Human fetal progenitor tenocytes for the treatment of tendon afflictions

Grognuz, Anthony

UNIVERSITÉ DE GENÈVE

Section des sciences pharmaceutiques — FACULTÉ DES SCIENCES

Professeur Olivier Bugnon

UNIVERSITÉ DE LAUSANNE

Département de l'appareil locomoteur — FACULTÉ DE BIOLOGIE ET MÉDECINE

Professeure Lee Ann Laurent-Applegate

Human Fetal Progenitor Tenocytes for the Treatment of Tendon Afflictions

THÈSE

présentée à la Faculté des sciences de l'Université de Genève

pour obtenir le grade de Docteur ès sciences, mention sciences pharmaceutiques

par

Anthony GROGNUZ

de

Poliez-Pittet (VD)

Thèse N° 4886

GENÈVE

Atelier ReproMail

2016

UNIVERSITÉ
DE GENÈVE

FACULTÉ DES SCIENCES

Doctorat ès sciences
Mention sciences pharmaceutiques

Thèse de *Monsieur Anthony GROGNUZ*

intitulée :

"Human Fetal Progenitor Tenocytes for the Treatment of Tendon Afflictions"

La Faculté des sciences, sur le préavis de Madame L. A. LAURENT-APPLEGATE, professeure associée et directrice de thèse (Département de l'appareil Locomoteur, Faculté de biologie et médecine, Université de Lausanne, Suisse) Monsieur O. BUGNON, professeur associé et codirecteur de thèse (Section des sciences pharmaceutiques), Monsieur L. SCAPOZZA, professeur ordinaire (Section des sciences pharmaceutiques), Madame M. BLANCO-PRIETO, professeure (Section de pharmacie et de technologie pharmaceutique, Université de Navarre, Pamplune, Espagne) et Monsieur W. RAFFOUL, professeur (Département de l'appareil locomoteur, Service de chirurgie plastique, reconstructive et de la main, Centre hospitalier universitaire vaudois, Lausanne, Suisse), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 14 janvier 2016

Thèse - 4886 -

Le Décanat

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".

Remerciements

Je profite de cette occasion pour exprimer ma gratitude envers toutes les personnes qui ont eu une implication dans cette thèse, ainsi que toutes les personnes qui m'ont côtoyé et qui m'ont apporté leur soutien durant ces cinq dernières années. Je tiens à remercier :

La Prof. Lee Ann Laurent-Applegate, ma directrice de thèse, qui m'a donné l'envie de réaliser une thèse et qui m'a chaleureusement accueilli dans son équipe lorsque j'ai terminé mes études et que j'ai décidé de débuter un doctorat. Elle m'a toujours témoigné une grande confiance et m'a donné la chance de mener mes recherches de manière très indépendante, tout en étant toujours présente pour discuter, me guider et m'encourager.

Le Prof. Olivier Bugnon, mon co-directeur de thèse, qui a supervisé ce projet et m'a invité dans son équipe de recherche où j'ai pu partager mes expériences avec d'autres doctorants. Je remercie aussi ses secrétaires, Nadia Martel et Dominique Hunziker, pour l'aide qu'elles m'ont apportée dans les tâches administratives au moment de rendre mon travail.

Les membres externes du jury de thèse qui ont accepté d'accorder une partie de leur précieux temps pour évaluer ce travail et y apporter des remarques qui ont permis de le rendre meilleur. Il s'agit de la Prof. Maria Blanco-Prieto et du Prof. Leonardo Scapozza. Je remercie aussi le Professeur Eric Allémann qui a accepté de tenir le rôle de Président du jury et de diriger la défense publique de la thèse.

Le Prof. Wassim Raffoul et le Prof. Alain Farron qui se sont toujours montrés disponibles pour discuter des aspects chirurgicaux en lien avec le traitement des tendons et avec le développement de nouvelles stratégies. Je remercie aussi le Prof. Raffoul d'avoir accepté d'évaluer la thèse en tant que membre interne du jury et d'apporter ainsi son expertise clinique.

Tous les membres de l'Unité de Thérapie Régénérative (UTR) avec qui j'ai travaillé durant ces cinq dernières années et qui sont devenus mes amis. Corinne Scaletta qui était présente tout au long du projet et qui est devenue ma coéquipière de tous les jours aussi bien au laboratoire que lors de la pause café. La Dr. Nathalie Hirt-Burri qui était aussi là dès le début de ma thèse et qui insuffle une ambiance chaleureuse à notre groupe. Murielle Michetti qui est arrivée dans l'équipe peu de temps après moi et qui a apporté beaucoup d'innovation et d'énergie dans notre équipe. Le Dr. Claude Schweizer qui a passé deux ans dans le groupe et qui était une source de connaissances. Les personnes qui ont rejoint l'Unité plus récemment et qui ont maintenu cette même ambiance agréable de travail dans notre équipe ; la Dr. Paris Jafari, la Dr. Marjorie Flahaut, la Dr. Annick Jannerat et la Dr. Virginie Philippe.

Les personnes qui ont effectué un passage de plus ou moins longue durée au sein de l'UTR. Je remercie tout spécialement Tiffany Thébault et Barbara Brandani, nos deux apprenties laborantines, qui ont effectué un stage et m'ont aidé lors de différentes expériences en lien avec ce projet. Daniele Angelella qui a effectué son travail de Master en Médecine avec moi. Le Dr. Swenn Krähenbühl et le Dr. Ilias Petrou avec qui j'ai eu la chance de collaborer et qui sont

devenus de bons amis. Sara Moufarrij et Lina Deghayli qui ont apporté beaucoup de rire et de bons moments dans notre équipe.

Le Prof. Dominique Pioletti qui m'a ouvert les portes de son Laboratoire de Biomécanique Orthopédique (LBO) et qui a toujours été disponible pour discuter des aspects de biomécanique. Dans son équipe, j'aimerais particulièrement remercier Sandra Jaccoud, qui est devenue une amie, pour nos nombreuses collaborations et discussions, ainsi que Virginie Kokocinski pour sa précieuse aide dans les tâches administratives. Je remercie aussi tous les membres de ce groupe avec qui j'ai passé d'excellents moments à l'EPFL et lors de sorties de travail. Parmi eux, je remercie tout particulièrement le Dr. Philippe Abdel-Sayed, la Dr. Ulrike Kettenberger, Tanja Hausherr et Valérie Malfroy qui étaient présent depuis le début de ma thèse et qui sont devenus de bons amis. J'ai pu partager d'excellents moments avec eux et leurs conseils étaient toujours les bienvenus.

Les membres du MSRU (Unité de Recherche Musculo-Squelettique) du Tierspital à Zurich qui ont participé à l'étude *in vivo*. Il s'agit de la Prof. Brigitte von Rechenberg, la Dr. Karina Klein, la Dr. Katja Nuss, le Dr. Peter Kronen, la Dr. Dagmar Verdino et Aymone Lenisa. Je remercie tout particulièrement le Dr. Salim Darwiche qui est devenu un très bon ami et qui a supervisé cette étude.

Le BIOP (plateforme d'imagerie), le FCCF (plateforme de cytométrie de flux), le HCF (plateforme d'histologie) et l'ATME (atelier de mécanique) de l'EPFL qui m'ont apporté un soutien de qualité dans les différentes techniques et réalisations. J'adresse un remerciement particulier à la Dr. Jessica Sordet-Dessimoz et Gianni Mancini du HCF et à Marc Jeanneret et Laurent Chevalley de l'ATME pour leur disponibilité.

La Dr. Danielle Martinet du laboratoire de cytogénétique du CHUV pour son aide dans le caryotypage et dans l'évaluation de la stabilité génétique des cellules.

John Robert de Profil Export qui a suivi tout le projet et m'a invité à Lyon pour découvrir son entreprise et me transmettre tout le matériel nécessaire pour mon étude sur les matrices décellularisées.

Eric Quenneville de Biomomentum pour le partage de ses connaissances dans le domaine de la biomécanique.

La Dr. Angela Ciuffi de l'Institut de Microbiologie du CHUV qui m'a offert l'opportunité d'enseigner dans le cadre d'un module destiné aux étudiants de Bachelor en Biologie.

Le Dr. Olivier Dormond, le Dr. Marc Dufour, le Dr. Laurent Waselle et Catherine Pythoud, qui travaillaient au Pavillon 3 du CHUV quand j'ai débuté ma thèse et qui donnaient une excellente atmosphère à ce bâtiment dans lequel j'ai passé 2 années. Les membres du LAD (Laboratoire suisse d'analyse du dopage), du CPC (Centre de production cellulaire) et les autres personnes travaillant à l'EPCR qui nous ont accueillis chaleureusement à Epalinges lors du déménagement de notre laboratoire.

Mes amis, sur qui j'ai pu compter aussi bien dans les hauts que dans les bas, durant cette étape de vie que représente le doctorat.

Aurélie qui a partagé sa bonne humeur et son sourire avec moi et qui a été très présente durant ces derniers mois de thèse qui furent aussi les plus intensifs.

Finalement, je remercie chaleureusement ma famille qui est toujours là pour moi et qui m'a apporté un soutien inconditionnel durant ce doctorat et tout au long de ma vie.

Préface

Durant mes études de pharmacie, j'ai eu la chance de réaliser mon projet de Master en collaboration avec l'Unité de Thérapie Régénérative du CHUV sous la direction de la Prof. Lee Ann Laurent-Applegate. Lors de ces 5 mois, j'ai pu découvrir les bases de la thérapie cellulaire et je suis devenu familier avec les fibroblastes progéniteurs fœtaux.

J'ai trouvé ces cellules extrêmement intéressantes. En partant d'un don d'organe unique, il était possible de créer une banque cellulaire permettant la création de millions de pansements biologiques. Les cellules étaient utilisées sur de simples matrices en collagène et pouvaient être délivrées très simplement sur une blessure telle que la brûlure. Les résultats obtenus dans le traitement de grands brûlés m'ont beaucoup impressionné puisque les patients guérissaient avec une diminution des cicatrices et retrouvaient une excellente mobilité.

A la fin de mes études, j'ai décidé de m'immerger un peu plus profondément dans le domaine des thérapies cellulaires et j'ai demandé s'il était possible de rejoindre l'Unité de Thérapie Régénérative pour y effectuer un doctorat. J'ai été très heureux d'apprendre que la Prof. Laurent-Applegate avait pu trouver des fonds pour un projet et qu'elle m'acceptait dans son équipe.

L'Unité de Thérapie Régénérative travaille sur le développement de thérapies innovatrices pour les différents tissus musculo-squelettiques et c'est ainsi que j'ai commencé à travailler sur le tendon et sur les ténocytes progéniteurs fœtaux humains.

Résumé

Les tendons permettent la transmission de forces créées au niveau des muscles jusque vers les os et jouent ainsi un rôle important dans le système locomoteur. Ils interviennent également dans la protection des tissus auxquels ils sont liés en fonctionnant tels des tampons capables d'absorber l'énergie et de la transmettre. Malheureusement, les blessures au niveau de ces tissus sont fréquentes et existent sous différentes formes. Avec la démocratisation du sport, les blessures de type dégénératif (tendinopathies) ont fortement augmenté durant les dernières décennies. Elles sont douloureuses et peuvent détériorer la qualité de vie du patient. Elles rendent aussi le tendon plus fragile et plus sujet à des blessures aigües telles que des ruptures ou des avulsions. De nombreuses zones anatomiques sont concernées, notamment le pied (tendon d'Achille), le genou (tendon rotulien, bandelette ilio-tibiale, tendon du biceps fémoral) et l'avant-bras (tendons fléchisseurs et extenseurs du poignet et des doigts). Dans tous les cas, l'impact est important tant au niveau professionnel que dans la vie quotidienne.

La prise en charge de la blessure varie en fonction de sa gravité et s'étend de l'approche conservatrice, telle que de la physiothérapie, jusqu'à l'approche chirurgicale. La guérison est très lente et le résultat est souvent insuffisant. Il est en effet fréquent de voir survenir des pertes de mobilité, ainsi que de nouvelles ruptures au niveau de l'ancienne blessure, malgré la thérapie. Ces effets négatifs sont dus au processus de guérison lui-même, durant lequel un tissu cicatriciel est créé pour restaurer la fonction du tendon, mais malheureusement avec des qualités inférieures au tissu d'origine. En parallèle, un problème de disponibilité de matériel peut aussi être rencontré. Pour de nombreuses blessures, comme par exemple pour celles au niveau des tendons de la main, seule une autogreffe est actuellement envisageable. La mise en place d'alternatives de qualité serait utile pour le chirurgien.

Ces dernières années, l'attrait pour les thérapies régénératives avec le but d'améliorer la guérison des tendons a augmenté. Une revue de la littérature présentée au début du travail permet de connaître les possibilités en matière de thérapies cellulaires pour les blessures des tendons. Comme la création du tissu cicatriciel est l'un des problèmes majeurs lors de la guérison, l'idée d'utiliser une thérapie à base de cellules fœtales est évoquée. En effet, il est reconnu que le tissu fœtal guérit différemment qu'un tissu adulte et que le processus mène à une régénération en absence de cicatrice. Cette observation est valable pour de nombreux tissus, y compris les tendons et le processus repose sur les cellules elles-mêmes et non sur l'environnement *in utero*.

Basé sur ces observations, il a donc été décidé d'évaluer les caractéristiques d'une lignée de ténocytes progéniteurs fœtaux humains (hFPTs), dans le cadre de cette thèse. Ces cellules sont obtenues à partir d'un don d'organe unique lors d'une interruption volontaire de grossesse tardive (14 semaines de gestation). L'obtention des cellules entre dans le cadre d'un programme national de transplantation enregistré auprès de Swissmedic et en adéquation avec la loi sur la transplantation de 2007. Les différents protocoles sont approuvés par un comité d'éthique. C'est une personne de l'équipe médicale et indépendante de l'étude qui informe la patiente de la possibilité d'effectuer un don d'organe. Le sujet est abordé uniquement 24 heures avant la

procédure, au moment où son choix a déjà été arrêté et la patiente doit donner son consentement éclairé par écrit. Elle peut en outre décider de se retirer de l'étude à tout moment et sans justification.

Une biopsie d'environ 1 mm^3 a été prélevée et disséquée à partir du tendon d'Achille et la mise en culture dans des boîtes de Pétri a permis l'attachement et la prolifération des cellules. Il a ainsi été possible de créer une banque cellulaire de qualité GMP (Bonnes Pratiques de Fabrication) d'une cinquantaine d'ampoules contenant chacune 10 millions de cellules à la fin du passage 1. Il est possible de conserver les cellules dans les vapeurs d'azote liquide pour une conservation à long-terme et la récupération des cellules est bonne lors de leur ensemencement. Les cellules ont pu être utilisées en GLP (Bonnes Pratiques de Laboratoires) pour les amplifier et les tester. L'amplification de ces cellules est rapide en comparaison aux ténocytes adultes et elle est stable jusque dans des passages élevés, bien qu'il s'agisse d'une lignée primaire. Aucun ralentissement de la prolifération n'est observé avant le passage 10 et la morphologie, ainsi que le caryotype, sont normaux à passage 12. A partir du don unique, il est potentiellement possible d'obtenir environ $2x10^{14}$ cellules en passage 6 (qui correspondrait à une utilisation clinique), ce qui est suffisant pour des dizaines de millions de traitements futurs. Le phénotype de ténocytes a été confirmé par des proliférations en 2D et en 3D et il a été démontré que les cellules sécrètent plusieurs molécules importantes du tendon, telles que le collagène I, le scleraxis et la tenomoduline. Le phénotype ténogénique est très stable et il n'y a pas de dépôts minéraux ou lipidiques lors de culture avec des milieux spécifiques connus pour induire la différentiation d'autres types cellulaires. Les hFPTs ont en outre montré une caractéristique intéressante, puisqu'ils sont capables de stimuler l'activité des ténocytes adultes en co-culture. Ce point pourrait être utile dans le but d'améliorer et d'accélérer la guérison des tendons. En effet, l'homéostasie du tendon repose sur le comportement adapté des ténocytes adultes capables de maintenir une balance équilibrée entre synthèse et destruction de la matrice extracellulaire. La modulation de ces cellules pourrait donc être intéressante d'un point de vue thérapeutique.

Ces différents résultats encourageants ont dirigé la poursuite du projet vers la recherche de systèmes pour administrer les cellules. Dans la revue présentée en début de thèse, il est déjà suggéré que différents systèmes devraient être développés. Des cellules resuspendues sous forme injectable seraient utiles dans les situations relativement bénignes, alors que des substituts de tendon pourraient être utilisés lors de lésions plus graves nécessitant une réparation chirurgicale.

Pour la formulation sous forme injectable, il a été décidé de travailler avec des hydrogels à base d'acide hyaluronique, une protéine de la classe des glycosaminoglycanes rencontrée naturellement dans le tendon. Il existe de nombreux produits commerciaux ayant reçu une autorisation de mise sur le marché et le fait de travailler avec une formulation déjà enregistrée facilite une potentielle utilisation ultérieure chez l'homme. Toujours dans l'esprit d'une administration clinique future, il a été décidé de resuspendre les cellules dans le gel en absence de milieu ou de toute autre solution soutenant la croissance. Les tests effectués sur différents gels ont montré une relation contraire entre la viscosité et le taux de survie des cellules. D'un côté, le but est de trouver une formulation permettant une survie maximale des cellules, mais de l'autre, il est aussi nécessaire de trouver une

formulation suffisamment visqueuse, à la fois pour maintenir les cellules en suspension dans le gel et pour éviter un écoulement hors de la zone traitée. Les analyses effectuées à différentes températures et différents temps de conservation ont démontré qu'il était possible d'aboutir à une formulation présentant un bon compromis entre survie des cellules et viscosité. De plus il était possible de conserver la préparation durant 3 jours à une température réfrigérée. Ceci est intéressant puisqu'il est possible de préparer une formulation sur demande du médecin et qu'il y a ensuite une certaine flexibilité dans le délai d'utilisation. Le conditionnement en seringue rend en outre l'utilisation extrêmement simple pour le praticien.

Malheureusement, si une formulation injectable est idéale pour les blessures les plus simples, elle l'est moins pour les blessures plus graves où un produit solide est nécessaire afin d'apporter un support mécanique suffisant. Dans la cadre de cette thèse, il a été a décidé de créer des supports à base de matrice extracellulaire (ECM), car ces derniers offrent une alternative souvent mieux tolérée comparée aux matrices synthétiques. Différents types de tissu peuvent être utilisés et c'est le tendon fléchisseur superficiel du doigt du cheval qui a été choisi comme base dans ce projet, en raison de son homologie avec le tendon humain. De plus, et toujours dans le but d'une future utilisation clinique, il est possible d'obtenir une traçabilité exhaustive du matériel (auprès de fournisseurs agréés pour l'industrie alimentaire qui éliminent les tendons). Plusieurs solutions sont testées pour décelluláriser le tissu, afin d'éliminer les épitopes immunogènes liés aux cellules. Différentes techniques d'analyse sont employées pour évaluer la qualité de la décellularisation et de la conservation de la structure ainsi que des propriétés biomécaniques. Les résultats obtenus au cours de cette thèse ne permettent pas d'obtenir un substitut de tendon pour le moment. Cependant, il a été possible de créer des supports de taille réduite entièrement décellularisés et procurant des caractéristiques mécaniques intéressantes (bien qu'encore largement inférieure à celles d'un tendon natif). De plus, ces matrices se montrent biocompatibles avec les hFPTs qui peuvent adhérer à leur surface. De nombreuses évolutions sont nécessaires pour obtenir un réel substitut de tendon, cependant de bonnes bases ont été posées en vue de futurs développements.

Finalement, suite aux résultats prometteurs obtenus *in vitro* et à la disponibilité d'une formulation injectable, les cellules ont été évaluées *in vivo* dans un modèle animal. Cette étape est fondamentale pour s'assurer de la sécurité d'un traitement dans des conditions aussi proche que possible d'une utilisation finale chez l'homme et en présence d'un système immunitaire actif. Le modèle a été développé avec l'équipe de l'unité de recherche musculo-squelettique du Tierspital de Zurich et consiste en une blessure non transfixiante au niveau du tendon rotulien du lapin. La taille suffisante de ce tendon et son accès aisé sont à l'origine de ce choix. Il a été décidé d'évaluer les hFPTs avec la formulation injectable retenue lors des tests sur les gels d'acide hyaluronique, mais avec une concentration adaptée au modèle. Il a également été décidé de procéder en deux phases dans cette étude animale, afin de pouvoir valider ou adapter le modèle animal à la fin de la première phase. Seule la première phase a été réalisée dans le cadre de cette thèse. Les résultats, obtenus six semaines après l'intervention, ont montré que le processus de guérison n'était pas terminé et que le tissu était hypercellulaire et hypervascularisé. Des macrophages étaient présents dans le site lésé, mais la présence de ces derniers est normale dans le processus de guérison et de

remodelage du tissu. Aucune réaction immunitaire anormale n'a été détectée, excepté dans les zones où du matériel de suture n'avait pas encore été résorbé (autres types de cellules immunitaires, telles que des cellules géantes à corps étranger). Ces résultats sont prometteurs en matière de sécurité et le modèle est intéressant. Cependant le temps de guérison devrait être prolongé pour la seconde phase de l'étude, afin de pouvoir évaluer et comparer la qualité du tissu final, une fois le processus de guérison plus avancé, voire terminé. En effet, le but d'utiliser les hFPTs serait de lutter contre la formation du tissu cicatriciel et l'observation de celui-ci devrait être effectuée une fois le processus complet terminé.

Globalement, les résultats obtenus durant cette thèse sont encourageants et posent des bases solides dans le but de développer de nouveaux traitements pour les blessures des tendons. Néanmoins, un long travail reste à faire pour démontrer une parfaite sécurité et une bonne efficacité de ces cellules et pour développer des produits pouvant être utilisés réellement en clinique.

TABLE OF CONTENT

Chapter I

–

Introduction

Foreword

The first chapter is an overall introduction which aims to prepare the reading of the following chapters of the thesis. The focus is placed on the tendon and its pathologies, alongside potential treatments.

Structure and Composition of Tendon

Tendons are dense-connective tissues consisting of specialized fibroblastic cells called tenocytes scattered within an extracellular matrix. They are characterized by a relatively poor vascularization, which gives them an overall brilliant white aspect. They play an important role in our body by binding muscles to bones (exceptionally a muscle to another muscle, e.g. rectus abdominis) and permit storing the force created in the first to transmit it to the second. With their collagen organization and due to their low and mainly anaerobic metabolism, they are able to carry loads and maintain tension for long periods without risk of ischemia or necrosis (1). With their elaborated structure, they not only transmit forces but also act as buffers against tension and compression by absorbing external forces from various directions and thus they protect muscles against tears (1-3).

Tendons differ considerably in shape depending on their location, varying from flat to ribbon-shaped or cylindrical (1, 2, 4, 5). Their length is also dependant on the location and muscles dedicated to create powerful forces are usually elongated by short but wide tendons (e.g. quadriceps) while muscles responsible for fine movements are usually linked to long and thin tendons (e.g. hand flexor tendons) (1).

The zone of transition from tendon to muscle is called the myotendinous junction (MTJ) and the location where the tendon inserts into bone is known as the enthesis (Fig. 1), which can be either fibrous (same dense connective tissue structure as in tendon mid-substance) or fibrocartilaginous (with a transition composed of four zones from an uncalcified tendinous structure to a calcified cartilaginous-bony tissue) (6-8).

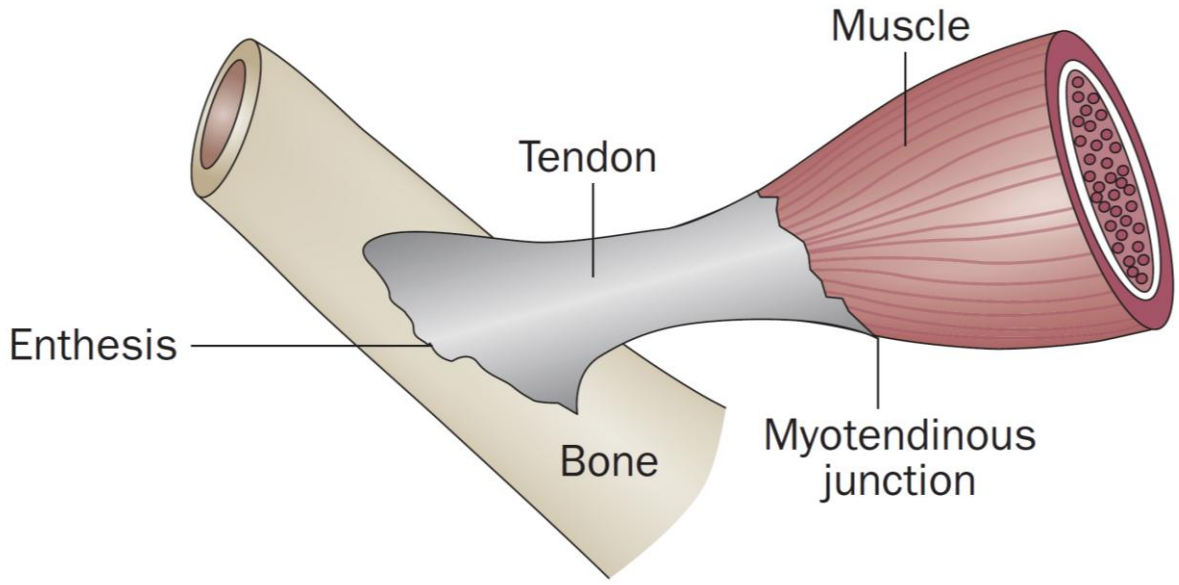

Figure 1: Tendon within the musculoskeletal system. Reprinted by permission from Macmillan Publishers Ltd: Nature Review Rheumatology, Nourissat et al., 11(4):225, copyright 2015.

The tendon is a white poorly vacularized tissue connecting a muscle to a bone. The myotendinous junction is the transition from muscle to tendon and the enthesis is the position where tendon usually becomes fibrocartilaginous to insert into bone.

Structure

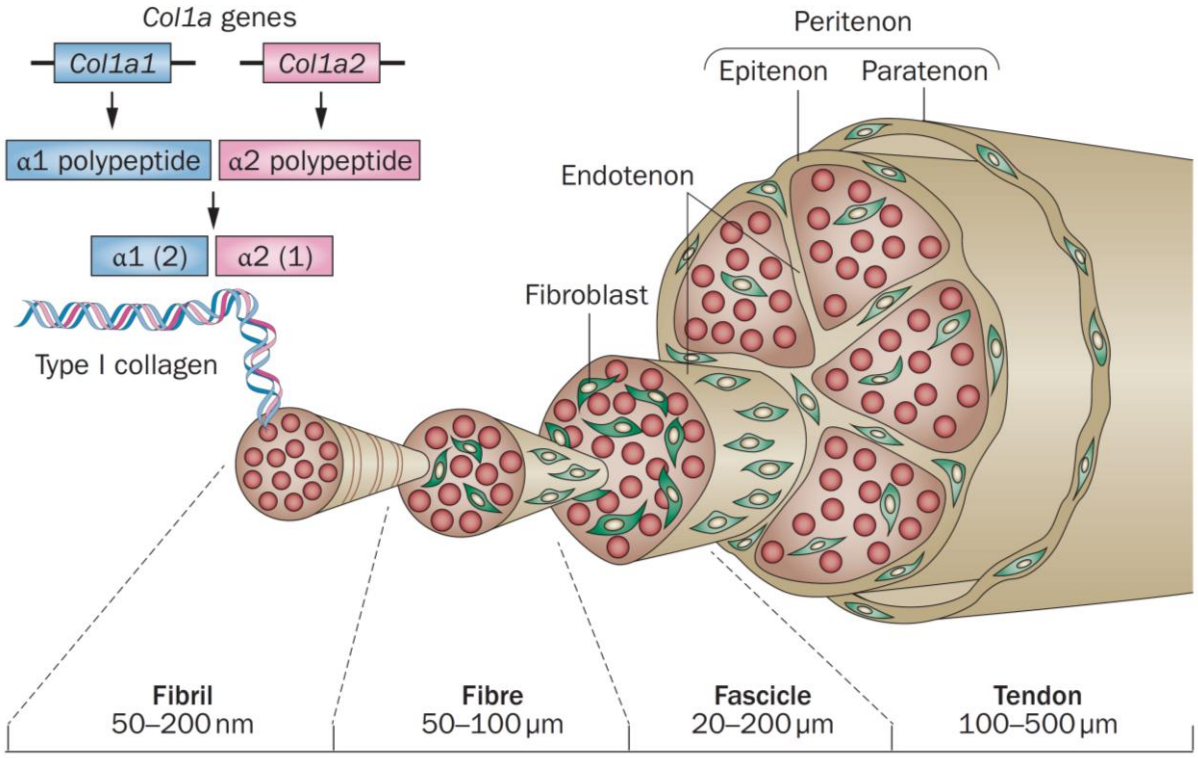

Figure 2: Tendon structure. Reprinted by permission from Macmillan Publishers Ltd: Nature Review Rheumatology, Nourissat et al., 11(4):225, copyright 2015.

The tendon structure is based on a vast organization of collagen, which represents 60−80% of the dry mass. With more than 90% of the total collagen, collagen I is the most important type in tendon. Collagen molecules are composed of 3 helix subunits and aggregate together to form collagen fibrils, recognizable with their striated pattern. Fibrils bind together to form collagen fibers. The tenocytes are tendon specialized fibroblasts disseminated between collagen fibers. Collagen fibers are bundled together by the surrounding endotenon to form fascicles. Finally, the fascicles are recovered by the epitenon on the periphery of tendon. The tendon can be surrounded by a paratenon or other surrounding tissues.

Tendons are mainly composed of collagen (60−80% of dry mass) and rely on a very hierarchical organization of this molecule within the tissue (1, 2, 4, 9-11). The tenocytes, which are the tendon specific fibroblasts, are responsible to regulate the fibrillogenesis (5). The manner to describe the aggregation and organization of collagen within tendon can vary between authors. The recent reviews from Mienaltowski and Birk and from Nourissat *et al.* use the same elegant manner to explain this organization (Fig. 2) (9, 10). Collagen α chains are rich in glycine (1/3 of aminoacids) as well as proline and hydroxyproline and adopt a left-handed helical configuration (angulation due to glycine, without h-bonds). Three α chains supercoil to form a right-handed superhelix collagen molecule (stabilized by inter-chain h-bonds) and give the collagen its type (9). Proteoglycans and glycosaminoglycans appear between the alpha-chains (1). Collagen molecules assemble together to form collagen fibrils highly recognizable with their striated structure and a periodicity of 67 nm. The fibrils are heterotypic with molecules of different types forming one fibril. Fibrils are bundled together to form a collagen fiber. Bundles of fibers, with elongated

tenocytes in between, are organized as fascicles and are surrounded by a loose connective tissue known as the endotenon (9). The collagen fibers are mainly oriented longitudinally, but fibers can also present transversal variations and this can give a wavy pattern to the tendon at rest (1, 9, 12). To form the tendon, the different fascicles are then bundled together by the epitenon, another connective tissue contiguous to the endotenon (9). These two sheaths permit a good gliding between fascicles and they allow vascularization, lymphatics and innervation deep into the tendon structure (1, 5, 11).

