detergent that efficiently lysing cells and solubilizing cytoplasmic components, particularly in dense organs (UYGUN, 2010). The expected effectiveness for SDS during decellularization seems related with the complexity of the organ in question, i.e. 24 h of incubation at 0.1-1% SDS concentrations (HREBIKOVA; DIAZ; MOKRY, 2015); or at 0.01% initial concentration (KAKABADZE; KAKABADZE, 2015).

However, SDS may compromise ECM structure and collagen integrity when applied in high concentrations (KEANE; SWINEHART; BADYLAK, 2015). By contrast, the mild non-ionic Triton X-100 action during decellularizations include a moderate effect upon tissue architecture, resulting in minimal damages to the ECM structure (HREBIKOVA; DIAZ; MOKRY, 2015). Considered one of the bests detergents to eliminate lipids from tissues (CRAPO; GILBERT; BADYLAK, 2011), Triton X-100 especially acts upon lipid-lipid and lipid-protein interactions (YAM et al., 2016). Mild action of Triton X-100 allows its application in a broad range of time during decellularizations, from 6 h to 14 days if improving cell removal, or even less if demanded to remove residual SDS (KAKABADZE; KAKABADZE, 2015).

A glance over the ECM shows that its 3D structural organization and composition, are related to multiple cellular functionalities (OEFNER et al., 2015; PARK et al., 2015; XING et al., 2015). A proper decellularization provides biological scaffolds preserving biocompatibility, biodegradability, and mechanical resistance of their ECM (DE KEMP et al., 2015; XU et al., 2015). Therefore, functional ECM on scaffolds are inductive materials for tissues and organs regenerations (PAN et al., 2016), since recellularized with appropriate cells, including an optimal seeding method, and a physiologically relevant culture method (SCARRIT, 2015).

Static and conventional two-dimensional (2D) culture recellularizations are based on passive introduction of cell suspension into 3D scaffolds with effectiveness limited by low seeding and minimal cell penetration (FU et al., 2014b). However, 3D cell culture has been considered an excellent tool to mimic natural morphological and/or functional features of cells and tissues *in vivo*, i.e. fusion of cytotrophoblasts into syncytiotrophoblasts (MCCONKEY et al., 2016). Besides, 3D cultures are dynamic, performed by a Rotary Cell Culture System (RCCS), and improved by microgravity effect simulation that alter cells perception of gravitational direction which is vital for cell movements (TANG et al., 2017; WOLF; KLEIS, 2016).

Searching for new sources of biomaterials for tissue engineering purposes, placentas that are usually discarded after birth without damage to the donors were used in this study to produce 3D scaffolds for further 3D culture recellularization.

2 MATERIALS AND METHODS

2.1 Samples

This study was approved by Ethical Committee on Animal Use (Protocol ID.: 5669271015) of School of Veterinary Medicine and Animal Science of University of Sao Paulo, Sao Paulo, Brazil. In total, 112 mouse placentas (*Mus musculus*, C57BL/6) of 18.5 days of gestation were collected. Native placentas were weighed using an analytical balance (PA214CP, OHAUS) and measured in diameter using a Vernier caliper. Ten placentas were washed three times with phosphate-buffered saline (PBS; 136.9 mM of NaCl, 26.8 mM of KCl, 14.7 mM of KH_2PO_4 and 8.1 mM of $Na_2HPO4.7H_2O$; pH 7.2) for 5 min each, including 0.5% antibiotics (Penicillin-streptomycin, 15140130, Gibco). Just after decellularization, the placentas were then submitted to the experimental analysis.

2.2 Placental scaffolds production

Mouse placental decellularization lasted five days at room temperature, and mechanical force induced by orbital agitation of 150 r/min (TS-2000A, VDRL Multifunctional Shaker, Biomixer). The first three days of decellularization included SDS (13-1313-01, LGC Biotecnologia) considering its potent action on the cellular extraction. SDS was diluted in distillated water, with 0.5% antibiotics, and used at 0.01%, 0.1% and 1% concentration, one day each, as proposed both to bovine, and human placentas (KAKABADZE; KAKABADZE, 2015). Subsequently, 1% Triton X-100 (13-1315-05, LGC Biotecnologia) in distillated water with 0.5% antibiotics was applied in order to finalize cell content extraction and to remove the residual SDS. Finally, placental scaffolds were washed three times in PBS with 0.5% antibiotics for 15 min each, and sterilized in ultraviolet light for 2 h.

2.3 Recellularization by 3D dynamic suspension cell culture

Mouse embryonic fibroblasts (SCRC-1049™, ATCC®) were reseed on placental scaffolds in order to evaluate their potential for recellularization. Fibroblasts were thawed, centrifuged, and suspended in fibroblast proliferating medium (FPM) containing: 88% Dulbecco's Modified Eagle Medium (DMEM, 12100046, Gibco), 10% fetal bovine serum (FBS, 10Bio500, LGC Biotecnologia), 1% non-essential amino acids (MEM NEAA, BR30238-01, LGC Biotecnologia), 1% amino acids (BME, B6766, Sigma), and 1 µL/mL antibiotics (Amikacin sulphate 16.68 µg/µL, Novafarma). Fibroblasts were placed on a culture flask, and cultured in an incubator, under humidified atmosphere of 5% CO_2 in air at 37ºC. Medium was changed every 48 h, and cells were passaged at 80% confluence, until the sixth passage. Recellularization was performed in six days by dynamic suspension in a 3D Rotary Cell Culture System (RCCS, RCCS-4SC, Synthecon) under rotational speed of 13 r/min. First, ten placental scaffolds were placed in a 10mL sterile culture rotating wall vessel (RWV) bioreactor, and cultured in the rotating system 48 h prior to recellularization, to stabilize the biomaterial in the culture environment contending FPM. Afterwards, fibroblasts were dissociated with Tryple (12563-029, Gibco), suspended in 10mL FPM at density of 3 x 10^6 cells, and added to the vessel containing placental scaffolds. Medium addition was performed with a syringe while emptying bubble in the vessel with another syringe. Closing the sterile valves, vessel was mounted to the rotator base, and placed in 5% CO_2, 37ºC incubator. Medium was changed every 24 h.

2.4 Histology

Native and decellularized, and then recellularized 3D scaffolds were fixed in 4% paraformaldehyde (PFA) for 48 h, dehydrated in crescent concentrations of ethanol. Later on, samples were diaphanized in xylene, paraffin embedded, and sectioned (5 µm) in microtome (RM2265, Leica). Microsections were transferred to glass slides, and Hematoxylin-Eosin (HE, nuclei presence), Masson’s Trichrome (connective tissue conformation), Colloidal Iron and combined Alcian Blue – Periodic Acid-Schiff (PAB, glycosaminoglycans) stains were performed. The results were visualized using a light microscopy (Eclipse 80i, Nikon) and photographed using a digital camera (DS-Ri Color Digital Camera, Nikon).

2.5 Immunohistochemistry for paraffin-embedded tissues (IHC-P)

To analyze ECM proteins in native and decellularized 3D scaffolds, sections (5 µm) of 4% PFA fixed and paraffin-embedded samples were transferred to poli-L-lysine (p8920, Sigma) coated glass slides. Then, tissues were treated with xylene, hydrated with decrescent ethanol concentrations, and rinsed with distilled water. Antigen retrieval was performed by heat-induced in citrate buffer (1.83 mM of monohydrate citric acid and 8.9 mM of sodium citrate tribasic dehydrate, pH 6.0). The endogenous peroxidase was blocked with 3% hydrogen peroxide in distillated water, at dark chamber cooling to the room temperature. Later on, nonspecific protein interaction was blocked with 2% bovine serum albumin (BSA) in tris-buffered saline (TBS; 2.0 mM of Trizma base and 1.36 mM of sodium chloride, pH 7.5). Microsections were then incubated with the following primary antibodies: anti-collagen I (600-401-103S, 1:400, Rockland), anti-collagen III (sc-8779, 1:100, Santa Cruz Biotechnology), anti-collagen IV (1-CO083-0, 1:500, Quartett), anti-laminin subunit alpha-2 (bs-8561R, 1:200, Bioss Antibodies) and anti-fibronectin (NBP1-91258, 1:200, Novus Biologicals), overnight in humidity chamber at 4ºC. For negative controls, irrelevant anti-mouse IgG (M5284, Sigma) or anti-rabbit IgG (ab27478, Abcam) substituted the primary antibodies. The reaction was detected with Dako Advance HRP kit (K6068, Dako, USA), developing the color with DAB+ Substrate Chromogen System (K3468, Dako). A counter-staining was proceeded with hematoxylin. Washes with 0.2% BSA in TBS were performed at each step after primary antibody incubation. The slides were mounted and visualized using an Eclipse 80i microscope and photographed using a DS-Ri Color Digital Camera.

2.6 DAPI stain in frozen sections (DAPI)

Native and decellularized placentas were also analyzed for the presence of cellular nuclei by immunofluorescence. In both cases, samples were mounted in OCT embedding compound (4583-1, Sakura), frozen with liquid nitrogen vapor, and stored at -150º C. Tissue sections (40 µm) were produced using a cryostat (CM1520, Leica), thaw-mounted on to poli-L-lysine (p8920, Sigma) coated histological slides, and snap freeze at -150ºC. Then, they were air dried and fixed in ice-cold acetone at -20º C. Thawing at room temperature, samples were washed with TBS. The coverslips were

mounted with Prolong™ Gold Antifade Mountant with DAPI (P36931, Invitrogen) and visualized using a confocal microscope (FV1000, Olympus).

2.7 Whole-mounting immunofluorescence (Wm-IF)

Whole placental 3D scaffolds recellularized with fibroblasts were fixed in 4% PFA and washed with PBS. Once air dried, the samples were washed with TBS and incubated with primary antibodies diluted in 0.2% BSA in TBS in a humidified chamber at room temperature. The following primary antibodies were used: anti-β2 tubulin (sc-47751, 1:100, Santa Cruz Biotechnology) and anti-collagen IV (ab6586, Abcam). Newly washed with TBS, tissues were incubated with secondary antibodies goat anti-mouse Alexa Fluor 488 (ab150113, Abcam) and goat anti-rabbit Alexa Fluor 568 (A-11011, Invitrogen) at 1:500 dilution in .0.2% BSA in TBS. After another wash with TBS, nuclear stain was provided applying Pierce DAPI Nuclear Counterstains (62248, 1:10.000, Thermo Scientific). The results were collected in a series of optical sections using confocal microscope FV1000, Olympus.

2.8 Scanning Electron Microscopy (SEM)

Native, decellularized and recellularized placental samples were pre-fixed in 4% PFA for 48 h, and post-fixed in 1% osmium tetroxide for 90 min. Then, they were dehydrated in crescent concentrations of ethanol and dried using a critical point (EM CPD300, Leica). Later on, the samples were transferred and fixed on stubs and gold metalized (EMITECH K550). Visualizations were obtained using a scanning electron microscope (LEO 435VP SEM, LEO Electron Microscopy Ltd.).

2.9 Reminiscent genomic DNA quantification

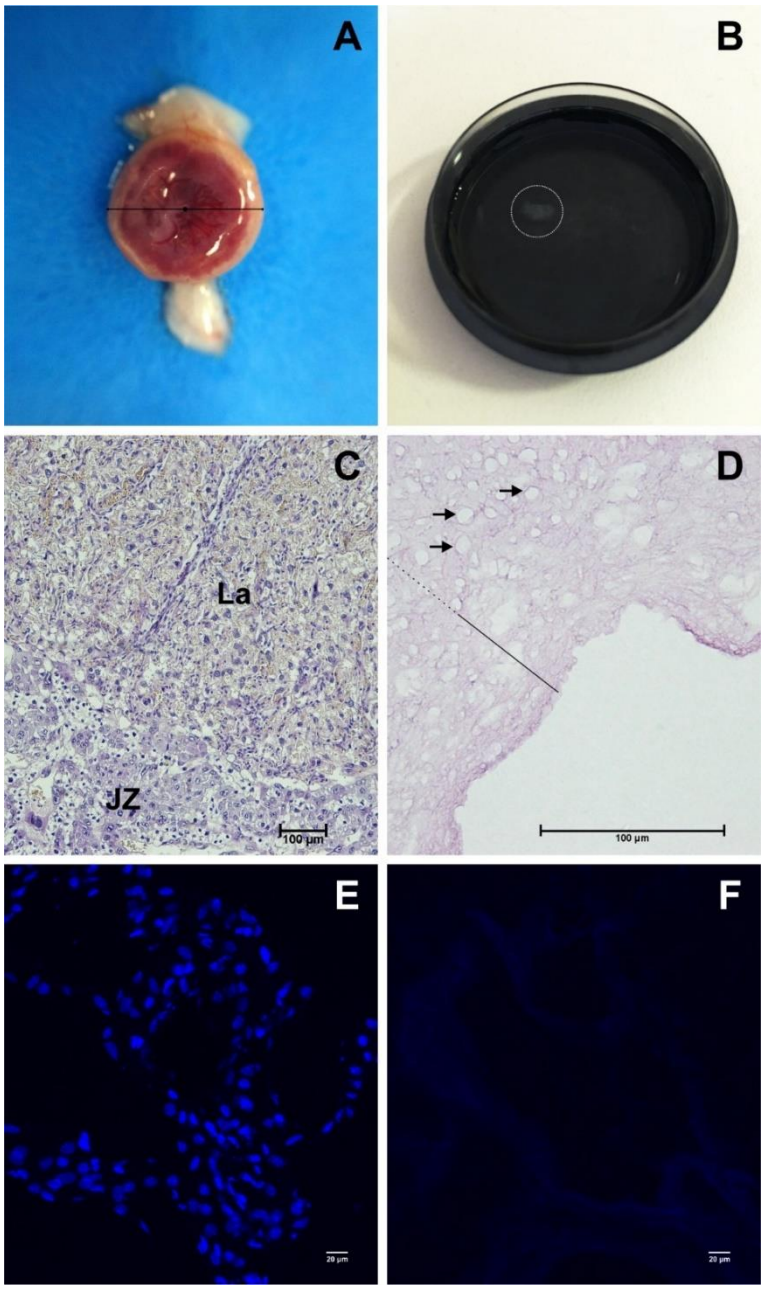
Decellularized placentas were prepared as previously described (BARRETO et al., 2017), and the genomic DNA quantification was performed with a Quant-iT™ DNA PicoGreen® dsDNA Reagent (P7581, Invitrogen) according to the manufacturer's

instructions. The final products were analyzed by spectrophotometry measuring the absorbance at 530 nm in a microplate reader (μQuantTM, BioTek).

3 RESULTS

We efficiently decellularized mouse placentas at E18.5 employing crescent concentrations of SDS (0.01, 0.1 and 1%) and 1% Triton X-100. Using this technique, we produced a cell-free placental ECM with structure and important aspects of its composition well-preserved. After decellularization, native placentas (Figure 1A) lost about 51.97% (±2.27) in weight and 54.26% (±6.06) in diameter resulting on scaffolds with a mean diameter of 0.43 (±0.05) centimeters (Figure 1B). Compared to the native condition (Figure 1C, E), mouse placental scaffolds were absenting of cell nuclei when stained by HE (Figure 1D) and DAPI (Figure 1F), and only 9.42 ± 5.2 ng dsDNA per mg of ECM persisted. After decellularization, placental regions lost their natural appearance remaining certain characteristics in its ECM spatial configuration, i.e. former fetal vessels structure which were helpful to estimate labyrinthine region (Figure 1D).