The tendon can further be surrounded by five different types of structures which give them ideal gliding abilities (only tendons with linear movement are deprived of such structures) (1). Long tendons often go through canals covering grooves and notches which are called fibrous sheaths or retinacula (e.g. flexor and extensor tendons of the hand and foot). These fibrous sheaths can be reinforced by a reflexion pulley which helps to maintain the tendon in its canal when it faces a change in direction. When a very efficient lubrification is needed (in some parts of the hand or of the foot), synovial sheaths can be found around the tendon, with synovial fluid between the parietal and visceral sheets composing this structure. Paratenon is a loose aerolar fibrillar connective tissue which is found in many tendons (e.g. Achilles tendon). It is not a real synovial sheath, but it allows good gliding by acting as an elastic sleeve. Finally, tendon bursae are found near bony prominences to reduce friction and protect the tendon (e.g. subacromial bursae) (1).

Similarities with Ligaments

Tendons present many similarities with ligaments which are responsible to bind a bone to another bone. Both tissues present a low cell to matrix ratio and their composition is very similar with cells lying within a highly organized matrix composed mostly of collagen. Ligaments are sometimes reported to contain slightly more water (60−70% for ligaments) than tendons (50 to 60%) (9), but tendons are often considered to also contain approximately 70% of water (2, 4). The collagen is of utmost importance in these tissues and it represents 60−80% of the dry mass in tendons and more than 80% in ligaments. However, these small differences in composition, coupled with some variations in morphology, allow each tissue to have its own specificity. The most important variation is certainly the differences in crimping patterns of their collagen fibers. Whereas tendon collagen fibers are mainly longitudinally oriented, there are regions in ligaments where subsets of fibers run obliquely to the other longitudinally oriented fibers. This can explain the difference in roles between tendons which must transmit and sustain high forces and ligaments which must stabilize the joint by preventing a too high range of motion between bones. This also explains the fact that when loaded, tendons become more rapidly stiffer and transmit ideally the forces while ligaments allow slightly more laxity (9).

Composition

The tendons are composed of approximately 70% water (2, 4). The tenocytes are dispersed in a well-organized extracellular matrix and the cell/matrix ratio is low for a connective tissue. While collagens are the most frequent proteins and account for 60−80% of the dry mass, the organization of the tissue relies on the good interaction of collagen I with other collagen as well as with many other matrix and cell proteins (1, 2, 4, 9-11).

Cells

The great majority of cells present in tendon are tenocytes (90−95%) which are specialized tendon fibroblasts responsible for extracellular matrix deposition. However, other cell types can also be found in small quantities, notably vascular cells in capillaries, synovial cells belonging to sheaths surrounding tendons and chondrocytes in enthesis. Inflammation cells can also eventually be found in diseased tendons (1, 2). Tendon Stem/Progenitor Cells (TSPCs) are multipotent cells that can be found in low levels in tendon. Their characteristics are close to the ones of Mesenchymanl Stem Cells (MSCs), but with a higher expression of tenogenic molecules such as scleraxis, tenomodulin or tenascin-C (13).

Collagens

There are 28 different types of collagens, themselves encoded by 45 genes, and several of them are found in tendon. With the help of other molecules (such as fibril-associated collagens with interrupted triple helix (FACITs), small leucine-rich proteoglycans or glycoproteins), the different collagens arrange themselves in different manners to give rise to various organizations (9).

Collagens I, II, III, V and XI are part of the fibril-forming class of collagens and have been found in tendons and in ligaments (9). Collagen I represents by far the most abundant collagen with approximately 60−80% of the dry mass of the tendon and up to 95% of the total collagen (1, 2, 4, 9-11). Collagen III represents 5 to 10% of the total collagen content (9). It is necessary during tendon development for a regular collagen I fibrillogenesis (14). After tendon injuries, it is increased during the first phases of the healing process (15). Collagen II (which is very present in cartilage) is found at the enthesis (9, 16) and also around some gliding systems such as pulleys (4). Collagen V and XI are present in very low amounts, but they are very important in fibril development as their absence leads to altered collagen fibril (9).

Collagens XII and XIV can be found both in tendons and ligaments. They are FACITs which associate with collagen fibrils and help in interactions with different molecules (9). In a chick model, Collagen XIV is highly expressed during fetal development, but there is a net decrease after birth (17). Collagen type IX is also part of the FACIT family and it interacts with collagen II and can be found in cartilaginous regions of tendon (4, 9).

Collagen IV is a basement membrane collagen and is implicated in the interface between tissues (9). Collagen VI is a beaded filament-forming collagen implicated in different aspects such as cell

proliferation, migration, differentiation and apoptosis. It is also implicated in the development, homeostasis and repair of tendons and its absence leads to dysregulation in fibrillogenesis (9, 18). Finally, Collagen XIII is a transmembrane collagen found particularly in the myotendinous junction (9).

Elastin

Elastin is another fibrous protein of the tendon. It is less present than that of collagen and only represents 1−2% of the extracellular matrix dry weight. It is rich in glycin and proline like collagen, but not in hydroxyproline. Elastin can elongate up to 70−150% of its length and gives flexibility to the tendon. It also seems responsible in helping collagen fibers to recover their crimp after elongation (1, 4, 19).

Proteoglycans

Proteoglycans are encompassed within and between fibrils and fibers of collagen (1, 20). They represent less than 1% of the dry weight of tendon, but they are of great importance (4, 20). They are large molecules obtained by the binding of a core protein with glycosaminoglycan (GAG) chains and can be divided into two families. The small leucine-rich proteoglycans possess a small core protein (about 40 kDa) and 1 to 4 GAG chains attached to it. The large proteoglycan possess a core protein more than 5 times larger and multiple GAG chains. In regions subjected to tension, small proteoglycans are far more present with 90% vs 10% of large proteoglycans. In compressive regions, large proteoglycans are more common and the distribution is usually 50%-50% (20).

Small proteoglycans are particularly important in tendon development and organization. They regulate fibrillogenesis of collagen, they have the ability to regulate growth factor signalling (transforming growth factor, TGF and epidermal growth factor, EGF) and they can influence cell proliferation. Mice with a deficiency in either decorin (most present proteoglycan in tendon) (21), biglycan (22), fibromodulin (23) or lumican (24) present abnormal collagen organization in tendons. Biglycan and fibromodulin have also shown an importance in tendon stem/progenitor cell (TSPC) niches (13).

The large proteoglycans are negatively charged and can trap a high content of water. This permits the creation of a viscous environment that plays different roles such as good protection against compression, adapted environment for collagen fiber stretching, good diffusion of hydrosoluble molecules and cell migration (20). Aggrecan and versican are both found in tendon, particularly in zones of compression.

Glycosaminoglycans (GAGs)

Glycosaminoglycans are linear polymers with repeated disaccharides. Chondroitin sulphate, dermatan sulphate and keratin sulphate are bound to a core protein to form the proteoglycans

presented above. They are less present in tendons than in other connective tissue such as for cartilage and their concentration varies from 0.2% of the dry mass in tensional zones (mainly dermatan sulfate) to 3.5−5% in pressure zone or enthesis (mainly chondroitin sulfate, but keratin sulfate is also more frequent in pressure zones). Heparan sulphate and heparin (also sulphated) are found mostly in the myotendinous junction. Hyaluronan constitutes around 6% of the total GAG amount in tendon and it differs from the other GAGs as it is larger and it is not bound to a core protein nor is it sulphated (20).

Glycoproteins

Glyoproteins are formed of a core protein to which a glycan is bound and they seem implicated in mechanical stability. They are 10 to 20 times smaller than proteoglycans and also represent less than 1% of the dry weight of tendon (1). Different adhesive glycoproteins are found within tendons, such as fibronectin, thrombospondin, tenacin-C and undulin. Laminin is found in vascular regions and within the myotendinous junction (1).

Fibronectin is found on collagen surfaces (11). It is not specific to tendon, but it is important in adhesion and binds cells and many proteins of the extracellular matrix (4). It is increased in case of tendon injury and helps to give strength to a wound by permitting crosslinking of fibronectin and collagen (4). Thrombospondin and tenomodulin are relatively specific to tendon and their genes were identified as the best tendon-selective genes in both rat and human mature tendon (25). The role of tenomodulin in tendon was probably the most studied among glycoproteins. It was shown that it was regulated by scleraxis, a vital transcription factor in tendon development (26). The presence of tenomodulin is necessary for tendon maturation and tenocyte proliferation and its absence lead to defects in tendon structure (27, 28). Tenascin-C is frequent in tendon. It has sometimes been proposed as a specific tendon marker, but it should probably not be labelled as such since its expression is also elevated in other tissues such as cartilage (25). It is regulated by mechanical loading and could play a role in collagen alignment (2).

Anorganic Molecules

Some anorganic molecules are implicated in tendon metabolism and can be found in small amounts within the extracellular matrix. They represent less than 0.2% of the dry mass (1). Among them, calcium is the most present and it is found in higher amounts at the enthesis than in the midsubstance (1). The presence in the midsubstance is augmented in case of calcifying tendinopathy (29). Copper is important in the formation of collagen crosslinks and manganese participate to enzymatic reaction during synthesis of the extracellular matrix (1).

Biomechanics of Tendon

During a muscular contraction, the muscle shortens and exerts a force on the tendon which elongates. Due to its viscoelastic capacity, the tendon can then go back to its initial length when the muscle is released. The viscoelastic behavior combines both an elastic capacity (reversible and conservative) and a viscous capacity (irreversible and dissipative) and it seems to rely on the vast organization of collagen with other interacting molecules and with the presence of water. This leads tendons to be compliant and more deformable at low loadings, but to become stiffer and more efficient to transmit forces when loads increase (9, 11). The biomechanical behavior of tendon can be well visualized with a stress-strain curve (Fig. 3).

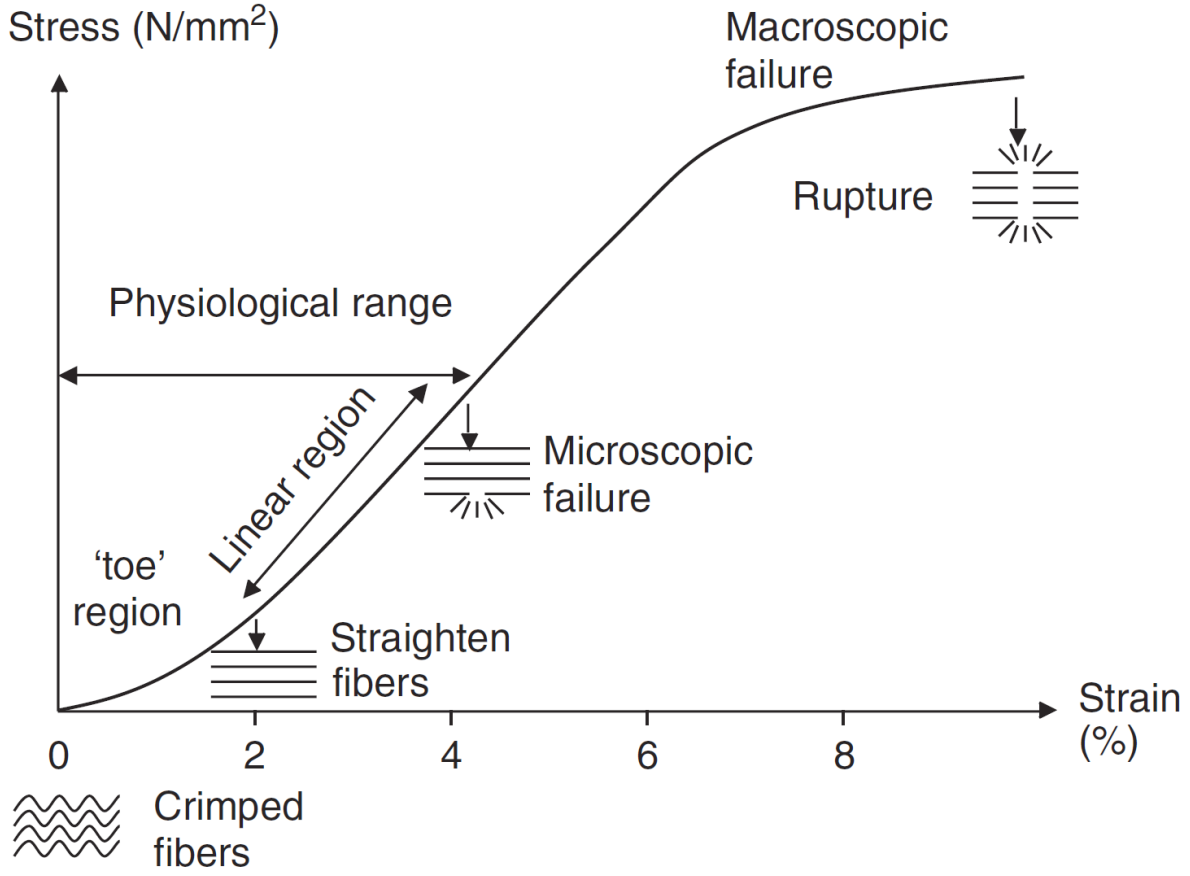

Figure 3: Tendon stress strain curve. Reprinted from Journal of Biomechamics, 39/9, Wang JH, Mechanobiology of tendon, Page No. 1567, Copyright (2006), with permission from Elsevier.
The stress-strain curve presents an initial toe region up to approximately 2% of strain, followed by a linear region between approximately 2% and 4%. The slope of the curve in this region is known as the Young's modulus (or elastic modulus) and gives information about the rigidity of the tissue. Over 4% strain, some microscopic tears occur and beyond 8% to 10% strain, macroscopic tears occur with finally a rupture of the tendon. The area under the curve from initial strain to point of failure in considered as the strain energy density.

To obtain such a curve, it is possible to use a tensile testing machine. A tendon is fixed between two clamps and elongated up to rupture. During elongation (in mm), a captor records the increasing tension (N). The elongation can be normalized to strain (%) based on the initial length

and the tension normalized to stress (MPa) based on the cross section, which allows a comparison between samples of various dimensions. The stress-strain curve of tendon presents an initial toe region up to approximately 2% of strain. In this region, the normally crimped fibers are stretched to become more linear and molecules interacting with collagens provide viscoelastic properties. There is then a linear functional region between approximately 2% and 4% where the fibers are completely straightened. There is a slippage within and then between collagen fibrils. The slope of the curve in this region is known as the Young's modulus or elastic modulus (MPa) and provides information on the rigidity of the tissue. Some authors prefer to present the stiffness which depends on the dimension of the sample and represents a ratio between force and elongation (N/mm). Over 4% strain, fibrils continue to slip until fibril molecules slip away and create some microscopic tears in tendon fibers and then macroscopic tears with a rupture of the tendon beyond 8% to 10% strain. Finally, the point of failure is considered as the point of ultimate tensile stress and strain. The area under the curve from initial strain to point of failure is considered as the strain energy density (J/mm^3) (9, 11). The values vary from tendon to tendon and the Young's modulus is generally between 500 MPa and 1500 MPa (30-34).

Mechanobiology in Tendon

Tendons not only transmit forces as they are also a mechanosensitive structure able to feel mechanostimuli through tenocytes and adapt their behavior (11). Mechanical forces are required for tendon homeostasis and an absence of forces impairs tendon properties (35). Indeed, an absence of mechanical forces is linked with a decrease in collagen synthesis in tendon and this effect is seen rapidly (less than 10 days) (36). In opposition, genes coding for important tendon molecules such as scleraxis, collagen type 1 and tenomodulin are increased in various situations where loading is applied on tendons or cells (10). Nevertheless, excessive loading can become deleterious for the tendon. It was shown that TSPCs, which are present in small quantity in tendon, maintain a tenocyte phenotype within strains not exceeding the physiological range of 4%. With strains of 8%, TSPCs have been reported to change phenotype towards non-tenogenic lineages. This could in part explain the appearance of calcifications, lipid accumulations or mucoid degeneration seen in tendinopathies (37).

Mechanostimulation is also important in tendon healing. Complete unloading and overloading are both deleterious in tendon healing. Controlled loading is often recommended and it promotes tendon homeostasis and repair at the midtendon. On the other hand, it inhibits tendon repair at the enthesis and cast immobilization should be preferred when this location is injured (10, 38).

The mechanisms of mechanotransduction are not totally understood. Different structures such as integrins can sense the force and the signal can then be converted chemically. In tendon, it has been well demonstrated that mechanical forces can be converted in TGF-β and influence the TGF-β–SMAD2/3 pathway (39). The fibroblast growth factor - extracellular signal-regulated kinase/mitogen-activated protein kinase (FGF–ERK/MAPK) pathway is influenced by mechanical stimulations too (10). Cyclic strains were also shown to positively regulate other

cytokines such as prostaglandin E2 (PGE2), interleukin-6 (IL-6) and IL-1β (40) and transcription factors including early growth response proteins 1 and 2 (EGR1and EGR2) (41).

Tendon Afflictions and Healing

Tendon afflictions are common and affect a wide and heterogeneous population, with impact at workplace and in leisure activities. In developed countries, they are responsible for a non-negligible burden in society. From a large joint clinical data-base, it has been shown that for 100.4 million injuries of any kind reported in 2006/2007 in the United States, about 61.2 million are musculoskeletal and among them, 44% are due to sprains and strains of soft tissue including ligaments or tendons (42).
Tendon afflictions can appear in multiple locations and in various forms. On one hand, there are overuse degenerative injuries referred to as tendinopathies. On the other hand, there are acute afflictions characterized by partial or total tears or avulsions. The main recognized factor inducing tendinopathies is abnormal loading. This phenomenon can be due to excess sport, but also to physiological exercise or repetition of specific movements in work. On the contrary, acute injuries are generally due to one specific event where tendon is submitted to an overload (10). The frontier between chronic and acute injuries is not always clear and tendinopathies are often a predisposition to spontaneous rupture (43, 44). Pure acute injuries without any predisposition also exist and are often due to accidental lacerations. The hand tendon system is particularly exposed in this specific situation (45, 46).

Tendinopathies

In absence of histopathological evaluation, the term tendinopathy should be used to speak about clinical symptoms associated with painful overused tendons (generally associated with impairment). The term tendinitis (implying inflammation) which has been widely used should be abandoned, because the process is mainly degenerative and inflammatory responses are not commonly seen (40, 47).

Tendinopathies have augmented during the last decades. Frequent injuries to tendons include the Achilles, the patellar, the tractus iliotibialis, the biceps femoris (Hamstring syndrome), the rotator cuff, the common wrist extensors (tennis elbow) and the common wrist flexors (golfer's elbow) (48, 49). Kannus *et al.* found that the overall prevalence of tendons presenting degenerative changes among the control tendons of different types was of 34% (43).

Healthy tendons present a regular structure thanks to the tenocytes which are responsible for the secretion and maintenance of the extracellular matrix with a fine balance between synthesis and catabolism (1). In case of tendinopathy, the fine structure is compromised and becomes weaker. Histopathological evaluation permits to differentiate various types of tendinopathies. Among them, the majority are due to tendinosis (50). In this case, the histopathological observations of overused tendons generally present a disorganized extracellular matrix with separation of collagen

fibers, decrease in fiber diameter and in density, but increase in type III collagen and in glycosaminoglycans. Simultaneously, hypercellularity and neovascularization are detected, but inflammation and granulation tissues are not common and are normally only seen in tendon ruptures. Subcategories of tendinosis showing lipoid degeneration or calcifications between collagen fibers can also be found (2, 47, 51). The expression of many genes is modified during tendinopathy, notably collagens, matrix metalloproteinases (MMPs), tissue inhibitors of matallopreinase (TIMPs), bone morphogenetic proteins (BMPs) and a number of genes implicated in signalling pathways such as Wnt, TGF-β or FGF (40). Inflammation is rarely marked in tendon itself (40), but the surrounding tissues can be involved. Paratenonitis (peritendinitis) is the term employed to describe the inflammation state of the sheets surrounding the tendon, whether there is a single layer sheath (tenosynovitis) or a double-layer sheath (tenovaginitis) (47).

Altered cell differentiation is sometimes thought to play a role in the aetiology of tendinopathies (52), as calcification and appearance of lipid droplets (normally not seen in tenocytes) are factors found in tendinopathies which can precede spontaneous rupture of tendons (43). Excessive intensity in loadings could be responsible as TSPCs have been shown to change phenotype when stressed beyond physiological range (37). The frequency of loadings can also have an impact. In the case of acute loadings, the net balance of collagen levels is negative for up to 18−36 hours after exercise and then becomes positive up to 72 hours. Thereafter, repeated loadings without sufficient rest can lead to an amplification of the collagen breakdown (53).

The healing of tendinopathy is time consuming for recovery of a normal structure and disappearance of pain. This can be linked with a lower metabolism in tendon compared to other tissue. The turnover of total collagen is more than twice than that of muscle (54) and the oxygen consumption is 7.5 times lower than in muscles (55).

Rheabilitation therapies are desired and eccentric exercise, which consists of extensive stretching, seems the most efficient treatment. It is possible to take nonsteroidal anti-inflammatory drugs (NSAIDs) to diminish pain, but little evidence is demonstrated and a long-term prescription should be avoided. Corticosteroids can also provide short-term pain relief, but should not be used in the long term as they can lead to less resistant tendons more prone to ruptures. Other techniques have been tested with controversial results, as for example Cryotherapy, extracorporeal shock wave therapy, therapeutic ultrasounds, phonophoresis, low intensity laser therapy, topical nitroglycerin or injection of substances like heparin, dextrose, aprotinin, polidocanol or sulphated glycosaminoglyacans (56, 57). Platelet-Rich Plasma (PRP) has been used largely for treatment of tendon injuries, but the results are extremely divergent. This could be due to the preparation of the formulation which varies greatly from one study to another (58-60). Other biologics and cellular therapies have been tested more over the last years. They could become future conventional treatments, but none has demonstrated its supremacy until now and further research is needed (61).

Acute Injuries

Acute injuries can arise either within previously degenerative states or in absence of predisposition. A tear is created within the structure and can be partial or total. If the tear occurs at the enthesis, the term avulsion is used. In case of tendon rupture or avulsion, a natural healing takes place through a long process composed of three overlapping phases (2, 5, 15).

A first inflammatory phase starts immediately following injury with a preponderant role of macrophages. This phase was precisely described for various tissues and can be divided in two subphases (62, 63). The early steps of the inflammatory phase are characterized by the release of pro-inflammatory factors by M1 macrophages (classical activated macrophages) with eradication of invading microorganisms, and promotion of type I immune responses. During this phase, the expression of pro-inflammatory molecules such as tumor necrosis factor α (TNFα), IL-1β and cyclooxygenase 2 (COX 2) is highly increased (64). The secondary inflammatory phase relies on anti-inflammatory M2 macrophages (alternatively activated macrophages) which sustain tissue repair and are involved in debris scavenging, angiogenesis, connective tissue remodelling, and resolution of inflammation. There is a concomitant release of growth factors acting on neovascularisation such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), as well as profibrotic factors like TGF-β and connective tissue growth factor (CTGF). Such factors have an important impact on the final scar. Macrophages recruited after the inflammation phase present lower expression of such factors and are not implicated in scar formation (62).

After a few days, the second step consists in a proliferative phase. There is the production of a matrix mostly composed of type III collagen and in part under the control of tenocytes and macrophages. The wound is hypercellular and an extensive blood vessel network is developed (2, 5, 15). It is still not totally clear where the cells that are implicated come from. Extrinsic healing relies on the intervention of cells from the environmental tissues, such as tendon sheaths fibroblasts and inflammatory cells which invade the healing site. Such healing is not desired and often leads to the formation of adhesion and thus impaired tendon gliding. It also leads to a disorganized collagen matrix, scar tissue and diminution of biomechanical characteristics (2, 15, 65). Intrinsic healing relies on tenocytes from the tendon itself. The outcomes are better with less scar tissue, less adhesions and a better reorganization of the collagen fibers (2, 15, 65). Both mechanisms probably participate in the process. TSPCs could also play a role in healing and they can be found in the midsubsance as well as in the surrounding paratenon (10).

The last step is the remodelling phase which takes place after one or two months. Extracellular matrix proteins are still produced, but with an increase in type I collagen deposition and the reorganization and optimization of the fiber alignment. With time, the cell density and activity diminishes and the overall procedure can take as long as one year or more (5, 15). However, the structure and mechanical properties found before injury are never attained again. Moreover, the scar tissue and the adhesions created during the process can lead to decreased mobility and higher risk of rerupture (2, 5, 15).

On the chemical aspect, TGF-β signalling seems to have an implication in scar tissue formation. During development, wounds heal without scar and present low levels of TGF-β1 and TGF-β2, but TGF-β3 is abundant (66). It is the contrary in adult healing with TGF-β1 and TGF-β2 abundant early in the healing process and TGF-β3 only induced late and in low amounts. Thus, TGF-β1 and TGF-β2 seem implicated in scar formation, while TGF-β3 seems favored in situations of scar-free healing (67). Tendon associated proteins such as collagens, tenomodulin, tenascin-C and proteoglycans show increased expression after tendon injury (68). Many other proteins have a modified expression of their gene during healing, notably BMPs, MMPs and TIMPs, as well as numerous growth factors and exhaustive data can be found in the literature (64, 67, 68). It can be noted that in absence of SMAD3, growth differentiation factor (GDF-5) or EGR1, the repair process cannot be completed (10).

Despite the ability of tendon to heal naturally, a surgical intervention is often necessary for extended injuries. Depending on the pathology and on the location, it can vary between tenotomy, tenodesis, suture, transfer, augmentation, graft or even prosthetic implants. The final outcomes are often disappointing. Reruptures are very frequent independently of surgical or non-surgical management (10). Reruptures of rotator cuff have been reported between 20% and 70% in the literature (69) and the complications, such as adhesions, are frequent and can be seen in 30% to 40% of the repaired or unrepaired hand tendons (70).

Controlled mobilization is often recommended in parallel, because it has been shown that mechanical stimulation leads to fewer adhesions and increased strength. However, it can inhibit tendon repair at the enthesis and therefore, cast immobilization should be preferred when this location is injured (10, 15, 38, 71).

Cell Therapies

Cell Culture

A biopsy from tissue is required to start cell culture. The tissue can be mechanically or enzymatically disrupted to allow cells to disassociate from the tissue and adhere to the cell culture material (Petri dish or flask) for adherent cell types. There are other cell types that can grow in suspension. Basically, all cell types require nutrients (culture medium), adequate physical conditions (incubator with a controlled temperature and CO_2) and sterile conditions (laminar flow hood). With time, the cells can proliferate and the medium must be replenished regularly (2−3 times per weeks for many cell types). We refer to "full confluency" when the cells cover all the surface of the flask. The cells can be detached from the flask with an enzyme (e.g. trypsin) to be used or reseeded in new flasks for further expansion. Each cycle of amplification (seeding, proliferation and detachment) is referred as a passage and allows for an exponential increase in cell number. If the cell type is sufficiently resistant, it is possible to create a cell bank and cryopreserve the cells in vials in the vapor of liquid nitrogen for long-term storage. This stock offers an off-the-shelf availability when the cells are required.

Cell Sources

Cell therapies have received much interest during the last decades for the treatment of various pathologies. Stem cells have certainly received the most attention and were employed in a multitude of studies, even including human trials for tendon treatment with bone marrow-derived mesenchymal stem cells (BM-MSCs) and adipose-derived stem cells (ASCs) (72, 73). Stem cells are undifferentiated cells, with self-renewing capacity (symmetric cell division) and differentiation capacity (asymmetric cell division) (74-77). The cells are generally not used with their native phenotype, but are expanded and differentiated in vitro to produce large cell numbers (74). The culture is nevertheless demanding and expensive, because growth factors or specific nutrients must be employed to guide the cells in the desired phenotype (75). It is important that once differentiated, these cells maintain the acquired phenotype and do not dedifferentiate, but there is some concern for this aspect. For the tendon, there are still questions about the stability of MSC to act as tenocytes and the undesired formation of calcifications was seen in different studies (13, 78).

Fetal progenitor cells are another cell source. In Switzerland, like in many other countries, fetal cells are considered as an organ donation from a voluntary pregnancy interruption. The Unit of Regenerative Therapy has been working with these cells since 1993 and developed a National Transplantation Program which was originally registered with the Swiss Federal Office of Public Health.

The protocols have all been approved by an Ethics Committee. There is a medical doctor independent from the research team who informs the mother donor about the possibility to make an organ donation and this information is given only 24 hours before intervention, when the decision has already been confirmed by the patient. The patient must give her written informed consent and can change her mind at any moment without any justification. The cells are harvested from specific differentiated tissues and cell banks of skin, bone, cartilage, disc, muscle and tendon are created with conditions respecting the requirements for clinical application

For safety reason, the mother donor is controlled for serology at 1 and 3 month after organ donation to assure no seroconversion. The fact that all the cells within one bank come from the same biopsy is advantageous for security testing, as it is thus possible to proceed to extensive screening of a dedicated portion of the bank to assure the absence of pathogens such as bacteria (including mycoplasms), yeast, viruses (both animal and human depending on the cell culture additives) or prions. As fetal cells are already differentiated with their own specific phenotype, it is not necessary to direct the growth with specific growth factors and the culture conditions are very basic. With their good proliferation abilities, it is possible to create cell banks of very large sizes and sufficient to provide cells for hundreds of thousands of treatments (79-81). The cell bank is based on only one organ donation, but it has been demonstrated that cell banks from different donors established in the same manner showed low heterogeneity (79).

The use of fetal cells is not new. For example, the fetal cell line WI-38 was developed in 1961 (82) and used to create vaccines since 1962 (83). The fetal cell line MRC-5 was characterized in 1970 (84) and was also used to produce vaccines. Both these fetal cell lines are still used for the same purpose even today (e.g. Varivax® based on both cell lines), which demonstrates the remarkable stability of such cells.

In parallel to their exceptional cell banking and preservation abilities, fetal cells present very interesting healing properties. Indeed, tissue of animals and humans for midgestation fetuses (>24-week gestation for human) are known to heal with absence of scar tissue (85-87). The process is moreover more rapid than for newborn or adult healing (87, 88).The differences in mechanism remain mainly unknown, but it is due to the fetal tissue itself and not to the *in utero* environment (89-92). Interestingly, this can be observed for various tissues including skin (86, 87), bone (93), cartilage (94) and also tendon (95). The fetal cells seem to be responsible for this scarless regeneration (96). All these aspects taken together make fetal cells a valuable choice for the treatment of pathologies where fibrotic-scar tissue is created.

Concerning transplantation, there is evidence that fetal cells have the ability to modulate immune reaction. One of the most important aspects is the fact that fetal cells are tolerated during pregnancy, despite the recognition of paternal human leukocyte antigen C (HLA-C) (97). Fetal fibroblasts and keratinocytes present also an immunosuppressive activity and are able to inhibit lymphoproliferation (98).

In our Unit, the most advanced clinical research has been made on skin fetal progenitor fibroblasts. It was possible to obtain excellent functional and esthetic results in pediatric patients treated for burns or wounds with biological bandages containing these cells (99, 100). It was also possible to stimulate healing and wound closure in adults suffering from refractory venous ulcers (101). Furthermore, the repeated application of bandages did not induce immune reactions which confirms a good tolerance from the host towards these cells.