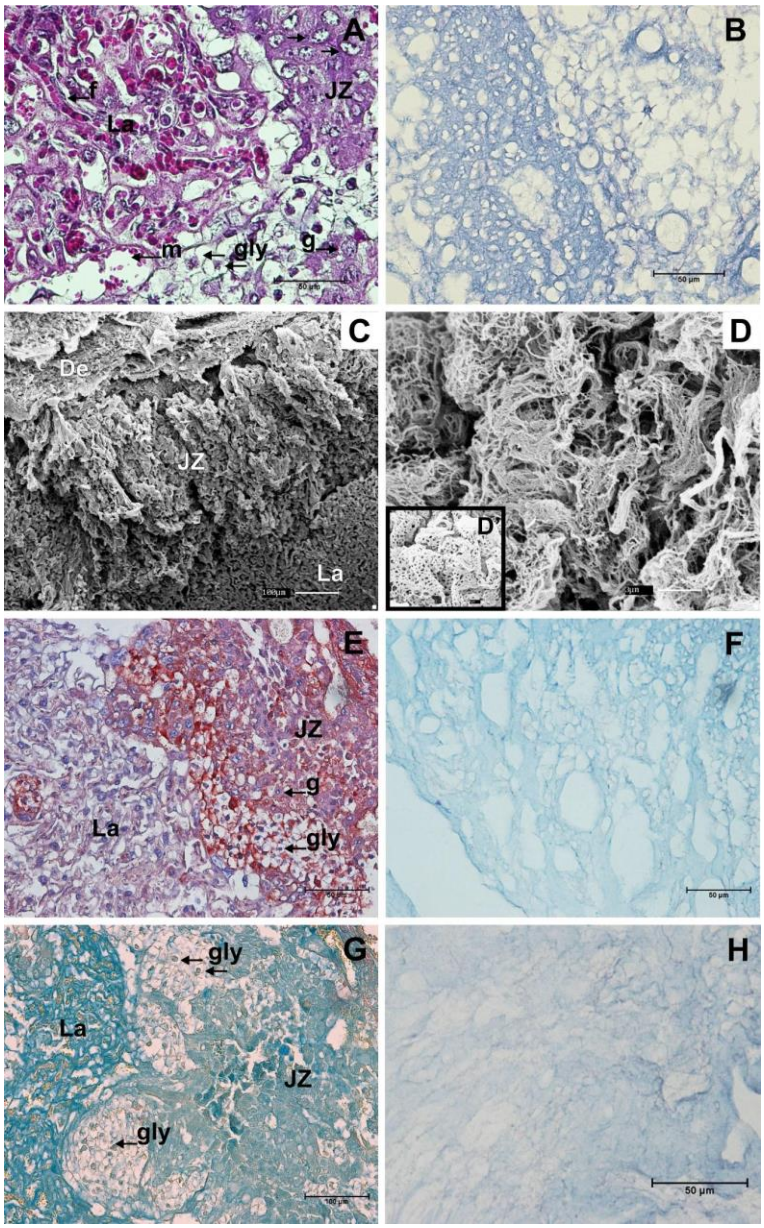
Figure 1 - Decellularization of whole mouse placentas. Mouse placentas before (A) and after (B) decellularization process. Small scaffold floating in PBS on a 35 mm Petri dish (B). In Hematoxylin-Eosin stained tissue cell nuclei and other contents are normally visualized in native placental regions (C), but absent on the acellular ECM (D). In the decellularized ECM spatial configuration, placental regions were estimated considering the labyrinth (fill line) extension containing former fetal vessels structure (fv, arrows) and its continuous relationship with junctional zone and decidua (dashed line), respectively. The contrast between the nuclei presence (blue points stained) and absence were also noted in DAPI stains of native (E) and decellularized (F) placental tissues. JZ, junctional zone; La, labyrinth. Scale Bars = 100 µm (A, B), 20 µm (C, D)



Fonte: (ROMAGNOLLI, P., 2018).

The Masson's Trichome stain evidenced a native placental tissue occupied by its cellular contents (Figure 2A). By contrast, after decellularization only a greater amount of a well-structured ECM was present (Figure 2B). The complex ultrastructural organization in native placental tissues were captured by SEM (Figure 2C). In decellularized placentas, the intricacy of fibrillar ECM proteins was observed in different arrangements among placental regions. In the junctional zone delicate fibrils were interspersed by former vessels structures (Figure 2D) while labyrinthine fibrils were thicker and honeycomb-like structured (Figure 2D'). Regarding general components, they were mostly similar among their distinct regions, both in native and decellularized mouse placental ECM. The proteoglycans distribution was accessed by PAB (Figure 2E, F) and Colloidal Iron stains (Figure 2G, H). Thereby, proteoglycans (blue stained) were widespread throughout labyrinth, junctional zone and decidua, both in native (Figure 2E, F) and decellularized placental ECM (Figure 2G, H).

Figure 2 - Structural analysis of mouse placenta. In the Masson's Trichome stain (A, B) the new spatial configuration is presented by an abundant and well-shaped decellularized placental extracellular matrix (ECM) (B), after removal of usual cellular components (A). Ultrastructural analysis of mouse placenta by Scanning Electron Microscopy (C, D, D'). The complex arrangement of the native placental regions (C) is also observed through cellular gaps on the scaffolds (D, D'). In-between the meshwork fibrils in the junctional zone, the angioarchitecture pattern were preserved (D), while a fibrillar honeycomb-like structural arrangement was observed in the labyrinth (D'). Proteoglycans were blue-colored in mouse placental ECM native placenta by combined Alcian Blue-PAS (E) and Colloidal Iron (G) stains, and similarly maintained decellularization (F, H). De, decidua; JZ, junctional zone; La, labyrinth; f, fetal capillary; g, trophoblast giant cell; gly, trophoblast glycogen cell; m, maternal blood space. Scale Bars = 1 µm (D'), 3 µm (D), 50 µm (A, B, E, F, H), 100 µm (C, G)



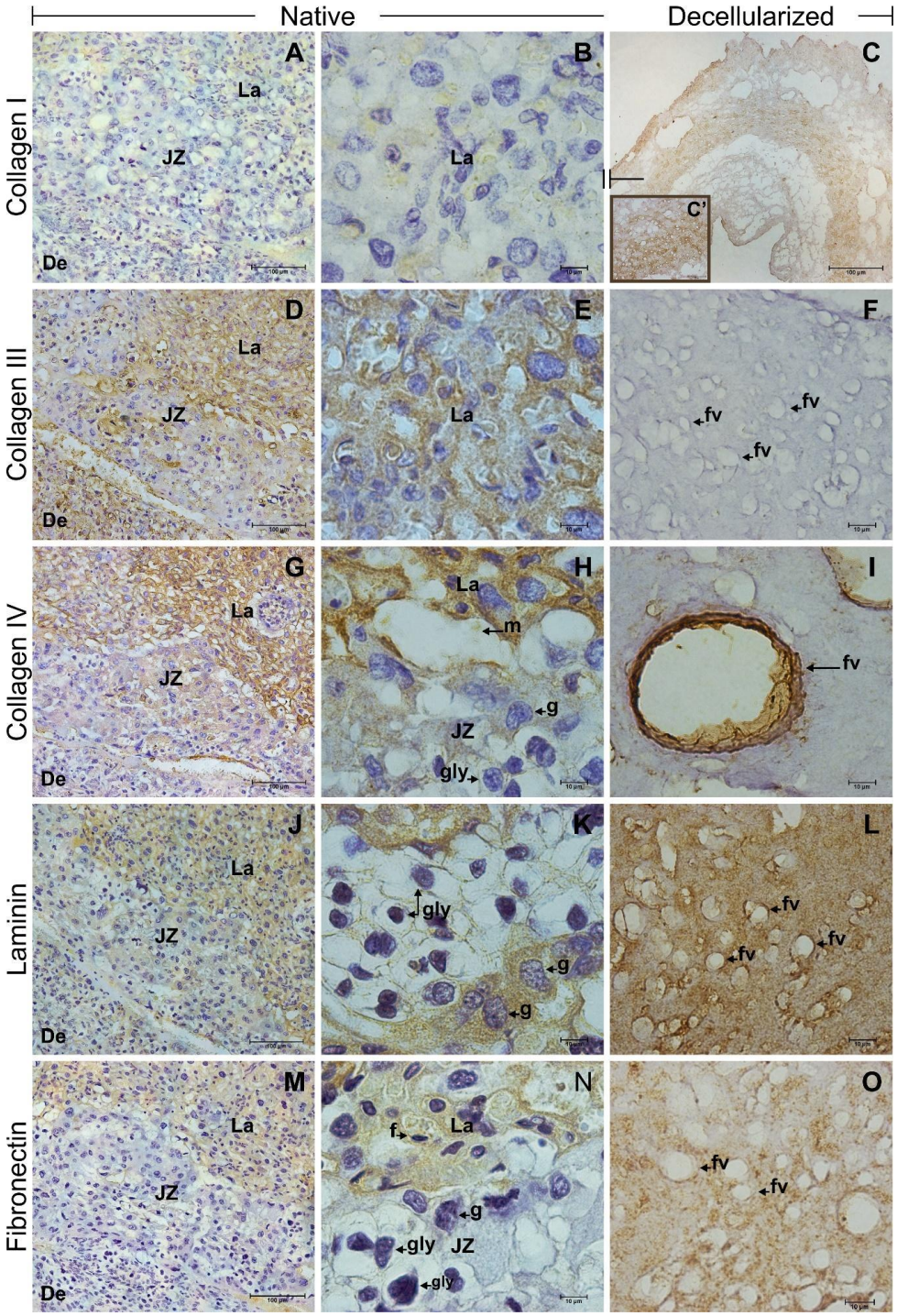
Fonte: (ROMAGNOLLI, P., 2018).

By immunohistochemistry, differences in intensity and localization of analyzed protein were evident when comparing native and decellularized placentas (Figure 3).

The collagen I in the stroma of native placentas at E18.5 was low present both in labyrinth and junctional zone and absent in the decidua, though (Figure 3A, B). However, the scattered presence of collagen I in the ECM was evidenced after decellularization in all placenta regions (Figure 3C). Collagen III was largely detected in a strong presence within labyrinth and decidua, when compared to junctional zone (Figure 3D, E). Unexpectedly, collagen III was almost absent in the decellularized placenta (Figure 3F).

Data concerning the basement membrane showed that collagen IV had a strong presence in labyrinth, intermediate in junctional zone, and weak in decidua (Figure 3G, H). Similar intensities for collagen IV were detected in decellularized placentas mainly surrounding their former vessels (Figure 3I). Laminin was observed at an intermediate level in labyrinth being almost absent in junctional zone and weak in decidua (Figure 3J, K). Interestingly, laminin was spread in intermediate level through the decellularized placental regions (Figure 3L). Fibronectin was detected at intermediate levels within labyrinth of native (Figure 3M, N) and decellularized (Figure 5H) placentas. In native, fibronectin was not expressed in junctional zone and decidua (Figure 3M, N).

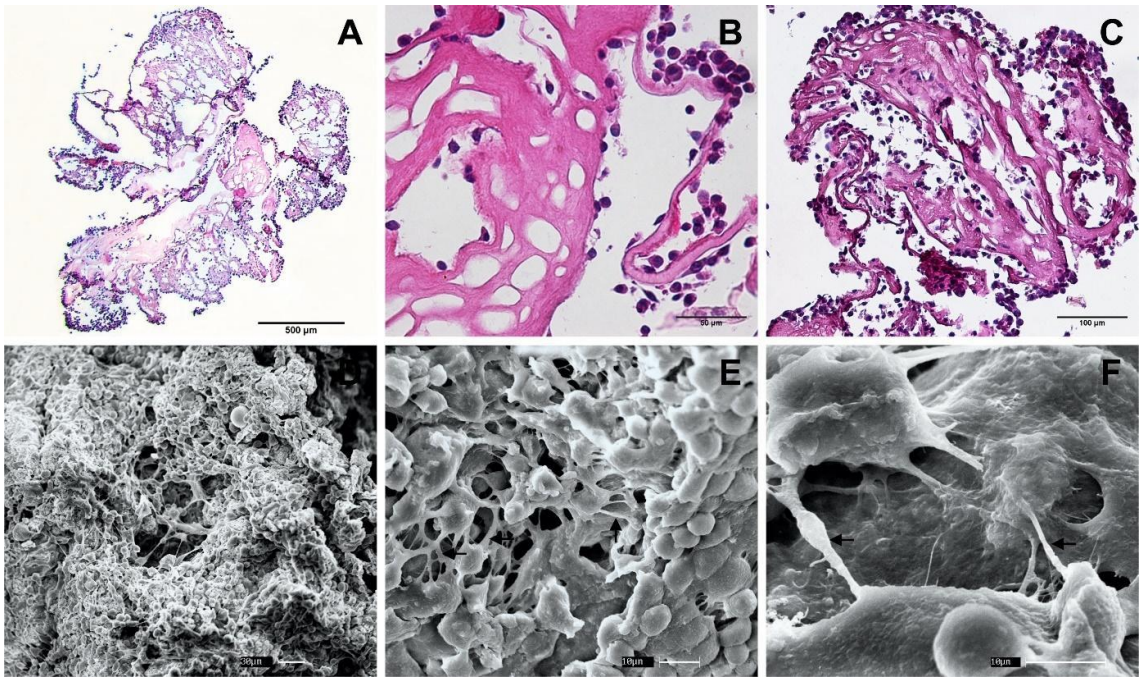
Figure 3 - Immunohistochemistry for collagens I, III and IV, laminin and fibronectin in native and decellularized mouse placenta. Collagen I was lightly visualized in native (A, B) and unexpectedly strong spread in the ECM after decellularization (C, C'). Unlike, the collagen III observed in native (D, E;) was absent in the scaffolds (F). Collagen IV (G, H), laminin (J, K), and fibronectin (M, N) had similar distribution in the native placental regions, except by the fibronectin absence in the junctional zone (N). In the acellular ECM, the mainly collagen IV (I), but also laminin (L) and fibronectin (O) surrounded the former fetal vessels. De, decidua; JZ, junctional zone; La, labyrinth; f, fetal capillary; fv; former fetal vessel; g, trophoblast giant cell; gly, trophoblast glycogen cell; m, maternal blood space. Scale Bars = 100 µm (A, d, D, G, J, M), 50 µm (C', F, I, L), 10 µm (B, D, H, K, N, O)



Fonte: (ROMAGNOLLI, P., 2018).

When testing the bioactivity properties of placental scaffolds for recellularizations, fibroblasts 3D culture resulted in a repopulation of placental scaffolds. HE stains showed fibroblasts attached to the scaffold surface since day three (Figure 4A, B), subsequently proliferating and migrating to occupy inner positions at day six (Figure 4C). The 3D placental scaffolds provided a microenvironment for massive cellular anchoring (Figure 4D). Preserving their rounded morphology as consequence of suspension culture, fibroblasts reseeded on placental scaffolds developed both cell-to-cell interactions (Figure 4E), and cell-matrix adhesions (Figure 4F).