The regulatory aspect is an evolving field and it has been marked by some changes in 2007−2008. The European Union (EU) adapted its regulation on advanced therapy medicinal products (ATMPs) and the text was adopted in all European member states in 2008. Switzerland also modified its Transplantation Act in July of 2007 (Federal Act on the Transplantation of Organs, Tissues and Cells of 8 October 2004, RS 810.21) to align with the EU regulation upgrade. The creation of new cell banks was necessary and was realized in 2009 from one new organ donation. The federal program is now registered with Swissmedic, the Swiss Agency for Therapeutic Products, and also regulated by its specific Biobank. The human fetal progenitor tenocytes evaluated throughout this thesis come from this cell bank.

References

1. Kannus P. Structure of the tendon connective tissue. Scand J Med Sci Sports. 2000;10(6):312-20.

2. Sharma P, Maffulli N. Tendon injury and tendinopathy: healing and repair. J Bone Joint Surg Am. 2005;87(1):187-202.

3. Jozsa L, Kannus P, Balint JB, Reffy A. Three-dimensional ultrastructure of human tendons. Acta Anat (Basel). 1991;142(4):306-12.

4. O'Brien M. Structure and metabolism of tendons. Scand J Med Sci Sports. 1997;7(2):55-61.

5. Voleti PB, Buckley MR, Soslowsky LJ. Tendon healing: repair and regeneration. Annu Rev Biomed Eng. 2012;14:47-71.

6. Benjamin M, Toumi H, Ralphs JR, Bydder G, Best TM, Milz S. Where tendons and ligaments meet bone: attachment sites ('entheses') in relation to exercise and/or mechanical load. J Anat. 2006;208(4):471-90.

7. Apostolakos J, Durant TJ, Dwyer CR, Russell RP, Weinreb JH, Alaee F, et al. The enthesis: a review of the tendon-to-bone insertion. Muscles Ligaments Tendons J. 2014;4(3):333-42.

8. Thomopoulos S, Williams GR, Gimbel JA, Favata M, Soslowsky LJ. Variation of biomechanical, structural, and compositional properties along the tendon to bone insertion site. J Orthop Res. 2003;21(3):413-9.

9. Mienaltowski MJ, Birk DE. Structure, physiology, and biochemistry of collagens. Adv Exp Med Biol. 2014;802:5-29.

10. Nourissat G, Berenbaum F, Duprez D. Tendon injury: from biology to tendon repair. Nat Rev Rheumatol. 2015;11(4):223-33.

11. Wang JH. Mechanobiology of tendon. J Biomech. 2006;39(9):1563-82.

12. Elliott DH. Structure and Function of Mammalian Tendon. Biol Rev Camb Philos Soc. 1965;40:392-421.

13. Bi Y, Ehirchiou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W, et al. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. Nat Med. 2007;13(10):1219-27.

14. Liu X, Wu H, Byrne M, Krane S, Jaenisch R. Type III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular development. Proc Natl Acad Sci U S A. 1997;94(5):1852-6.

15. James R, Kesturu G, Balian G, Chhabra AB. Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. J Hand Surg Am. 2008;33(1):102-12.

16. Thomopoulos S, Genin GM, Galatz LM. The development and morphogenesis of the tendon-to-bone insertion - what development can teach us about healing. J Musculoskelet Neuronal Interact. 2010;10(1):35-45.

17. Young BB, Gordon MK, Birk DE. Expression of type XIV collagen in developing chicken tendons: association with assembly and growth of collagen fibrils. Dev Dyn. 2000;217(4):430-9.

18. Izu Y, Ansorge HL, Zhang G, Soslowsky LJ, Bonaldo P, Chu ML, et al. Dysfunctional tendon collagen fibrillogenesis in collagen VI null mice. Matrix Biol. 2011;30(1):53-61.

19. Butler DL, Grood ES, Noyes FR, Zernicke RF. Biomechanics of ligaments and tendons. Exerc Sport Sci Rev. 1978;6:125-81.

20. Yoon JH, Halper J. Tendon proteoglycans: biochemistry and function. J Musculoskelet Neuronal Interact. 2005;5(1):22-34.

21. Danielson KG, Baribault H, Holmes DF, Graham H, Kadler KE, Iozzo RV. Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. J Cell Biol. 1997;136(3):729-43.

22. Ameye L, Young MF. Mice deficient in small leucine-rich proteoglycans: novel in vivo models for osteoporosis, osteoarthritis, Ehlers-Danlos syndrome, muscular dystrophy, and corneal diseases. Glycobiology. 2002;12(9):107R-16R.

23. Svensson L, Aszodi A, Reinholt FP, Fassler R, Heinegard D, Oldberg A. Fibromodulin-null mice have abnormal collagen fibrils, tissue organization, and altered lumican deposition in tendon. J Biol Chem. 1999;274(14):9636-47.

24. Chakravarti S, Magnuson T, Lass JH, Jepsen KJ, LaMantia C, Carroll H. Lumican regulates collagen fibril assembly: skin fragility and corneal opacity in the absence of lumican. J Cell Biol. 1998;141(5):1277-86.

25. Jelinsky SA, Archambault J, Li L, Seeherman H. Tendon-selective genes identified from rat and human musculoskeletal tissues. J Orthop Res. 2010;28(3):289-97.

26. Shukunami C, Takimoto A, Oro M, Hiraki Y. Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes. Dev Biol. 2006;298(1):234-47.

27. Murchison ND, Price BA, Conner DA, Keene DR, Olson EN, Tabin CJ, et al. Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. Development. 2007;134(14):2697-708.

28. Docheva D, Hunziker EB, Fassler R, Brandau O. Tenomodulin is necessary for tenocyte proliferation and tendon maturation. Mol Cell Biol. 2005;25(2):699-705.

29. Jozsa L, Lehto M, Kvist M, Balint JB, Reffy A. Alterations in dry mass content of collagen fibers in degenerative tendinopathy and tendon-rupture. Matrix. 1989;9(2):140-6.

30. Carlson GD, Botte MJ, Josephs MS, Newton PO, Davis JL, Woo SL. Morphologic and biomechanical comparison of tendons used as free grafts. J Hand Surg Am. 1993;18(1):76-82.

31. Lichtwark GA, Wilson AM. In vivo mechanical properties of the human Achilles tendon during one-legged hopping. J Exp Biol. 2005;208(Pt 24):4715-25.

32. Maganaris CN, Paul JP. In vivo human tendon mechanical properties. J Physiol. 1999;521 Pt 1:307-13.

33. Pridgen BC, Woon CY, Kim M, Thorfinn J, Lindsey D, Pham H, et al. Flexor tendon tissue engineering: acellularization of human flexor tendons with preservation of biomechanical properties and biocompatibility. Tissue Eng Part C Methods. 2011;17(8):819-28.

34. Johnson GA, Tramaglini DM, Levine RE, Ohno K, Choi NY, Woo SL. Tensile and viscoelastic properties of human patellar tendon. J Orthop Res. 1994;12(6):796-803.

35. Wang JH, Guo Q, Li B. Tendon biomechanics and mechanobiology--a minireview of basic concepts and recent advancements. J Hand Ther. 2012;25(2):133-40; quiz 41.

36. de Boer MD, Selby A, Atherton P, Smith K, Seynnes OR, Maganaris CN, et al. The temporal responses of protein synthesis, gene expression and cell signalling in human quadriceps muscle and patellar tendon to disuse. J Physiol. 2007;585(Pt 1):241-51.

37. Zhang J, Wang JH. Mechanobiological response of tendon stem cells: implications of tendon homeostasis and pathogenesis of tendinopathy. J Orthop Res. 2010;28(5):639-43.

38. Killian ML, Cavinatto L, Galatz LM, Thomopoulos S. The role of mechanobiology in tendon healing. J Shoulder Elbow Surg. 2012;21(2):228-37.

39. Maeda T, Sakabe T, Sunaga A, Sakai K, Rivera AL, Keene DR, et al. Conversion of mechanical force into TGF-beta-mediated biochemical signals. Curr Biol. 2011;21(11):933-41.

40. Jelinsky SA, Rodeo SA, Li J, Gulotta LV, Archambault JM, Seeherman HJ. Regulation of gene expression in human tendinopathy. BMC Musculoskelet Disord. 2011;12:86.

41. Eliasson P, Andersson T, Hammerman M, Aspenberg P. Primary gene response to mechanical loading in healing rat Achilles tendons. Journal of applied physiology (Bethesda, Md : 1985). 2013;114(11):1519-26.

42. United States Bone and Joint Initiative. Musculoskeletal Injuries. The Burden of Musculoskeletal Diseases in the United States. Rosemont, IL: American Academy of Orthopaedic Surgeons; 2011. p. 129-79.

43. Kannus P, Jozsa L. Histopathological changes preceding spontaneous rupture of a tendon. A controlled study of 891 patients. J Bone Joint Surg Am. 1991;73(10):1507-25.

44. Maffulli N, Barrass V, Ewen SW. Light microscopic histology of achilles tendon ruptures. A comparison with unruptured tendons. Am J Sports Med. 2000;28(6):857-63.

45. Tuncali D, Yavuz N, Terzioglu A, Aslan G. The rate of upper-extremity deep-structure injuries through small penetrating lacerations. Ann Plast Surg. 2005;55(2):146-8.

46. de Jong JP, Nguyen JT, Sonnema AJ, Nguyen EC, Amadio PC, Moran SL. The incidence of acute traumatic tendon injuries in the hand and wrist: a 10-year population-based study. Clin Orthop Surg. 2014;6(2):196-202.

47. Maffulli N, Khan KM, Puddu G. Overuse tendon conditions: time to change a confusing terminology. Arthroscopy. 1998;14(8):840-3.

48. Paavola M, Kannus P, Järvinen M. Epidemiology of Tendon Problems in Sport. In: Maffulli N, Renström P, Leadbetter W, editors. Tendon Injuries. London: Springer; 2005. p. 32-9.

49. Gaida JE, Alfredson H, Kiss ZS, Bass SL, Cook JL. Asymptomatic Achilles tendon pathology is associated with a central fat distribution in men and a peripheral fat distribution in women: a cross sectional study of 298 individuals. BMC Musculoskelet Disord. 2010;11:41.

50. Maffulli N, Wong J, Almekinders LC. Types and epidemiology of tendinopathy. Clin Sports Med. 2003;22(4):675-92.

51. Reinking M. Tendinopathy in athletes. Phys Ther Sport. 2012;13(1):3-10.

52. de Mos M, Koevoet WJ, Jahr H, Verstegen MM, Heijboer MP, Kops N, et al. Intrinsic differentiation potential of adolescent human tendon tissue: an in-vitro cell differentiation study. BMC Musculoskelet Disord. 2007;8:16.

53. Magnusson SP, Langberg H, Kjaer M. The pathogenesis of tendinopathy: balancing the response to loading. Nat Rev Rheumatol. 2010;6(5):262-8.

54. Gerber G, Gerber G, Altman KI. Studies on the metabolism of tissue proteins. I. Turnover of collagen labeled with proline-U-C14 in young rats. J Biol Chem. 1960;235:2653-6.

55. Vailas AC, Tipton CM, Laughlin HL, Tcheng TK, Matthes RD. Physical activity and hypophysectomy on the aerobic capacity of ligaments and tendons. Journal of applied physiology: respiratory, environmental and exercise physiology. 1978;44(4):542-6.

56. Childress MA, Beutler A. Management of chronic tendon injuries. Am Fam Physician. 2013;87(7):486-90.

57. Rees JD, Wilson AM, Wolman RL. Current concepts in the management of tendon disorders. Rheumatology (Oxford). 2006;45(5):508-21.

58. Baksh N, Hannon CP, Murawski CD, Smyth NA, Kennedy JG. Platelet-rich plasma in tendon models: a systematic review of basic science literature. Arthroscopy. 2013;29(3):596-607.

59. Paoloni J, De Vos RJ, Hamilton B, Murrell GA, Orchard J. Platelet-rich plasma treatment for ligament and tendon injuries. Clin J Sport Med. 2011;21(1):37-45.

60. Taylor DW, Petrera M, Hendry M, Theodoropoulos JS. A systematic review of the use of platelet-rich plasma in sports medicine as a new treatment for tendon and ligament injuries. Clin J Sport Med. 2011;21(4):344-52.

61. Docheva D, Muller SA, Majewski M, Evans CH. Biologics for tendon repair. Adv Drug Del Rev. 2015;84:222-39.

62. Lucas T, Waisman A, Ranjan R, Roes J, Krieg T, Muller W, et al. Differential roles of macrophages in diverse phases of skin repair. J Immunol. 2010;184(7):3964-77.

63. Chazaud B. Macrophages: supportive cells for tissue repair and regeneration. Immunobiology. 2014;219(3):172-8.

64. Manning CN, Havlioglu N, Knutsen E, Sakiyama-Elbert SE, Silva MJ, Thomopoulos S, et al. The early inflammatory response after flexor tendon healing: a gene expression and histological analysis. J Orthop Res. 2014;32(5):645-52.

65. Schulze-Tanzil G, Al-Sadi O, Ertel W, Lohan A. Decellularized tendon extracellular matrix-a valuable approach for tendon reconstruction? Cells. 2012;1(4):1010-28.

66. Ferguson MWJ, O'Kane S. Scar-free healing: from embryonic mechanisms to adult therapeutic intervention. Philosophical Transactions of the Royal Society B: Biological Sciences. 2004;359(1445):839-50.

67. Juneja SC, Schwarz EM, O'Keefe RJ, Awad HA. Cellular and molecular factors in flexor tendon repair and adhesions: a histological and gene expression analysis. Connect Tissue Res. 2013;54(3):218-26.

68. Jelinsky SA, Li L, Ellis D, Archambault J, Li J, St Andre M, et al. Treatment with rhBMP12 or rhBMP13 increase the rate and the quality of rat Achilles tendon repair. J Orthop Res. 2011;29(10):1604-12.

69. Aurora A, McCarron J, Iannotti JP, Derwin K. Commercially available extracellular matrix materials for rotator cuff repairs: state of the art and future trends. J Shoulder Elbow Surg. 2007;16(5 Suppl):S171-8.

70. Aydin A, Topalan M, Mezdegi A, Sezer I, Ozkan T, Erer M, et al. [Single-stage flexor tendoplasty in the treatment of flexor tendon injuries]. Acta Orthop Traumatol Turc. 2004;38(1):54-9.

71. Gimbel JA, Van Kleunen JP, Williams GR, Thomopoulos S, Soslowsky LJ. Long durations of immobilization in the rat result in enhanced mechanical properties of the healing supraspinatus tendon insertion site. J Biomech Eng. 2007;129(3):400-4.

72. Ellera Gomes JL, da Silva RC, Silla LM, Abreu MR, Pellanda R. Conventional rotator cuff repair complemented by the aid of mononuclear autologous stem cells. Knee Surg Sports Traumatol Arthrosc. 2012;20(2):373-7.

73. Lee SY, Kim W, Lim C, Chung SG. Treatment of Lateral Epicondylosis by Using Allogeneic Adipose-Derived Mesenchymal Stem Cells: A Pilot Study. Stem Cells. 2015;33(10):2995-3005.

74. Bara JJ, Richards RG, Alini M, Stoddart MJ. Concise review: Bone marrow-derived mesenchymal stem cells change phenotype following in vitro culture: implications for basic research and the clinic. Stem Cells. 2014;32(7):1713-23.

75. Chamberlain G, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells. 2007;25(11):2739-49.

76. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315-7.

77. Maxson S, Lopez EA, Yoo D, Danilkovitch-Miagkova A, Leroux MA. Concise review: role of mesenchymal stem cells in wound repair. Stem Cells Transl Med. 2012;1(2):142-9.

78. Awad HA, Boivin GP, Dressler MR, Smith FN, Young RG, Butler DL. Repair of patellar tendon injuries using a cell-collagen composite. J Orthop Res. 2003;21(3):420-31.

79. Quintin A, Hirt-Burri N, Scaletta C, Schizas C, Pioletti DP, Applegate LA. Consistency and safety of cell banks for research and clinical use: preliminary analysis of fetal skin banks. Cell Transplant. 2007;16(7):675-84.

80. Applegate LA, Scaletta C, Hirt-Burri N, Raffoul W, Pioletti D. Whole-cell bioprocessing of human fetal cells for tissue engineering of skin. Skin Pharmacol Physiol. 2009;22(2):63-73.

81. Applegate LA, Weber D, Simon J-P, Scaletta C, Hirt-Burri N, de Buys Roessingh A, et al. Organ Donation and Whole-Cell Bioprocessing in the Swiss Fetal Progenitor Cell Transplantation Platform. In: Saidi. RF, editor. Organ Donation and Organ Donors: Issues, Challenges and Perspectives. Hauppauge NY: Nova Science Publishers, Inc.; 2013. p. 125-48.

82. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Exp Cell Res. 1961;25:585-621.

83. Hayflick L, Plotkin SA, Norton TW, Koprowski H. Preparation of poliovirus vaccines in a human fetal diploid cell strain. American journal of hygiene. 1962;75:240-58.

84. Jacobs JP, Jones CM, Baille JP. Characteristics of a human diploid cell designated MRC-5. Nature. 1970;227(5254):168-70.

85. Adzick NS, Longaker MT. Scarless fetal healing. Therapeutic implications. Ann Surg. 1992;215(1):3-7.

86. Ihara S, Motobayashi Y, Nagao E, Kistler A. Ontogenetic transition of wound healing pattern in rat skin occurring at the fetal stage. Development. 1990;110(3):671-80.

87. Longaker MT, Whitby DJ, Adzick NS, Crombleholme TM, Langer JC, Duncan BW, et al. Studies in fetal wound healing, VI. Second and early third trimester fetal wounds demonstrate rapid collagen deposition without scar formation. J Pediatr Surg. 1990;25(1):63-8; discussion 8-9.

88. Adzick NS, Harrison MR, Glick PL, Beckstead JH, Villa RL, Scheuenstuhl H, et al. Comparison of fetal, newborn, and adult wound healing by histologic, enzyme-histochemical, and hydroxyproline determinations. J Pediatr Surg. 1985;20(4):315-9.

89. Dang C, Ting K, Soo C, Longaker MT, Lorenz HP. Fetal wound healing current perspectives. Clin Plast Surg. 2003;30(1):13-23.

90. Bullard KM, Longaker MT, Lorenz HP. Fetal wound healing: current biology. World J Surg. 2003;27(1):54-61.

91. Favata M, Beredjiklian PK, Zgonis MH, Beason DP, Crombleholme TM, Jawad AF, et al. Regenerative properties of fetal sheep tendon are not adversely affected by transplantation into an adult environment. J Orthop Res. 2006;24(11):2124-32.

92. Longaker MT, Whitby DJ, Ferguson MW, Lorenz HP, Harrison MR, Adzick NS. Adult skin wounds in the fetal environment heal with scar formation. Ann Surg. 1994;219(1):65-72.

93. Longaker MT, Moelleken BR, Cheng JC, Jennings RW, Adzick NS, Mintorovich J, et al. Fetal fracture healing in a lamb model. Plast Reconstr Surg. 1992;90(2):161-73.

94. Namba RS, Meuli M, Sullivan KM, Le AX, Adzick NS. Spontaneous repair of superficial defects in articular cartilage in a fetal lamb model. J Bone Joint Surg Am. 1998;80(1):4-10.

95. Beredjiklian PK, Favata M, Cartmell JS, Flanagan CL, Crombleholme TM, Soslowsky LJ. Regenerative versus reparative healing in tendon: a study of biomechanical and histological properties in fetal sheep. Ann Biomed Eng. 2003;31(10):1143-52.

96. Lorenz HP, Lin RY, Longaker MT, Whitby DJ, Adzick NS. The fetal fibroblast: the effector cell of scarless fetal skin repair. Plast Reconstr Surg. 1995;96(6):1251-61.

97. Ober C. HLA and pregnancy: the paradox of the fetal allograft. Am J Hum Genet. 1998;62(1):1-5.

98. Zuliani T, Saiagh S, Knol AC, Esbelin J, Dreno B. Fetal fibroblasts and keratinocytes with immunosuppressive properties for allogeneic cell-based wound therapy. PLoS One. 2013;8(7):e70408.

99. Hohlfeld J, de Buys Roessingh A, Hirt-Burri N, Chaubert P, Gerber S, Scaletta C, et al. Tissue engineered fetal skin constructs for paediatric burns. Lancet. 2005;366(9488):840-2.

100. De Buys Roessingh AS, Hohlfeld J, Scaletta C, Hirt-Burri N, Gerber S, Hohlfeld P, et al. Development, characterization, and use of a fetal skin cell bank for tissue engineering in wound healing. Cell Transplant. 2006;15(8-9):823-34.

101. Ramelet AA, Hirt-Burri N, Raffoul W, Scaletta C, Pioletti DP, Offord E, et al. Chronic wound healing by fetal cell therapy may be explained by differential gene profiling observed in fetal versus old skin cells. Exp Gerontol. 2009;44(3):208-18.

Chapter II

–

Cell Therapies for Tendons: Old Cell Choice for Modern Innovation

Foreword

The chapter II consists of a review written with Dr. Ilias G. Petrou as equal principal co-author and published in Swiss Medical Weekly. The aim of this paper was to make an inventory of the different cell therapies that can be used for the treatment of tendon afflictions. Human Fetal Prognitor Tenocytes (hFPTs) are cited for the first time in the literature and the idea to use them for the treatment of tendon pathologies is proposed.

Established in 1871
Swiss Medical Weekly
Formerly: Schweizerische Medizinische Wochenschrift
The European Journal of Medical Sciences

Review article: Medical intelligence | Published 7 August 2014, doi:10.4414/smw.2014.13989
Cite this as: Swiss Med Wkly. 2014;144:w13989

Cell therapies for tendons: Old cell choice for modern innovation

Ilias G. Petrou[*], *Anthony Grognuz*[*], *Nathalie Hirt-Burri, Wassim Raffoul, Lee Ann Applegate*

Plastic, Reconstructive and Hand Surgery, Unit of Regenerative Medicine, University Hospital of Lausanne, Switzerland

* Considered as equal principal co-authors

Summary

Although tissue engineering and cell therapies are becoming realistic approaches for medical therapeutics, it is likely that musculoskeletal applications will be among the first to benefit on a large scale. Cell sources for tissue engineering and cell therapies for tendon pathologies are reviewed with an emphasis on small defect tendon injuries as seen in the hand which could adapt well to injectable cell administration. Specifically, cell sources including tenocytes, tendon sheath fibroblasts, bone marrow or adipose-derived stem cells, amniotic cells, placenta cells and platelet-derivatives have been proposed to enhance tendon regeneration. The associated advantages and disadvantages for these different strategies will be discussed and evolving regulatory requirements for cellular therapies will also be addressed. Human progenitor tenocytes, along with their clinical cell banking potential, will be presented as an alternative cell source solution. Similar cell banking techniques have already been described with other progenitor cell types in the 1950's for vaccine production, and these "old" cell types incite potentially interesting therapeutic options that could be improved with modern innovation for tendon regeneration and repair.

Key words: *cell therapy; progenitor; tenocytes; hand surgery; flexor tendon; neotendon; tendon regeneration; tendon tissue engineering*

Introduction

Tendon disorders are frequent and create functional and productivity problems in sports and in the workplace. Incomplete healing of tendon injuries not only leads to disability, but can also be associated with debilitating pain. Accidents with acute tendon laceration of the hand are particularly common. Approximately 1,775 new cases are reported in Switzerland each year with a mean insurance cost of 23,843 CHF [1]. The Swiss hand surgeon, Claude Verdan, was the first to introduce the classification of flexor tendon injuries, separating them into five anatomical zones [2]. There are also associated pathologies of tendons and ligaments that can frequently occur with hand overuse, degenerative diseases and aging. These can promote joint instability and arthritis and eventually may require surgical

Figure 1

Tendon structure, histology and cell culture.
Ultrastructure of tendon showing longitudinal fibres and histological sections stained with haematoxylin and eosin depicting extracellular matrix colored in pink (~80% composition) and cellular components mainly with tenocytes stained in blue (~20% composition)(horse tendon for illustration). Primary cultures from tissue can be developed by culturing cells in minimal medium (DMEM + 10% FBS + 1% glutamine) for approximately 1 week in a monolayer culture to provide "fibroblast-like" pure tenocyte populations (human progenitor tenocytes).

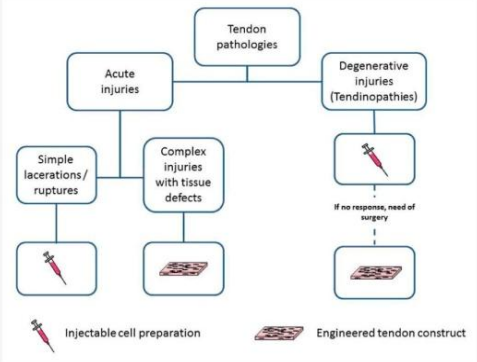

Figure 2

Tendon pathologies and therapeutic strategy.
Tendon pathologies can be either acute or degenerative. Degenerative therapies could be treated with biological cellular therapies with injectable forms to delay degenerative responses and more invasive surgery. Acute injuries, depending on their severity, could be treated with either non-invasive to invasive technique with cells in viscous biogels to complex neo-tissue constructs.

intervention. Following the pioneering endeavors of tendon surgery, modern therapeutic strategies with primary suture and precocious-controlled motion have been developed with good but not always ideal results.

On the pathophysiological level, intrinsic tenocyte regenerative response, adequate nutrition and prevention of adhesions are some of the key elements that have to be considered in order to achieve an optimal functional result after tendon injury. Despite early surgical treatment and mobilisation, adhesions are frequent and secondary tendon rupture is still a major concern. Joint instability is usually the consequence of trauma to bone and degeneration of cartilage. Tendon transfers are used to stabilise the joint, but treatment failures are frequent where there is tendon degeneration. It has been proposed that cell stimulation could prevent surgical failure. Tendon regeneration is a time consuming procedure because of slow metabolism in adult tenocytes. Immediately following an injury an inflammatory phase takes place, during which chemo-attractive substances are released to recruit and stimulate tenocytes. After a few days and during the first few weeks, the second step of healing is controlled by tenocytes and macrophages and there is a high deposition of matrix, mostly composed of type 3 collagen. After one or two months, a third phase appears with the extracellular matrix (ECM) continuing to be produced but with a higher amount of type 1 collagen. The tissue is reorganised and becomes more and more aligned. This process takes as long as one year or more, with the cell density and activity diminishing with time [3, 4].

The architecture and orientation of the collagen fibres of the tendon are of paramount importance, providing an exquisite anatomical structure assuring elasticity, mobility and tensile strength and therefore becoming a functional tissue with unique biomechanical qualities (fig. 1A & B). The role of the tendon is to provide correct transmission of forces between muscle and bone and to protect tension and compression of structures upon forces. This conjunctive tissue can come in various forms and lengths [3, 5, 6]. Interestingly, the composition of tendon is mostly ECM at 80% and cells at approximately 20% [7] with 90–95% of the cells being tenocytes [3, 5]. A minor portion of chondrocytes, synovial, vascular and tendon stem cells can also be detected. The longitudinal cells present in histological sections (fig. 1B) represent tenocytes and are distributed fairly uniformly throughout the ECM and when cultured from primary tissue form “fibroblastic-like” cells in 2–D

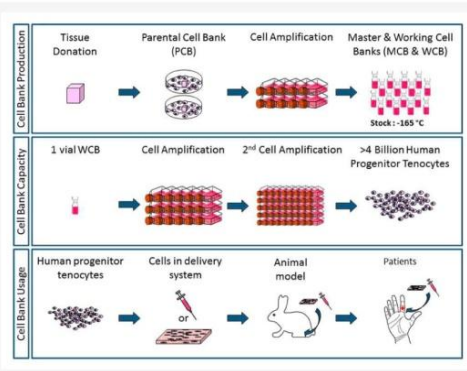

Figure 3

Progenitor human tenocyte cell bank production, capacity and usage for off-the-shelf therapeutic options for tendinopathies. From one single tendon tissue (2 mm^3, organ donation of foetal Achilles tendon tissue at 14 weeks gestation), clinical cell banks can be produced including parental primary cultures, Master and Working Cell Banks that contain 200 vials each of progenitor tenocytes at low passages (P. 3–6). Expansion of cells produces high quantities of cells that can be stocked in vapor phase of liquid nitrogen (–165 °C) providing cell stocks for hundreds of thousands of patients. Cells can be administered in hydrogels or on other delivery systems for testing in animal models and pre-clinical patient treatments in the process for therapeutic agent development.

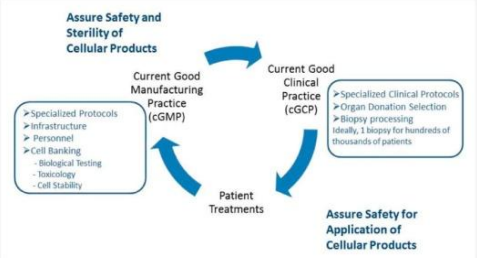

Figure 4

Good Clinical Practice and Good Manufacturing Practice associated with cellular therapeutics.

Current Good Clinical Practices (cGCP) are necessary to begin the process of cellular therapies and tissue engineering with detailed protocols for organ donation and biopsy processing. Tissue and cellular handling then needs to abide by Current Good Manufacturing Processes (cGMP) which include specific protocols for infrastructure, equipment and personnel. cGCP is then needed for appropriate administration of cellular therapies. Thus, the entire process requires high collaboration between biologists, technicians, nurses and surgeons.

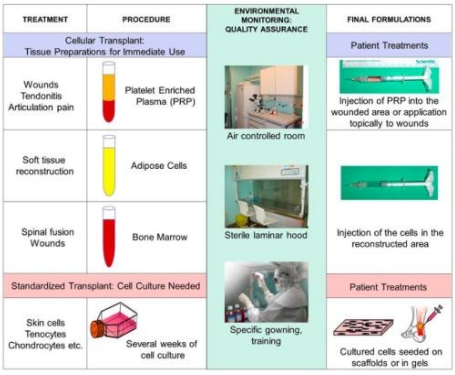

Figure 5

Regulatory requirements for cellular therapy products.

Regulatory requirements have become more extensive for cellular therapies and tend towards conservative drug development in their production, pre-clinical testing and clinical trials. Blood products also have to be produced under cGMP and only in institutions with a license for manipulation. Organs, cells and tissues (PRP, adipose, bone marrow, skin, cartilage, tendon etc.) need to be processed with environmental monitoring and full quality assurance with cGMP processing through to the final formulation and delivery to the patient.

culture (fig. 1C). The ECM is mainly composed of collagen fibres representing 65–80% of tendon dry matter with Type I and III being the most frequent (95% Type I) [6]. Elastic fibres (1–2%), proteoglycans along with glycoproteins (less tan 1%) and inorganic molecules (less than 0.2%) are the remaining elements of the ECM [5].

In the case of injury, the healed tendon cannot restore its exact natural structure, resulting in the formation of scar tissue which weakens its overall functional abilities [8]. In clinical practice it is frequently observed that patient recovery is long and full strength mobilisation takes almost 3 months after the time of the injury to return. Tendon injuries can be classified as acute or degenerative conditions (fig. 2). Acute injuries associated with partial ruptures or fissures could be treated first with injectable functional cell populations before turning towards more invasive tissue replacement. Tendinopathies with degenerative conditions could be stabilised with injectable biological cell therapy solutions in order to delay any surgical procedures. With tissue defects, the length of time before primary repair, the failure of the primary closure and enhanced chances of re-rupture are common problems encountered. Under these circumstances, the use of tendon grafts or transfers is sometimes necessary (fig. 2). However, these procedures are associated with donor site morbidity (if autologous graft), increased operating time, risk of infection and higher cost.

These drawbacks have promoted translational research in the search for solutions of off-the-shelf tendon engineered constructs and cellular therapies that could be available whenever and wherever needed. The optimal result of new therapeutic strategies would be the achievement of a scar-free regenerated tissue. Cadaveric de-cellularised tendons are routinely used and new tissue engineering strategies would be important for clinical use. As for any tissue engineering therapeutic strategy, the cell source and type is crucial. The implicated cells should be easy to collect, easily and rapidly cultured and expanded, and should possess maximum stability of the desired phenotype which would assure patient safety upon implantation. In addition, they should also possess a high tendon formation potential without triggering an immunological reaction of the recipient patient.

In this short review on innovation we will present the existing cell sources used in cellular therapies of tendon with their advantages and drawbacks, and we will present the potential use of human allogenic progenitor tenocytes for the repair and enhancement of tissue regeneration of the tendon.

Embryonic cell sources for tendon tissue engineering applications

Embryonic stem cells (ESCs) have been proposed as a cell source for tendon repair and regeneration. These are undifferentiated cells which are able to create all cell types and the original tissue source is the early age embryo (around five days following ovum fertilisation). The derived cells are “totipotent” until eight cell divisions occur. Then, approximately 2 weeks post-fertilisation, the cells become pluripotent and can no longer develop into another embryo, but they can develop into every cell type with proper stimulation (i.e. growth factors). All of these stages of development up to 8 weeks post-fertilisation are considered to be an embryo.

A tendon model derived from ESCs was developed successfully and grafted in a rat. Maturation and differentiation of the cells into a tenocyte-type was achieved and observed 30 days after implantation. Using the same cells in a fibrin gel to treat rat patellar tendons led to better structural and functional results than the controls with fibrin gel only. The cells survived for 4 weeks and activated the intrinsic tendon regeneration procedure with no presence of ectopic tissue or teratomas. However, ESCs have the potential to differentiate into many phenotypes and the presence of factors like GDF5 and BMP2 in the wound could potentially lead to the formation of bone or cartilage tissues [9].

In a large animal model with induced horse flexor tendonitis (collagenase gel – physical defect), the injection of ESCs to the injured tendon led to an important clinical amelioration. The double-blinded experiment using MRI as well as ultrasound analysis showed improved structural changes in the animals treated with ESCs over placebo [10] but could not define the specific mechanism of amelioration through exogenous cell transplant, local cytokine or immunological modulation or simply by the stimulation of environmental endogenous horse cells.

The use of ESCs remains fairly complicated on the technical front with high concerns in terms of consistency and security of the cells as they could dedifferentiate when introduced into an *in vivo* environment. Cultures of these stem cell types are technically very demanding since the amount of tissue to begin the culture is very small (<100 cells) and up-scaling the stem cells in an undifferentiated state requires many growth factor supplements. In addition, in many countries ethical issues for the use of embryonic stem cells have been associated with specific directives and licenses to allow cell manipulation and research. More sophisticated techniques of cell encapsulation and cellular cloning could provide strategies to improve the delivery of the correct cell population and therefore provide an allogenic (cells not from the same patient) cell source ready-for-use, which would address safety issues for the patient.

Adult cell sources for tendon tissue engineering applications

Various adult cell sources are under current research and development to achieve tendon regeneration. The tissue engineering strategies can thereby be described depending on the differentiation level of the cells used and on the original tissue source. In addition to the above described embryonic and allogenic cell source, autologous cells have also been proposed and various adult stem cell populations have been envisioned for tendon repair. Cell sources implemented include: i) isolated, cultured “mesenchymal cells” (MSCs) from bone marrow (BM-MSCs), adipose tissue (AD-MSCs); ii) fully differentiated cell lineages such as tenocytes, and; iii) blood-derivatives such as platelets or plasma-enriched platelet preparations (PRP).

Fresh blood or bone marrow have been extensively used for over 40 years, since the beginning of the tissue engineering era. Although cells from fresh bone marrow or blood are accessible without much effort, allotransplantation remains difficult as these transplantations could lead

to an acute graft-versus host disease [11, 12]. This is why modern cell-based therapeutic techniques focus on the use of specific "purified" and culture expanded lineages. Interestingly, Osiris Therapeutics demonstrated in 1997 that BM-MSCs isolated by density gradient, purified to eliminate non-MSC cell sources and expanded in cell culture, could be used as a secure allogenic cell source for different clinical affections [13]. The first clinical trials using BM-MSCs allogenic stocked cells for burns and wounds will take place at the University of Miami as they were awarded a Department of Defense and Armed Forces Institute of Regenerative Medicine Grant (DOD-AFIRM) for tissue repair (http://med.miami.edu/news/miller-school-physician-scientists-receive-3–million-defense-grant-to-treat/). Combining stem cells with autografting techniques used in burn wounds may help in diffusion of the necessary nutrients, growth factors and oxygen for successful transplantation. Likewise, adipose cells derived from the stromal fraction have been shown to improve diffusion and help overall tissue homeostasis in a mouse model to date [14]. Additionally, other stem cell sources such as tonsil mesenchymal cells can aid in the preparation of skin engineered grafts [15] and full lymphatic vascular systems can be linked to skin grafts with the development of lymphatic capillaries constructed *in vitro* [16]. Especially for burn patients these techniques need to be continually improved and optimised to allow shorter production and earlier delivery times to the patient [17].

Both adipose-derived and bone-marrow-derived MSCs have been used for tendon repair and regeneration potential in animal models including rats, horses, and sheep [18–21]. Only slight improvements have been reported in these models and the regeneration quality cannot be compared to that seen intrinsically in foetal tendon [10, 22, 23]. These cell types have also been used on acellularised allogenic tendons or on various scaffolds and tested in animal models. The optimal goal is to produce a bioengineered construct with high force before failure, adequate tensile stiffness and absence of calcifications [24–27]. So far in human trials, autotransplantation of mononuclear stem cells extracted from the iliac crest has given encouraging results in terms of safety and ability to enhance intrinsic tendon regeneration [28].

Each cell type possesses different advantages and disadvantages. MSCs isolated from different tissue could present immunological advantages for autologous use. As only one out of every 100,000 cells derived from bone marrow is a stem cell, a technically demanding procedure of isolation and expansion is needed. AD-MSCs are more readily available (>100–fold compared to BM-MSC) and have better growth capacity *in vitro*. Although the use of MSCs has shown tendon regenerative properties, the formation of calcifications in almost all the experimental models prevents their establishment as a successful bioengineered therapeutic agent for tendons. Recently discovered tendon stem cells [29] could potentially allow regeneration with less calcification. Unfortunately it is difficult to harvest these cells in an autologous tendon.

It is possible that differentiated cells will be more competent in the *in vivo* environment due to their higher production of specific components of the extracellular matrix and lower de-differentiation potential. Cell choices from local tendon environment such as tenocytes, and tendon sheath fibroblasts were proposed [27] and comparisons between adipose-derived MSC and bone-marrow derived MSC to these two differentiated cell types were assessed for their qualities both *in vitro* and *in vivo*. As all cell types were viable and seemed to deposit matrix, differentiated cell sources might avoid calcification formation and prove more useful. However, adult tendon cells present the same issue as tendon stem cells and an autologous source does not yet seem technically imaginable.

Another recent popular cell source for tendon regeneration is platelets. Platelet rich plasma (PRP) can be used alone or with biocompatible scaffolds, providing better structural results resulting in increased functional outcomes [30, 31]. Until now, PRP is used as an autotransplantation as there is always a small but existing risk of immunological reactions in the case of allotransplantation [32]. On the one hand there is still strong controversy regarding the real positive effect that this therapeutic agent can obtain [33] but on the contrary the isolation and preparation can be done rapidly and inexpensively [34].

Progenitor cell sources in tissue engineering applications

Placenta, umbilical cord or even amniotic liquid contain various types of foetal progenitor cells that have been employed in bioengineering [35–37]. Besides these above mentioned tissues, most foetal cell research is based on specific material derived at the latter end of the first trimester (11 to 14 weeks of gestation). At this stage, cell lineages with tissue specific cells can be established. The tissue can be considered an organ donation when the mother-donor is contacted only after her decision to proceed with a voluntary pregnancy interruption, when she (and her partner) gives an informed consent and when there is no payment [38]. This procedure is authorised by legislation in most countries.

The use of foetal tissues or cells in research began in the laboratories of immunologists and neurologists. The polio vaccine, which led to a Nobel Prize for Medicine in 1954 to American immunologists, was developed using cultures of human foetal cells which are still used in contemporary vaccine development today. Transplantation of foetal neural cells has been used to treat conditions such as Huntington's [39, 40] or Parkinson's disease [41]. Similarly, foetal transplants have been used for spinal cord affections or injuries by providing encouraging results in motor function recovery and also by offering a safe transplantation procedure for patients [42–45]. Moreover, human foetal liver cells have been used for more than 25 years to treat severe immunodeficiency, haematological disorders and congenital disorders of metabolism [46].

Recently, liver failures and diabetes have been targeted by foetal cell therapy strategies [47]. Better understanding of developmental embryology has substantially helped technological progress. Studies have also revealed important inductive signals and transcription factors that can play crucial roles in the differentiation of hepatocytes and b-cells from various stem and progenitor cell types [48]. It should be noted that human foetal liver cells were suc-

cessfully isolated to treat end-stage liver disease. In a case report it was shown that the patient model for end-stage liver disease (MELD) score improved significantly within the first 18 months of follow-up [49]. In another study including 25 patients, clinical and biochemical parameters improved, patients did not present with hepatic encephalopathy and their mean MELD score decreased during 6 months of observation [50].

In recent years, three-dimensional biological bandages, developed from human foetal skin progenitor cells have been used to treat burns in children and also for chronic wounds [51, 52]. With this particular technique, cells from one dedicated cell bank can be expanded to produce over 35 x 10^9 tissue engineering skin constructs (9 x12 cm) providing an off-the shelf cell-based therapy [53]. Following this approach, human foetal bone cells [54] and chondro-progenitor cells [55] have been used as potential regenerative agents for human skeletal tissue, and depending on delivery systems, the cells can be used either in injectable techniques for difficult to treat areas or on scaffolds for cavity filling [56]. Surprisingly, tendon tissue engineering using foetal progenitor cells has barely begun to be investigated. Lately, our group has studied the characteristics of human progenitor tenocytes for a better comprehension of their biology *in vitro* (http://www.unil.ch/webdav/site/fbm/shared/recherche/FBM_Day_2012/FBMDAY_ABSTRACTBOOK_2012_FINAL.pdf, abstract on p.93) and to evaluate their potential use for tendon tissue engineering and for the production of a biocompatible neo-tendon. A short description follows below.

Potential of human progenitor tenocytes for tendon tissue engineering applications

Up to now, it has been shown that the determining factor in cell therapies is the cell choice and technical specifications that are related to their collection, culture, expansion, storage and stability. In addition, therapeutically, the cells should have high tissue regenerative properties, produce low or no immunological induced reactions, and have no pro-inflammatory issues. In Figure 3, a clinical cell bank of human progenitor tenocytes is depicted that can be developed in a very short time period from one single organ donation of achilles heel tendon (~2 mm^3). These cells have been developed in a registered transplantation programme in Switzerland since 2007 (08.2007, protocol #62/07: Development of foetal cell banks for tissue engineering) [34]. The primary culture or parental cell bank can be produced in less than 14 days using a simple medium as nutrient (Dulbecco's Modified Eagles Medium [DMEM] supplemented with 10% foetal bovine serum and 1% glutamine) (fig. 3A). Master and working cell bank vials (MCB & WCB) of 1–10^{10} cells can be stored at –165 °C in the vapor phase of liquid nitrogen for at least 5 years with no incidence on stability (fig. 3B). Other progenitor cell types have been shown to remain stable for 20 years to date (i.e. skin). From the original 2 mm^3 of tissue, it would be possible to develop around 200 vials of a MCB and an equivalent quantity of WCB vials. The overall potential of one, unique organ donation can thus be illustrated in that at least 35 x 10^9 treatments could be produced from the clinical cell bank. The cells produced could be delivered either in hydrogels to allow minimal invasive options or within a scaffold to provide possibilities for larger defect repair and regeneration (fig. 3C).

Evolving regulatory requirements for cellular therapies

The main reason why cellular therapies have not been implemented more rapidly in the clinical setting is because of the fluctuating regulatory requirements that have surrounded cellular products over the last years. The EU regulation on advanced therapy medicinal products (ATMP) was adopted in all European member states in 2008 and Switzerland modified its Transplantation Act in July of 2007 (Federal Act on the Transplantation of Organs, Tissues and Cells of 8 October 2004, RS 810.21). Since these dates, all cellular products must be in compliance with Current Good Clinical Practice (cGCP) guidelines for their procurement and collection, and with Current Good Manufacturing Procedures (cGMP) for the production and manufacturing of cells and tissues. These new regulations impose strict criteria for the production of cellular products and the environment for which they are produced (fig. 4 & 5). The necessary requirements can be assimilated to the industry that produces vaccines for world-wide use. As a result, cellular products and therapies used in early clinical trials require hospital settings that have appropriate licensed clean-rooms for stocking and manufacturing of cells; a major task that takes time and extremely high budgets. Most of the major hospitals in Switzerland have invested in facilities or are investing in them to assure cellular therapeutic options. These types of clean rooms and quality assurance (QA) systems associated with them are now necessary even for PRP preparations following a recent decision by SwissMedic (August, 2013: http://www.swissmedic.ch/zulassungen/00196/01956/index.html?lang=fr). All preparations of blood and serum derivatives for the treatments of tendinopathies are obliged to be considered as medicinal products under the interpretation of the Therapeutic Products Act (Federal Act on Medicinal Products and Medical Devices of 15 December 2000, TPA, RS 812.21). Consequently, preparations can only be prepared in appropriate laboratories that have a license for manufacturing and fabrication must respect "GMP on a small industrial scale", which are edited by SwissMedic in Pharmacopoea Helvetica (11.1, chapter 20.1 and 21.1). Clinical indications that are regularly treated with these PRP and associated preparations include achilles tendinopathy, patellar tendinopathy ('jumper's knee'), rotator cuff disorders, calcaneal and plantar fasciitis in the foot, muscle strains, ligament sprains, articular cartilage injury and even for preparation before hair transplants. In the field of hand surgery, the conditions including lateral epicondylitis ('tennis elbow'), medial epicondylitis (golfer's elbow), osteoarthritis, acute flexor/extensor tendon rupture/laceration or even hand rejuvenation techniques will now need to follow these new strict regulations. Consequently, any product destined to be transferred to patients, which is composed of organs, cells and living tissues from autologous, allogenic or xenogeneic sources (PRP, MSCs, skin progenitor cells, tenocytes) will need to follow environmental monitoring and different degrees of GMP manufacturing. Manufacturing will

include all manipulations ranging from simple tissue and cell separation techniques (i.e. centrifugations) to the more complex manipulations of cell culturing and extensive cell separation (fig. 5). With the advancement of technology and the ever increasing interest in tendon regeneration research, tendon pathologies could be included in more complex cell-based therapeutic strategies in the near future.

Benefits for patient care with these new innovative therapies offer promise in repairing, replacing or restoring the functionality of damaged tissues and in the management of pain. In order to provide potential cell-based therapies to increasing patient numbers, it will be necessary to optimise the cell choice for isolation, proliferation and stability to assure the highest patient security. In addition, all of these new innovative therapies will evolve in relation to regulatory requirements and more importantly adapted technical specifications (fig. 4). Biologists and technicians, in their roles as "laboratory surgeons", will need to work even more closely with clinicians to adapt innovative cell therapeutics to the patient.

Acknowledgements: We would like to thank the Foundation S.A.N.T.E and Foundation Family Sandoz for financing our Progenitor Transplantation Program. We also would like to thank the Orthopaedic Hospital Foundation for support for the doctoral thesis of one of the students (AG).
Funding: Foundation Sandoz Family, Foundation S.A.N.T.E., Foundation Orthopedic Hospital

Correspondence: *Prof. Lee Ann Laurent-Applegate, Unit of Regenerative Therapy, Service of Plastic and Reconstructive Surgery, Department of Musculoskeletal Medicine, CHUV / EPCR02 / Ch. Croisettes 22, CH-1066 Epalinges, Switzerland, Lee.Laurent-Applegate[at]chuv.ch*

References

1 Service de centralization des statistiques de l'assurance accident LAA. www.unfallstatistik.ch

2 Verdan CE. Half a century of flexor-tendon surgery. Current status and changing philosophies. J Bone Joint Surg Am. 1972;54(3):472–91.

3 Sharma P, Maffulli N. Tendon injury and tendinopathy: healing and repair. J Bone Joint Surg Am. 2005;87(1):187–202.

4 Voleti PB, Buckley MR, Soslowsky LJ. Tendon healing: repair and regeneration. Annual Rev Biomed Eng. 2012;14:47–71.

5 Kannus P. Structure of the tendon connective tissue. Scand J Med Sci Sports. 2000;10(6):312–20.

6 O'Brien M. Structure and metabolism of tendons. Scand J Med Sci Sports. 1997;7(2):55–61.

7 Tai CC, Williams A. Ligaments and tendons, in Basic Orthopaedic Sciences, Ed. Ramachandram, CRC Press 2006, chapter 8, pp. 71–78.

8 James R, Kesturu G, Balian G, Chhabra AB. Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. J Hand Surg Am. 2008;33(1):102–12

9 Chen X, Song XH, Yin Z, Zou X-Y, Wang L-L, Hu H, et al. Stepwise differentiation of human embryonic stem cells promotes tendon regeneration by secreting fetal tendon matrix and differentiation factors. Stem Cells. 2009;27(6):1276–1287.

10 Watts AE, Yeager AE, Kopyov OV, Nixon AJ. Fetal derived embryonic-like stem cells improve healing in a large animal flexor tendonitis model. Stem Cell Res Ther. 2011;2(1):4.

11 Ferrara JL, Cooke KR, Teshima T. The pathophysiology of acute graft-versus-host disease. Int J Hematol. 2003;78(3):181–7.

12 Petersdorf EW, Shuler KB, Longton GM, Spies T, Hansen JA. Population study of allelic diversity in the human MHC class I-related MIC-A gene. Immunogenetics. 1999;49(7–8):605–12.

13 Mack GS. Osiris seals billion-dollar deal with Genzyme for cell therapy. Nature Biotech. 2009;27(2):106–7.

14 Klar AS, Güven S, Biedermann T, Luginbühl J, Böttcher-Haberzeth S, Meuli-Simmen C, Meuli M, Martin I, Scherberich A, Reichmann E. Tissue-engineered dermo-epidermal skin grafts prevascularized with adipose-derived cells. Biomaterials. 2014;35(19):5065–78.

15 Böttcher-Haberzeth S, Biedermann T, Klar AS, Pontiggia L, Rac J, Nadal D, et al. Tissue engineering of skin: human tonsil-derived mesenchymal cells can function as dermal fibroblasts. Pediatr Surg Int. 2014;30(2):213–22.

16 Daniela Marino, Joachim Luginbühl, Simonetta Scola, Martin Meuli, Ernst Reichmann. Bioengineering Dermo-Epidermal Skin Grafts with Blood and Lymphatic Capillaries. Sci Transl Med. 2014;6(221): 221ra14 DOI: 10.1126/scitranslmed.3006894.

17 Pontiggia L, Klar AS, Böttcher-Haberzeth S, Biedermann T, Meuli M, Reichmann E. Optimizing in vitro culture conditions leads to a significantly shorter production time of human dermo-epidermal skin substitutes. Pediatr Surg Int. 2013;29(3):249–56.

18 Ju YJ, Muneta T, Yoshimura H, Koga H, Sekiya I. Synovial mesenchymal stem cells accelerate early remodeling of tendon-bone healing. Cell Tissue Res. 2008;332(3):469–78.

19 Lacitignola L, Crovace A, Rossi G, Francioso E. Cell therapy for tendinitis, experimental and clinical report. Vet Res Commun. 2008;32:s33–38.

20 Nixon AJ, Dahlgren LA, Haupt JL, Yeager AE, Ward DL. Effect of adipose-derived nucleated cell fractions on tendon repair in horses with collagenase-induced tendinitis. Am J Vet Res. 2008;69(7):928–37.

21 Smith RK. Mesenchymal stem cell therapy for equine tendinopathy. Disabil Rehabil. 2008;30(20–22):1752–8.

22 Favata M, Beredjiklian PK, Zgonis MH, Beason DP, Crombleholme TM, Jawad AF, Soslowsky LJ. Regenerative properties of fetal sheep tendon are not adversely affected by transplantation into an adult environment. J Orthop Res. 2006;24(11):2124–32.

23 Beredjiklian PK, Favata M, Cartmell JS, Flanagan CL, Crombleholme TM, Soslowsky LJ. Regenerative versus reparative healing in tendon: a study of biomechanical and histological properties in fetal sheep. Annals of Biom Eng. 2003;31(10):1143–52.

24 Kryger GS, Chong AKS, Costa M, Pham H, Bates SJ, Chang J. A comparison of tenocytes and mesenchymal stem cells for use in flexor tendon tissue engineering. J Hand Surg. 2007;32(5):597–605.

25 Ouyang HW,Goh JCH, Thambyah A, Teoh SH, Lee EH. Knitted polylactide-co-glycolide scaffold loaded with bone marrow stromal cells in repair and regeneration of rabbit achilles tendon. Tissue Eng. 2003;9(3):431–9.

26 Chong AKS, Ang AD, Goh JCH, Hui JHP, Lim AYT, Lee EH, et al. Bone marrow derived mesenchymal stem cells influence early tendon-healing in a rabbit Achilles tendon model. J Bone Joint Surgery A. 2007;89(1):74–81.

27 Gulotta LV, Kovacevic D, Packer JD, Deng XH, Rodeo SA. Bone marrow-derived mesenchymal stem cells transduced with scleraxis improve rotator cuff healing in a rat model. Am J Sports Med. 2011;39(6):1282–9.

28 Ellera Gomes JL, Canquerini da Silva R, Silla LMR, Abreu MR, Pellanda R. Conventional rotator cuff repair complemented by the aid of mononuclear autologous stem cells. Knee Surg Sports Traumatol Arthrosc. 2012;20(2):373–7.

29 Bi Y, Ehirchiou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W, et al. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. Nature Med. 2007;13(10):1219–27.

30 Fernández-Sarmiento JA, Domínguez JM, Granados MM, Morgaz J, Navarrete R, Carrillo JM, et al. Histological study of the influence of plasma rich in growth factors (PRGF) on the healing of divided Achilles tendons in sheep. J Bone Joint Surg Am. 2013;95(3):246–55.

31 Sato D, Takahara M, Narita A, Yamakawa J, Hashimoto J, Ishikawa H, Ogino T. Effect of platelet-rich plasma with fibrin matrix on healing of intrasynovial flexor tendons. J Hand Surg Am. 2012;37(7):1356–63.

32 Cid J, Harm SK, Yazer MH. Platelet transfusion – the art and science of compromise. Transfus Med Hemother. 2013;40(3):160–71.

33 Schepull T, Kvist J, Norrman H, Trinks M, Berlin G, Aspenberg P. Autologous platelets have no effect on the healing of human achilles tendon ruptures: a randomized single-blind study. Am J Sports Med. 2011;39(1):38–47.

34 Akhundov K, Pietramaggiori G, Waselle L, Darwiche S, Guerid S, Scaletta C, et al. Development of a Cost-Effective Method for Platelet Rich Plasma (PRP) Preparation for Topical Wound Healing. Ann Burns Fire Disasters. 2012;25(4):207–13.

35 Kaviani A, Guleserian K, Perry TE, Russel W, Jennings RW, Moritz M, et al. Fetal tissue engineering from amniotic fluid. J Am Coll Surg. 2003;196(4):592–7.

36 Kadner A, Hoerstrup SP, Tracy J, Breymann C, Maurus CF, Melnitchouk S, et al. Human umbilical cord cells: A new cell source for cardiovascular tissue engineering. Ann Thorac Surg. 2002;74:S1422–8.

37 Kaviani A, Perry TE, Barnes CM, Oh JT, Ziegler MM, Fishman SJ, et al. The placenta as a cell source in fetal tissue engineering. J Pediatr Surg. 2002;37:995–9.

38 Applegate LA, Weber D, Simon J-P, Scaletta C, Hirt-Burri N, de Buys Roessingh A, Raffoul W. Chapter 7 – Organ donation and whole-cell bioprocessing in the Swiss Fetal Progenitor Cell Transplantation Platform. Ed. RF Saidi, Organ Donation and Organ Donors. Nova Science Publishers; 2013. p.125–147.

39 Rosser AE, Bachoud-Lévi A-C. Clinical trials of neural transplantation in Huntington's disease. Prog Brain Res. 2012;200:345–71.

40 Schackel S, Pauly M-C, Piroth T, Nikkhah G, Döbrössy MD. Donor age dependent graft development and recovery in a rat model of Huntington's disease: Histological and behavioral analysis. Behav Brain Res. 2013;256:56–63.

41 Lindvall, O. Developing dopaminergic cell therapy for Parkinson's disease – give up or move forward? Mov. Disord. 2013;28(3):268–73.

42 Wirth ED 3rd, Reier PJ, Fessler RG, Thompson FJ, Uthman B, Behrman A, et al. Feasibility and safety of neural tissue transplantation in patients with syringomyelia. J Neurotrauma. 2001;18(9):911–29.

43 Akesson E, Piao JH, Samuelsson EB, Holmberg L, Kjaeldgaard A, Falci S, et al. Long-term culture and neuronal survival after intraspinal transplantation of human spinal cord-derived neurospheres. Physiol Behav. 2007;92(1–2):60–6.

44 Iwai H, Nori S, Nishimura S, Yasuda A, Takano M, Tsuji O, et al. Transplantation of neural stem/progenitor cells at different locations in mice with spinal cord injury. Cell Transplant. 2013 Aug 30. doi: 10.3727/096368913X670967. (Epub ahead of print)

45 Mothe, AJ, Tator CH. Review of transplantation of neural stem/progenitor cells for spinal cord injury. Int J Dev Neurosci. 2013;31(7):701–13.

46 Touraine JL, Raudrant D, Golfier F, Rebaud A, Sembeil R, Roncarolo MG, et al. Reappraisal of in utero stem cell transplantation based on long-term results. Fetal Diagn Ther. 2004;19(4):305–12.

47 Montanucci P, Pennoni I, Pescara T, Basta G, Calafiore R. Treatment of diabetes mellitus with microencapsulated fetal human liver (FH-B-TPN) engineered cells. Biomaterials. 2013;34(16):4002–12.

48 Zaret KS, Grompe M. Generation and regeneration of cells of the liver and pancreas. Science. 2008;322(5907):1490–4.

49 Gridelli B, Vizzini G, Pietrosi G, Luca A, Spada M, Gruttadauria S, et al. Efficient human fetal liver cell isolation protocol based on vascular perfusion for liver cell–based therapy and case report on cell transplantation. Liver Transpl. 2012;18(2):226–37.

50 Khan AA, Shaik MV, Parveen N, Rajendraprasad A, Aleem MA, Habeeb MA, et al. Human fetal liver-derived stem cell transplantation as supportive modality in the management of end-stage decompensated liver cirrhosis. Cell Transplant. 2010;19(4):409–18.

51 Hohlfeld J, de Buys Roessingh A, Hirt-Burri N, Chaubert P, Gerber S, Scaletta C, et al. Tissue engineered fetal skin constructs for paediatric burns. Lancet. 2005;366:840–2.

52 Ramelet A-A, Hirt-Burri N, Raffoul W, Scaletta C, Pioletti D, Offord E, et al. Chronic wound healing by fetal cell therapy may be explained by differential gene profiling observed in fetal versus old skin cells. Exp Gerontol. 2009;44:208–18.

53 Applegate LA, Weber D, Simon J-P, Scaletta C, de Buys Roessingh A, Raffoul W. Organ donation and whole-cell bioprocessing in the swiss fetal progenitor cell transplantation program. In: Organ donation and Organ donors: Issues, challenges and perspectives. Nova Science Publishers, Ed. Saidi RF. pp 125–147, 2013.

54 Pioletti DP, Montjovent MO, Zambelli PY, Applegate L. Bone tissue engineering using foetal cell therapy. Swiss Med Wkly. 2006;136(35–36):557–60.

55 Darwiche S, Scaletta C, Raffoul W, Pioletti DP, Applegate LA. Epiphyseal Chondroprogenitors Provide a Stable Cell Source for Cartilage Cell Therapy. Cell Medicine. 2012;4:23–32.

56 Tenorio DM, Scaletta C, Jaccoud S, Hirt-Burri N, Pioletti DP, Jaques B, Applegate LA. Human fetal bone cells in delivery systems for bone engineering. J Tissue Eng Regen Med. 2011;5(10):806–14.

Figures (large format)

Figure 1

Tendon structure, histology and cell culture.
Ultrastructure of tendon showing longitudinal fibres and histological sections stained with haematoxylin and eosin depicting extracellular matrix colored in pink (~80% composition) and cellular components mainly with tenocytes stained in blue (~20% composition)(horse tendon for illustration). Primary cultures from tissue can be developed by culturing cells in minimal medium (DMEM + 10% FBS + 1% glutamine) for approximately 1 week in a monolayer culture to provide "fibroblast-like" pure tenocyte populations (human progenitor tenocytes).

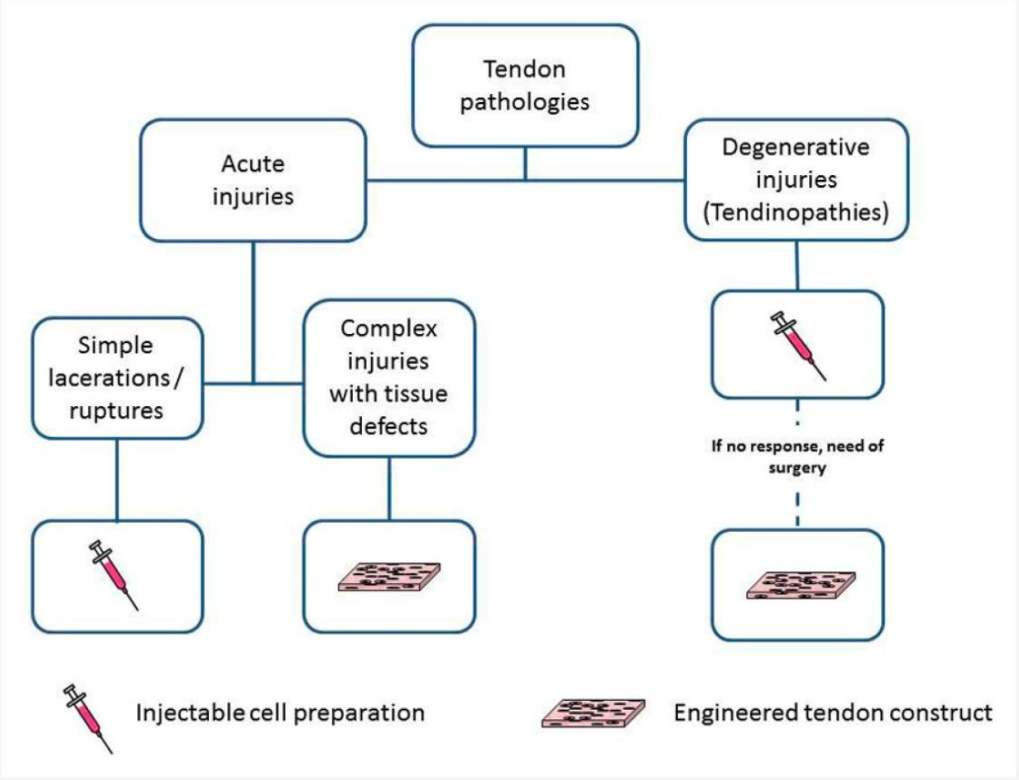

Figure 2

Tendon pathologies and therapeutic strategy.
Tendon pathologies can be either acute or degenerative. Degenerative therapies could be treated with biological cellular therapies with injectable forms to delay degenerative responses and more invasive surgery. Acute injuries, depending on their severity, could be treated with either non-invasive to invasive technique with cells in viscous biogels to complex neo-tissue constructs.