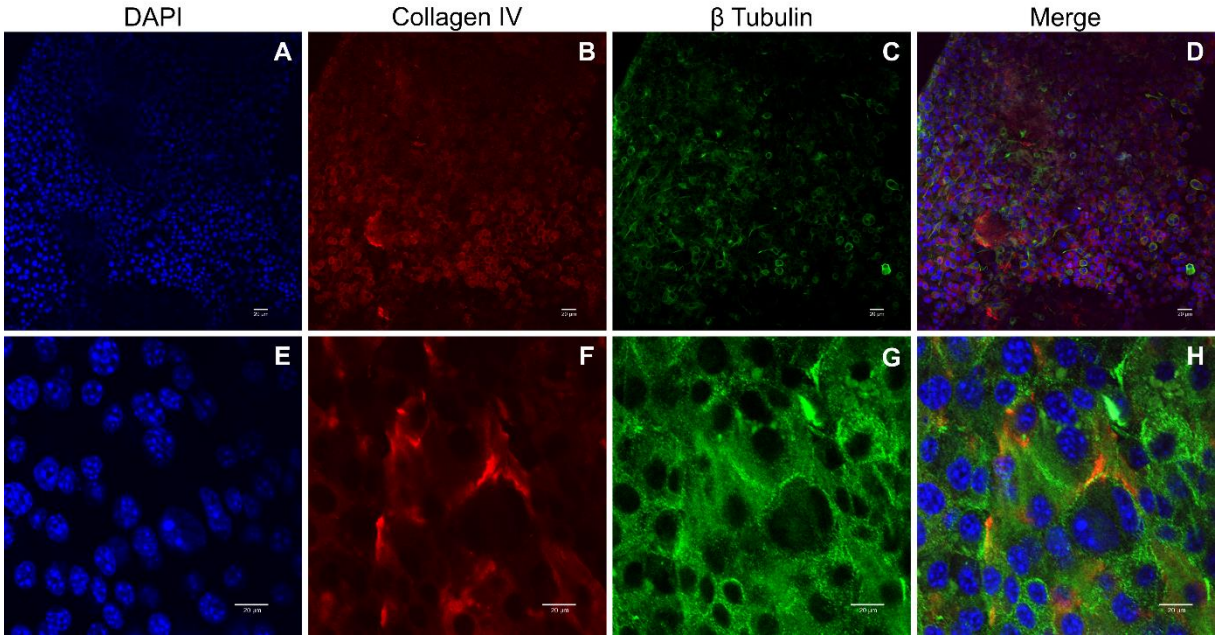
Figure 4 - Placental scaffolds recellularized with mouse embryonic fibroblasts in a dynamic three-dimensional culture system. Hematoxylin-Eosin stained placental scaffold after three (A, B) and six (C) days of recellularization. Initially, fibroblasts adhered to the outer surface of the scaffold (A, B), later migrating through the extracellular matrix to internal regions (C). Scanning electron microscopy analysis of three-dimensional fibroblast growth on placental scaffold under six days of dynamic culture (D, E, F). Fibroblasts are distributed throughout the placenta scaffold (D). Round-shaped, cells interact with each other (E), and adhere to the extracellular matrix by their cytoplasmic extensions (F, arrows). Scale Bars = 500 µm (A), 100 µm (C), 50 µm (B), 30 µm (D), 10 µm (D), 1 µm (D)



Fonte: (ROMAGNOLLI, P., 2018).

In spite of collagen IV presented in the decellularized placental ECM, the new cells extensively occupying placental scaffolds (Figure 5A, E) may producing or remodeling collagen IV due alteration of their distribution pattern (Figure 5B, F). Spatial organization and integration adopted during fibroblasts migration and occupation of the new 3D microenvironment were pivotal for repopulation of the placental scaffolds (Figure 5C, D, G, H).

Figure 5 - Whole-mount immunofluorescence on mouse placental scaffolds recellularized with mouse embryonic fibroblasts, using collagen IV (B, F) and β tubulin (C, G) antibodies. With a high cellular concentration, collagen IV present in placental scaffold (F, arrow) is obliterated by the large amount of cellular-derived collagen IV (B, F), during the reorganization of the fibroblasts on the scaffold surface. Cytoplasmic microtubules evolved in fibroblasts motility mechanism are characteristically expressing β Tubulin (C, G). Nuclei were stained with DAPI (A, E), and merge images produced for full view (D, H). Scale Bars = 20 µm



Fonte: (ROMAGNOLLI, P., 2018).

4 DISCUSSION

Our results showed a biological scaffold derived from a small size whole organ decellularization. The new approach of using mouse placentas in tissue engineering was designed including the most commonly detergents employed for removing cells from tissues and organs (FAULK et al., 2014; KEANE; SWINEHART; BADYLAK,

2015), in association with orbital agitation (FU et al., 2014a). SDS concentrations and time of exposure, as previously established for human and cow placentomes (KAKABADZE; KAKABADZE, 2015), were also favorable for mouse placentas decellularization. Finalized with Triton X-100 treatment (KEANE; SWINEHART; BADYLAK, 2015), decellularization resulted in well-preserved ECM within scaffolds. In association, both SDS and Triton X-100 provided less immunogenic scaffolds for further engraftments (PRICE et al., 2015). The weight and diameter losses in mouse placental scaffolds were the primary successful positive signs of cell removal (OLIVEIRA et al., 2013). The result also included the absence of cell nuclei as showed by histological analysis, in addition to low amount of reminiscent DNA as stated to a biological scaffold (CRAPO; GILBERT; BADYLAK, 2011; SYED et al., 2014). The structure of decellularized placental scaffolds revealed an acellular condition with minimal damage to the ECM (BADYLAK; FREYTES; GILBERT, 2009), as also stated (PATI et al., 2014).

Apart from fibrillar protein that gives the ECM structure, proteoglycans and glycosaminoglycans fill ECM spaces, close interacting with collagen fibrils, and acting as reservoir of several molecules, i.e. growth factors, enzymes and hormones (JUNQUEIRA; MONTES, 1983; SCOTT, 1986; WU et al., 2015). Thus, the Colloidal Iron and PAB stains evidenced that the proteoglycans were maintained on the scaffolds after decellularization at similar levels and distribution as in native placentas.

On the scaffolds, collagen I was almost absent, contrasting with those observed in the native placentas. Otherwise, collagen I could be detected in those regions of decellularized placenta where it was absent in native tissue. The interstitial collagens I and III fibrils are associated in soft tissues, both expressed in mouse and human placentas (CAMERON et al., 2002; MALAK et al., 1993), where the collagen I fibrils are structural components, and collagen III are related to the assembly and thickness of the fibrils (SPIESS; ZORN, 2007). In human placentas, the presence of collagen I fibrils are masked by the encasement of the collagen III (AMENTA et al., 1986), being only exposed when collagen III is removed, i.e. by 8 M urea in dermis (FLEISCHMAJER et al., 1990) or by SDS in our model. Therefore, during decellularization SDS may have modified and disrupted structurally the collagen III fibrils (FAULK et al., 2014), thus justifying their absence on the scaffolds, while it gave

to the collagen I a better exposition. Alterations of this co-expression may change the mechanical properties of the fibrils (ASGARI et al., 2017).

For collagen IV, its presence was overspread in all native placental regions, as predicted for mouse placentas (SAYLAM et al., 2002; THOMAS; DZIADEK, 1993). Abundant in all of basement membranes, the maintenance of collagen IV on the placental scaffolds is a suitable characteristic for structural support to cell adhesion and migration (OEFNER et al., 2015). Besides, the absence of collagen IV is correlated to the rupture of native ECM structure (PÖSCHL et al., 2004; SCHENKE-LAYLAND et al., 2007), and its presence on placental scaffolds indicates structural integrity and functionality, thus favoring cell attachments.

Detected in native mouse placentas, fibronectin attaches the interstitial collagen system to the basement membranes (AMENTA et al., 1986; GIACHINI et al., 2008; SAYLAM et al., 2002). Persisting to decellularization, fibronectin may facilitate adhesion to multiple cell types, making the scaffolds suitable for tissue culture (LONDONO; BADYLAK, 2014).

The laminin had similar distribution as collagen IV. This protein is localized on the lamina lucida of the basement membrane, situated in-between basal cell membrane and the lamina (AMENTA et al., 1986; GIACHINI et al., 2008; SAYLAM et al., 2002; THOMAS; DZIADEK, 1993). Also, it is an important element related to the organization and maintenance of vascular structures (LONDONO; BADYLAK, 2014).

Fibronectin, collagen IV and laminin are the main ECM basement membrane components (PÖSCHL et al., 2004), and their maintenance after decellularization is an important indicative of scaffolds bioactivity for future applications.

The 3D environment installed on placental scaffolds effectively supported cells, allowing cell repopulation (GILPIN et al., 2014), cell proliferation and migration (WILLEMSE et al., 2017). Surpassing the traditional and static 2D culture, the 3D culture environment can be further increased by dynamic mechanisms to reseed cell population (FU et al., 2014a; PAO et al., 2017). Indeed, using the RCCS, the embryonic mouse fibroblasts reseeded on placental scaffolds by 3D dynamic cell culture improved placental tissue recellularization such as in lung scaffolds, promoting both cell growth and differentiation (CRABBÉ et al., 2015). The natural morphology preserved by

mouse embryonic fibroblasts attached to ECM scaffolds is another benefit of RCCS. Natural cellular morphology is an expected characteristic when mechanical cellular damage is reduced by maintained a low shearing force, within the fluid during rotating culture, and also avoiding bubbles due to turbulence caused by the rotary motion (Pao et al., 2017).

Small size whole-placental scaffolds may contribute in studies upon cell-to-cell and cell-to-ECM interactions (FRANCZYK et al., 2017; KEANE; BADYLAK, 2014; SCHNEIDER et al., 2016), providing a biological 3D microenvironment for cell culture (ROBINS et al., 2011). It can be highly advantageous to observe placental scaffolds as a promise source of 3D microenvironment ready to be used in Regenerative Medicine routine, i.e. human placentas and cow placentomes scaffolds as auxiliary liver connections to the systemic circulation (KAKABADZE; KAKABADZE, 2015), and as support to hepatic tissue in acute liver failure (KAKABADZE et al., 2017). In fact, cow placentomes are interesting alternatives to develop large-scale scaffolds with complex vascular architecture (BARRETO et al., 2017). A small whole placental scaffold may improve the mouse placenta model (CARTER, 2007), placing an acellular 3D microenvironment in the panel of trophoblast studies, overcoming the traditional monolayers (RAI; CROSS, 2015). Trophoblast were also seen as a model of primary syncytiotrophoblast, based on 3D dynamic culture (MCCONKEY et al., 2016). However, may unifying 3Ds placental environments with dynamic 3D culture assays, a new model in trophoblast research can raise. A model in which trophoblasts could be seeded, cultured and studied on their natural environment, or even had their development accompanied since their cellular precursors, i.e. murine fibroblasts (KUBACZKA et al., 2015).

In conclusion, placental scaffolds can be produced by decellularization with preserved ECM structure and composition, and it can be recellularized, serving as a 3D environment for tissue engineering and cellular biology purposes.

ACKNOWLEDGEMENTS

We warmly thank Anthony Carter from University of Southern Denmark, and Phelipe O. Favaron from University of Sao Paulo, for fruitful discussions. We are very

grateful to Karl Klisch from University of Zürich for providing ECM antibodies. We thank Rose E. G. Rici from Advanced Center of Image Diagnosis of University of Sao Paulo for microscopy analysis supporting. We have received grants from Sao Paulo Research Foundation (14/50844-3) and from National Council for Scientific and Technological Development (467476/2014-4).

REFERENCES

AMENTA, P. S. et al. The extracellular matrix is an integrated unit: Ultrastructural localization of collagen types I, III, IV, V, VI, fibronectin, and laminin in human term placenta. **Collagen and Related Research**, v. 6, p. 125–152, 1986.

ASGARI, M. et al. *In vitro* fibrillogenesis of tropocollagen type III in collagen type I affects its relative fibrillar topology and mechanics. **Scientific Reports**, v. 7, n. 1, p. 1392, 2017.

BADYLAK, S. F.; FREYTES, D. O.; GILBERT, T. W. Extracellular matrix as a biological scaffold material: Structure and function. **Acta Biomaterialia**, v. 5, p. 1–13, 2009.

BARRETO, R. DA S. N. et al. Decellularized bovine cotyledons may serve as biological scaffolds with preserved vascular arrangement. **Journal of Tissue Engineering and Regenerative Medicine**, n. November, p. 1–9, 2017.

CAMERON, G. J. et al. Structure of type I and type III heterotypic collagen fibrils: An X-ray diffraction study. **Journal of Structural Biology**, v. 137, p. 15–22, 2002.

CARTER, A. M. Animal models of human placentation - A review. **Placenta**, v. 21, n. Suppl., p. 41–47, 2007.

CRABBÉ, A. et al. Recellularization of decellularized lung scaffolds is enhanced by dynamic suspension culture. **PLoS One**, v. 10, n. 5, p. 1–17, 2015.

CRAPO, P. M.; GILBERT, T. W.; BADYLAK, S. F. An overview of tissue and whole organ decellularization processes. **Biomaterials**, v. 32, n. 12, p. 3233–3243, 2011.

DE KEMP, V. et al. Tissue engineering for human urethral reconstruction: systematic review of recent literature. **PLoS One**, v. 10, n. 2, p. e0118653, 2015.

FAULK, D. M. et al. The effect of detergents on the basement membrane complex of a biologic scaffold material. **Acta Biomaterialia**, v. 10, p. 183–193, 2014.

FLEISCHMAJER, R. et al. Dermal collagen fibrils of type I and type III collagen molecules. **Journal of Structural Biology**, v. 105, p. 162–169, 1990.

FRANCZYK, M. et al. Extracellular matrix proteins in healthy and retained placentas, comparing hemochorial and synepitheliochorial placentas. **Placenta**, v. 50, p. 19–24, 2017.

FU, R. H. et al. Decellularization and recellularization technologies in tissue engineering. **Cell Transplantation**, v. 23, p. 621–630, 2014a.

FU, R. H. et al. Decellularization and recellularization technologies in tissue engineering. **Cell Transplantation**, v. 23, n. 4–5, p. 621–630, 2014b.

GIACHINI, F. R. C. et al. Maternal diabetes affects specific extracellular matrix components during placentation. **Journal of Anatomy**, v. 212, p. 31–41, 2008.

GILPIN, S. E. et al. Enhanced lung epithelial specification of human induced pluripotent stem cells on decellularized lung matrix. **Annals of Thoracic Surgery**, v. 98, n. 5, p. 1721–1729, 2014.

HREBIKOVA, H.; DIAZ, D.; MOKRY, J. Chemical decellularization: a promising approach for preparation of extracellular matrix. **Biomedical papers**, v. 159, n. 1, p. 12–17, 2015.

JUNQUEIRA, L. C.; MONTES, G. S. Biology of collagen-proteoglycan interaction. **Archivum Histologicum Japonicum**, v. 46, n. 5, p. 589–629, 1983.

KAKABADZE, A.; KAKABADZE, Z. Prospect of using decellularized human placenta and cow placentome for creation of new organs: targeting the liver (Part I: Anatomic study). **Transplantation Proceedings**, v. 47, p. 1222–1227, 2015.

KAKABADZE, Z. et al. Decellularized human placenta supports hepatic tissue and allows rescue in acute liver failure. **Hepatology**, 6 dez. 2017.

KATARI, R.; PELOSO, A.; ORLANDO, G. Tissue engineering and regenerative medicine: semantic considerations for an evolving paradigm. **Frontiers in Bioengineering and Biotechnology**, v. 2, p. 1–6, 2015.

KEANE, T. J.; BADYLAK, S. F. Biomaterials for tissue engineering applications. **Seminars in Pediatric Surgery**, v. 23, p. 112–118, 2014.

KEANE, T. J.; SWINEHART, I.; BADYLAK, S. F. Methods of tissue decellularization used for preparation of biologic scaffolds and *in vivo* relevance. **Methods**, v. 84, p. 25–34, 2015.

KITAOKA, M. et al. Immunohistochemical localization of procollagen types I and III during placentation in pregnant rats by type-specific procollagen antibodies. **The Journal of Histochemistry and Cytochemistry**, v. 42, n. 11, p. 1453–1461, 1994.

KUBACZKA, C. et al. Direct induction of trophoblast stem cells from murine fibroblasts. **Cell Stem Cell**, v. 17, n. 5, p. 557–568, 2015.

LONDONO, R.; BADYLAK, S. F. Biologic scaffolds for regenerative medicine: Mechanisms of *in vivo* remodeling. **Annals of Biomedical Engineering**, v. 43, n. 3, p. 577–592, 2014.

MALAK, T. et al. Confocal immunofluorescence localization of collagen types I, III, IV, V and VI and their ultrastructural organization in term human fetal membranes. **Placenta**, v. 14, p. 385–406, 1993.

MCCONKEY, C. A. et al. A three-dimensional culture system recapitulates placental syncytiotrophoblast development and microbial resistance. **Science advances**, v. 2, n. 3, p. e1501462, 2016.

METHE, K. et al. An alternative approach to decellularize whole porcine heart. **BioResearch Open Access**, v. 3, n. 6, p. 327–338, 2014.

OEFNER, C. M. et al. Collagen type IV at the fetal-maternal interface. **Placenta**, v. 36, p. 59–68, 2015.

OLIVEIRA, A. C. et al. Evaluation of small intestine grafts decellularization methods for corneal tissue engineering. **PLoS One**, v. 8, n. 6, p. e66538, 2013.

PAN, J. et al. In-vivo organ engineering: Perfusion of hepatocytes in a single liver lobe scaffold of living rats. **International Journal of Biochemistry and Cell Biology**, v. 80, p. 124–131, 2016.

PAO, S. I. et al. Effect of microgravity on the mesenchymal stem cell characteristics of limbal fibroblasts. **Journal of the Chinese Medical Association**, v. 80, n. 9, p. 595–607, 2017.

PARK, Y.-B. et al. Effect of chondrocyte-derived early extracellular matrix on chondrogenesis of placenta-derived mesenchymal stem cells. **Biomedical Materials**, v. 10, n. 3, p. 35014, 2015.

PATI, F. et al. Printing three-dimensional tissue analogues with decellularized extracellular matrix bioink. **Nature Communications**, v. 5, p. 3935, 2014.

PÖSCHL, E. et al. Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development. **Development**, v. 131, n. 7, p. 1619–1628, 2004.

PRICE, A. P. et al. Automated decellularization of intact, human-sized lungs for tissue engineering. **Tissue Engineering: Part C**, v. 21, p. 94–103, 2015.

RAI, A.; CROSS, J. C. Three-dimensional cultures of trophoblast stem cells autonomously develop vascular-like spaces lined by trophoblast giant cells. **Developmental Biology**, v. 398, n. 1, p. 110–119, 2015.

ROBINS, J. C. et al. Bioengineering anembryonic human trophoblast vesicles. **Reproductive Sciences**, v. 18, n. 2, p. 128–135, 2011.

SAYLAM, C. et al. Distribution of fibronectin, laminin and collagen type IV in the materno-fetal boundary zone of the developing mouse placenta. Experimental study. **Archives of Gynecology and Obstetrics**, v. 266, n. 2, p. 83–85, 2002.

SCARRIT, M. E. A review of cellularization strategies for tissue engineering of whole organs. **Frontiers in Bioengineering and Biotechnology**, v. 3, n. March, p. 1–17, 2015.

SCHENKE-LAYLAND, K. et al. Collagen IV induces trophoectoderm differentiation of mouse embryonic stem cells. **Stem cells**, v. 25, n. 6, p. 1529–1538, 2007.

SCHNEIDER, K. H. et al. Decellularized human placenta chorion matrix as a favorable source of small-diameter vascular grafts. **Acta Biomaterialia**, v. 29, p. 125–134, 2016.

SCOTT, J. E. **Proteoglycan-collagen interactions**. Novartis Foundation Symposium. **Anais**...1986

SPIESS, K.; ZORN, T. M. T. Collagen types I, III, and V constitute the thick collagen fibrils of the mouse decidua. **Microscopy Research and Technique**, v. 70, p. 18–25, 2007.

SYED, O. et al. Evaluation of decellularization protocols for production of tubular small intestine submucosa scaffolds for use in oesophageal tissue engineering. **Acta Biomaterialia**, v. 10, n. 12, p. 5043–5054, 2014.

TANG, Y. et al. The combination of three-dimensional and rotary cell culture system promotes the proliferation and maintains the differentiation potential of rat BMSCs. **Scientific Reports**, v. 7, n. 1, p. 1–15, 2017.
THOMAS, T.; DZIADEK, M. Differential expression of laminin, nidogen and collagen IV genes in the midgestation mouse placenta. **Placenta**, v. 14, n. 6, p. 701–13, 1993.

UYGUN, B. E. et al. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. **Nature medicine**, v. 16, n. 7, p. 814–820, 2010.

WILLEMSE, J. et al. From organoids to organs: Bioengineering liver grafts from hepatic stem cells and matrix. **Best Practice and Research: Clinical Gastroenterology**, v. 31, n. 2, p. 151–159, 2017.

WOLF, D. A.; KLEIS, S. J. Principles of analogue and true microgravity bioreactors to tissue engineering. In: **Effect of Spaceflight and Spaceflight Analogue Culture on Human and Microbial Cells**. New York, NY: Springer New York, 2016. p. 39–60.

WU, Z. et al. Decellularized scaffolds containing hyaluronic acid and EGF for promoting the recovery of skin wounds. **Journal of materials science. Materials in medicine**, v. 26, n. 1, p. 5322, 2015.

XING, Q. et al. Decellularization of fibroblast cell sheets for natural extracellular matrix scaffold preparation. **Tissue Engineering Part C: Methods**, v. 21, n. 1, p. 77–

87, 2015.

XU, Y. et al. Preparation and characterization of bone marrow mesenchymal stem cell-derived extracellular matrix scaffolds. **Journal of Biomedical Materials Research Part B: Applied Biomaterials**, v. 103, n. 3, p. 670–678, 2015.

YAM, G. H.-F. et al. Decellularization of human stromal refractive lenticules for corneal tissue engineering. **Scientific reports**, v. 6, n. April, p. 26339, 2016.

3 MOUSE PLACENTAL SCAFFOLDS: A MODEL TO CULTURE HEPATOCYTE-LIKE CELLS INDUCED FROM MOUSE EMBRYONIC STEM CELLS IN A THREE-DIMENSIONAL DYNAMIC AND ROTATING SYSTEM

Patricia Romagnolli, Rodrigo da Silva Nunes Barreto, Rose Eli Grassi Rici, Maria Angelica Miglino

University of Sao Paulo

Correspondence: Maria Angelica Miglino, University of Sao Paulo, Av. Prof. Orlando Marques de Paiva, 87, University City, Butantã, CEP: 05508-270, Sao Paulo, Brazil. Email: miglino@usp.br

ABSTRACT

Multidisciplinary researches are searching for both new resources of biomaterials and potential hepatocytes, in order to supply drug tests, cell therapies, and cell transplantation for liver diseases. Placenta can be eligible as a new model in tissue engineering, due to its rich extracellular matrix (ECM) and availability after birth. The evaluation of placental as potential biomaterial, placental mouse scaffolds were produced by decellularization with 0.01, 0.1 and 1% SDS, and 1% Triton X-100, and they were valued by means of structure and composition. Afterwards, scaffolds were co-cultured in a rotating system with mouse embryonic fibroblasts. Placental scaffolds presented a cell-free ECM with structure and composition well-preserved and proteoglycans widespread, containing 9.42 ± 5.2 ng dsDNA per mg of ECM. Collagen I, weak in natives, clearly appears in ECM after recellularization. On the other hand, collagen III well observed in native placental tissue, was absent on scaffolds. This interesting observation may have been found due to the solubilization SDS-induced of the collagen III fibrils during decellularization. Fibronectin was well-observed in placental scaffolds whereas laminin and collagen IV were strongly expressed. Placental scaffolds as potential for repopulation was confirmed after the 3D recellularization culture, using a Rotary Cell Culture System (RCCS). Providing suitable scaffolds, newly 3D recellularization culture was performed to induce hepatic fate differentiation from mouse embryonic stem cells (mES), simulating liver embryonic development events by adding growth factors. As a result, a high index of cells adhered, proliferated and migrated through outer and inner ECM surface. Activin A and Wnt3a (d0-6) induced primitive endoderm fate, and BMP4 and FGF2 (d6-10) differentiation-induced generate definitive endoderm cells. FGF1, FGF4 and FG8b (d10-14) induced hepatoblast phenotype cells labelling AFP and CK7. HGF and FS-

288 (d14-23) induced to hepatocyte-like cells positive for CK18 and Alb markers. Glycogen storage was observed by PAS-D staining and indicating hepatocyte-like cells also. Differentiated heterogeneous cell hepatic lineage, confirmed mouse placental scaffolds as a useful model to support recellularization and simultaneous differentiation into hepatic fate, simulating phases of embryonic development.

Keywords: mouse placental scaffolds; recellularization; rotating 3D culture; embryonic stem cells; hepatocyte-like cells

1 INTRODUCTION

It is well noted that there is a global demand for therapies on liver diseases which in turn represents the scope of Regenerative Medicine, increasing the basic biomedical research, and placing cell therapy and tissue/organ engineering as main methods in experimental, basic and clinical trials (KHOLODENKO; YARYGIN, 2017).

Liver is an important organ required for metabolic activities, nutrient storage, and detoxification, with recognized ability of regeneration which in turn can be compromised if severely damaged or by inhibition of resident hepatocyte proliferation (SONG et al., 2017; XU et al., 2016).

High morbidity and mortality are associated with liver diseases although Tissue Engineering and Regenerative Medicine are offering alternative methods for liver pathology treatment demand such as cell transplantation aiming repopulating liver tissue with hepatocytes and propel own liver regeneration capacity (KHOLODENKO; YARYGIN, 2017).

Hepatocytes are parenchymal and stable cells, remaining quiescent in the G0 phase of the cell cycle (TAO et al., 2017), although proliferating in cases of hepatic injury (EZQUER et al., 2017), toward hepatic restoration and normalization (HUANG; RUDNICK, 2014). Still, the extensive parenchymal destruction, or pre-existing conditions prevent hepatic tissue restoration (ALVAREZ-SOLA et al., 2017). Facing liver diseases, therapies based on hepatocytes transplantation compensate liver

injuries, although still restricted by the inadequate donor shortage and the inability of hepatocytes to proliferate *in vitro* (HU; LI, 2015).

The investment in functional hepatocytes derived from non-hepatic origin such as embryonic stem (ES) cells (CHINZEI et al., 2002) with potential to differentiate into 200 cell types of the adult body (BHARTIYA, 2015), including hepatocytes (YAMAMOTO et al., 2003), improved a new liver cell source (OGOKE; OLUWOLE; PARASHURAMA, 2017). *In vitro* differentiation contributes to hepatocytes for clinical transplantations into the diseased liver, besides early drug discovery and toxicology screening (GABRIEL et al., 2012; GOUON-EVANS et al., 2006).

Embryonic stages of development have been recapitulated by *in vitro* ES differentiation (JONES et al., 2002), simulating the signaling pathways of the early embryo in ES cell cultures, including hepatocytes (GOUON-EVANS et al., 2006).

Initially, generating embryoid bodies from ES, hepatic differentiation evolved to protocols directly signaling embryo development under adherent culture conditions (SILLER et al., 2015). Therefore, recent protocols have now added growth factors for sequential induction of ES into primitive streak, primitive and definitive endoderm, toward hepatocyte-like cells cultures, i.e. in mouse, rat and human (PAUWELYN et al., 2011; ROELANDT et al., 2010; SONG et al., 2009).

Other important aspect concerning to cell differentiation involves the environment in which hepatocyte are generate, once conventional static two-dimensional (2D) cultures affect hepatocytes organization, shape, polarity, and functionality (NELSON et al., 2010). However, newly three-dimensional (3D) culture has providing spheroid or organoid hepatocytes, a 3D aggregate that increased cell-cell homotypic interactions (OGOKE; OLUWOLE; PARASHURAMA, 2017), essential for the proper function of liver tissues and maturation of liver cells (ISHIKAWA et al., 2011) with surgical applications *in vivo* pre-clinical and clinical trials (SHRI et al., 2017).

Moreover, a series of methods have also been developing a 3D environment for hepatocyte culture, including extracellular matrix (ECM) sandwich or hydrogel, and 3D natural or synthetically derived scaffolds (GODOY et al., 2013)

Natural or biological scaffolds are those produced by decellularization of organs and tissues, discharging resident cells, and maintaining the ultrastructure and

composition of the native ECM (FU et al., 2014). Natural scaffolds from animal models were produced by decellularization of whole liver of rats (NAVARRO-TABLEROS et al., 2015; SHUPE et al., 2010; UYGUN, 2010), mouse (WANG et al., 2014), and pigs (BAO et al., 2015). Nevertheless, scaffolds derived from human liver have been produced from hepatic lobes (MAZZA et al., 2015), or tissue fragments (MATTEI et al., 2017), and are also limited shortage of donor organs (XIANG et al., 2015).

Considering the bioengineering objectives, placentas constitute one optimal source for scaffolds production once they are usually discarded after birth (SCHNEIDER et al., 2016) thus not requiring invasive techniques to be harvested (LEONEL et al., 2018). These advantages have been introducing the usage of placentas in the context of bioengineering as a source of scaffolds as auxiliary liver connections to the systemic circulation of the recipient (KAKABADZE; KAKABADZE, 2015), supporting hepatic tissue and allowing to rescue in acute liver failure (KAKABADZE et al., 2017). Later on, we confirmed decellularized bovine cotyledons as an interesting alternative to develop large-scale scaffolds with complex vascular architecture (BARRETO et al., 2017).

Therefore, we aimed to use mouse placental scaffolds as a 3D environment intended for simultaneous ES cells recellularization and differentiation into hepatocytes-like cells, in a 3D rotating culture, and by means of simulating stages of embryonic development.

2 MATERIALS AND METHODS

The study was approved by the Ethical Committee on Animal Use of the School of Veterinary Medicine and Animal Science at the University of Sao Paulo, Sao Paulo, Brazil (Protocol Nr. 5669271015).

2.1 Placental scaffolds

The placental scaffolds production was described previously (Unpublished manuscript)[1]. Briefly, mouse placental decellularization lasted for five consecutive days, at room temperature and orbital agitation (150 rev/min). In the first three days, the placentas were treated with sodium dodecyl sulfate (SDS, 13-1313-01, LGC Biotecnologia), diluted in distillated water with 0.5% of antibiotics (penicillin-streptomycin solution, 15140130, Gibco), and used at 0.01%, 0.1% and 1%, one day each. Triton X-100 1% (13-1315-05) was applied during the last two days, finalizing cell contends extraction, and removing residual SDS. Decellularized placentas were washed three times with phosphate buffer solution (PBS; 136.9 mM of NaCl, 26.8 mM of KCl, 14.7 mM of KH2PO4 and 8.1 mM of Na2HPO4.7H2O; pH 7.2) with antibiotics, 15 min each, and sterilized in UV light for 2 h.

2.2 Mouse embryonic stem cells preparation

Initially, mouse embryonic fibroblasts (MEFs) (SCRC-1049™, ATCC®) were centrifuged and suspended in MEFs proliferating medium containing 88% Dulbecco's Modified Eagle Medium (DMEM high, 12100046, Gibco), 10% fetal bovine serum (FBS, 10Bio500, LGC Biotecnologia), 1% non-essential amino acids (MEM NEAA, BR30238-01, LGC Biotecnologia), 1% amino acids (BME, B6766, Sigma, USA), and 1 µL/mL Amikacin sulfate (16.68 µg/µL) (Novafarma, BRA). Cells are placed on a culture dish and cultured in an incubator under humidified atmosphere of 5% CO2 in air at 37ºC. Medium was changed every 48 h, and MEFs were passaged at 80% confluence, until passage six. MEFs were treated with 10 µg/mL Mitomycin C (M4287, Sigma), and plated (5 x 105 cells/mL) on 0.1% gelatin (G1890, Sigma) coated dishes. Mouse embryonic stem cells (mES), previously established line H106 (BAQIR; SMITH, 2003) were kindly provided by Prof. Joaquim M. Garcia (Sao Paulo State University, BRA). mES cell were centrifuged and suspended in mES proliferating medium containing 76.8% DMEM low (BR30002-05, LGC Biotecnologia), 20% Knock OutTM DMEM (10829018, Gibco), 1% sodium pyruvate (P8636, Sigma), 1% MEM NEAA, 1% L-

[1] ROMAGNOLLI, P.; BARRETO, R. DA S. N.; FRATINI, P.; MESS, A. M.; MIGLINO, M. A. **Mouse placental scaffolds: a three-dimensional environment model for recellularization**. Finalizado para submissão.

glutamine (BR30034-01, LGC Biotecnologia), 0.2% 110 µM 2-mercaptoethanol (21985023, Life Technologies), 1 µL/mL Leukemia Inhibitory Factor from mouse (mLIF, L5158, Sigma), and 1 µL/mL Amikacin. mES were plated (1.5 x 105 cell/mL) on mitomycin-treated MEFs, and cultured in 5% CO2, 37ºC incubator. Medium was changed every 24 h, and mES cells were split 1:6 each 48-72 h by dissociation with 1X Tryple (12563-029, Gibco). Prior to recellularization, mES cells were cultured for one passage on 0.1% gelatin coated dishes to reduce the presence of MEFs.