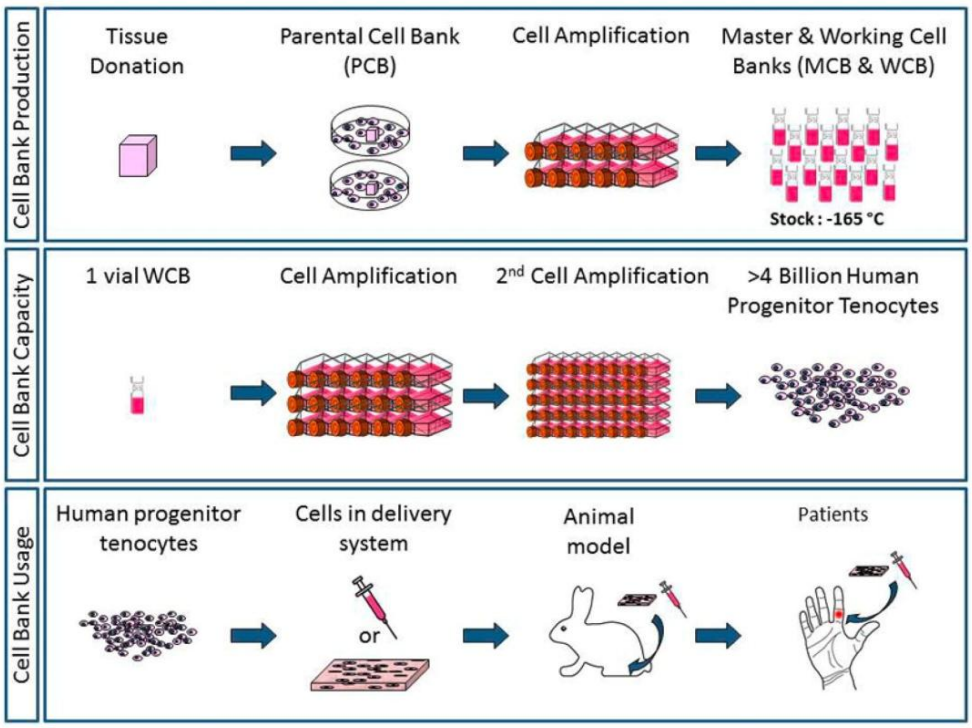

Figure 3

Progenitor human tenocyte cell bank production, capacity and usage for off-the-shelf therapeutic options for tendinopathies.
From one single tendon tissue (2 mm^3, organ donation of foetal Achilles tendon tissue at 14 weeks gestation), clinical cell banks can be produced including parental primary cultures, Master and Working Cell Banks that contain 200 vials each of progenitor tenocytes at low passages (P. 3–6). Expansion of cells produces high quantities of cells that can be stocked in vapor phase of liquid nitrogen (–165 °C) providing cell stocks for hundreds of thousands of patients. Cells can be administered in hydrogels or on other delivery systems for testing in animal models and pre-clinical patient treatments in the process for therapeutic agent development.

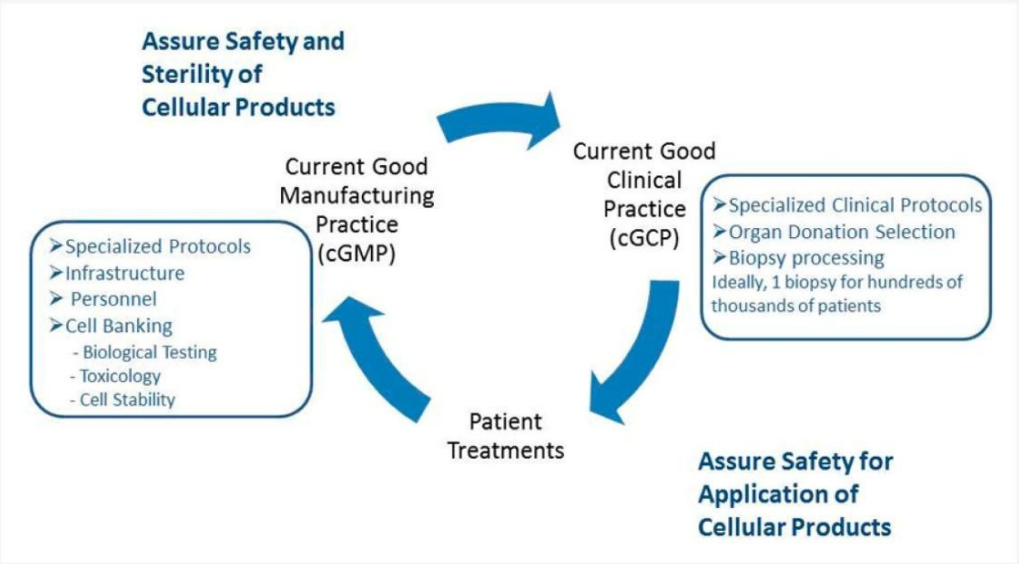

Figure 4

Good Clinical Practice and Good Manufacturing Practice associated with cellular therapeutics.
Current Good Clinical Practices (cGCP) are necessary to begin the process of cellular therapies and tissue engineering with detailed protocols for organ donation and biopsy processing. Tissue and cellular handling then needs to abide by Current Good Manufacturing Processes (cGMP) which include specific protocols for infrastructure, equipment and personnel. cGCP is then needed for appropriate administration of cellular therapies. Thus, the entire process requires high collaboration between biologists, technicians, nurses and surgeons.

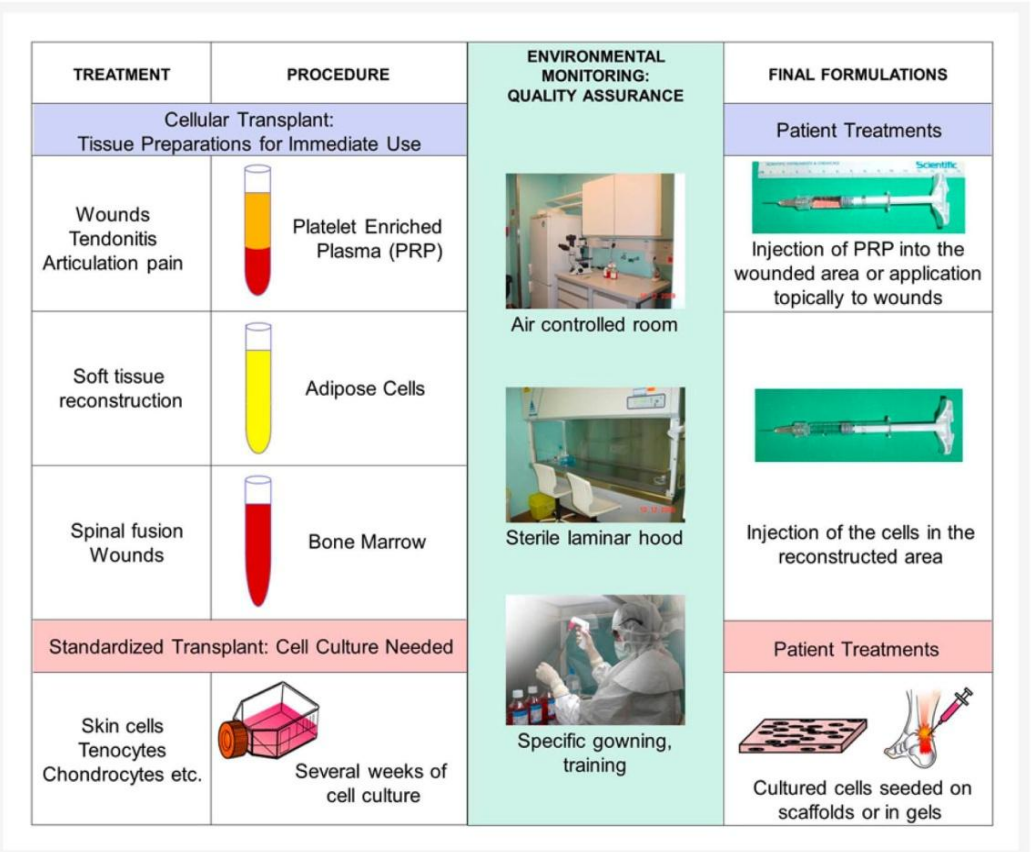

Figure 5

Regulatory requirements for cellular therapy products.

Regulatory requirements have become more extensive for cellular therapies and tend towards conservative drug development in their production, pre-clinical testing and clinical trials. Blood products also have to be produced under cGMP and only in institutions with a license for manipulation. Organs, cells and tissues (PRP, adipose, bone marrow, skin, cartilage, tendon etc.) need to be processed with environmental monitoring and full quality assurance with cGMP processing through to the final formulation and delivery to the patient.

CHAPTER III

–

HUMAN FETAL PROGENITOR TENOCYTE CHARACTERIZATION FOR REGENERATIVE MEDICINE

Foreword

The chapter III presents a summary of the results obtained in vitro with human Fetal Progenitor Tenocytes (hFPTs) and was accepted as a research article for publication in Cell Transplantation. The subject for proposed use of the cells and creation of the cell bank are broached, as well as the conservation and recovery abilities necessary for clinical cell banking. The cell growth and morphology are described for 2D and 3D culture, with identification of some important markers. Different assays to evaluate the homogeneity and stability of the cells are presented. The effect on adult tenocytes is evaluated through a coculture assay and preliminary delivery systems are assessed.

Human Fetal Progenitor Tenocytes for Regenerative Medicine

Accepted 23/06/2015 for publication in Cell Transplantation

Available online 24/06/2015 as early Epub: http://dx.doi.org/10.3727/096368915X688515

Reprinted in this thesis with the kind permission of Cognizant Communication Corporation

A. Grognuz[1], C. Scaletta[1], A. Farron[2], W. Raffoul[1] and L.A. Applegate[1]*

1) Unit of Regenerative Therapy, Service of Plastic, Reconstructive and Hand Surgery, Department of Musculoskeletal Medicine, University Hospital of Lausanne, Switzerland

2) Service of Orthopaedics and Traumatology, Department of Musculoskeletal Medicine, University Hospital of Lausanne, Switzerland.

***Address for Correspondence:**
Professor Lee Ann Laurent-Applegate
University Hospital of Lausanne (CHUV)
Service of Plastic, Reconstructive and Hand Surgery
Unit of Regenerative Therapy (UTR)
EPCR/Croisettes 22
1066 Epalinges
SWITZERLAND
E-mail: Lee.Laurent-Applegate@chuv.ch
Telephone: + 41 21 314 35 10
Fax: + 41 21 887 84 14

Abstract

Tendon injuries are very frequent and affect a wide and heterogeneous population. Unfortunately, the healing process is long with outcomes that are not often satisfactory due to fibrotic tissue appearance, which leads to scar and adhesion development. Tissue engineering and cell therapies emerge as interesting alternatives to classical treatments. In this study, we evaluated human fetal progenitor tenocytes (hFPTs) as a potential cell source for treatment of tendon afflictions, as fetal cells are known to promote healing in a scarless regenerative process. hFPTs presented a rapid and stable growth up to passage 9, allowing to create a large cell bank for off-the-shelf availability. hFPTs showed a strong tenogenic phenotype with an excellent stability, even when placed in conditions normally inducing cells to differentiate. The karyotype also indicated a good stability up to passage 12, which is far beyond that necessary for clinical application (passage 6). When placed in coculture, hFPTs had the capacity to stimulate human adult tenocytes (hATs), which are responsible for the deposition of a new extracellular matrix during tendon healing. Finally, it was possible to distribute cells in both porous or gel scaffolds with an excellent survival, thus permitting a large variety of applications (from simple injections to grafts acting as filling material). All of these results are encouraging in the development of an off-the-shelf cell source capable of stimulating tendon regeneration for the treatment of tendon injuries.

Key words: Cell banking; Fetal cell therapy; Tendon healing; Tendon injuries; Tenocytes.

Introduction

Tendons have the role to transmit forces from muscle to bone and protect the surrounding tissues by working as buffers against tension and compression. Unfortunately, tendon injuries are very common and affect wide and heterogeneous populations, which leads to potential loss of activity due to decreased mobility and chronic pain. Tendon injuries can be divided into two main categories depending on their acute or chronic nature; acute afflictions usually include traumatic partial or total tears or avulsions, while chronic afflictions are due to an overuse degenerative process presently referred to as tendinopathies (1). Although there is a wide range of tendinopathies, the majority are due to tendinosis (2). In this case, the histopathological observations of overused tendons generally present a disorganized extracellular matrix with separation of collagen fibers, decrease in fiber diameter and in density, but increase in type III collagen and in glycosaminoglycans. Simultaneously, hypercellularity and neovascularization are detected, but inflammation and granulation tissues are not common and are normally only seen in tendon ruptures. Subcategories of tendinosis showing lipoid degeneration or calcifications between collagen fibers can also be found (1, 3, 4). However, the separation between acute and degenerative tendon injuries is not always clear as spontaneous ruptures appear almost always from degenerative states, and thus, tendinopathies can be considered as a predisposition to tendon ruptures (5, 6). In many countries, with the democratization of sport practice, the number of overuse injuries has augmented during the last decades, and frequent sport-related injuries to tendons include the Achilles, the patellar, the tractus iliotibialis, the biceps femoris (Hamstring syndrome), the rotator cuff, the common wrist extensors (tennis elbow) and the common wrist flexors (golfer's elbow) (7, 8). From a large joint clinical database, it has been shown that for 100.4 million injuries of any kind reported in 2006/2007 in the US, about 61.2 million are musculoskeletal, and among them, 44% are due to sprains and strains of soft tissue including ligaments or tendons (9). Taking results globally in the US, surgical interventions to repair tendons were estimated at 51,000 per year for rotator cuff tendons, 44,000 for Achilles tendon, and 42,000 for patellar tendon (10). Acute injuries to the hand tendon system also represent numerous cases (11). Globally, a variety of tendons are implicated with frequent impact on work or leisure.

The conventional treatments depend on the gravity of the injury, but range from conservative treatments with physiotherapy or infiltration and extend to surgical interventions such as tenotomy, tenodesis, suture, transfer, graft or even prosthetic implants. Controlled mobilization is recommended in parallel because it has been shown that mechanical stimulation leads to fewer adhesions and increased strength (12). Regardless, tendon healing is always long, and this process can be divided in three overlapping steps. At first the inflammatory phase starts immediately following injury with the synthesis of chemoattractive substances by erythrocytes and inflammatory cells to attract and stimulate tenocytes. After a few days, the second step consists in the production of matrix mostly composed of type III collagen and under the control of tenocytes and macrophages. The last step takes place after 1 or 2 months; extracellular matrix proteins are still produced, but with an increase in type I collagen deposition and the

reorganization and optimization of the fiber alignment. With time, the cell density and activity diminishes and the overall procedure can take as long as one year or more. Unfortunately, even if long, the healing process is not perfect, and the natural structure is never attained again. Instead, scar tissue and adhesions remain and can lead to decreased mobility and rerupture (1, 12, 13).

Along with diminished healing outcomes, another issue is the potential lack of material for tissue replacement. For example, in the case of hand tendon injuries, a graft is frequently needed. The harvest of the palmaris longus or plantaris tendon is the prime choice as other graft sites potentially lead to disability. Nevertheless, these two sources are vestigial muscles, and thus are sometimes absent in individuals (14, 15) and even if present are often not adequate (15, 16).

To counter the poor healing outcomes and the potential lack of material, new strategies must be explored and tested. The field of regenerative therapy has encountered much interest lately. Off-the-shelf methods of cellular therapies able to stimulate tendon regeneration while preventing scar tissue maintenance would be of particular interest. It has been well documented in animals and humans that the early to midgestation fetuses (<24-week gestation for human) heal wounds through a mechanism that differs from adult tissue and is more rapid and leads to a regenerated tissue without scar (17), which is intrinsic to the fetal tissue itself and not to the fetal environment (18-20). It was shown in fetal animal models that bone (21), articular cartilage (22) and tendon (23) have the same ability as the skin to heal without scar. A recent study highlighted the change in expression of multiple genes during tendon development, which could explain the different type of healing (24). In the same manner, the use of cells harvested from fetal tissue has proven to be positive in wound healing. Two clinical trials including pediatric patients suffering from burns or wounds obtained excellent functional and esthetical results with the use of biological bandages composed of fetal skin progenitor cells. The damaged sites stimulated by fetal fibroblasts healed so well that the autografts initially intended were finally not necessary (25, 26). In another study on adults suffering from refractory venous ulcers, the use of fetal skin progenitor cells also improved the healing (27). Along with their ability to stimulate healing, fetal cells present other interesting characteristics. They are already tissue specific and thus have innate good phenotypic stability. Moreover, with their high proliferation capacity, it is notably possible to create large cell stocks (cell banks) showing a high level of consistency and safety with respect to stringent requirements for clinical practice. Indeed, as the cell bank can be based on only one donor tissue, it is possible to trace all levels of the process from tissue to cells to final preparations including extensive screening for infectious diseases (28, 29).

Therefore, fetal cells with their off-the-shelf availability and their capacity to stimulate healing in a scarless manner are a very attractive source for tissue regeneration. In this study, we evaluate the characteristics of human fetal progenitor tenocytes (hFPTs) that have undertaken clinical processing as these cells could be a potential safe and stable source of therapeutic cells available for tissue engineering and for treatment of tendon injuries.

Materials and Methods

Tissue Culture and Cell Banking

Human Fetal Progenitor Tenocyte Harvesting and Cell Banking

Human fetal progenitor tenocytes were isolated from the Achilles tendon of a male 14-week gestation organ donation according to a protocol approved by an ethics committee [University Hospital of Lausanne (CHUV), Ethics Committee Protocol No. 62/07: 14-week gestation organ donation, registered under the Federal Transplantation Program and its Biobank complying with the laws and regulations]. The procedure was already explained in detail for other tissues (28, 30). Briefly, the tendon biopsy of approximately 1 mm^3 was dissected by a fetal pathologist and placed in phosphate-buffered saline (PBS: NaCl 6.8 g/l, Na_2HPO_4 1.5 g/l, KH_2PO_4 0.4 g/l, Bichsel, Interlaken, Switzerland, code No. 100 0 324). Within several hours, the biopsy was transported to the certified human tissue laboratory for cell culture for clinical use. The biopsy was washed three times for 15 min in PBS with 1% penicillin-streptomycin (Pen-Strep: Gibco, Life Technologies Ltd., Paisley, UK, code No. 15140-122). The tissue was dissected into smaller fragments, placed into culture dishes of 10-cm diameter (TPP, Trasadingen, Switzerland), and cultured in clinical growth medium composed of Dulbecco's modified Eagle medium (DMEM: Gibco, code No. 41966-029) containing 25 mM dextrose, 1 mM sodium pyruvate, supplemented to 5.97 mM L-glutamine (L-glut: Gibco, code No. 25030-024) and with 10% clinical-grade fetal bovine serum (FBS: Invitrogen, Life Technologies Ltd, code No. 10101145), without antibiotic supplementation. The medium was changed every 3 to 4 days and first cell growth emitting from tissue was seen already at 24 h. These cells (passage 0) were grown to 50% confluence, detached with trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA: 0.05% trypsin-0.02% EDTA, Gibco, code No. 25300-054), distributed into 75-cm^2 flasks (TPP) at passage 1, and they were used to develop a Parental Cell Bank 11 days later when they reached full confluence. A total of 50 cryo-vials, each with 10 million cells in 1mL cryo-solution [50% DMEM, 40% FBS, 10% dimethyl sulphoxide (DMSO: Sigma, St.Louis, MO, USA, code No. D2438)], were frozen at a cooling rate of 1°C/min within a Nalgene cryo freezing container placed at -80°C. After 24 hours, cells were transferred to the vapor phase of liquid nitrogen (-165°C) for long-term storage. The developed process has provided a unique cell population with the associated cell deposits for patent application (WO 2013/008174 A1: European publication No. 2732030: European Collection of Cell Cultures, ECACC deposit reference 12070302).

Human Adult Tenocyte (hAT) Harvesting and Cell Banking

Adult tenocytes can be harvested from pieces of tendon resected during an operation. As Achilles tendon is generally not resected, it was decided to work with another anatomical source more readily available. Healthy abductor pollicis longus tendon of a 69-year-old male donor was obtained in the CHUV in accordance with the institutional policy on tissue donation and under the Department Biobank regulations (CHUV, Regulations for Biobank for Musculoskeletal Medicine Department No. 3-12-2012). Tissue was placed in PBS and directly transferred to the

laboratory. The tissue fragment was rinsed three times for 15 min in PBS with 5% Pen-Strep. It was then dissected into smaller fragments and transferred into dishes of 6-cm diameter with growth medium (further called standard growth medium) composed of DMEM containing 25 mM dextrose, 1 mM sodium pyruvate, 5.97 mM L-glut, and 10% laboratory-grade FBS (Sigma, code No. F7524), without antibiotic supplementation. The cells could be seen to migrate from the tissue after several days (5−7), and the medium was changed every 3 to 4 days. At confluency, cells were detached with trypsin-EDTA and grown in 75-cm^2 flasks up to passage 2 to develop a standard cell stock. Cells were trypsinized and distributed into 24 vials, each with 1 million cells in 1mL cryo-solution (50% DMEM, 40% FBS, 10% DMSO) and frozen in the same manner as for hFPTs (lower cell concentration to obtain sufficient number of vials). A parental adult cell bank composed of 24 million cells in passage 2 was therefore created.

Cell Growth and Morphology: 2D and 3D Conditions

Both human fetal progenitor and adult tenocytes were grown under the same conditions for 2D culture. Cells were expanded into tissue culture polystyrene flasks of 75-cm^2 with filter screw cap (TPP) placed in cell culture incubators at 37°C in a humidified atmosphere containing 5% CO_2. The standard growth medium was composed of DMEM with 25 mM dextrose, 1 mM sodium pyruvate, 5.97 mM L-glutamine, and 10% laboratory-grade FBS (Sigma, code No. F7524), free of antibiotic supplementation. It was changed every 3 to 4 days, and cells were trypsinized when reaching confluence. Seeding densities of 3,000 and 6,000 cells/cm^2 were tested, but as higher seeding densities did not accelerate the overall growth, cells were then seeded at the low density of 3,000 cells/cm^2. Cell growth of hFPTs in 2D standard culture conditions was analyzed between passage 4 and 15. The morphology was controlled and recorded every 3 to 4 days by imaging (CKX41 and E-330; Olympus, Tokyo, Japan). After 13−15 days of growth, cells of at least three flasks were trypsinized, pooled together, and counted. The series were repeated multiple times, and each passage was evaluated at least four times. Mean population doublings (PD) and doubling time (DT) were determined for each passage using the formulas:

$$PD = \frac{\ln\left(\frac{final\ cell\ number}{initial\ cell\ number}\right)}{\ln(2)} \quad (1)$$

$$DT = \frac{Time\ [d]}{PD} \quad (2)$$

The linearity of the lengthening in doubling time for late passages was evaluated statistically by determining the regression line with the root mean square error model and calculating the coefficient of determination (R^2) in excel software (Microsoft, Redmond, WA, USA).

hFPTs were also seeded in six wells at passages 4 and 9 and the same formulas were used to determine the shortest doubling time during the most rapid growth period occurring between day 1 or 2 and day 7 of culture (each time point in triplicate and experiments repeated three times).

For some experiments, the cells were also cultured as microtissue pellets with a 3D configuration. They were produced by centrifuging 500,000 cells for 10 min at 250 x g and then placing

individual tubes in cell culture incubators at 37°C in a humidified atmosphere containing 5% CO_2 with the cap unscrewed half a rotation to allow gas circulation. Standard growth medium was changed every 3 to 4 days. Some pellets were also produced with a supplementation of 284 µM L-ascorbic acid (Sigma, code No. A4544) to evaluate the impact on protein deposition.

Cell Conservation, Viability and Recovery

The cells were preserved in a standard cryo-solution (50% DMEM, 40% FBS, and 10% DMSO) for long-term conservation. The percentage of living cells at thawing was based on cell counting with a hemocytometer and the Trypan blue exclusion technique (Sigma, code No. T-8154). The cells were then plated in 75-cm^2 tissue culture flasks and counted when cells reached confluency (after 13 to 15 days in culture with a seeding density of 3,000 cells/cm^2). The results were compared to cells seeded at the same passage from trypsinization instead of thawing.

Tendon Marker Profiling

hFPTs and hATs were both evaluated in 2D culture to compare their morphology and the expression of characteristic markers. The cells were plated at 3,000 cells/cm^2 in 48-well plates and incubated under standard culture conditions for 72 h. Immunohistochemical stainings were then realized as follows: Permeabilization of membranes was made during treatment for 10 min in 0.1% Triton X-100 (Applichem, Darmstadt, Germany, code No. A1388). The slides were washed three times in PBS and blocked for 30 min in bovine serum albumin solution 1% (BSA: Sigma, code No. A7906), before incubation with primary antibody at 4°C overnight. Primary antibodies were diluted in BSA 1% and targeted type I collagen (1:1,000; Abcam, Cambridge, UK, code No. ab292), scleraxis (1:100; Abcam, code No. ab58655) and tenomodulin (N-14) (1:100; Santa Cruz Biotechnology, Heidelberg, Germany, code No. sc-49325). After three washes in PBS, the slides were incubated with their complementary secondary antibody for 40 min at room temperature, namely Alexa Fluor® 488 donkey anti-goat IgG (1:800 in PBS; Molecular Probes, Life Technologies Ltd, Paisley, UK code No. A-11055) or Alexa Fluor® 568 donkey anti-rabbit IgG (1:1,000 in PBS; Molecular Probe, code No. A-10042). The nuclei were then counterstained with 4,6-diamidino-2-phenylindole (DAPI: Roche, Rotkreuz, Switzerland, code No. 10236276001) and images were taken with an inverted microscope equipped for fluorescence (Olympus IX81) and a digital camera (Andor iXon). Control stainings were performed with the secondary antibodies only (primary antibodies replaced by 1% BSA) and were used as negative to adjust display of fluorescent signals within ImageJ software (NIH, Bethesda, MD, USA).

hFPTs and hATs were also grown in 3D pellet culture to evaluate their ability to produce tendon matrix. After 14 days, pellets were fixed in 4% (w/v) neutralized formalin solution (J.T. Baker, Deventer, The Netherlands, code No. 3933) for 24 h at 4°C, washed three times with PBS, and subsequently dehydrated and embedded in paraffin (Merck Millipore, Darmstadt, Germany, code No. 111609). The sections of 3 µm were then rehydrated and washed with PBS prior to staining.

Type I collagen, tenomodulin, and scleraxis immunohistochemical stainings were realized with the same method as described above for monolayer cells, but with an additional step consisting of antigen retrieval for 10 min in heated Tris (hydroxymethyl)aminomethane (Tris: 10 mM; Sigma, code No. T-6066)-EDTA (1 mM; Sigma, code No. 03680) at pH 9.0, before blocking the section. Instead of addition of DAPI, the slides with 3D pellet culture were then mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA, code No. H-1200). Control staining and treatment of images were done as explained above for 2D monolayer cells.

Cell Population Homogeneity and Stability: Flow Cytometry and Karyotyping

Population homogeneity and stability were evaluated at passages 3, 6, and 9 by flow cytometry. Cells were tagged with ethidium monoazide (EMA: 1 μg/ml, Molecular Probes, code No. E1374) to exclude the cells with damaged membranes. Primary monoclonal mouse antibodies targeting fibroblast/epithelial marker D7-FIB (1:50 in PBS; Abd Serotec, Dusseldorf, Germany, code No. MCA1399GT, clone D7-FIB), macrophage/neutrophil marker CD14 (1:10; Abd Serotec, code No. MCA2185T, clone MEM-18), hematopoietic and endothelial cell marker CD34 (1:50; Abd Serotec, code No. MCA547GT, clone QBEND/10), cell adhesion marker CD90 (1:10; Abd Serotec, code No. MCA90, clone F15-42-1) and the coreceptor of the transforming growth factor-β (TGF-β) superfamily CD105 (1:50; Abd Serotec, code No. MCA1557, clone SN6) were used. A secondary antibody, Alexa Fluor® 488 goat anti-mouse IgG (1:1,000 in PBS; Molecular Probes, code No. A-11001), was used in combination. Conjugated monoclonal antibodies were also used to tag human leukocyte antigens (HLA), which are implicated in transplant rejection. HLA-DR, DP, DQ antibody was coupled with fluorescein isothyocyanate (FITC) and HLA-A, B, C antibody with phycoerythrin (PE) (1:1; BD Pharmingen, San Diego, CA, USA, code No. 555558, clone Tu39 and 555553, clone G46-2.6). The non-immunogenic HLA-G protein was also tested with a primary monoclonal mouse antibody (1:10; Abcam, code No. ab52455, clone 4H84) and with the same secondary antibody. Negative controls were made with nonspecific antibodies of the same isotypes at equal concentrations (Abd Serotec, code No. MCA928EL, R&D Systems, Minneapolis, MN, USA, code No. IC002P and BD Pharmingen, code No. 555573). Data were obtained with a CyAN™ADP analyzer (Beckman Coulter, Indianapolis, IN, USA) and processed with FlowJo software (Ashland, OR, USA).

A conventional karyotype was realized in order to observe potential numerical or structural abnormalities and to identify low-degree mosaic conditions of the hFPTs. For these analyses, dividing cells were exposed for 1.5 h to colchicine (Sigma, code No. 27650) at a final concentration of 0.16 μg/ml to block cell division in metaphase. The cells were then exposed to a hypotonic shock with 37.5 mM KCl (Sigma, code No. 60121) for 20 min, fixed with 25% acetic acid (Sigma, code No. 320099) and 75% methanol (Sigma, code No. 179337) and spread onto a glass microscope slide. The metaphases were exposed for 10–20 s to trypsin (1% in EDTA), washed with 150 mM NaCl, and dried at room temperature. The slides were then stained with

Giemsa solution (2% in PBS; Merck Millipore, code No. 109204) for 8 min for visualization of the chromosomal G-band pattern. Karyotyping of hFPTs was accomplished with the observation of 60 metaphases at passages 3, 6, and 12. Forty supplementary metaphases were observed for chromosome 19 at passage 3.

Phenotypic Stability with Osteogenic- or Adipogenic-Inducing Conditions

hFPTs were plated at 3,000 cells per cm^2 in 12-well plates, alongside with human adipose-derived stem cells (ASCs) and human bone marrow-derived mesenchymal stem cells (BM-MSCs), both obtained in the CHUV in accordance with the institutional policy on tissue donation and under the Department Biobank regulations (CHUV, Ethics protocol No. 124/10: "Development of cell banks from bone marrow for the development of treatments and bioengineering" and Regulations for Biobank for Musculoskeletal Medicine Department of the CHUV No. 3-12-2012). The cells were grown for 4 days with standard growth medium. An osteogenic induction was then accomplished over 21 days using a medium composed of α-MEM (Gibco, code No. 22571-020), 10% FBS, 5.97 mM L-glut, 284 µM L-ascorbic acid, 5 mM β-Glycerophosphate (Sigma, code No. G9422) and 100 nM dexamethasone (Sigma, code No. D4902) which was changed every 3 to 4 days. Cells were then rinsed with deionized water and fixed in 4% formalin solution for 10 min at room temperature before Von Kossa staining (Sigma, code No. 85228, S-4019 and S-1648) or Alizarin red (pH 9) staining (Sigma, code No. A5533) to observe the production of mineralized matrix, with the first showing phosphate deposition and the second calcium deposition (31). An adipogenic induction was realized over 21 days using a medium composed of DMEM, 5.97 mM L-glut, 1% insulin-transferrin-selenite (ITS: final concentration: 10 mg/l bovine insulin, 5.5 mg/l human transferrin, and 5 µg/l sodium selenite; Sigma, code No. I3146), 1 µM dexamethasone, 100 µM indomethacin (Sigma, code No. I7378) and 100 µM 3-isobutyl-1-methylxanthine (IBMX: Sigma, code No. I5879). Cells were then rinsed with deionized water, fixed in 4% formalin solution for 10 min at room temperature and stained with Oil red O staining kit (Diapath, Martinengo, Italy, code No. 010303), according to the protocol of the manufacturer, to highlight the presence of neutral lipids.

Biocompatibility in Porous Scaffold and Gel Matrix

For evaluation of cell survival in porous scaffolds, hFPTs were trypsinized at passage 4, and solutions containing 250,000 cells in 500 µl standard growth medium were inoculated onto solid 2.25 cm x 3 cm commercial horse collagen membranes (TissuFleece E; Baxter, Nuremberg, Germany, code No. B2240100999999) and placed in culture dishes of 10-cm diameter. After 1 h of incubation at 37°C and 5% CO_2 to allow cells to attach to the membranes, standard growth medium was added in the dishes, which were then replaced in the incubator. At several time points up to day 14, the membranes were rinsed with PBS and 250 µl of LIVE/DEAD solution (Molecular Probes, code No. L-3224) added over the entire membranes. The membranes were

incubated for 30 min at room temperature, before images were taken with an IX81 Olympus fluorescent microscope and Andor iXon digital camera and treated within ImageJ.

For evaluation of cell survival in hydrogel, hFPTs were trypsinized at passage 4, rinsed with PBS, and resuspended in PBS at 1 million cells/ml (PBS was used because growth medium cannot be removed from the gel). 40 µl was distributed into wells of a 96-well plate, mixed with 60 µl of a commercial 0.8% hyaluronic acid (HA) gel (Sinovial 0.8%; IBSA, Lodi, Italy) with a displacing pipette to homogenize the suspension, and the plate was placed at 4°C overnight. After 24 and 72 h, 100 µl of LIVE/DEAD solution was added to the wells and mixed with the displacing pipette. Images were taken after 30 min of incubation at room temperature and treated within ImageJ.

Growth Stimulating Ability of hFPTs Towards hATs

A coculture assay was realized using hATs in a 12-well plate and γ-irradiated hFPTs in Transwell inserts placed just above the hATs (Costar, Corning Incorporated, Lowell, MA, USA, code No. 3460-COR) (Fig. 1).

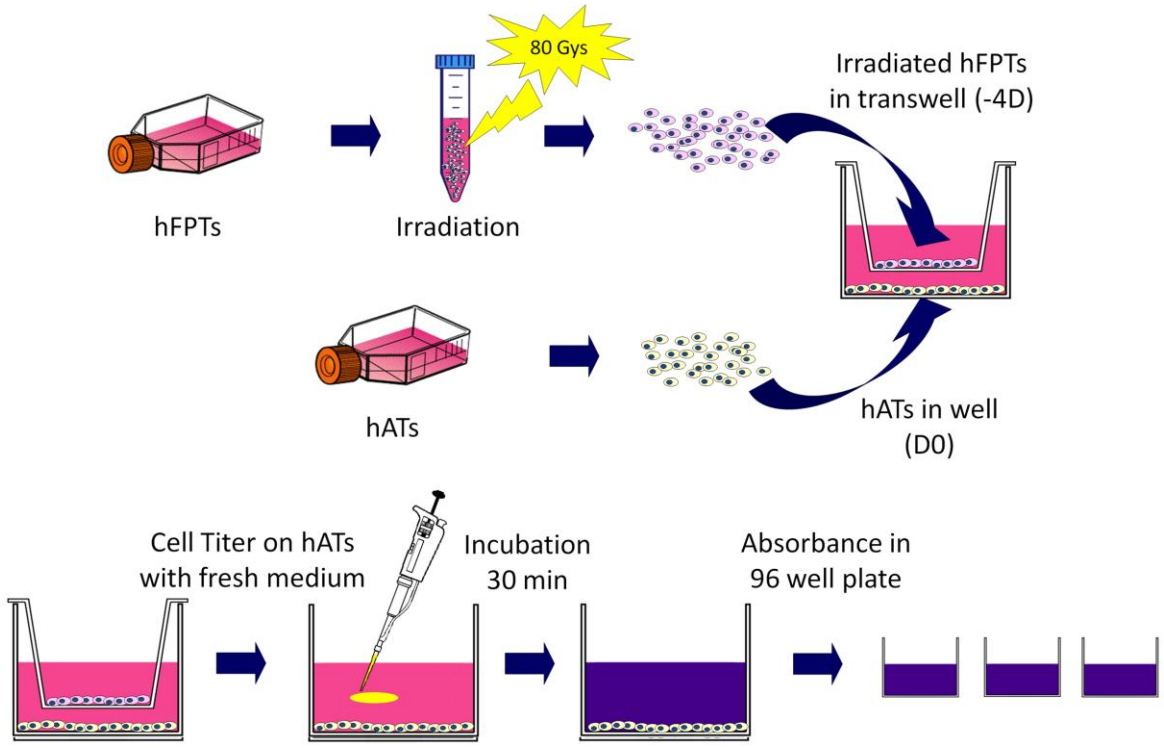

Figure 1: Coculture method for stimulation assay.
Coculture method employed to assess metabolic activity of human adult tenocytes (hATs) when stimulated by human fetal progenitor tenocytes (hFPTs). hFPTs irradiated (to block division) and placed in Transwell inserts 4 days (-4D) before hATs to already condition the medium. hATs seeded in wells on D0 for beginning of growth. Cell Titer assay done on hATs with fresh medium, and supernatant transferred into 96-well plate after reaction for absorbance reading at 492 nm.

The 0.4-µm polyester membrane at the bottom of Transwell allowed liquid diffusion while preventing any cell exchange. On day minus 4 (-4D), hFPTs were irradiated with a dose of 80 Gy (Cesium 137) to inactivate their cellular division capacity in order to maintain a stable amount of cells in Transwells throughout the experiment (parallel test without growth for four time points up to day 25). They were seeded in triplicates to test four conditions corresponding to 0, 10,000, 50,000, and 100,000 irradiated hFPTs per Transwell with a total of 2 ml of standard growth medium between the well and Transwell. On day 0 (D0), the wells were all seeded at a density of 3,000 cells/cm^2 with adult cells. The 4-day delay between the two seeding sessions was done to already have a conditioned medium on day 0. The activity of hATs was measured every 3 to 4 days up to day 17, and at each time point, the Transwells were placed apart in incubator with 1 ml of conditioned medium, which was preserved. The medium remaining in wells was discarded, 500 µl standard growth medium and 20 µl Cell Titer (Promega, Madison, WI, USA, code No. G3580) were added in each well (and in three supplementary empty wells for blanks), and the metabolic reaction took place during 30 min at 37°C. After 30 min, the reaction was stopped by rapidly transferring the supernatants to an empty 12-well plate. The supernatants were then transferred into a 96-well plate, and 10 minutes after the end of reaction, the absorbance was read at 492 nm (Tecan Infinite F50; Mannerdorf, Switzerland), the blank absorbance was subtracted, and the mean corrected absorbance was calculated for the three wells of each condition. The percentage increase in activity was also calculated for each condition in comparison to hATs without stimulation. The hATs were rinsed once with PBS, 1 ml of standard growth medium was added to the wells, and the corresponding Transwells with 1 ml conditioned medium were placed back in the plate and into the incubator until next time point. The whole experiment was performed three times successively, and the results are presented as mean of the three experiments with error bars indicating standard deviation. For each experiment and each condition, the average increase throughout the five time points was calculated, and the global increase per condition was then determined for the three experiments.

Results

Cell Harvesting, Cell Culture and Cell Banking

From one single fetal tendon biopsy, it was possible to isolate hFPTs and to expand them to obtain a clinical cell bank consisting of 50 vials of 10 million cells each at the end of passage 1. Starting from the adult pollicis longus tendon biopsy, it was possible to create a cell bank of 24 vials of 1 million cells each at the end of passage 2. Concerning the morphology, hFPTs maintained very similar spindle-shaped morphology from low passages up to passage 12 with highly aligned configuration when the density increased. In higher passages (passage 15), the cells were slightly larger, and there was weaker cellular alignment when the density increased (Fig. 2A). The mean doubling time of hFPTs during passage showed two different phases. From passage 3 to passage 9, the growth was very steady, and the mean doubling time was always more rapid than 4 days (mean = 3.75 days, SD 0.16 days) with a mean of 3.72 population doubling per

passage (SD 0.18 PD). The minimal doubling time was recorded between day 1 or 2 and day 7 and was also very similar between passage 4 (mean = 1.2 days SD 0.2 days, n = 3) and passage 9 (mean = 1.3 days, SD 0.1 days, n = 3). From passage 10 to passage 15, the proliferation rate slightly decreased linearly (R^2 = 0.94). For each passage, one population doubling took eight additional hours than in the previous passages to be achieved, which represents around 8.5% increase in doubling time (Fig. 2B). In comparison, hATs have already a longer doubling time at passage 4 with a mean of 4.40 days per doubling (SD 0.21 days, n = 4), which then slows down to 5.38 days (SD 0.99 days, n = 3) at passage 6 and 6.54 days (SD 0.21 days, n = 2) at passage 8 (Fig. 2C). It was shown that low seeding densities (3,000 cells/cm^2) could be implemented with hFPTs with results very similar to higher seeding densities (6,000 cells/cm^2) (Fig. 2D). Low seeding densities allowed for a rapid early expansion and development of a very large clinical cell bank.

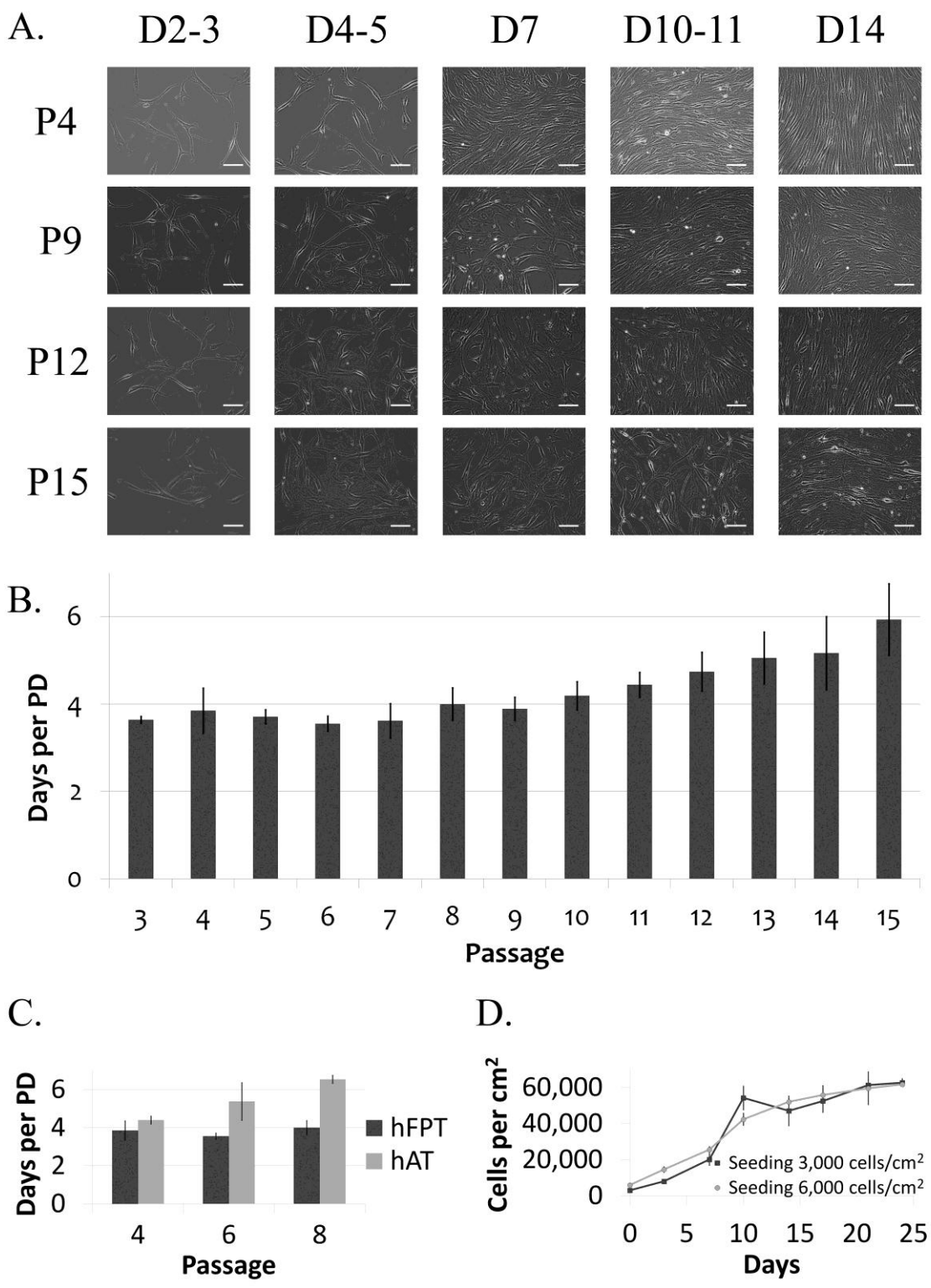

Figure 2: hFPT growth in monolayer culture.

Cellular morphology of human fetal progenitor tenocytes (hFPTs) during cellular growth from low seeding density to confluency during early (P4−9), mid (P12), and late passages (P15) (A). Doubling time of hFPTs accomplished from early passages to late end-of-passage culture. Each passage tested four times with average number of days to allow cell doubling presented with standard deviation (B). Doubling time of hFPTs compared to the one of adult tenocytes (hATs) at similar passages (C). Influence on cell growth depending on low to intermediate seeding densities represented by actual cell counts from 0 to 24 days of culture. Experimentation done in triplicate with average and standard deviation indicated (D). Scale bars: 100 µm.

Cell Conservation, Viability and Recovery

hFPTs conditioned in freezing medium and frozen at minus 80°C in passage 3 for 4 to 10 months presented a survival rate of 97.6% (SD 0.6%, n = 5) after thawing. When seeded in 75-cm^2 flasks at passage 4 for 14 days, they had a mean doubling time of 3.73 days (SD 0.13 days, n = 3) which is very close to what is found for the same passage from enzymatic detachment with 3.62 days (SD 0.42 days, n = 2). hFPTs frozen in the vapor phase of liquid nitrogen for 30 to 36 months presented a survival rate of 93.8% (SD 2.9%, n = 3) and a mean doubling time of 3.43 days (SD 0.21 days, n = 3).

Expression of Surface Markers in 2D and 3D Culture Conditions

When cultured in 2D monolayer culture, fetal progenitor and adult tenocytes presented a very similar spindle-shaped morphology, and both expressed the three different markers. The normally extracellular type I collagen was present in the cytoplasm, but not in the nucleus. The type II transmembrane protein tenomodulin was expressed over the entire surface. The transcription factor scleraxis was detectable as a light staining both within the nucleus and in close proximity (Fig. 3A).

Human progenitor and adult tenocytes had both the ability to form 3D pellets, and in such a configuration, they were able to deposit extracellular matrix during the creation of microtissues. The matrix-to-cell ratio was slightly higher in hFPT pellets with the nucleus appearing more distant with more matrix in between. Interestingly, type I collagen was only detectable in the extracellular matrix surrounding fetal progenitor cells, but not around adult cells. The supplementation of the medium with ascorbic acid allowed to detect type I collagen also with hATs, but still in lower quantity compared to hFPTs. Tenomodulin (extracellular domain) was positive both in progenitor and adult tenocytes. Scleraxis is not an extracellular matrix protein, but was also tested in 3D to evaluate its expression. Although visible in monolayer culture, we were not able to detect the presence of this transcription factor in 3D culture for either fetal progenitor or adult tenocytes (Fig. 3B). The addition of ascorbic acid did not change the expression of tenomodulin or scleraxis (data not shown).

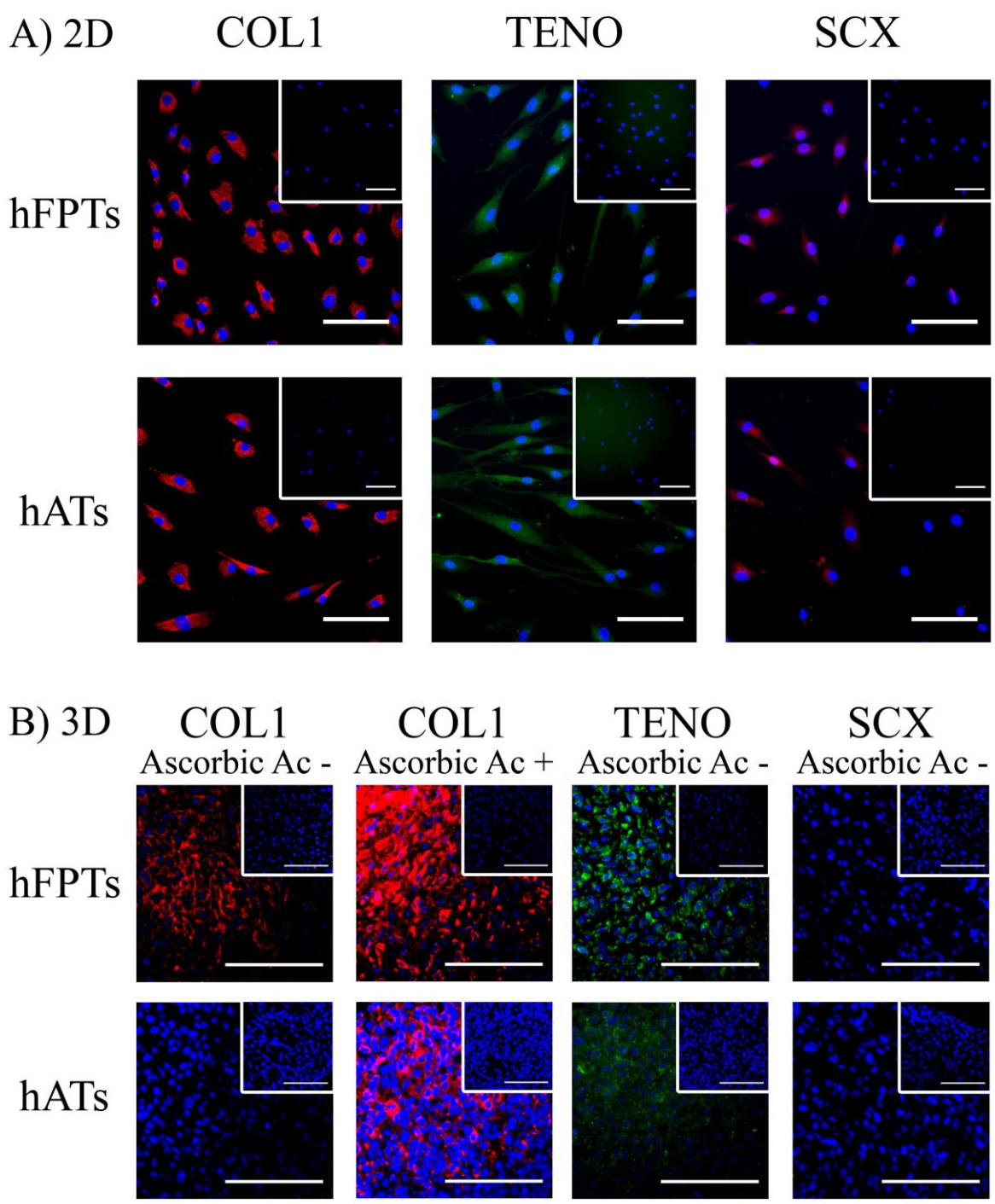

Figure 3: hFPT immunohistochemical staining in 2D and 3D (compared to hAT).
Immunohistochemical staining of human fetal progenitor tenocytes (hFPTs) and adult tenocytes (hATs) for collagen I (COL1), tenomodulin (TENO), and scleraxis (SCX) in 2D monolayer culture (A) and 3D pellet culture with (+) or without (-) ascorbic acid supplementation (B). Negative controls shown in upper right corner of images. Scale bars: 100 μm.

Cell Population Homogeneity and Stability: Flow Cytometry and Karyotyping

The cells showed a homogenous population distribution at all tested passages, and there was no shift in the expression of the observed surface markers. hFPTs stained positive for the fibroblast

marker D7-fib, for the cell adhesion marker CD90, and for the coreceptor of the TGF-β superfamily CD105. There was no discernible contaminating subpopulation enrichment, as seen by the negative macrophage/neutrophil marker CD14 and hematopoietic/endothelial cell marker CD34. As expected, the proteins of MHC class II (HLA- DR, DP, DQ) were not present, while the ones from MHC class I (HLA-A, B, C) were mildly positive. The particular MHC class I protein HLA-G was not present (Fig. 4A).

hFPTs presented a male karyotype as seen by the presence of both X and Y chromosomes, and the quality was very good at all passages tested with a resolution of 500 G bands. At passage 3, on the 60 metaphases tested, 57 were normal, and there was one nonclonal numerical abnormality observed during experimentation. Two metaphases presented structural abnormalities with a possible deletion of terminal short arm of chromosome 19. Therefore an additional 40 supplementary metaphases were tested for chromosome 19 without observation of other deletion. At passage 6, there was no clonal abnormality. Fifty-six metaphases were considered normal, one with numerical abnormality and three with structural abnormalities. At passage 12, the 60 metaphases presented all a normal karyotype. For each passage, the karyotype was always 46, XY. No mosaicism was detected at any passages, and with 60 metaphases tested for each passage, the risk of mosaicism remains smaller than 5% with a 0.95 confidence level as demonstrated by Hook (32) (Fig. 4B).

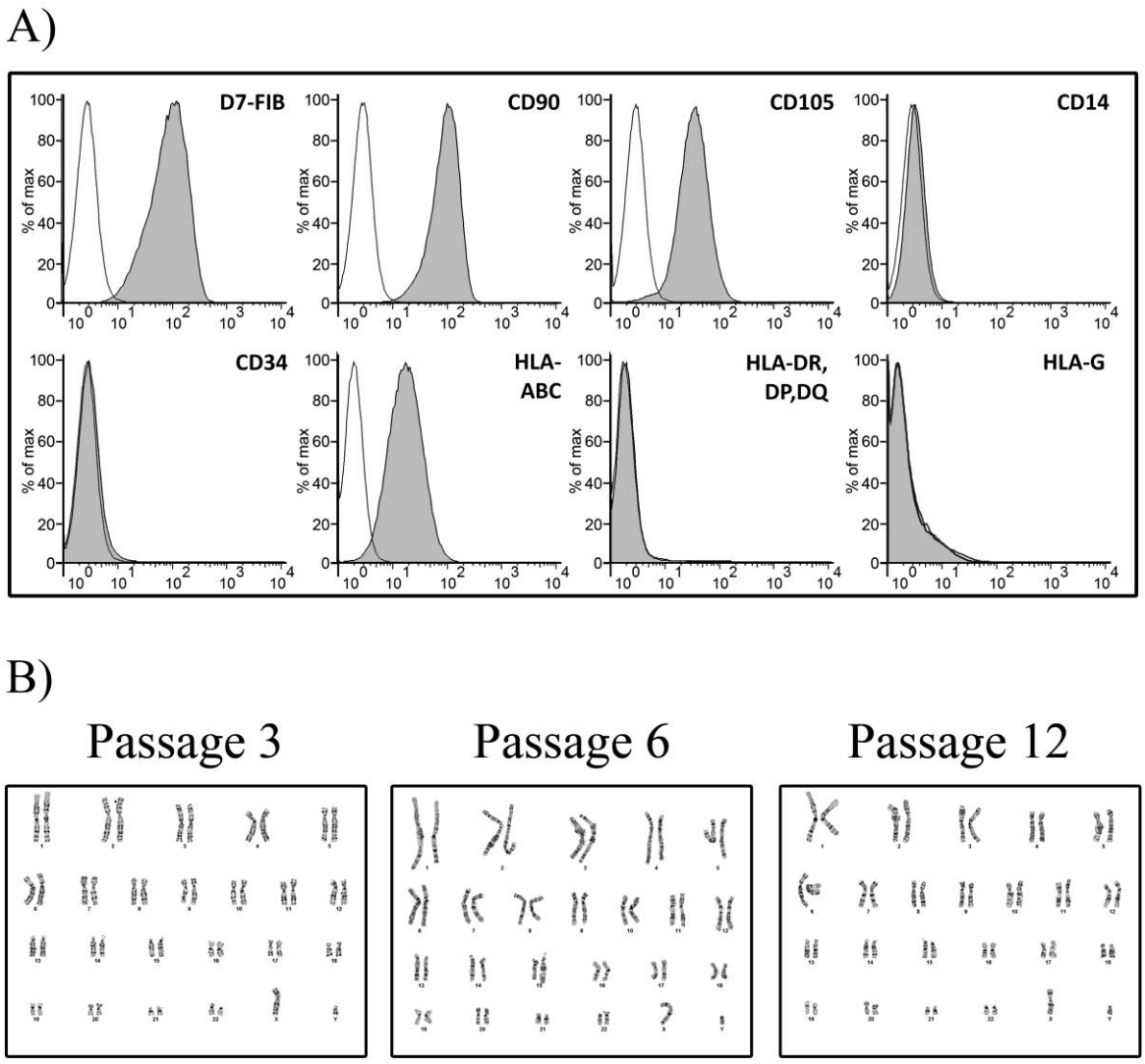

Figure 4: hFPT population homogeneity and genetic stability.

Cell surface proteins tested by flow cytometry for human fetal progenitor tenocytes (hFPTs) at passage 6 (A). Karyotyping of hFPTs at low and high passages with low cell culture seeding protocols (B).

Phenotypic Stability with Osteogenic- or Adipogenic-Inducing Conditions

After 3 weeks of osteogenic induction, hFPTs remained stable, and there was no mineral deposition as shown with the Alizarin red and the Von Kossa staining, which both remained negative. Both of the adult stem cells derived from adipose tissue and bone marrow (ASCs and BM-MSCs) cultured under the same conditions deposited phosphate and calcium matrix as expected. A similar observation was made for adipogenic induction with hFPTs being negative for the Oil red O staining, while ASCs and BM-MSCs presented lipid accumulation (Fig. 5).

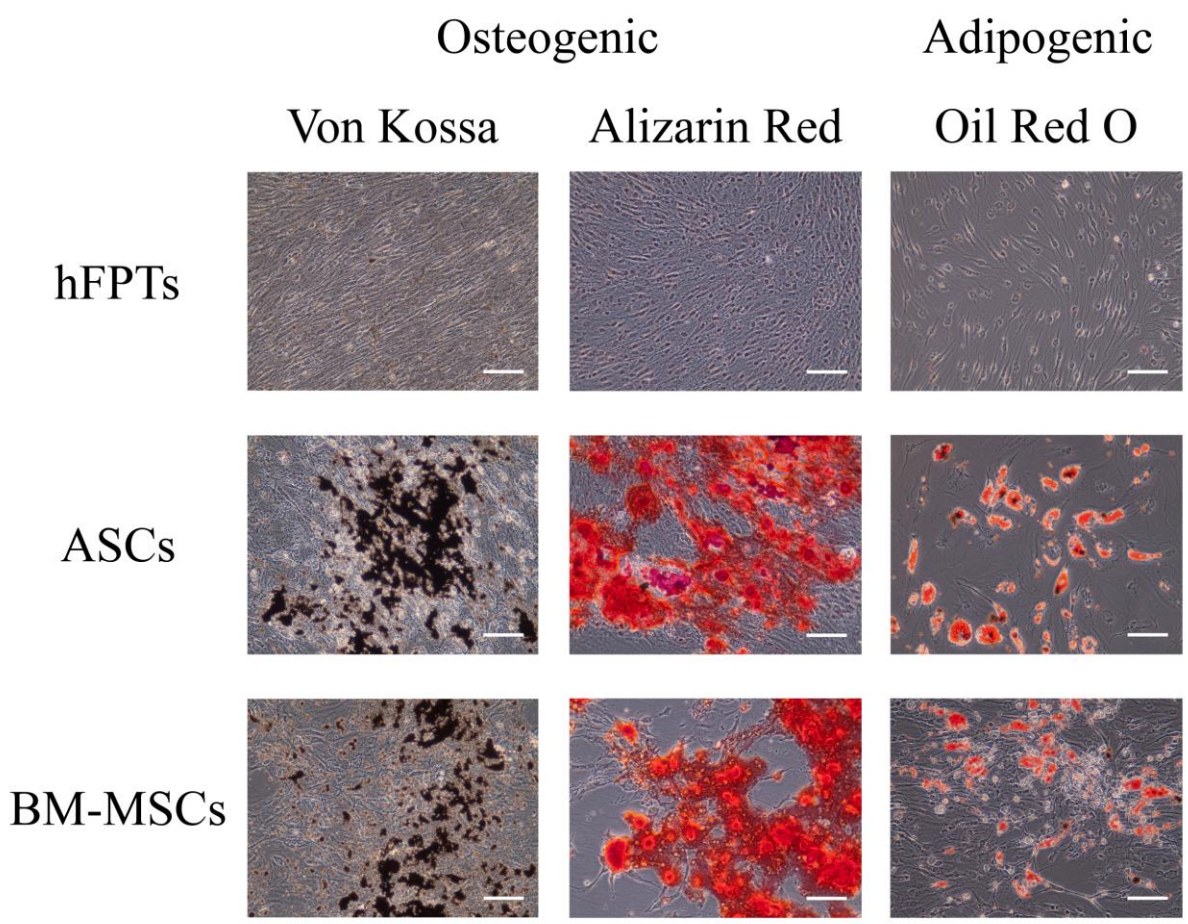

Figure 5: hFPT phenotypic stability with inducing conditions (compared to ASC and BM-MSC).
Osteogenic and adipogenic differentiations of fetal progenitor tenocytes (hFPTs), adipose-derived stem cells (ASCs), and bone marrow-derived mesenchymal stem cells (BM-MSCs) over 21 days of induction. Osteogenic induction yields calcified nodules or lipid accumulation for BM-MSCs and ASCs, which are in brown-black with Von Kossa staining (phosphate deposition) and in red with Alizarin red staining (calcium deposition). Adipogenic induction yields lipid accumulation for BM-MSCs and ASCs, which are in red with Oil red O staining. No matrix deposition or lipid accumulation seen for hFPTs. Scale bars: 100 μm.