2.3 Recellularization and differentiation three-dimensional rotating culture

Recellularization was performed in a three-dimensional Rotary Cell Culture System (RCCS-4SC, Synthecon), under rotational speed of 11 rev/min. Ten placental scaffolds were cultured in rotating system 48 h prior to recellularization in order to stabilize the biomaterial in the culture environment, with basal mES cells differentiation medium containing 57.58% DMEM low, 40% MCDB-201-water (M6770, Sigma), 0.25% insulin-transferrin-selenium (ITS, I3146, Sigma), 0.25% linoleic acid-bovine serum albumin (LA-BSA, L9530, Sigma), 1% L-ascorbic acid (A8960, Sigma), 0.2% 110 µM 2-mercaptoethanol, 0.2% dexamethasone (D4902, Sigma), 0.5% FBS, 0.02% insulin (I9278, Sigma,) and 1 µL/mL amikacin (16.68 µg/µL). Scaffolds were placed in a 10mL sterile Rotating Wall Vessel bioreactor (RWV), adding 10 mL of medium with a syringe, while emptying bubble in the vessel with another syringe. The sterile valves were closed, and the vessel was mounted to the rotator base of the system, placed in 5% CO2, 37ºC incubator. mES cell cultured in 0.1% gelatin coated dishes were dissociated with Tryple, suspended in basal mES cells differentiation medium, and added to the vessel containing the placental scaffolds at density of 3 x 106 cells. During recellularization, medium was supplemented with cytokines and grow factors for hepatic differentiation (PAUWELYN et al., 2011; ROELANDT et al., 2010): 20 µL/mL Activin-A (A4941, Sigma) and 10 µL/mL Wnt3α (1324-WN, R&D Systems) from d0-6, 2 µL/mL FGF2 (SRP6159, Sigma) and 10 µL/mL BMP4 (SRP3260, Sigma) from d6-10, 50 µL/mL FGF1 (SRP6159, Sigma), 1 µL/mL FGF4 (F8424, Sigma) and 10 µL/mL FGF8b (SRP 6162, Sigma) from d10-14, and 4 µL/mL HGF (H1404, Sigma) and 100 µL/mL Follistatin-288 (769-FS, R&D Systems) from d14-23. Medium was changed every 24 h, with the rotating culture system placed in 5% CO2, 37ºC incubator.

2.4 Histology

Recellularized placental scaffolds were fixed in 4% buffered paraformaldehyde (PFA) for 48 h, dehydrated in ethanol, diaphanized in xylene, paraffin embedded, sectioned (5 µm) in microtome (RM2265, Leica Biosystems), and transferred to glass slides. Hematoxylin-Eosin (HE) and Periodic Acid Schiff-Diastase (PAS-D) stains were performed to evaluate nuclei and glycogen storage, respectively. For controlling of glycogen, visualization sections were stained with Periodic Acid Schiff (PAS) without previous treatment with diastase. The results were visualized at light microscopy (Eclipse 80i, Nikon), and photographed in digital camera (DS-Ri Color Digital Camera, Nikon).

2.5 Immunofluorescence of frozen sections (IF-Fr)

Placental recellularized scaffolds were mounted in OCT embedding compound (4583-1, Sakura), frozen with liquid nitrogen vapor, and stored at -150º C. Tissue sections (10 µm) were produced using a cryostat (CM1520, Leica), thaw-mounted on to poli-L-lysine (p8920, Sigma) coated histological slides, and snap freeze at -150ºC. Samples were fixed in ice-cold acetone (-20º C) for 10 min, thawed at room temperature for 30 min, and washed three times with 1X Tris-Buffered Saline (TBS; 2.0 mM of Trizma base and 1.36 mM of sodium chloride, pH 7.5) for 5 min each. Samples were incubated with primary antibodies [Anti-Collagen IV (ab6586, 1:300, abcam), msAnti-Cytokeratin 18 (ab668, 1:100, abcam), and anti-Cytokeratin 7 (sc-70936, 1:100, Santa Cruz Biotechnology)] diluted in 0.2% bovine serum albumin (BSA) in TBS in a humidified chamber for 1 h at room temperature. Newly washed with TBS, were incubated with secondary antibodies: Goat Anti-Mouse IgG H&L (Alexa Fluor® 488), ab150113, 1:500, abcam; and Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488), ab150077, 1:500, abcam] in 0.2% BSA for another hour in the dark. After another washing with TBS, the coverslips were mounted with Prolong™ Gold Antifade Mountant with DAPI (P36931, Invitrogen), and visualized in confocal microscope FV1000, Olympus.

2.6 Immunohistochemistry for paraffin-embedded tissues (IHC-P)

To analyze ECM proteins in native and decellularized 3D scaffolds, sections (5 µm) of 4% PFA fixed and paraffin-embedded samples were transferred to poli-L-lysine (p8920, Sigma) coated glass slides. Then, tissues were treated with xylene, hydrated with decrescent ethanol concentrations, and rinsed with distilled water. Antigen retrieval was performed by heat-induced in citrate buffer (1.83 mM of monohydrate citric acid and 8.9 mM of sodium citrate tribasic dehydrate, pH 6.0). The endogenous peroxidase was blocked with 3% hydrogen peroxide in distillated water at dark chamber cooling to the room temperature. Later on, nonspecific protein interaction was blocked with 2% bovine serum albumin (BSA) in tris-buffered saline (TBS; 2.0 mM of Trizma base and 1.36 mM of sodium chloride, pH 7.5). Microsections were then incubated with the following primary antibodies (abcam®): anti-Oct-4 (ab181557, 1:500), anti-Nanog (ab80892, 1:400), anti-FOXA2 (ab108422, 1:500), anti-SOX17 (ab192453, 1:300), Anti-alpha 1 Fetoprotein (ab46799, 1:100), Anti-Mouse Serum Albumin (ab19196, 1:100), overnight in humidity chamber at 4ºC. For negative controls, irrelevant anti-mouse IgG (M5284, Sigma) or anti-rabbit IgG (ab27478, Abcam) substituted the primary antibodies. The reaction was detected with Dako Advance HRP kit (K6068, Dako, USA), developing the color with DAB+ Substrate Chromogen System (K3468, Dako). A counter-staining was proceeded with hematoxylin. Washes with 0.2% BSA in TBS were performed at each step after primary antibody incubation. The slides were mounted and visualized using an Eclipse 80i microscope and photographed using a DS-Ri Color Digital Camera.

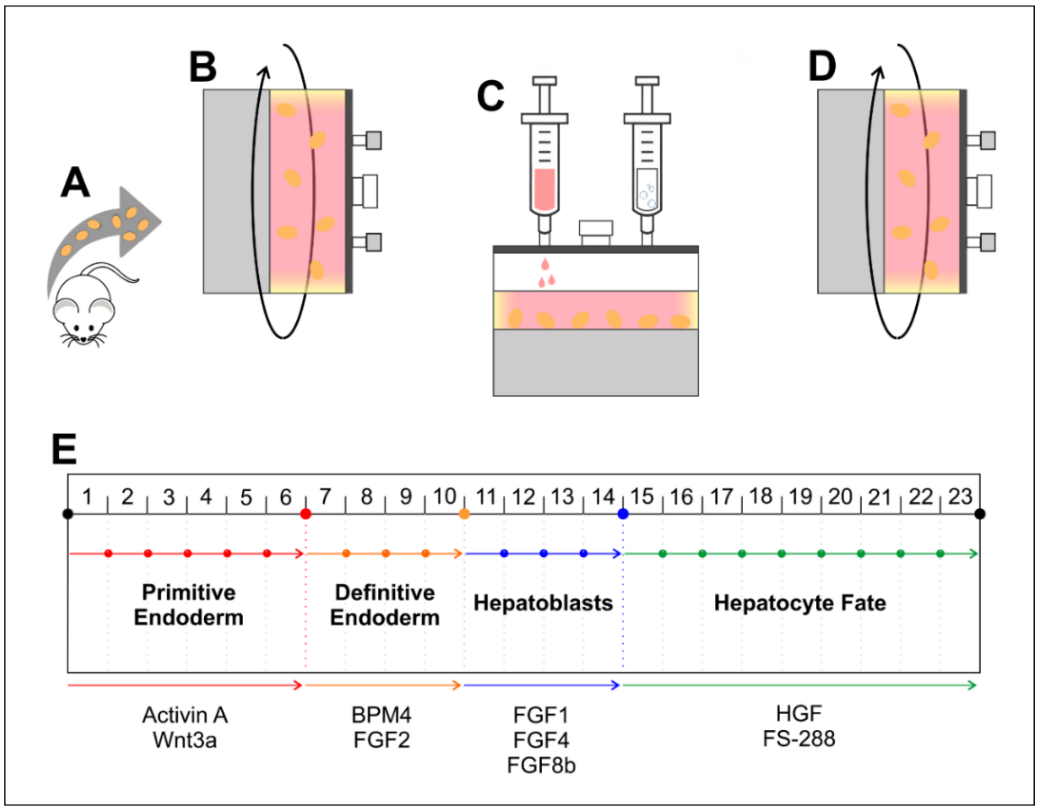
2.8 Scanning Electron Microscopy (SEM)

Recellularized placental scaffolds were fixed in 4% PFA for 48 h and in 1% osmium tetroxide for 90 min. Afterwards, samples were dehydrated in ethanol, dried using an automated critical point (EM CPD300, Leica), transferred and fixed on stubs and gold metalized (EMITECH K550). The results were visualized and photographed with a scanning electron microscope (LEO 435VP SEM, LEO Electron Microscopy Ltd).

3 RESULTS

In order to induce mES cells differentiation into hepatic fate on mouse placental scaffolds (Figure 1A), we performed the recellularization by dynamic and suspension 3D culture using a RCCS (Figure 1B, C, D). The simulation of embryonic events that was required to mES cells induction into hepatic lineage, it was developed into four phases protocol, adding growth factors as differentiation-specific inductors in the basal differentiation medium, including: Activin A and Wnt3a from d0-d6to inducing mES into primitive endoderm cells; followed by BMP4 and FGF2 from d6-d10 as definitive endoderm differentiation promoters; holding towards hepatoblasts. The medium was added at d10-d14 with FGF1, FGF2 and FGFb8 and finally, the protocol included HGF and Follistatin-288 (FS-288) to d14-d23 targeting the hepatocyte-like cells induction (Figure 1E).

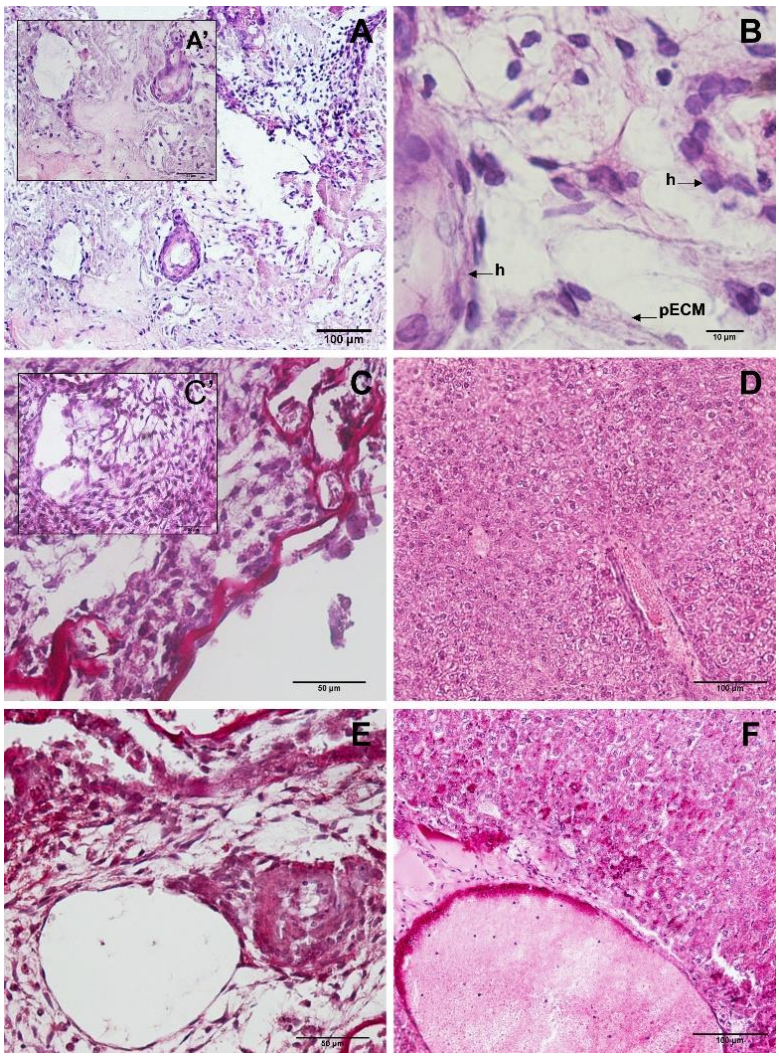
Figure 1 - Differentiation of hepatic lineage derived from mES cells on mouse placental scaffolds in a Rotating Cell Culture System (RCCS). Mouse placental scaffolds (A) were previously stabilized in basal medium in a rotating wall vessel (RWV) bioreactor (B). Embryonic cells were added, and cell differentiation was inducted towards primitive endoderm, definitive endoderm, hepatoblast and hepatocyte phenotypes respectively by addition of growth factors (E).



Fonte: (ROMAGNOLLI, P., 2018).

During 3D culture recellularization cells initially suspended in medium adhered to the scaffolds, proliferating and migrating throughout outer and inner surfaces of the acellular placental extracellular matrix (Figure 2A, B). By PAS-D staining, mES cells differentiated progeny positive to glycogen storage was observed in repopulating placental scaffold resembling hepatocyte-like cells (Figure 2C, D). Differentiated cells storing glycogen visualized on placental scaffold (Figure 2C) were indicative feature of functionality, as well as hepatocytes in mouse liver (Figure 2D). The glycogen stored levels observation was confirmed with PAS Diastase negative (Figure 2E, F).