Biocompatibility in Porous Scaffold and Gel Matrix

Twenty-four hours after seeding, hFPTs demonstrated a good biocompatibility with commercial horse collagen as indicated using the Live/Dead assay. The cells were homogeneously dispersed, they could attach to the scaffold, and more than 95% were metabolically active (green). Several time points were evaluated, and this viability was still found after 14 days. Once cells were successfully attached to the matrix (1 h), it was possible to rinse the membrane multiple times with PBS with no cell detachment seen. In contrast, cells seeded in PBS were mostly alive, but remained round and could not attach without standard growth medium. hFPTs could be homogeneously dispersed in a 0.8% HA gel by using a displacement pipette. They did not need growth medium and could be directly mixed in PBS or NaCl before being dispersed uniformly throughout a viscous gel matrix. More than 95% of the cells were alive and metabolically active

when conserved for 24 h at 4°C. After 72 h, there was an increase in dead cells, and the overall viability approached 70% (Fig. 6).

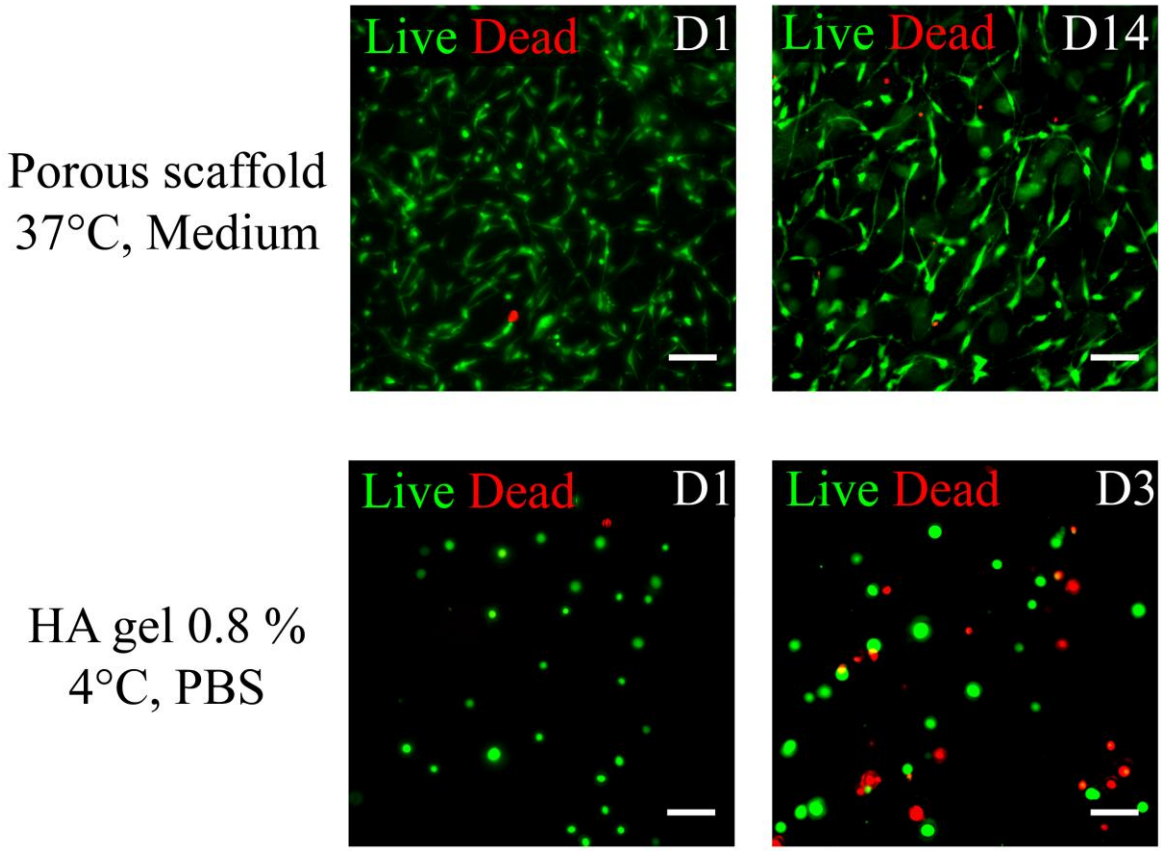

Figure 6: hFPT biocompatibility within delivery systems.
Biocompatibility of human fetal progenitor tenocytes (hFPTs) with porous collagen scaffold and hyaluronic acid gel (0.8%) the day following seeding (D1) and again after 14 days (D14) for porous scaffold and 3 days (D3) for gel. Metabolically active live cells appear in green and damaged dead cells in red. Scale bars: 100 µm.

Stimulating Ability of hFPTs Towards hATs

In vitro cell culture models for tendon regeneration can provide general results on dose response. Such models may lead to crucial preclinical information for critical assessment of potential responses of injury in vivo. The results within the coculture assay showed a dose-dependent stimulation of hFPTs on adult tenocytes. A quantity of 10,000 cells per Transwell seemed insufficient to stimulate adult tenocytes (0% increase, SD 2.3%, n = 3). But with higher amounts of cells, between day 3 and 17, there was a mean increase of 10% (SD 1.3%, n = 3) of the metabolic activity of adult tenocytes with 50,000 hFPTs per Transwell and of 25% (SD 3.8%, n = 3) with 100,000 hFPTs per Transwell, compared to an absence of hFPTs (Fig. 7). The increased global activity means that the number of adult cells was increased or that the metabolic activity per cell was increased, or that both were simultaneous.

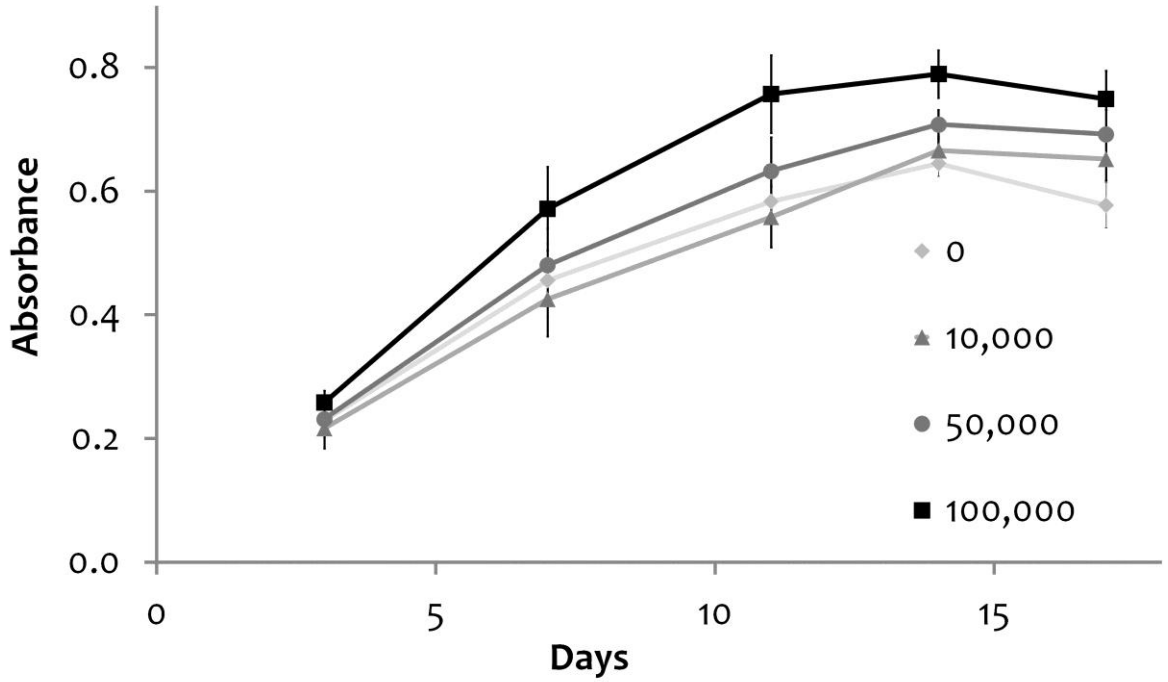

Figure 7: Stimulating ability of hFPTs.
Metabolic activity of human adult tenocytes (hATs) stimulated by various amounts of irradiated human fetal progenitor tenocytes (hFPTs) through a coculture with Transwell inserts. Experimentation done three times and in triplicate with average and standard deviation indicated.

Discussion

In case of tendon injury, the major negative outcomes are the presence of scar tissue and adherence between tendon and its surrounding structures. Fetal tendon heals in a different manner, without fibrotic tissue or adhesion. The differences seen between fetal regenerative healing and adult reparative healing relies probably on different cytokine profiles. The idea to use isolated cytokines is appealing; however, tissue healing is a complex process with multiple interactions between the different cytokines, and there is most likely a defined balance to be found to ameliorate tendon healing (23). Nevertheless, fetal cells, which naturally present such a tuned balance of cytokines and which moreover are supposed to be themselves the effectors of scarless healing (33), could be optimized for a unique cell source for therapeutic use.

To be compatible with a potential clinical use in the future, it is crucial to be in accordance with the laws and regulations. Tissues and cells for clinical use are regulated nationally in most countries worldwide. In the present case, the tissue was obtained as an organ donation, which is registered with Swissmedic (Swiss Agency for Therapeutic Products) in a Federal Transplantation Program and with respect to the Law for Transplantation RS810.21 of Switzerland and its related directives on research with human subjects. Moreover, it was possible to integrate current good manufacturing practice (GMP) for cell processing for the creation of the parental cell bank, which is of primary importance. Banking cells allows the creation of an off-the-shelf stock, which presents a major advantage to have cells available easily and rapidly for their use. In parallel to the

legal aspects, the cells are expected to present features such as good proliferation capacity, good stability, and possibility of long-term storage.

The culture of hFPTs was rapid with a minimal doubling time of approximately 30 h in maximal growth period and a mean doubling time in passage 10 still more rapid than for hAT in passage 4. hFPTs only required very basic culture conditions, without need of specific growth factors, which permits inexpensive and easier growth conditions compared to other cell types such as mesenchymal stem cells (MSCs), which necessitate demanding conditions for upscaling cells in an undifferentiated state or directing them in a specific phenotype. The overall rate of proliferation was very steady up to passage 9, even with low initial seeding densities. This long stability can probably be explained as hFPTs were obtained from a young tissue of 14-week gestation. Even if the cells had slightly slower proliferation at much higher passages, the morphology and karyotype accomplished at passage 12 did not show any abnormalities. A major advantage of progenitor cells is the possibility to expand them to create a large working cell bank and therefore an unlimited off-the-shelf reserve. An average of 3.72 population doublings per passage up to passage 9 means that at each passage, it is possible to increase the number of cells by a factor 13.18 fold (= $2^{3.72}$). If we imagine limiting all clinical work with the cells at passage 6 (two thirds of the observable signs of cell aging), this would lead to an increase by almost 400,000 fold (13.18^{5}) the initial number of cells banked at passage 1. Thus, we can estimate a total of 1.96 x 10^{14} cells at the end of passage 6 if the entire cell bank was expanded. Estimating approximately 1 million hFPTs per treatment protocol, it would be possible to create more than 100 million treatments (Fig. 8). These estimations are hypothetical, but are based on clinical experience with our hospital for treatment of burns and wounds (between 270,000 and 540,000 cells were used per 9 cm x 12 cm scaffold for biologic bandages for wound healing), and it shows the potential of such a cell line when correctly banked for clinical use (25-27).

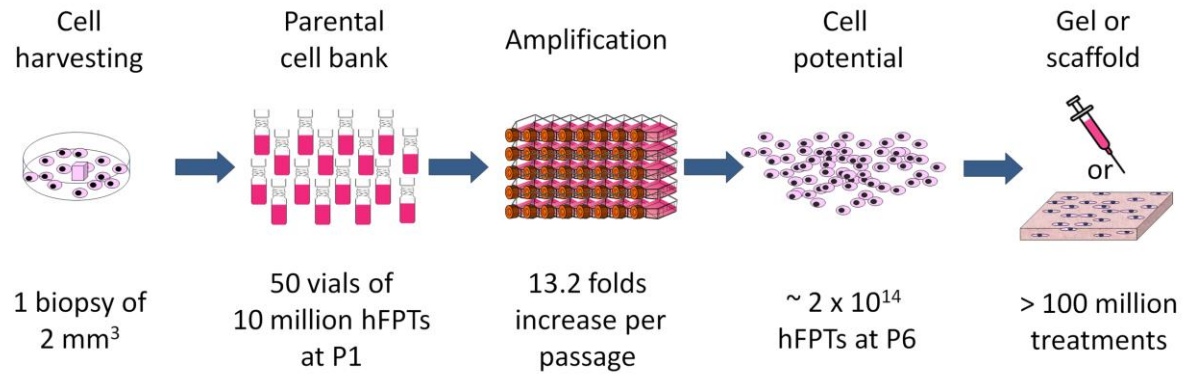

Figure 8: hFPT cell bank processing and capacity.
Starting with one biopsy from fetal Achilles tendon, it was possible to create a cell bank of 50 vials with 10 million human fetal progenitor tenocytes (hFPTs) each. Based on growth capacity of hFPTs, more than 100 million treatments could be potentially created with cells at the end of passage 6.

Our results show that it was possible to conserve the cells at -80°C for short-term conservation (with more than 95% survival over 4 to 10 months) or at -165°C in the vapor phase of liquid

nitrogen for long-term storage (with 90−95% survival over 30 to 36 months). Moreover, there was no delay in the growth of frozen cells as the doubling times to reach confluency were very close to what was found with cells cultured at the same passage from enzymatic detachment. These results were obtained with standard cryo-solution, and the period of conservation is probably much longer, as we have been able to conserve other progenitor cells at -165°C for more than 15 to 20 years with this manner (e.g., skin progenitor cells, unpublished data).

There is still no real specific tendon marker in the literature (34), so it is preferable to detect the presence of a pattern of several proteins which are not specific, but nevertheless characteristic of tendon tissue. It was the case in this study and the tenocyte status was confirmed for hFPTs with the presence of type I collagen, tenomodulin and scleraxis observable in monolayer culture, alongside with a morphology very similar compared to adult tenocytes. Concerning morphology, it is noticeable that fetal tenocytes present a more rounded morphology within tendon in vivo during development and at low ages (35). However, when placed in 2D culture, fetal progenitor tenocytes present a spindle-shaped morphology comparable to the one seen with older cells, even at low passages. Concerning the markers, type I collagen is an extracellular matrix component, and the strong cytoplasmic staining, which was observed, could be explained by the presence of type I procollagen. Type I procollagen shares the same amino acid sequence as type I collagen with the presence of propeptides at both terminal ends. It is synthesized in the cell before being converted to type I collagen by cleavage of propeptide during secretion in the extracellular matrix (36, 37). Interestingly, when cultured as microtissue pellets, there were some differences between fetal progenitor and adult cells. The global deposition of matrix was visually increased with hFPTs compared to hATs, and there was a deposition of type I collagen in hFPT pellets, while it was not possible to detect this protein in 3D culture from hATs cultured under the same conditions. The addition of ascorbic acid (implicated in collagen synthesis) allowed the synthesis of type I collagen in both fetal and adult pellets, but still in lower quantities for hATs than for hFPTs. Fetal and adult tendons are known to be able to secrete different types of matrix, notably in the case of tendon healing. In adult healing, type I collagen peaks only after several weeks to replace the temporary fixative matrix (1), while in fetal healing the regenerated tendon appears rapidly indiscernible from unwounded tendons (23). Thus, it is possible that human progenitor tenocytes secrete preferably type I collagen, while adult cells are more turned towards other proteins. Further investigations would be necessary to compare the differences between the extracellular matrix compositions, which could be linked with the regenerative properties of fetal cells versus the reparative abilities of adult cells, and a recent study in sheep highlighted differences in morphological and molecular changes from fetal development to adult (24). Tenomodulin and scleraxis have both proven to have an implication in tendon development, as mice lacking either one have shown defects in tendon structure (38, 39). In 3D culture, tenomodulin was secreted in both young and old cell lines. On the other hand, it was not possible to detect the presence of scleraxis in 3D culture in either fetal progenitor or in adult cells. Taylor *et al.* found a similar situation with a significantly reduced expression of scleraxis in 3D cell culture (34). They hypothesized that scleraxis is important in the early steps of tendon development, but that once the phenotype is acquired there is no further change in its expression.

The similar expression of scleraxis between normal and diseased tendons supports these assumptions (34).

The surface markers of hFPTs seen with flow cytometry indicated a homogenous cell population. As expected, the overall profile was similar to fibroblastic adherent cells (positive for D7-fib and CD90). Potential contaminating subpopulations like hematopoietic or endothelial cells were not found (CD34 and CD14), which is important as it is known that the reduction of such cells (e.g., T lymphocytes) prevents significant graft-versus-host disease reactions (40). There was no shift in the expression of the surface markers between low and high passages, indicating a good stability. The presence of CD105 was really interesting as it increases the stability of the cells in the tenogenic phenotype. Indeed, it has recently been demonstrated that a lack of this protein in tenocytes leads to more chondrogeneration in healing tendon than when this protein is present (41).

hFPTs exhibited, as expected, a lack of MHC class II proteins (HLA-DR, DP, DQ), while MHC class I (HLA-A, B, C) was mildly present. This profile is very close to the one of MSCs (42). T lymphocytes are known to drive allograft rejection through HLA recognition, and the presence of MHC class I proteins on the surface of a cell should normally trigger an immune reaction from $CD8^+$ T cells. Nonetheless, it has largely been proven that MSCs, even if they present MHC class I antigens, have immune-modulating effects and that they are able to be grafted with lack of immunological response. Indeed, they possess the ability to inhibit proliferation of T lymphocytes in vitro (42) and have also been well tolerated in vivo, notably in primates, even in the case of MHC mismatch (43, 44). Neonatal foreskin cells found in the commercial product Apligraft present also a similar MHC profile and have also been well tolerated (45). The tolerance seen with these examples would indicate that the mechanism is not fully understood and that some allogenic cell types, although presenting HLA proteins can be used for transplantation without immunosuppression. There is multiple evidence that fetal cells behave in the same manner with a capacity to modulate immune reaction. One of the most important observations supporting this is during gestation itself. It has been shown that even if paternal HLA-C are recognized during pregnancy, there is no harm to the fetus (46). The presence of HLA-G has been proposed as a potential modulator, as these proteins are elevated on fetal cells in contact with maternal tissue and have been shown to be able to inhibit different lymphocytes (47-49). hFPTs did not express these particular MHC class I proteins, but this is nonetheless not the only mechanism of immunomodulation in fetal tissue. Fetal fibroblasts and keratinocytes present an immunosuppressive activity and are able to inhibit lymphoproliferation, notably through their expression of indoleamine 2,3-dioxygenase (IDO) (50). Our past clinical trials have also illustrated an excellent tolerance for fetal cells. Biologic bandages containing fetal fibroblasts lead to improved outcomes in burn and wound healing, and even if there was no engraftment long term, multiple applications did not initiate immune reactions (25, 26).

Altered cell differentiation is sometimes thought to play a role in the aetiology of tendinopathies (51), as calcification and appearance of lipid droplets (normally not seen in tendon) are factors found in tendinopathies, which can precede spontaneous rupture of tendons (5). To use cell

therapy for tendon treatment, it is then of primary importance to have a cell line with a stable phenotype, to avoid drift to osteogenic or adipogenic phenotypes. While ASCs and BM-MSCs showed differentiation into osteogenic or adipogenic phenotypes under inducing conditions, hFPTs demonstrated a very high stability in their phenotype without any sign of mineral or lipid accumulation. These results are particularly encouraging as it could prevent the differentiation of the cells and the appearance of calcification or lipid accumulation often seen in vivo with other cell types. Mesenchymal stem cells, either from adipose tissue or from bone marrow, have been employed for tendon regeneration in various animal models (52), and even in a human clinical trial (53). In most of the cases, MSCs lead to improved outcomes, but they could not attain the quality found with fetal healing (23, 54). Furthermore, there is still concern about the stability of MSCs to act as tenocytes when used in tendon repair. In a study conducted by Awad *et al.*, the use of MSC-collagen composites improved the biomechanical parameters of the repaired tendon, but lead to the formation of bone in the repair site in 28% of the grafted tendons versus 0% in the natural repair control group (55). Bi *et al.* also warned that BM-MSCs expanded in vitro before transplantation into mice formed bone rather than tendon tissue and that the use of such cells could potentially lead to calcifications. In contrast, they showed that the use of the recently discovered tendon stem/progenitor cells (TSPCs) avoided this problem with a phenotype more tuned into tendon production. However, the harvesting of autologous TSPCs does not seem possible for clinical use as there is not an easily available source (56). Embryonic stem cells (ESCs) have also been tested in vivo, and Chen *et al.* have proposed a manner to first differentiate ESCs into MSCs before tendon treatment to avoid the risk of teratoma formation (57). More recently, induced pluripotent stem cells (iPSCs) improved the outcomes of tendon healing in a study (58). However, ESCs and iPSCs are also inducible cells and still present the same risk of dedifferentiation as MSCs. Differentiated cells such as human autologous tenocytes could present good stability, but as for TSPCs, there is a lack of an easily available source for harvesting, and this would also require a biopsy and lengthy culture period before clinical use would be possible. hFPTs present both advantages of a strong overall stability (phenotype and karyotype), along with an off-the-shelf availability whenever needed, which gives support for these cells as a potential source in clinical use.

Different clinically acceptable delivery systems would be critical to assure localized and targeted treatments. hFPTs had the ability to positively interact both with gel and porous matrix and therefore could provide interesting delivery modes that are routinely used in the clinic. On one side, injectable cell preparations capable of stimulating tendon regeneration could be particularly interesting to treat tendinopathies and simple acute injuries. The ability of hFPTs to survive in HA gel overnight (or even up to 3 days) at 4°C could provide an interesting logistic solution. It would be easy to prepare the formulation on the day before an intervention by removing cells from the frozen-stored bank, rinsing them with PBS or NaCl to eliminate undesired products, mixing them with gel and keeping the product at 4°C for transport. On the other hand, engineered tendon constructs would be useful for resistant degenerative or extended acute injuries, when filling bulk tissue defects with material is required (59). hFPTs can be uniformly and efficiently integrated into scaffold, and a rinsing step before delivery allows to remove

undesired products without detaching cells. The product can be delivered almost immediately or kept in culture for longer periods.

Tendon healing is known to be a long process in part driven by adult tenocytes. Our in vitro model of coculture has proven that hFPTs could stimulate adult tenocytes, which is interesting as an increase in their global activity could potentially accelerate the overall process. In addition, studies and literature indicate that wound stimulation provided by cell therapy with fetal progenitor cells could potentially provide a scarless healing with the absence of fibrotic tissue. An in vivo study to show safety and efficacy would be the next step to bring this cell therapy closer to the clinic.

Conclusion

hFPTs have been shown to be a unique stable cell source that can be produced under stringent current GMP manufacturing. They present a remarkable genetic and phenotypic stability and are able to conserve their tenogenic nature even in the presence of inductive conditions towards other phenotypes. The possibility to create rapidly a large cell bank to obtain off-the-shelf reserves is ideal for cell therapy and tissue engineering. Moreover, the cells show a good interaction both with porous and gel scaffolds for clinical preparations and delivery. As the cells are all derived from the same organ donation, it is also possible to include within the process extensive screening easily adaptable to GMP cell banking conditions. Finally, their ability to stimulate the activity of adult tenocytes is of importance as it could lead to a more rapid healing in vivo. The in vivo model chosen for further evaluation should also allow distinguishing whether rapid regenerative healing without scar could be attained all the while assuring safety and that no immune reaction would be elicited by these particular banked fetal progenitor tenocytes.

Acknowledgments

These studies were funded in part by the Orthopaedic Hospital Foundation of Lausanne and particularly for the support of the doctoral project (AG). We thank the Sandoz Family Foundation and the S.A.N.T.E Foundation for their continued support in our Transplantation Program. We thank Danielle Martinet for her help in karyotype analysis. The authors declare no conflicts of interest.

References

1. Sharma P, Maffulli N. Tendon injury and tendinopathy: healing and repair. J Bone Joint Surg Am. 2005;87(1):187-202.

2. Maffulli N, Wong J, Almekinders LC. Types and epidemiology of tendinopathy. Clin Sports Med. 2003;22(4):675-92.

3. Maffulli N, Khan KM, Puddu G. Overuse tendon conditions: time to change a confusing terminology. Arthroscopy. 1998;14(8):840-3.

4. Reinking M. Tendinopathy in athletes. Phys Ther Sport. 2012;13(1):3-10.

5. Kannus P, Jozsa L. Histopathological changes preceding spontaneous rupture of a tendon. A controlled study of 891 patients. J Bone Joint Surg Am. 1991;73(10):1507-25.

6. Maffulli N, Barrass V, Ewen SW. Light microscopic histology of achilles tendon ruptures. A comparison with unruptured tendons. Am J Sports Med. 2000;28(6):857-63.

7. Paavola M, Kannus P, Järvinen M. Epidemiology of Tendon Problems in Sport. In: Maffulli N, Renström P, Leadbetter W, editors. Tendon Injuries. London: Springer; 2005. p. 32-9.

8. Gaida JE, Alfredson H, Kiss ZS, Bass SL, Cook JL. Asymptomatic Achilles tendon pathology is associated with a central fat distribution in men and a peripheral fat distribution in women: a cross sectional study of 298 individuals. BMC Musculoskelet Disord. 2010;11:41.

9. United States Bone and Joint Initiative. Musculoskeletal Injuries. The Burden of Musculoskeletal Diseases in the United States. Rosemont, IL: American Academy of Orthopaedic Surgeons; 2011. p. 129-79.

10. Butler DL, Juncosa N, Dressler MR. Functional efficacy of tendon repair processes. Annu Rev Biomed Eng. 2004;6:303-29.

11. Tuncali D, Yavuz N, Terzioglu A, Aslan G. The rate of upper-extremity deep-structure injuries through small penetrating lacerations. Ann Plast Surg. 2005;55(2):146-8.

12. James R, Kesturu G, Balian G, Chhabra AB. Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. J Hand Surg Am. 2008;33(1):102-12.

13. Voleti PB, Buckley MR, Soslowsky LJ. Tendon healing: repair and regeneration. Annu Rev Biomed Eng. 2012;14:47-71.

14. Wehbe MA. Tendon graft donor sites. J Hand Surg Am. 1992;17(6):1130-2.

15. Jakubietz MG, Jakubietz DF, Gruenert JG, Zahn R, Meffert RH, Jakubietz RG. Adequacy of palmaris longus and plantaris tendons for tendon grafting. J Hand Surg Am. 2011;36(4):695-8.

16. Harvey FJ, Chu G, Harvey PM. Surgical availability of the plantaris tendon. J Hand Surg Am. 1983;8(3):243-7.

17. Adzick NS, Longaker MT. Scarless fetal healing. Therapeutic implications. Ann Surg. 1992;215(1):3-7.

18. Dang C, Ting K, Soo C, Longaker MT, Lorenz HP. Fetal wound healing current perspectives. Clin Plast Surg. 2003;30(1):13-23.

19. Bullard KM, Longaker MT, Lorenz HP. Fetal wound healing: current biology. World J Surg. 2003;27(1):54-61.

20. Favata M, Beredjiklian PK, Zgonis MH, Beason DP, Crombleholme TM, Jawad AF, et al. Regenerative properties of fetal sheep tendon are not adversely affected by transplantation into an adult environment. J Orthop Res. 2006;24(11):2124-32.

21. Longaker MT, Moelleken BR, Cheng JC, Jennings RW, Adzick NS, Mintorovich J, et al. Fetal fracture healing in a lamb model. Plast Reconstr Surg. 1992;90(2):161-73.

22. Namba RS, Meuli M, Sullivan KM, Le AX, Adzick NS. Spontaneous repair of superficial defects in articular cartilage in a fetal lamb model. J Bone Joint Surg Am. 1998;80(1):4-10.

23. Beredjiklian PK, Favata M, Cartmell JS, Flanagan CL, Crombleholme TM, Soslowsky LJ. Regenerative versus reparative healing in tendon: a study of biomechanical and histological properties in fetal sheep. Ann Biomed Eng. 2003;31(10):1143-52.

24. Russo V, Mauro A, Martelli A, Di Giacinto O, Di Marcantonio L, Nardinocchi D, et al. Cellular and molecular maturation in fetal and adult ovine calcaneal tendons. J Anat. 2015;226(2):126-42.

25. Hohlfeld J, de Buys Roessingh A, Hirt-Burri N, Chaubert P, Gerber S, Scaletta C, et al. Tissue engineered fetal skin constructs for paediatric burns. Lancet. 2005;366(9488):840-2.

26. De Buys Roessingh AS, Hohlfeld J, Scaletta C, Hirt-Burri N, Gerber S, Hohlfeld P, et al. Development, characterization, and use of a fetal skin cell bank for tissue engineering in wound healing. Cell Transplant. 2006;15(8-9):823-34.

27. Ramelet AA, Hirt-Burri N, Raffoul W, Scaletta C, Pioletti DP, Offord E, et al. Chronic wound healing by fetal cell therapy may be explained by differential gene profiling observed in fetal versus old skin cells. Exp Gerontol. 2009;44(3):208-18.

28. Quintin A, Hirt-Burri N, Scaletta C, Schizas C, Pioletti DP, Applegate LA. Consistency and safety of cell banks for research and clinical use: preliminary analysis of fetal skin banks. Cell Transplant. 2007;16(7):675-84.

29. Applegate LA, Scaletta C, Hirt-Burri N, Raffoul W, Pioletti D. Whole-cell bioprocessing of human fetal cells for tissue engineering of skin. Skin Pharmacol Physiol. 2009;22(2):63-73.

30. Applegate LA, Weber D, Simon J-P, Scaletta C, Hirt-Burri N, de Buys Roessingh A, et al. Organ Donation and Whole-Cell Bioprocessing in the Swiss Fetal Progenitor Cell Transplantation Platform. In: Saidi. RF, editor. Organ Donation and Organ Donors: Issues, Challenges and Perspectives. Hauppauge NY: Nova Science Publishers, Inc.; 2013. p. 125-48.

31. Puchtler H, Meloan SN, Terry MS. On the history and mechanism of alizarin and alizarin red S stains for calcium. J Histochem Cytochem. 1969;17(2):110-24.

32. Hook EB. Exclusion of chromosomal mosaicism: tables of 90%, 95% and 99% confidence limits and comments on use. Am J Hum Genet. 1977;29(1):94-7.