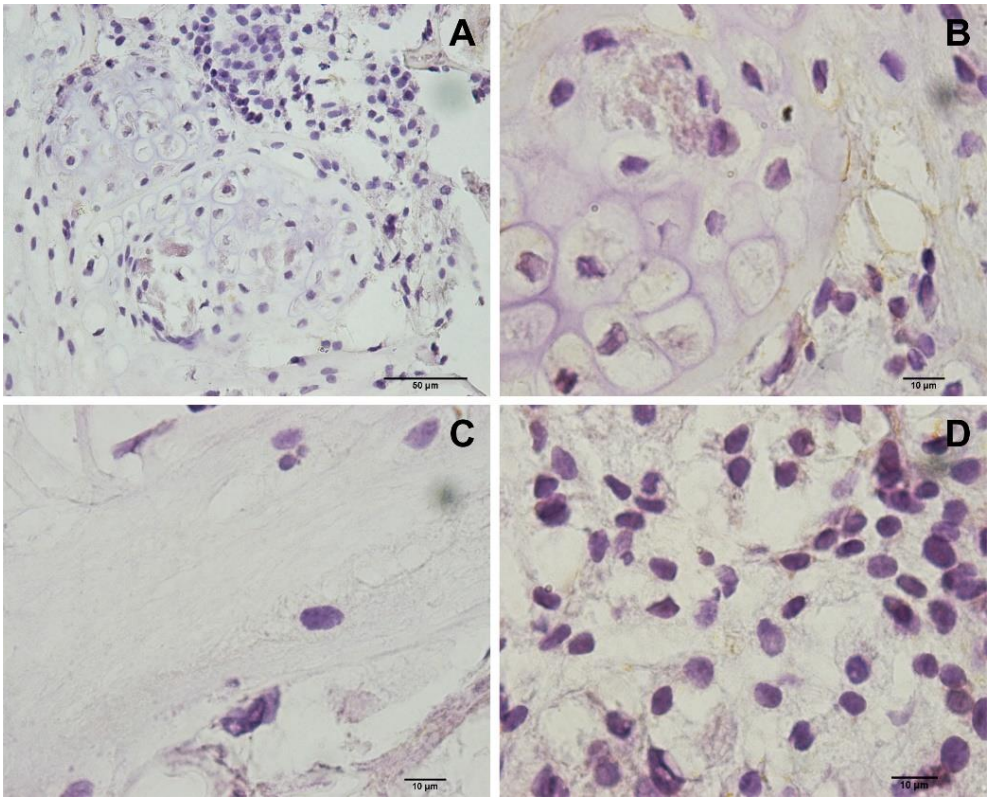
Figure 2 - Hepatic cells differentiated from mouse embryonic stem cells on mouse placental scaffolds. Hematoxylin-Eosin staining visualization of cell repopulation spread by placental scaffold (A, A'), widespread in the extracellular matrix (ECM) (B). Glycose storage visualized by Periodic Acid Schiff-Diastase staining in induced mES cells hepatocyte progeny (C) resembling natural hepatocytes on mouse liver tissue (D). Levels of glycogen were confirmed by Periodic Acid Schiff without previous Diastase treatment both for recellularized placenta (E), and mouse liver tissues (F). h, hepatic cells; pECM, placental ECM. Scale Bars = 10 µm (B), 50 µm (A', C, E), 100 µm (A, D, F)



Fonte: (ROMAGNOLLI, P., 2018).

The absence of mES cells and primitive endoderm cells on placental scaffolds after recellularization were representative of successful induction throughout the two first phases simulating liver embryonic development in 3D cultured for hepatic differentiation (Figure 3 A, B). mES was presumed absent due to its negative observation of the pluripotency Oct4 (Figure 3A) and Nanog (3B) markers. Thus, resulting differentiated hepatic lineage cells also lost their positivity to Foxa2 (Figure 3C) and Sox17 (Figure 3D) both markers typically presented by primitive endoderm cells. Given such result, the addition of Activin A and Wnt3 from d0-d6 and BMP4 and FGF2 from d6-d10 was considered efficient to guide mES pluripotency until definitive endoderm phase

Figure 3 - Immunohistochemistry of mouse placental scaffold recellularized with hepatic cells differentiated from mouse embryonic stem cells. Absence of pluripotent cells showed by negative Oct4 (A) and Nanog (B). Absence positive staining for Foxa2 (C) and Sox17 (D) immunostaining, indicating that cells have passed through primitive endoderm stage. Scale Bars = 10 µm (C, D), 50 µm (A, B)



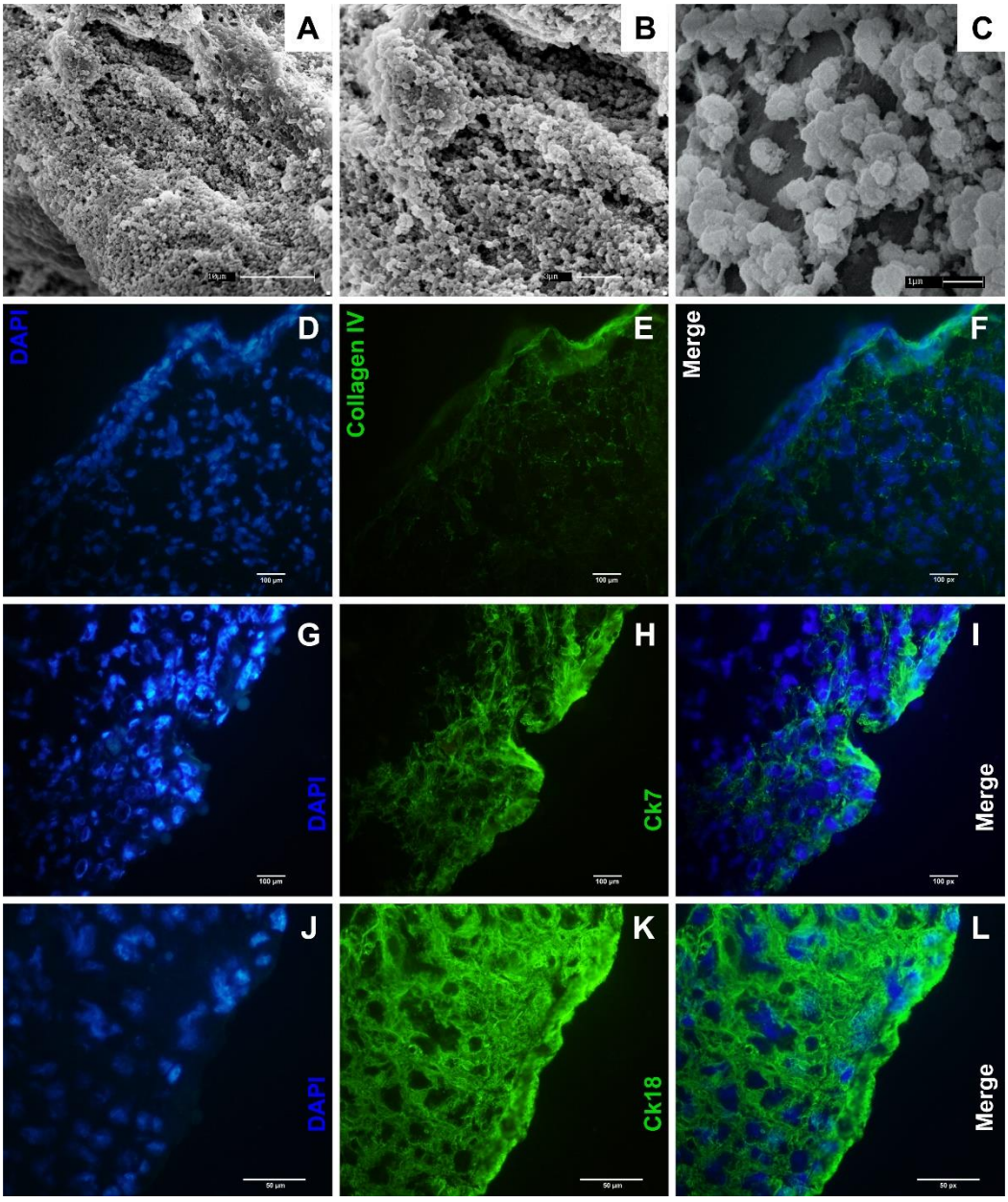
Fonte: (ROMAGNOLLI, P., 2018).

Ultrastructural aspects evolving the relationship between recellularized cells and placental scaffold ECM resulting from 3D culture recellularization were visualized by SEM (Figure 4A, B, C). Simultaneously to the events induced by growth factors

during the distinct phases of differentiation culture, cells occupied the placental scaffold 3D surface (Figure 4A). Through adhesion, proliferation and migration, cells recellularizing the placental scaffold were observed both on the outer surface and in recesses that lead to access inner spaces of placental ECM (Figure 4B). Finally, by ultrastructural analysis, it was possible to observe adhesion and modulation of the differentiated hepatic lineage cells to the placental scaffold ECM (Figure 4C). The collagen type IV preserved in the scaffolds ECM may have been an adjuvant in cell adhesion and migration (Figure 4D, E, F).

By immunofluorescence staining was observed the mES commitment to hepatic fate on placental scaffolds, as result of simultaneous differentiation and recellularization 3D culture induced by growth factors (Figure 4G-L). Thus, due FGF1, FGF2 and FGF8b induction from d10-d14 of differentiation, primitive endoderm cells were guided to hepatoblast phenotype. Although, this condition should have been overcome by the final phase of differentiation when hepatoblasts differentiating into hepatocytes. Instead, few cells on recellularized mouse placental scaffolds remained positive for the CK7 hepatoblast marker (Figure 4G, H, I) resulting in a heterogenous hepatic cell population. Finally, hepatocyte-like cells positive for CK18 marker were observed by the end of the 3D recellularization culture, as expected for HGF and FS-288 induction (Figure 4J, K, L).

Figure 4 - Mouse placental scaffolds recellularized with hepatic cells differentiated from mouse embryonic stem cells in 3D culture. Aspects of differentiation by Scanning Electron Microscopy (A-C) and Immunofluorescence (D-L). Cells proliferated and migrated through both outer (A), and inner scaffold surfaces (B), using the extracellular matrix as anchor points for adhesion (C). Differentiated hepatic cells (D) and collagen type IV in placental scaffold (E) relationship (F). Hepatic lineage repopulating placental scaffolds (G, H, J, K) positive for CK7 hepatoblast marker (H, I) and hepatocyte-like marker CK18 (K, L). Scale Bars = 1 µm (C), 3 µm (B), 10 µm (A), 50 µm (J, K), 50 PX (L), 100 µm (D, E, G, H), 100 Px (F, I)

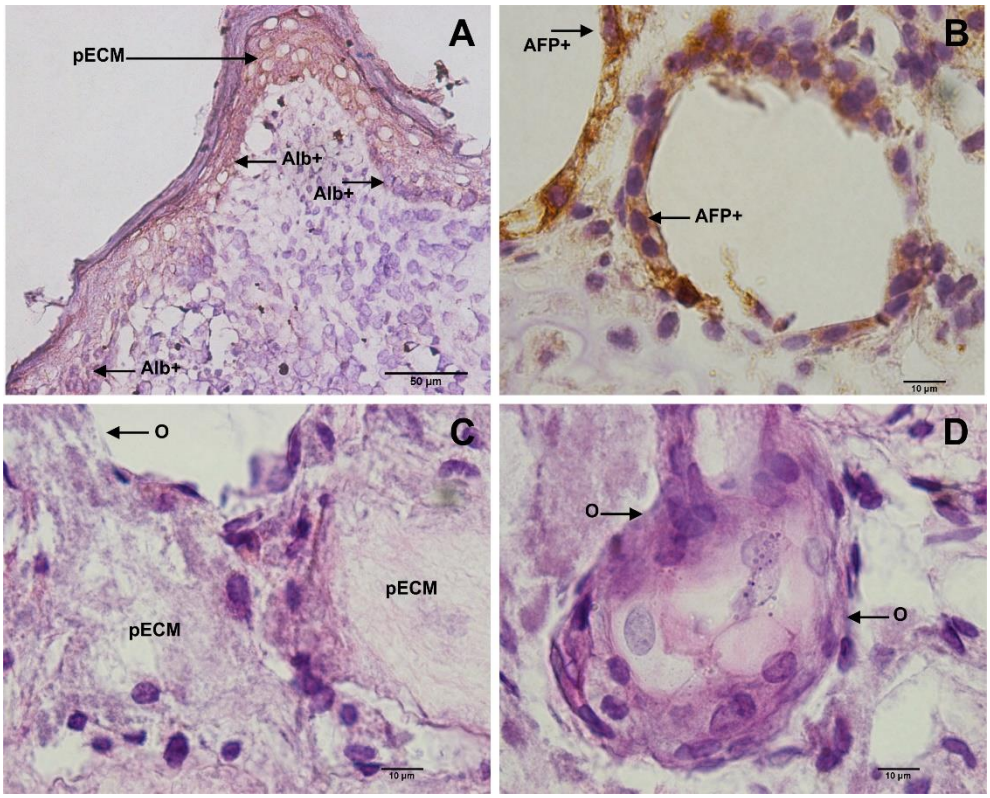


Fonte: (ROMAGNOLLI, P., 2018).

Immunohistochemistry staining findings also reveals positive signs of cell heterogenicity among final hepatic cell population on placental scaffold after recellularization (Figure 5A, B). Thus, positive staining for Alb were associated to the cells resembling hepatocyte-like cells and their circumjacent ECM (A) while positive

AFP cells were associated to hepatoblasts (B). Correlated histologic findings showed that AFP$^+$ cells were preferentially lining natural openings existent in the placental scaffolds (A, C, D).

Figure 5 - Immunohistochemistry labelling of hepatic cells differentiated from mouse embryonic stem cells on mouse placental scaffolds. Positive cells for Alb (A) and AFP (B) markers. AFP positive cells (A) preferentially lining placental ECM natural openings (B, C, D). AFP$^+$, alpha 1 fetoprotein positive cells; Alb$^+$, albumin positive cells; o, natural openings in placental extracellular matrix (ECM); pECM; placental ECM. Scale Bars = 10 µm (B, C, D), 50 µm (A)



Fonte: (ROMAGNOLLI, P., 2018).

4 DISCUSSION

Combining decellularization and recellularization specific techniques, we substituted native cells in mouse placentas for the hepatic lineage, promoting the placental extracellular matrix as a valid model to be used for biomaterials production instead of discarding after birth.

We achieved our differentiation results by following the timeline presented in recognized protocols to recapitulate embryonic stages of liver development which suggested positive signs for mES commitment to hepatic lineage. Thus, the relative heterogenous cells repopulating mouse placental scaffolds showed us immunologic and histologic labels for AFP, CK7, CK18, Alb and glycogen storage. It is most likely that they are cells displaying hepatocyte functionalities by Alb secretion and glycogen storage Finally, using a rotating system 3D culture to promote simultaneous differentiation and recellularization, we believe that the flow of cells accessing the scaffolds was favored. Moreover, by releasing from gravitational force and from restrictions of a monolayer, cell proliferation and migration, as well as cell-cell and cell-matrix seems to be favored.

Pluripotent embryonic stem cells as a source for hepatocyte-like cells differentiation were also adopted for other researches (BAHARVAND et al., 2006; CHINZEI et al., 2002; GOUON-EVANS et al., 2006; JONES et al., 2002; PAUWELYN et al., 2011; SILLER et al., 2015; WANG et al., 2012; YAMAMOTO et al., 2003).

The induction of ES differentiation into hepatic fate was performed in several protocols including distinct growth factors in addition to the culture medium in order to increase the rate of specific cell type and inducing cells into a lineage of interest (BISWAS; HUTCHINS, 2007). Initially mES were differentiated into embryoid bodies (EB) and from this stage on, the growth factors were included to the culture medium to induce hepatic fate, such as acidic fibroblast growth factor (FGF1), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF) (BAHARVAND et al., 2006; CHINZEI et al., 2002).

In order to recellularize placental scaffolds simultaneously with induction of mES to hepatocyte-like cells differentiation, we avoided EB generation and we designed our protocols based on recapitulation of embryonic stages of development (JONES et al., 2002). Thus, it mimics the signaling pathways of the early embryo in ES cell cultures (GOUON-EVANS et al., 2006) by including growth factors to the medium culture according specific concentrations, location and time (PAUWELYN et al., 2011). In this way, we adopted it as reference for grows factor addition the usage of Activin-A and Wnt3a from d0-6 to differentiate primitive endoderm; FGF2 and BMP4 from d6-10 to definitive endoderm specification; FGF1, FGF4 and FGF8b from d10-14 to

establishment of hepatoblast phenotype; and HGF and Follistatin-288 until to d23 to induce hepatocyte maturation and proliferation (PAUWELYN et al., 2011; ROELANDT et al., 2010).

Respecting and recognition are given to the value of all research and knowledge generated by the conventional 2D cultures (static) used as support environment to generate hepatocyte-like cells (GOUON-EVANS et al., 2006; SONG et al., 2009; TOUBOUL et al., 2010; YU et al., 2012). However, the 3D cell culture method adopted in our experiment was consonant with promising technologies absorbed by tissue engineered bringing new insights for cell culture, including induction of stem cells to hepatic fate. Recently, natural 3D matrices constituting of tissue engineered scaffolds have been used as important models for studies of cell-cell and cell-matrix interactions (HAKKINEN et al., 2011), demonstrating cell responses more similar to *in vivo* behavior when compared to 2D culture (EDMONDSON et al., 2014). Thus, differentiation of human embryonic stem cells into hepatocytes was performed into 3D space of natural or synthetically derived scaffolds (GODOY et al., 2013), from commercial (BAHARVAND et al., 2006) or natural sources (MAZZA et al., 2015). Hepatic differentiation has been also performed prefunding cells suspended in medium into the 3D environment of liver acellular ECM of scaffolds obtained by decellularization (PAN et al., 2016; WANG et al., 2014; XU et al., 2016) even though resulting in scattered cell colonies in the parenchyma and blood vessels (XU et al., 2016).

Later on, we performed our scaffold recellularization 3D culture using the RCCS as a tool to achieve hepatocyte-like derived from ES. Rotating bioreactors were originally developed by National Aeronautics and Space Administration (NASA) to evaluate cell growth and functions, and cell-cell interactions in a simulated microgravity of quiescent environment (BROWN et al., 2003). Such assumptions are important when considering that simulated microgravity does not alter cells perception of gravitational direction thus avoiding consequent loss of intracellular metabolic activity response (TANG et al., 2017; WOLF; KLEIS, 2016). Apparently, only few experiments were performed for hepatic cells generation from mES cells using rotating bioreactor for cell culture (ZHANG et al., 2013) or recellularization of polymer scaffolds (WANG et al., 2012). Moreover, it does not include the recellularization of natural 3D scaffolds with hepatic cells derived from mES differentiation at once.

Activin A and Wnt3a from d0-6 and BMP4 and FGF2 from d6-10 were added to the medium during recellularizaton in order to induce differentiation of pluripotent and primitive endoderm cells, respectively. Thus, ultimate cell population on recellularized placental scaffolds showed negative labells for the genes associated with pluripotency (Oct4 and Nanog) and endoderm (Foxa2 and Sox17) (DELAFOREST et al., 2011; PAUWELYN et al., 2011; ROELANDT et al., 2010; SONG et al., 2009; YU et al., 2012). Since 2006, protocols using ES cells identified the role of activin A and Wnt3 signaling during the establishment of primitive streak which ultimately leads to endoderm specification expression of Sox17 and Foxa2 markers (DELAFOREST et al., 2011; OGOKE; OLUWOLE; PARASHURAMA, 2017; SCHWARTZ et al., 2014; SILLER et al., 2015; SONG et al., 2009; TOUBOUL et al., 2010). BMP4 and FGF2 are secreted by embryonic adjacent cardiac mesoderm and septum transversum mesenchyme, respectively (ROELANDT et al., 2010). BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived endoderm (GOUON-EVANS et al., 2006; TOUBOUL et al., 2010) while FGF2 also promotes prehepatic endoderm (LEMAIGRE, 2009).

FGF1, FGF4 and FG8b in medium culture from d10-14 induced endoderm cells to hepatoblast phenotype and proliferation through placental scaffolds. Positive immunolabelling for CK7 and AFP hepatoblasts phenotypic markers (CHAUDHARI et al., 2016; YU et al., 2012) indicates the presence of immature hepatocyte-like cells (PAUWELYN et al., 2011). In the early stages of the differentiation toward maturation, the intermediate hepatocytes are positive for CK7 becoming negative at the late stages (TAKIYA et al., 2013). Hepatic differentiation cell protocols based on embryonic development simulation are challenged by their results exhibiting immature hepatic phenotype and persistently expressing hepatoblasts phenotypic markers (GODOY et al., 2013; SCHWARTZ et al., 2014).

Finally, from d14-23 HGF and FS-288 were added to the recellularization medium. As consequence, hepatocyte-like cells positive for CK18 and Alb markers were observed on repopulated placental scaffolds. HGF is a hepatotrophic factor (NELSON et al., 2010; STARZL; FUNG, 2010) that stimulates hepatoblast proliferation and liver regeneration (VOJDANI et al., 2015). HGF is employed as inductor of hepatocyte maturation and expansion in protocols simulating embryonic development while FS-288 act over bipotential hepatoblasts to favor hepatic over cholangiocyte

differentiation (PAUWELYN et al., 2011; ROELANDT et al., 2010; SONG et al., 2009). Alb and CK18 are characteristic markers of adult hepatocytes (GODOY et al., 2013; SCHWARTZ et al., 2014; SHRI et al., 2017; YAMAMOTO et al., 2003). Although, when expressed at low levels, Alb is one of the best characterized markers of nascent hepatic cells (SI-TAYEB; LEMAIGRE; DUNCAN, 2010).

In conclusion, we applied mouse placental scaffolds as a useful model to simultaneous recellularization and differentiations of MEs into hepatic fate via a protocol that simulates major stages of embryonic liver development in a rotating and dynamic 3D culture system.

ACKNOWLEDGEMENTS

Our gratitude to the Advanced Center of Image Diagnosis (CADI) of the School of Veterinary Medicine and Animal Science of the University of Sao Paulo, for microscopy supporting. We have received grants from Sao Paulo Research Foundation (14/50844-3) and from National Council for Scientific and Technological Development (467476/2014-4)

REFERENCES

ALVAREZ-SOLA, G. et al. Engineered fibroblast growth factor 19 protects from acetaminophen-induced liver injury and stimulates aged liver regeneration in mice. **Cell Death and Disease**, v. 8, n. 10, 2017.

BAHARVAND, H. et al. Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems *in vitro*. **International Journal of Developmental Biology**, v. 50, n. 7, p. 645–652, 2006.

BAO, J. et al. Hemocompatibility improvement of perfusion-decellularized clinical-scale liver scaffold through heparin immobilization. **Scientific Reports**, v. 5, n. June, p. 1–13, 2015.

BAQIR, S.; SMITH, L. C. Growth restricted *in vitro* culture conditions alter the imprinted gene expression patterns of mouse embryonic stem cells. **Cloning Stem Cells**, v. 5, n. 3, p. 199–212, 2003.

BARRETO, R. DA S. N. et al. Decellularized bovine cotyledons may serve as biological scaffolds with preserved vascular arrangement. **Journal of Tissue Engineering and Regenerative Medicine**, p. 1–9, 2017.

BHARTIYA, D. Intricacies of pluripotency. **Journal of Stem Cells & Regenerative Medicine**, v. 11, n. 1, p. 2–6, jan. 2015.

BISWAS, A.; HUTCHINS, R. Embryonic stem cells. **Stem Cells and Development**, v. 16, n. 2, p. 213–22, 23 abr. 2007.

BROWN, L. A et al. Maintenance of liver functions in rat hepatocytes cultured as spheroids in a rotating wall vessel. ***In vitro*** **Cellular & Developmental Biology. Animal**, v. 39, n. 1–2, p. 13–20, 2003.

CHAUDHARI, P. et al. Expression kinetics of hepatic progenitor markers in cellular models of human liver development recapitulating hepatocyte and biliary cell fate commitment. **Experimental Biology and Medicine**, v. 241, n. 15, p. 1653–1662, 2016.

CHINZEI, R. et al. Embryoid-body cells derived from a mouse embryonic stem cell line show differentiation into functional hepatocytes. **Hepatology**, v. 36, n. 1, p. 22–29, 2002.

DELAFOREST, A. et al. HNF4A is essential for specification of hepatic progenitors from human pluripotent stem cells. **Journal of Cell Science**, v. 124, n. 19, p. e1–e1, 2011.

EDMONDSON, R. et al. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. **ASSAY and Drug Development Technologies**, v. 12, n. 4, p. 207–218, 2014.

EZQUER, F. et al. Administration of multipotent mesenchymal stromal cells restores liver regeneration and improves liver function in obese mice with hepatic steatosis after partial hepatectomy. **Stem Cell Research & Therapy**, v. 8, n. 1, p. 20, 2017.

FU, R. H. et al. Decellularization and recellularization technologies in tissue engineering. **Cell Transplantation**, v. 23, n. 4–5, p. 621–630, 2014.

GABRIEL, E. et al. Differentiation and selection of hepatocyte precursors in suspension spheroid culture of transgenic murine embryonic stem cells. **PLoS ONE**, v. 7, n. 9, p. e44912, 2012.

GODOY, P. et al. Recent advances in 2D and 3D *in vitro* systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. Archives of Toxicology, v. 87, n. 8, p. 1315–153

GOUON-EVANS, V. et al. BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm. **Nature Biotechnology**, v. 24, n. 11, p. 1402–1411, 2006.

HAKKINEN, K. M. et al. Direct comparisons of the morphology, migration, cell adhesions, and actin cytoskeleton of fibroblasts in four different three-dimensional extracellular matrices. **Tissue Engineering Part A**, v. 17, n. 5–6, p. 713–724, 2011.

HU, C.; LI, L. *In vitro* culture of isolated primary hepatocytes and stem cell-derived hepatocyte-like cells for liver regeneration. **Protein & Cell**, v. 6, n. 8, p. 562–574, 2015.

HUANG, J.; RUDNICK, D. A. Elucidating the metabolic regulation of liver regeneration. **American Journal of Pathology**, v. 184, n. 2, p. 309–321, 2014.

ISHIKAWA, M. et al. Reconstitution of hepatic tissue architectures from fetal liver cells obtained from a three-dimensional culture with a rotating wall vessel bioreactor. **Journal of Bioscience and Bioengineering**, v. 111, n. 6, p. 711–718, 2011.

JONES, E. A et al. Hepatic differentiation of murine embryonic stem cells. **Experimental Cell Research**, v. 272, p. 15–22, 2002.

KAKABADZE, A.; KAKABADZE, Z. Prospect of using decellularized human placenta and cow placentome for creation of new organs: Targeting the liver (Part I: Anatomic study). **Transplantation Proceedings**, v. 47, p. 1222–1227, 2015.

KAKABADZE, Z. et al. Decellularized human placenta supports hepatic tissue and allows rescue in acute liver failure. **Hepatology**, 6 dez. 2017.

KHOLODENKO, I. V.; YARYGIN, K. N. Cellular mechanisms of liver regeneration and cell-based therapies of liver diseases. **BioMed Research International**, v. 2017, 2017.

LEMAIGRE, F. P. Mechanisms of liver development: concepts for understanding liver disorders and design of novel therapies. **Gastroenterology**, v. 137, n. 1, p. 62–79, 2009.

LEONEL, L. C. P. C. et al. Decellularization of placentas: Establishing a protocol. **Brazilian Journal of Medical and Biological Research**, v. 51, n. 1, p. 1–12, 2018.

MATTEI, G. et al. Decellularized human liver is too heterogeneous for designing a generic extracellular matrix mimic hepatic scaffold. **Artificial Organs**, v. 41, n. 5, 2017.

MAZZA, G. et al. Decellularized human liver as a natural 3D-scaffold for liver bioengineering and transplantation. **Scientific reports**, v. 5, p. 13079, 2015.

NAVARRO-TABLEROS, V. et al. Recellularization of rat liver scaffolds by human liver stem cells. **Tissue Engineering Part A**, v. 21, n. 11–12, p. 1929–1939, 2015.

NELSON, L. J. et al. Low-shear modelled microgravity environment maintains morphology and differentiated functionality of primary porcine hepatocyte cultures. **Cells Tissues Organs**, v. 192, n. 2, p. 125–140, 2010.

OGOKE, O.; OLUWOLE, J.; PARASHURAMA, N. Bioengineering considerations in liver regenerative medicine. **Journal of Biological Engineering**, v. 11, n. 1, p. 1–16, 2017.

PAN, J. et al. *In-vivo* organ engineering: Perfusion of hepatocytes in a single liver lobe scaffold of living rats. International **Journal of Biochemistry and Cell Biology**, v. 80, p. 124–131, 2016.

PAUWELYN, K. et al. Culture of mouse embryonic stem cells with serum but without exogenous growth factors is sufficient to generate functional hepatocyte-like cells. **PLoS One**, v. 6, n. 8, p. e23096, 2011.

ROELANDT, P. et al. Human embryonic and rat adult stem cells with primitive endoderm-like phenotype can be fated to definitive endoderm, and finally hepatocyte-like cells. **PLoS One**, v. 5, n. 8, p. 1–11, 2010.

SCHNEIDER, K. H. et al. Decellularized human placenta chorion matrix as a favorable source of small-diameter vascular grafts. **Acta Biomaterialia**, v. 29, p. 125–134, 2016.

SCHWARTZ, R. E. et al. Pluripotent stem cell-derived hepatocyte-like cells. **Biotechnology Advances**, v. 32, n. 2, p. 504–513, 2014.

SHRI, M. et al. Hanging drop, a best three-dimensional (3D) culture method for primary buffalo and sheep hepatocytes. **Scientific Reports**, v. 7, n. 1, p. 1–13, 2017.

SHUPE, T. et al. Method for the decellularization of intact rat liver. **Organogenesis**, v. 6, n. 2, p. 134–136, 2010.

SI-TAYEB, K.; LEMAIGRE, F. P.; DUNCAN, S. A. Organogenesis and development of the liver. **Developmental Cell**, v. 18, n. 2, p. 175–189, 2010.

SILLER, R. et al. Small-molecule-driven hepatocyte differentiation of human pluripotent stem cells. **Stem Cell Reports**, v. 4, n. 5, p. 939–952, 2015.

SONG, Z. et al. Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. **Cell Research**, v. 19, n. 11, p. 1233–1242, 2009.

SONG, Z. et al. Mechanosensing in liver regeneration. **Seminars in Cell and Developmental Biology**, v. 71, p. 153–167, 2017.

STARZL, T. E.; FUNG, J. J. Themes of liver transplantation. **Hepatology**, v. 51, p. 1869–1884, 2010.

TAKIYA, C. M. et al. Liver resident stem cell. In: **Resident Stem Cells and Regenerative Therapy**. [s.l.] Elsevier, 2013. p. 177–203.

TANG, Y. et al. The combination of three-dimensional and rotary cell culture system promotes the proliferation and maintains the differentiation potential of rat BMSCs. **Scientific Reports**, v. 7, n. 1, p. 1–15, 2017.

TAO, Y. et al. Liver regeneration: analysis of the main relevant signaling molecules. **Mediators of Inflammation**, v. 2017, 2017.

TOUBOUL, T. et al. Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. **Hepatology**, v. 51, n. 5, p. 1754–1765, 2010.

UYGUN, B. E. et al. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. **Nature medicine**, v. 16, n. 7, p. 814–820, 2010.

VOJDANI, Z. A. V et al. The influence of fibroblast growth factor 4 on hepatogenic capacity of Wharton's jelly mesenchymal stromal cells. **Romanian Journal of Morphology & Embryology**. v. 56, n. 3, p. 1043–1050, 2015.

WANG, X. et al. Decellularized liver scaffolds effectively support the proliferation and differentiation of mouse fetal hepatic progenitors. **Journal of Biomedical Materials Research - Part A**, v. 102, n. 4, p. 1017–1025, 2014.

WANG, Y. et al. Rotating microgravity-bioreactor cultivation enhances the hepatic differentiation of mouse embryonic stem cells on biodegradable polymer scaffolds. **Tissue Engineering Part A**, v. 18, n. 21–22, p. 2376–2385, 2012.

WOLF, D. A.; KLEIS, S. J. Principles of analogue and true microgravity bioreactors to tissue engineering. In: **Effect of Spaceflight and Spaceflight Analogue Culture on Human and Microbial Cells**. New York, NY: Springer New York, 2016. p. 39–60.

XIANG, J. X. et al. Liver regeneration using decellularized splenic scaffold: A novel approach in tissue engineering. **Hepatobiliary and Pancreatic Diseases International**, v. 14, n. 5, p. 502–508, 2015.

XU, B. et al. Assessing rat liver-derived biomatrix for hepatic tissue engineering with human fetal liver stem cells. **Advances in Tissue Engineering and Regenerative Medicine**, v. 1, n. 3, p. 1–9, 2016.

YAMAMOTO, H. et al. Differentiation of embryonic stem cells into hepatocytes: Biological functions and therapeutic application. **Hepatology**, v. 37, n. 5, p. 983–993, 2003.

YU, Y. et al. Hepatocyte-like cells differentiated from human induced pluripotent stem cells: Relevance to cellular therapies. **Stem Cell Research**, v. 9, n. 3, p. 196–207, 2012.

ZHANG, S. et al. Efficient large-scale generation of functional hepatocytes from mouse embryonic stem cells grown in a rotating bioreactor with exogenous growth factors and hormones. **Stem Cell Research and Therapy**, v. 4, n. 6, 2013.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

Researchers from multiple areas around the World are searching for solutions to diseases and injuries that may compromise health and life. In several fields of knowledge technologies has been produced in order to integrate these distinct areas for the common benefit. It is within this scope that Regenerative Medicine acts, aiming act on the restoration of organs and tissues, from both molecular and cellular levels, until the magnification of what can be seen with the naked eye. However, Regenerative Medicine needs to be supplied with materials and techniques that allow its full development. Therefore, the importance of Tissue Engineering lies in supply products and technologies to support the applications of '*medicine that regenerates*'.

The main objective of this research was based on the use of placentas, that are organs of extreme value, produced naturally and in great quantity, but which are discarded insofar as they fail to fulfill their primary purpose. Knowing the biological characteristics of the Placentas, we glimpsed their application for the benefits of biomaterials production, since the promisingly structure and rich composition in the placental extracellular matrix waved us positively.

Adopting the mouse placentas as a model, we started our journey towards mouse placental scaffolds production, by optimizing and standardizing an efficient protocol of decellularization. The challenge was before us, with minimal references about placentas decellularization, and none for mouse placentas. Completing successfully the phase of decellularization, as well as the analysis regarding its structure and composition, we had produced one of the few models of placental scaffolds developed, and unique for mouse. Then, we directed our efforts and scientific trials testing the bioactivity of mouse placental scaffolds receiving cells from a diverse nature of their original population. And another great challenge was in front of us, as knowing the limitations faced by the scientific community to obtain high rates of cellular repopulation on scaffolds, regardless of their nature. In addition, the best-effect procedures for reintroducing cells into scaffolds were beyond the morphological possibilities of our specimens. That's because the cell infusion by any vascular via was

not a plausible reality for mouse placental scaffolds with a mean of 0.9 cm in diameter. And the multidisciplinary concept presented us the current device ‘Rotary Cell Culture System (RCCS)’. Having a highly technological product originally produced by NASA, we performed the recellularization of our small placental scaffolds with embryonic mouse fibroblasts. The results were highly satisfactory, both for the reseeded cells behavior, adherence and proliferation in the new environment, and for validate the three-dimensional cell culture system at our disposal for further experiments.

With previous results, we used the mouse placental scaffolds as a source of biomaterial already validated, and we launched the new challenge: to recellularize the scaffolds with embryonic stem cells simultaneously to hepatocytes differentiation. Many protocols had already been tested with this intent, contemplating the most varied methods and materials. To differentiate hepatocytes also have had been used pluripotent stem cells both from other species, and the induced ones. Besides, protocols instituted for hepatic fate differentiation also included cells from later phases of development, such as endoderm cells, hepatoblasts, and immature or quiescent hepatocytes, considered as 'hepatic stem cells'. Among this diversity, the main challenge remained, due experiments resulting in a heterogeneous cell population.

Among the diversified possibilities of growing hepatocytes from embryonic stem cells, in 2006 a new modality proposed the simulation of the main embryonic events by specific growth factors signaling. Continuing the main liver embryonic phases, the protocols included the induction of pluripotent cells into primitive endoderm, and from there following the pathways of differentiation towards definitive endoderm, hepatoblasts, and culminating into hepatocytes specification. And this was our choice.

We have joined our unique mouse placental scaffolds both with the cellular models that revolutionized the history of medicine (stem cells), and the three-dimensional system of last generation culture, putting into practice one of the latest models for hepatic cells differentiation to reproduce what exists in nature.

The cell population drifting into the culture medium not only adhered to the scaffolds but also proliferated in its matrix and migrated into the scaffolds. The stem cells originally added to the culture followed the embryonic development stages signaled by growth factors, developing into primitive and definitive endoderm cells, hepatoblasts, and hepatocyte-like cells. After 23 days, we have obtained a

heterogeneous population, constituted by hepatic lineage, yet not homogeneous matured.

We have come a long way, and now we are able to state placentas in-between the valuable tools for scaffolds production, suitable to receive distinct cell types, either by direct recellularization or by differentiation. We observed that both 3D rotating culture system, and 3D matrices on scaffolds are favorable for the development of the cell's natural morphology, also for cells adhesion, proliferation and migration.

We have concluded that the future is still open to the challenges, both for the improvement of the technique that we performed to reproduce hepatocytes homogeneously, and for the culture of several other cell types under the same conditions.

We hope to contribute with our results for the development of knowledge and, above all, we hope to learn even more.

REFERENCES

ALESHCHEVA, G. et al. Scaffold-free tissue formation under real and simulated microgravity conditions. **Basic and Clinical Pharmacology and Toxicology**, v. 119, p. 26–33, 2016.

ALLAN, B. Closer to nature: new biomaterials and tissue engineering in ophthalmology. **British Journal of Ophthalmology**, v. 83, p. 1235–1240, 1999.

BADYLAK, S. F. et al. Engineered whole organs and complex tissues. **The Lancet**, v. 379, n. 9819, p. 943–952, 2012.

BADYLAK, S. F.; TAYLOR, D.; UYGUN, K. Whole-organ tissue engineering: decellularization and recellularization of three-dimensional matrix scaffolds. **Annual Review of Biomedical Engineering**, v. 13, p. 27–53, 2011.

BAHARVAND, H. et al. Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems *in vitro*. **International Journal of Developmental Biology**, v. 50, n. 7, p. 645–652, 2006.

BALDWIN, J. et al. *In vitro* pre-vascularization of tissue-engineered constructs A co-culture perspective. **Vascular Cell**, v. 6, p. 13, 2014.

BARRETO, R. DA S. N. et al. Decellularized bovine cotyledons may serve as biological scaffolds with preserved vascular arrangement. **Journal of Tissue Engineering and Regenerative Medicine**, p. 1–9, 2017.

BHARTIYA, D. Intricacies of pluripotency. **Journal of Stem Cells & Regenerative Medicine**, v. 11, n. 1, p. 2–6, jan. 2015.

BISWAS, A.; HUTCHINS, R. Embryonic stem cells. **Stem cells and Development**, v. 16, n. 2, p. 213–22, 23 abr. 2007.

CARTER, A. M. Animal models of human placentation - A review. **Placenta**, v. 21, n. Suppl., p. 41–47, 2007.

CARTER, A. M. Sources for comparative studies of placentation I. Embryological collections. **Placenta**, v. 29, n. 1, p. 95–98, 2008.

COAN, P. M.; FERGUSON-SMITH, A. C.; BURTON, G. J. Ultrastructural changes in the interhaemal membrane and junctional zone of the murine choriollantoic placenta across gestation. **Journal of Anatomy**, v. 207, n. 6, p. 783–796, 2005.

DA ANUNCIAÇÃO, A. R. A. et al. Extracellular matrix in epitheliochorial, endotheliochorial and haemochorial placentation and its potential application for regenerative medicine. **Reproduction in Domestic Animals**, v. 52, n. 1, p. 3–15, 2017.

DE KEMP, V. et al. Tissue engineering for human urethral reconstruction: systematic review of recent literature. **PLoS One**, v. 10, n. 2, p. e0118653, 2015.

DEBELS, H. et al. Dermal matrices and bioengineered skin substitutes. **Plastic and Reconstructive Surgery Global Open**, v. 3, n. 1, p. e284, 2015.

FRANCIPANE, M. G.; LAGASSE, E. Toward organs on demand: breakthroughs and challenges in models of organogenesis. **Current Pathobiology Reports**, v. 4, n. 3, p. 77–85, 2 set. 2016.

FREED, L. E. et al. Advanced tools for tissue engineering: scaffolds, bioreactors, and signaling. **Tissue engineering**, v. 12, n. 12, p. 3285–3305, 2006.

FU, R. H. et al. Decellularization and recellularization technologies in tissue engineering. Cell Transplantation, v. 23, n. 4–5, p. 621–630, 2014.

GABRIEL, E. et al. Differentiation and selection of hepatocyte precursors in suspension spheroid culture of transgenic murine embryonic stem cells. **PLoS One**, v. 7, n. 9, p. e44912, 2012.

HOSSEINKHANI, M.; MEHRABANI, D.; KARIMFAR, M. H. Tissue engineered scaffolds in regenerative medicine. **World Journal of Plastic Surgery**, v. 3, p. 6–8, 2014.

HUANG, J. et al. Tissue engineering and regenerative medicine in basic research: A year in review of 2014. **Tissue Engineering Part B: Reviews**, v. 21, n. 2, p. 167–176, abr. 2015.

JANIK, H.; MARZEC, M. A review: Fabrication of porous polyurethane scaffolds. **Materials Science & Engineering C**, v. 48, p. 586–591, 2015.

JONES, E. A et al. Hepatic differentiation of murine embryonic stem cells. **Experimental Cell Research**, v. 272, p. 15–22, 2002.

KAKABADZE, A.; KAKABADZE, Z. Prospect of using decellularized human placenta and cow placentome for creation of new organs: targeting the liver (Part I: Anatomic study). **Transplantation Proceedings**, v. 47, p. 1222–1227, 2015.

KAKABADZE, Z. et al. Decellularized human placenta supports hepatic tissue and allows rescue in acute liver failure. **Hepatology**, 6 dez. 2017.

KATARI, R.; PELOSO, A.; ORLANDO, G. Tissue engineering and regenerative medicine: semantic considerations for an evolving paradigm. **Frontiers in Bioengineering and Biotechnology**, v. 2, p. 1–6, 2015.

KEANE, T. J.; BADYLAK, S. F. Biomaterials for tissue engineering applications. **Seminars in Pediatric Surgery**, v. 23, p. 112–118, 2014.

LEISER, R.; KAUFMANN, P. Placental structure: in a comparative aspect. **Experimental and Clinical Endocrinology & Diabetes**, v. 102, p. 122–134, 1994.

LEONEL, L. C. P. C. et al. Decellularization of placentas: Establishing a protocol. **Brazilian Journal of Medical and Biological Research**, v. 51, n. 1, p. 1–12, 2018.

LONDONO, R.; BADYLAK, S. F. Biologic scaffolds for regenerative medicine: Mechanisms of *in vivo* Remodeling. **Annals of Biomedical Engineering**, v. 43, n. 3, p. 577–592, 2014.

MESS, A. M.; CARTER, A. M. Evolution of the interhaemal barrier in the placenta of rodents. **Placenta**, v. 30, n. 10, p. 914–918, 2009.

NAVARRO-TABLEROS, V. et al. Recellularization of rat liver scaffolds by human liver stem cells. **Tissue Engineering Part A**, v. 21, n. 11–12, p. 1929–1939, 2015.

NELSON, L. J. et al. Low-shear modelled microgravity environment maintains morphology and differentiated functionality of primary porcine hepatocyte cultures. **Cells Tissues Organs**, v. 192, n. 2, p. 125–140, 2010.

O'BRIEN, F. J. Biomaterials & scaffolds for tissue engineering. **Materials Today**, v. 14, n. 3, p. 88–95, 2011.

PARK, Y.-B. et al. Effect of chondrocyte-derived early extracellular matrix on chondrogenesis of placenta-derived mesenchymal stem cells. **Biomedical Materials**, v. 10, n. 3, p. 35014, 2015.

PAUWELYN, K. et al. Culture of mouse embryonic stem cells with serum but without exogenous growth factors is sufficient to generate functional hepatocyte-like cells. **PLoS One**, v. 6, n. 8, p. e23096, 2011.

BAKHSHAYESH, A. R. et al. Recent advances on biomedical applications of scaffolds in wound healing and dermal tissue engineering. **Artificial Cells, Nanomedicine, and Biotechnology**, v. 12, p. 1–15, 2017.

REING, J. E. et al. Degradation products of extracellular matrix affect cell migration and proliferation. **Tissue Engineering: Part** A, v. 15, n. 3, p. 605–614, 2009.

REING, J. E. et al. The effects of processing methods upon mechanical and biologic properties of porcine dermal extracellular matrix scaffolds. **Biomaterials**, v. 31, n. 33, p. 8626–8633, 2010.

RHEE, S. Fibroblasts in three dimensional matrices: Cell migration and matrix remodeling. **Experimental and Molecular Medicine**, v. 41, n. 12, p. 858–865, 2009.

ROELANDT, P. et al. Human embryonic and rat adult stem cells with primitive endoderm-like phenotype can be fated to definitive endoderm, and finally hepatocyte-like cells. **PLoS One**, v. 5, n. 8, p. 1–11, 2010.

SCHNEIDER, K. H. et al. Decellularized human placenta chorion matrix as a favorable source of small-diameter vascular grafts. **Acta Biomaterialia**, v. 29, p. 125–134, 2016.

SCHWARTZ, R. E. et al. Pluripotent stem cell-derived hepatocyte-like cells. **Biotechnology Advances**, v. 32, n. 2, p. 504–513, 2014.

SILLER, R. et al. Small-molecule-driven hepatocyte differentiation of human pluripotent stem cells. **Stem Cell Reports**, v. 4, n. 5, p. 939–952, 2015.

TAKATA, K.; FUJIKURA, K.; SHIN, B.-C. Ultrastructure of the rodent placental labyrinth: A site of barrier and transport. **Journal of Reproduction and Development**, v. 43, n. 1, p. 13–24, 1997.

TEODORI, L. et al. Native extracellular matrix: A new scaffolding platform for repair of damaged muscle. **Frontiers in Physiology**, v. 5, p. 1–9, 2014.

VARLEY, M. C.; MARKAKI, A. E.; BROOKS, R. A. Effect of rotation on scaffold motion and cell growth in rotating bioreactors. **Tissue Engineering Part A**, v. 23, n. 11–12, p. 522–534, 2017.

WANG, Y. et al. Rotating microgravity-bioreactor cultivation enhances the hepatic differentiation of mouse embryonic stem cells on biodegradable polymer scaffolds. **Tissue Engineering Part A**, v. 18, n. 21–22, p. 2376–2385, 2012.

WATSON, E.; CROSS, J. Development of structures and transport functions in the mouse placenta. **Physiology**, v. 20, p. 180–193, 2005.

XU, Y. et al. Preparation and characterization of bone marrow mesenchymal stem cell-derived extracellular matrix scaffolds. **Journal of Biomedical Materials Research Part B: Applied Biomaterials**, v. 103, n. 3, p. 670–678, 2015.

YAMAMOTO, H. et al. Differentiation of embryonic stem cells into hepatocytes: Biological functions and therapeutic application. **Hepatology**, v. 37, n. 5, p. 983–993, 2003.