33. Lorenz HP, Lin RY, Longaker MT, Whitby DJ, Adzick NS. The fetal fibroblast: the effector cell of scarless fetal skin repair. Plast Reconstr Surg. 1995;96(6):1251-61.

34. Taylor SE, Vaughan-Thomas A, Clements DN, Pinchbeck G, Macrory LC, Smith RK, et al. Gene expression markers of tendon fibroblasts in normal and diseased tissue compared

to monolayer and three dimensional culture systems. BMC Musculoskelet Disord. 2009;10:27.

35. Tuite DJ, Renstrom PA, O'Brien M. The aging tendon. Scand J Med Sci Sports. 1997;7(2):72-7.

36. Hulmes DJ. Building collagen molecules, fibrils, and suprafibrillar structures. J Struct Biol. 2002;137(1-2):2-10.

37. Banos CC, Thomas AH, Kuo CK. Collagen fibrillogenesis in tendon development: current models and regulation of fibril assembly. Birth Defects Res C Embryo Today. 2008;84(3):228-44.

38. Murchison ND, Price BA, Conner DA, Keene DR, Olson EN, Tabin CJ, et al. Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. Development. 2007;134(14):2697-708.

39. Docheva D, Hunziker EB, Fassler R, Brandau O. Tenomodulin is necessary for tenocyte proliferation and tendon maturation. Mol Cell Biol. 2005;25(2):699-705.

40. Prentice HG, Janossy G, Price-Jones L, Trejdosiewicz LK, Panjwani D, Graphakos S, et al. Depletion of T lymphocytes in donor marrow prevents significant graft-versus-host disease in matched allogeneic leukaemic marrow transplant recipients. Lancet. 1984;1(8375):472-6.

41. Asai S, Otsuru S, Candela ME, Cantley L, Uchibe K, Hofmann TJ, et al. Tendon Progenitor Cells in Injured Tendons Have Strong Chondrogenic Potential: The CD105-Negative Subpopulation Induces Chondrogenic Degeneration. Stem Cells. 2014;32(12):3266-77.

42. Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringden O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. Exp Hematol. 2003;31(10):890-6.

43. Devine SM, Bartholomew AM, Mahmud N, Nelson M, Patil S, Hardy W, et al. Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. Exp Hematol. 2001;29(2):244-55.

44. Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol. 2002;30(1):42-8.

45. Streit M, Braathen LR. Apligraf--a living human skin equivalent for the treatment of chronic wounds. Int J Artif Organs. 2000;23(12):831-3.

46. Ober C. HLA and pregnancy: the paradox of the fetal allograft. Am J Hum Genet. 1998;62(1):1-5.

47. Carosella ED, Moreau P, Lemaoult J, Rouas-Freiss N. HLA-G: from biology to clinical benefits. Trends Immunol. 2008;29(3):125-32.

48. Rouas-Freiss N, Marchal RE, Kirszenbaum M, Dausset J, Carosella ED. The alpha1 domain of HLA-G1 and HLA-G2 inhibits cytotoxicity induced by natural killer cells: is

HLA-G the public ligand for natural killer cell inhibitory receptors? Proc Natl Acad Sci U S A. 1997;94(10):5249-54.

49. Piccinni MP. T cell tolerance towards the fetal allograft. J Reprod Immunol. 2010;85(1):71-5.

50. Zuliani T, Saiagh S, Knol AC, Esbelin J, Dreno B. Fetal fibroblasts and keratinocytes with immunosuppressive properties for allogeneic cell-based wound therapy. PLoS One. 2013;8(7):e70408.

51. de Mos M, Koevoet WJ, Jahr H, Verstegen MM, Heijboer MP, Kops N, et al. Intrinsic differentiation potential of adolescent human tendon tissue: an in-vitro cell differentiation study. BMC Musculoskelet Disord. 2007;8:16.

52. Longo UG, Lamberti A, Petrillo S, Maffulli N, Denaro V. Scaffolds in tendon tissue engineering. Stem Cells Int. 2012;2012:517165.

53. Ellera Gomes JL, da Silva RC, Silla LM, Abreu MR, Pellanda R. Conventional rotator cuff repair complemented by the aid of mononuclear autologous stem cells. Knee Surg Sports Traumatol Arthrosc. 2012;20(2):373-7.

54. Young M. Stem cell applications in tendon disorders: a clinical perspective. Stem Cells Int. 2012;2012:637836.

55. Awad HA, Boivin GP, Dressler MR, Smith FN, Young RG, Butler DL. Repair of patellar tendon injuries using a cell-collagen composite. J Orthop Res. 2003;21(3):420-31.

56. Bi Y, Ehirchiou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W, et al. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. Nat Med. 2007;13(10):1219-27.

57. Chen X, Song XH, Yin Z, Zou XH, Wang LL, Hu H, et al. Stepwise differentiation of human embryonic stem cells promotes tendon regeneration by secreting fetal tendon matrix and differentiation factors. Stem Cells. 2009;27(6):1276-87.

58. Xu W, Wang Y, Liu E, Sun Y, Luo Z, Xu Z, et al. Human iPSC-derived neural crest stem cells promote tendon repair in a rat patellar tendon window defect model. Tissue Eng Part A. 2013;19(21-22):2439-51.

59. Petrou IG, Grognuz A, Hirt-Burri N, Raffoul W, Applegate LA. Cell therapies for tendons: old cell choice for modern innovation. Swiss Med Wkly. 2014;144:w13989.

CHAPTER IV

–

STABILITY ENHANCEMENT USING HYALURONIC ACID GELS FOR DELIVERY OF HUMAN FETAL PROGENITOR TENOCYTES

Foreword

The last chapter highlighted interesting in vitro results obtained with human fetal progenitor tenocytes (hFPTs) and this cell source could be useful in the treatment of tendon afflictions. In the present chapter, we compare formulations of hFPTs within different commercial hyaluronic acid gels with the aim to find a formulation that would allow for good stability and delivery of the cells. The rheological characteristics of the gels are evaluated and the cell survival is assessed for different temperatures and periods of storage. This chapter was accepeted as a research article for publication in Cell Medicine.

Stability Enhancement Using Hyaluronic Acid Gels for Delivery of Human Fetal Progenitor Tenocytes

Accepted 01/01/2015 for publication in Cell Medicine

Reprinted in this thesis with the kind permission of Cognizant Communication Corporation

A. Grognuz[1], C. Scaletta[1], A. Farron[2], D.P. Pioletti[3], W. Raffoul[1] and L.A. Applegate[1]*

1) Unit of Regenerative Therapy, Service of Plastic, Reconstructive and Hand Surgery, Department of Musculoskeletal Medicine, University Hospital of Lausanne, Switzerland

2) Service of Orthopaedics and Traumatology, Department of Musculoskeletal Medicine, University Hospital of Lausanne, Switzerland

3) Laboratory of Biomechanical Orthopedics, Institute of Bioengineering, EPFL, Lausanne, Switzerland

***Address for Correspondence:**
Professor Lee Ann Laurent-Applegate
University Hospital of Lausanne (CHUV)
Service of Plastic, Reconstructive and Hand Surgery
Unit of Regenerative Therapy (UTR)
EPCR/Croisettes 22
1066 Epalinges
SWITZERLAND
E-mail: Lee.Laurent-Applegate@chuv.ch
Telephone: + 41 21 314 35 10
Fax: + 41 21 887 84 14

Abstract

Tendon afflictions are very common and their negative impact is high both at the workplace and in leisure activities. Tendinopathies are increasing in prevalence and can lead to tendon ruptures, where healing is a long process with outcomes that are often disappointing. Human fetal progenitor tenocytes (hFPTs) have been recently tested in vitro as a potential cell source to stimulate tendon regeneration. The aim of the present study was to compare different commercial HA gels which could be used to resuspend hFPTs in a formulation that would allow for good delivery of the cells. No medium or growth supplement was used in the formulation in order to make it therapeutically dispensable. These conditions are stringent for cells, but surprisingly we found that different formulations could allow a good survival for up to 3 days when stored at 4 degrees (refrigerator stable). The gels must allow a good survival of the cells in parallel with a good stability of the preparation over time and sufficient viscosity to remain in place if deposited on a wounded location. Moreover, the cells must conserve their ability to attach and to proliferate. hFPTs were able to survive and to recover from all of the tested gels, but some products showed some advantages over others in terms of survival and viscosity. Finally, the Ostenil Tendon HA gel fulfilled all of the requirements and presented the best compromise between a good survival and sufficient rheological characteristics to create an interesting cell delivery system.

Key words: Cell therapy; Hyaluronic acid gel; Tendon healing; Human fetal progenitor tenocytes; Cell stability.

Introduction

Tendon afflictions are very common and various anatomical areas are affected, including the Achilles tendon, various hand tendons and the rotator cuff, all of which are among the major tendons exposed to injury. In the United States alone, more than 25 million sprains and strains of soft tissues such as tendons and ligaments are reported yearly (1) with an impact that is significant both at the workplace and in leisure activities. Our population is becoming older and aging is correlated with an increase in chronic degeneration of tendons, presently referred to as tendinopathies (2, 3). Elderly people are not the only ones affected, and with the democratization of sport practice, tendinopathies have been increasing in younger individuals during recent decades as well (4, 5).

Tendon homeostasis relies on good behavior of tenocytes, which are the cells responsible for the secretion of all extracellular matrix components and the maintenance of a fine balance between synthesis and catabolism of the extracellular matrix (6). In the case of acute loadings, the net balance of collagen levels is negative for up to 18−36 hours after exercise and then becomes positive up to 72 hours. Thereafter, repeated loadings without sufficient rest can lead to an amplification of the collagen breakdown (7). The extracellular matrix becomes more disorganized and weaker and many changes in the structure are detectable (8). Tendinopathies are not only painful, they also make tendons more prone to ruptures (9). In cases of rupture, tendon healing relies on a cascade of events. Tenocytes have an important role as they are responsible for formation of new extracellular matrix. Unfortunately, the quality of the new tissue never reaches that of the original tissue prior to injury and scar tissue and adhesions are frequent, which often leads to decreased mobility and reruptures (10-12).

Therefore, tenocytes have an important role both in tendinopathies and in tendon ruptures and their modulation would be very interesting to improve the outcomes obtained. Human fetal progenitor fibroblasts have shown excellent results in modulating regeneration of tissues such as skin towards a less fibrotic process (13, 14). We recently tested human fetal progenitor tenocytes (hFPTs) as a potential cell source for tendon regeneration and showed that it was possible to create cell banks with very high numbers of cells and with respect to good manufacturing practice (GMP), which is a prerequisite for potential clinical use (15). These cells were shown to have stimulating abilities towards adult tenocytes, along with very good genotypic and phenotypic stability, and the banks allow the availability of an off-the-shelf cell stock with cells easily accessible and rapidly for use. Such results are promising, but to be delivered to the afflicted site, the cells need a support scaffold such as a matrix or a gel. Seeding cells within gels presents the advantage of being easily injectable and, as surgery is not required for their use, they could be particularly useful for treatment of tendinopathies and simple acute injuries. The presence of medium with serum or other growth factor supplement is not feasible in a preparation destined to be injected into the body. Therefore, we decided to work with formulations containing only hFPTs, phosphate-buffered saline (PBS), and hyaluronic acid (HA) gels. HA is a glycosaminoglycan which is naturally present in numerous tissues, including tendons and numerous gels based on this molecule are commercially available with a market authorization for

injection. The variation of the degree of crosslinking of HA in the gel leads to characteristics which can be very different, notably the viscosity of the gel (16). In this study, we decided to compare a selection of HA gels in search for one formulation that, on one hand, would allow for survival of hFPTs and, on the other hand, would have good rheological characteristics, with the aim of creating a preparation that can be used for therapeutic interventions.

Materials and Methods

Cell Culture

hFPTs were grown in a monolayer in polystyrene tissue culture flasks of 75 cm^2 with filter screw caps (TPP, Trasadingen, Switzerland, code No. 90076) and were placed in cell culture incubators at 37°C in a humidified atmosphere containing 5% CO_2. They were seeded at a density of 3,000 cells/cm^2 at passage 5 from a cryopreserved vial from our cell bank. hFPTs were isolated from the Achilles tendon of a male 14-week gestation organ donation according to a protocol approved by the State Ethics Committee (University Hospital of Lausanne - CHUV, Ethics Committee Protocol N° 62/07: 14-week gestation organ donation, registered under the Federal Transplantation Program and its Biobank complying with the laws and regulations). The detailed cell banking procedures were described previously (15, 17). The medium was changed every 3 to 4 days and was composed of standard growth medium composed of Dulbecco's modified Eagle medium (DMEM: Gibco, Life Technologies Ltd., Paisley, UK, code No. 41966-029) containing 25 mM dextrose, 1 mM sodium pyruvate, and supplemented with 5.97 mM L-glutamine (L-glut: Gibco, code No. 25030-024) and with 10% fetal bovine serum (FBS: Sigma-Aldrich, St. Louis, MO, USA, code No. F7524), without antibiotic supplementation. When full confluence was reached, the cells were detached with TrypleE (Gibco, code No. 12605-010), counted with a hemocytometer and transferred in desired amounts into new tubes. They were then centrifuged at 250 x g, the supernatants were discarded and they were rinsed twice with PBS (NaCl 6.8 g/l, Na_2HPO_4 1.5 g/l, KH_2PO_4 0.4 g/l; Bichsel, Interlaken, Switzerland, code No. 100 0 324) to eliminate all residue in the medium. hFPTs were then resuspended with PBS to the desired concentration and were ready to be used at passage 6 for the different experiments.

Comparison of Cell Survival in Commercial HA Gels

hFPT survival was evaluated in seven different commercial gels containing HA in various amounts (Table 1).

Table 1: Selection of commercial HA gels.

Gel	Concentration in HA	Manufacturer
Hyalgan	1%	KELA Pharma, St-Niklaas, Belgium
Ostenil	1%	TRB Chemedica, Haar, Germany
Ostenil Plus	2%	TRB Chemedica, Haar, Germany
Ostenil Tendon	2%	TRB Chemedica, Haar, Germany
Sinovial	0.8%	IBSA, Lodi, Italy
Sinovial	1.6%	IBSA, Lodi, Italy
Synolis V-A	2%	Anteis, Plan-les-Ouates, Switzerland

hFPTs were initially tested with a concentration of 40,000 cells per 100 µl of gel. A cell suspension of 4 million cells/ml in PBS was prepared and 10 µl was dispatched in 9 wells of 96-well plates (Corning Inc., Corning, NY, USA, code No. 3596) (40,000 cells per well). There were as many plates prepared as conditions tested. The cells of each well were then resuspended with 90 µl of a specific gel and homogenized with a displacement pipette (Microman, Gilson, Middleton, WI, USA, code No. F148504). One plate was evaluated directly after resuspension (0 d). The preservation was then evaluated at room temperature (RT) and at 4°C after 24 hours. As the results were better at 4°C (mean survival of 75% for 4°C compared to 66% at RT), other plates were prepared to test the preservation at the 4°C temperature for multiple time points (1 d, 2 d, 3 d, and 7 d). The cells did not receive any medium or growth supplement during the preparation for the storage conditions. The survival was evaluated with a LIVE/DEAD solution (Molecular Probes, Life Technologies Ltd., Paisley, UK, code No. L-3224). On the desired day, 100 µl of LIVE/DEAD solution (prepared according to the manufacturer's instructions) was added to the wells and mixed with the displacing pipette. After 30 minutes of incubation at RT and with protection from light, images were taken with an inverted microscope equipped for fluorescence (IX81; Olympus, Tokyo, Japan) and a digital camera (iXon; Andor Technology Ltd., Belfast, UK). Green and red channels were recorded on a stack of 11 layers covering a total depth of 600 µm for each gel (60-µm space between slices) in the lower third portion. The final image was obtained within ImageJ (National Institutes of Health, Bethesda, MD, USA) through a standard deviation z-projection to obtain one layer composed of two color channels and converted to 16 bits. The final images were then analyzed with Cell Profiler (Broad Institute, Cambridge, MA, USA) to obtain the number of live and dead cells and a ratio representing the cell survival; calculations were done for each image. The identification of primary objects (live and dead cells) within the two color layers was done for a diameter of 10 to 120 pixels with automatic threshold strategy and distinction of clumped objects based on the shape with dividing lines based on intensity. Each identification was controlled manually to check the accuracy of the method. The image processing and analysis are summarized in Figure 1. As there were less visible

cells in some conditions, on day 7 the bottoms of the wells were also analyzed for sedimentation and an image was taken for each gel on a single layer with both colors.

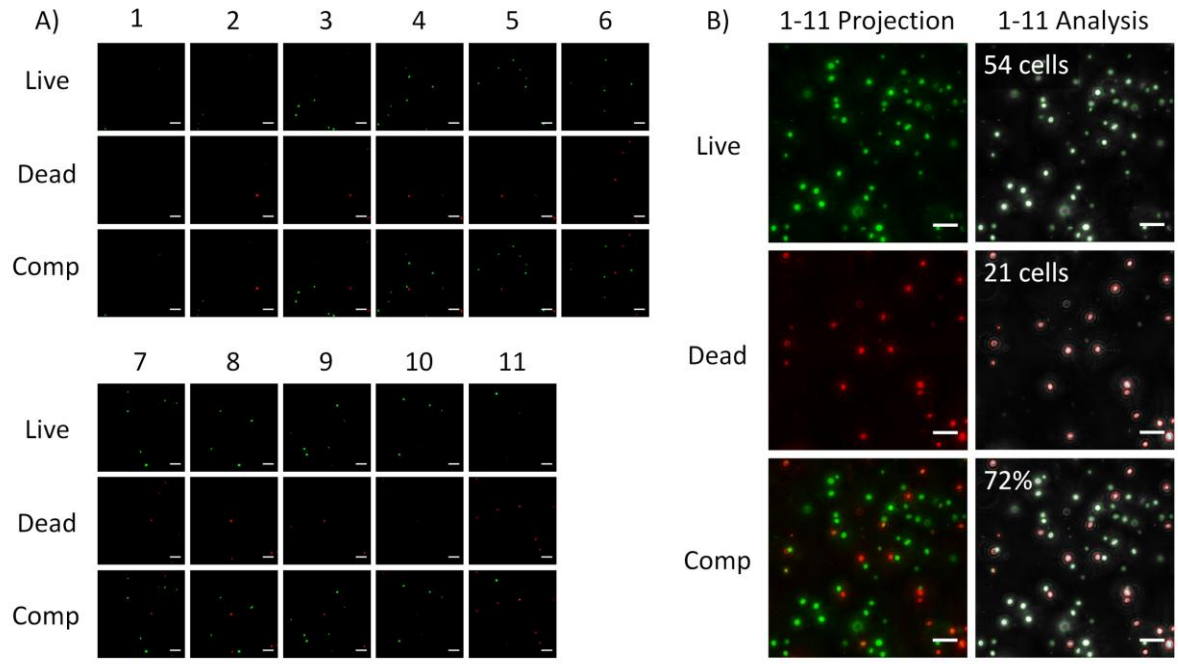

Figure 1: Image processing and analysis.
A) For the different gels, a stack of pictures was taken with multiple layers (either 11 or 3 layers depending on the concentration, always with 60-µm interspace between 2 layers) and 2 color channels (green for live cells and red for dead cells). A composite image with the two channels was created by merging the 2 color channels (comp). B) For each channel, the multiple slices were merged through a standard deviation z-projection within ImageJ software to obtain one projection representing the cells present in the whole portion analyzed (600 µm in the present case with 11 layers, 120 µm for 3 layers). The live and the dead cells were detected (circled in green or red) and counted automatically within Cell Profiler. The two color channels were fused on the same image and the ratio of live cells was indicated. Scale bars: 100 µm.

Rheology on HA Gels

The dynamic viscosity of each gel was measured with a Rheometer System CVOR 150 CE/WIN (Bohlin Instruments GmbH, Pforzheim, Germany) within a plate-plate system at RT. The diameter of the rotating plate was 10 mm, the distance between the plates was 800 µm and it was possible to place 80 µl of gel in-between. A shear rate of 1 Hz was applied for 20 seconds of pre-charge and the dynamic viscosity was then recorded at the same shear rate for 20 seconds and measured every 2 seconds. The measurements were then repeated twice with fresh gel. Finally, for each gel, the points were plotted as the mean of the three replicates. The dynamic viscosity was obtained by calculating the mean of the 11 points obtained from the 3 replicates and the standard deviation was calculated based on the same points.

Comparison of Cell Survival as a Function of Viscosity

A table summarizes the survival results after resuspension and after 3 days at 4°C (Table 2). The results were presented such that more than 85% of live cells were represented by "+++", from 60 to 85% "++" and for less than 60% "+". The degrees of viscosity were indicated in Table 2 as follows: the gels with a viscosity higher than 50 Pas were represented by "+++", from 10 to 50 Pas "++" and for less than 10 Pas "+". In parallel, a linear correlation was determined by plotting the survival results after 1 day of storage at 4°C as a function of the viscosity of the gels in excel software (Microsoft, Redmond, WA, USA). The root mean square error was used for the determination of the regression equation and the coefficient of determination (R^2).

Evaluation of Cell survival and Recovery with Increased Cell Concentration and Packaging in Syringes

The survival of hFPTs was then evaluated with improved formulations at a density of 500,000 cells per 100 µl of gel and with packaging within syringes. The same gels as above were used, except for Hyalgan which was not available for this experiment (due to supply problem). The cells were resuspended at a concentration of 50 million cells/ml in PBS and 80 µl was dispatched in wells of a 48-well plate. The cells of each well were resuspended with 720 µl of a specific gel and homogenized with a displacement pipette to reach the final concentration. The cell suspension was then simply aspirated with the syringe (without needle) to reach 500 µl, the air bubbles were eliminated, a cap was placed on the syringe and the final preparation with 2.5 million cells in a total volume of 500 µl was kept at 4°C for 72h.

The survival was evaluated with a LIVE/DEAD assay following a similar protocol as in the previous assay. For each gel, 100 µl of preparation was extruded through a 22-G needle in a well of a 96-well plate and 100 µl of the LIVE/DEAD solution was added. The only difference was a recording on only 3 layers representing a depth of 120 µm instead of 600 µm as the concentration was higher than previously accomplished. The images were also taken in the lower third portion and with the same interspace of 60 µm between slices, as in the previous experiment.

In parallel, a recovery assay was performed in monolayer culture. For each gel, 100 µl was diluted to 35 ml of standard growth medium and hFPTs were then dispatched in 12 wells of 2 six-well plates (evaluation of 4 time points in triplicate for each gel) with 2 ml per well to obtain a seeding of approximately 3,000 cells/cm^2 at passage 6 (each solution was counted to know the actual number of seeded cells). The plates were incubated at 37°C and at days 4, 7, 11 and 14, the cells were counted for 3 wells for each gel. The cells were rinsed with PBS, detached with TrypleE and transferred to a 15-ml tube. The TrypleE was inactivated by the addition of an equal volume of medium. The cells were centrifuged at 250 x g, the supernatant was discarded, and the cells were resuspended in an adapted volume to be counted with a hemocytometer. On the same days, the medium was replenished in the remaining wells. The population doublings (PDs) were

determined at each time point (equation 1) and the results were plotted as PDs as a function of time (PDs allow to avoid biases due to slight variations in seeding densities).

$$PD = \frac{\ln\left(\frac{final\ cell\ number}{initial\ cell\ number}\right)}{\ln(2)} \quad (1)$$

The results from hFPTs grown in six-wells at passage 6 directly from monolayer culture and which had never had contact with any gel were used as the control.

Results

Comparison of Cell Survival in Commercial HA Gels

Figure 2 presents the cell survival of hFPTs within the different gels for the different conditions tested.

Directly after cell resuspension within gel, the survival was determined to be excellent with Hyalgan, Sinovial 0.8% and Ostenil with 94%, 93%, and 88% live cells, respectively. There was a weaker proportion of live cells within Sinovial 1.6% and Ostenil Tendon with 75% and 74% live cells, respectively. Finally, Synolis V-A and Ostenil Plus presented a relatively weak survival directly after resuspension with just as many cells alive as dead (respectively 57% and 50% live cells).

After 24 hours of storage the cell survival was better for every gel stored at 4°C compared to RT, except for Ostenil Plus. Taking the results of all gels together, the mean survival was 66% (ranging from 40% to 85%) at RT and 75% (ranging from 54% to 91%) at 4°C. Hyalgan presented only a limited number of visible cells after 24 hours at RT, but no decrease in cell number was seen with the storage at 4°C.

After 48 hours at 4°C, the survival rates followed the same trend as for 24 hour storage at 4°C. There was again a decreased number of resuspended cells for Hyalgan.

After 72 hours at 4°C, the same trend was seen except for a drop in survival for Ostenil Plus and Sinovial 0.8%. There was a decreased number of resuspended cells both for Hyalgan and Sinovial 0.8%.

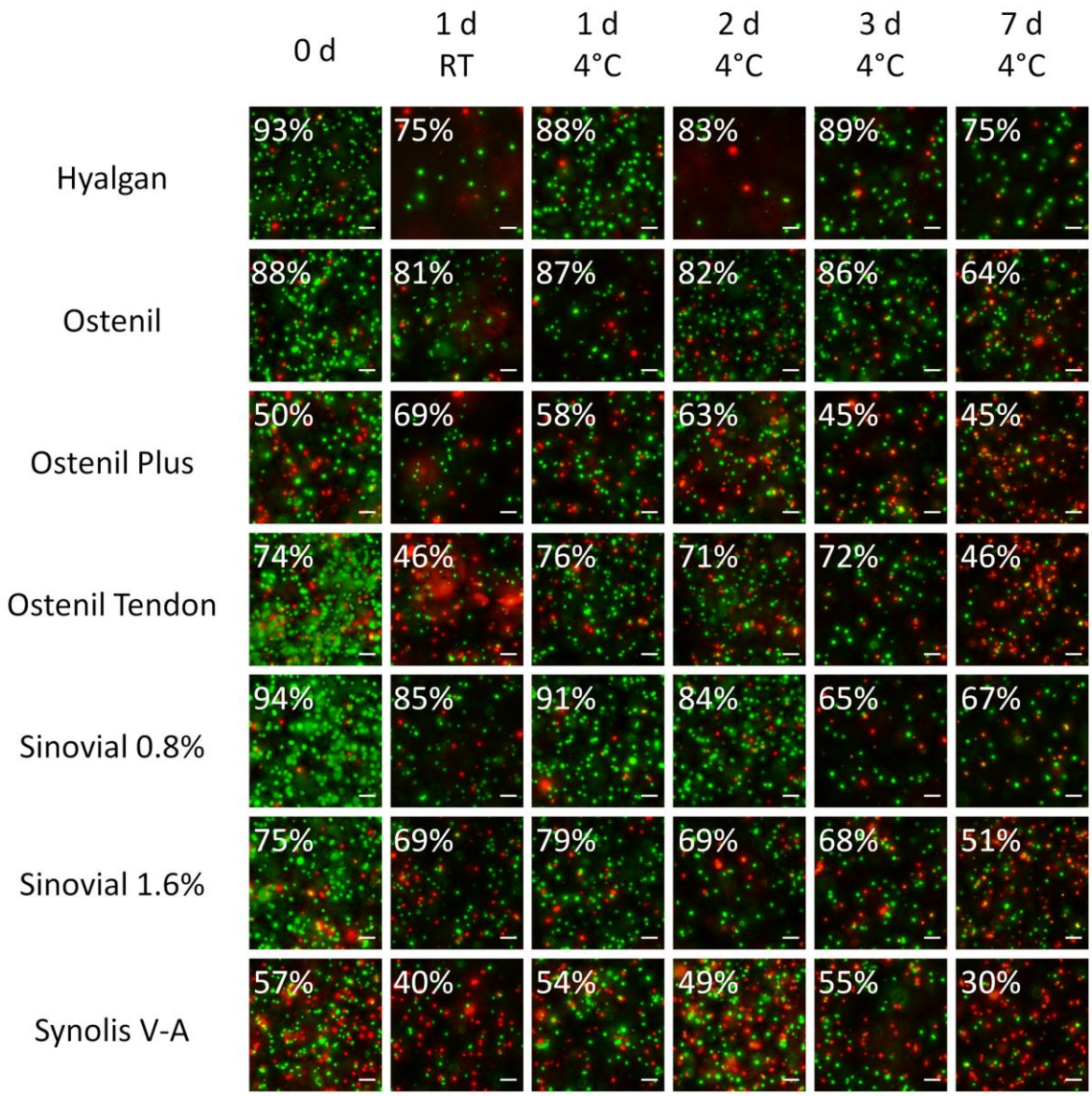

Figure 2: Cell survival of hFPTs within different commercial HA gels.
The images present the survival of hFPTs within HA gel for different conditions with various time points (0 to 7 days) and temperatures (RT or 4°C) of storage. The live cells appear in green (metabolically active), while the dead cells appear in red (membrane ruptured). Each image was obtained by projection of 11 slices covering a depth of 600 µm into one final image. The percentage indicates the ratio of live cells among the total number of cells for each image. Scale bars: 100 µm.

After 1 week at 4°C, the survival dropped for the other gels, namely Hyalgan, Ostenil, Ostenil Tendon, Sinovial 1.6% and Synolis V-A. The observation of the bottom of the wells (Fig. 3) showed that many more sedimented cells stayed at the bottom of the wells with Hyalgan and Sinovial 0.8% than with the other gels. There was also sedimentation with Ostenil, but to a lesser extent.

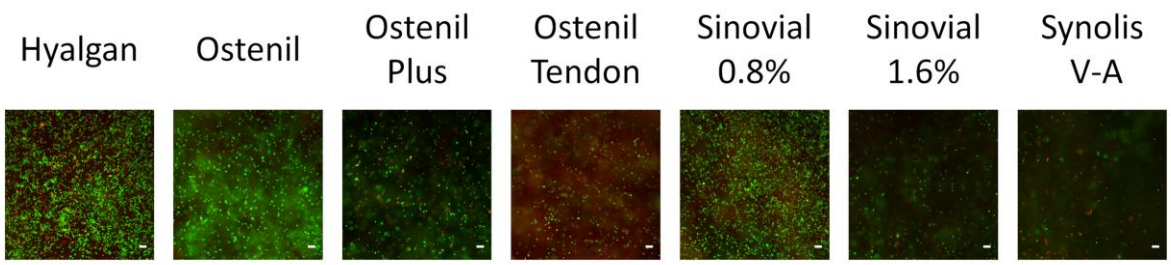

Figure 3: Sedimentation of hFPTs at the bottom of wells after 7 days at 4°C.
After 7 days of storage, the presence of sedimented cells at the bottom of the wells was more pronounced in Hyalgan and Sinovial 0.8% and to a more limited degree in Ostenil. Each image was obtained as one single layer with both color channels. The other gels were not affected. Scale bars: 100 μm.

Rheology

After 20 seconds of precharge, the recording of the dynamic viscosity during 20 seconds was seen to be very stable and reproducible between the triplicates for each gel. Hyalgan and Sinovial 0.8% presented very low degrees of viscosity, which were at 1.1 Pas and 3.2 Pas, respectively. Ostenil and Sinovial 1.6% were slightly more viscous with viscosities of 11.3 Pas and 12.2 Pas, respectively. Ostenil Plus and Ostenil Tendon had intermediate viscosities of 64.5 Pas and 60.4, respectively. Finally, Synolis V-A was the most viscous with 122.0 Pas (Fig. 4).

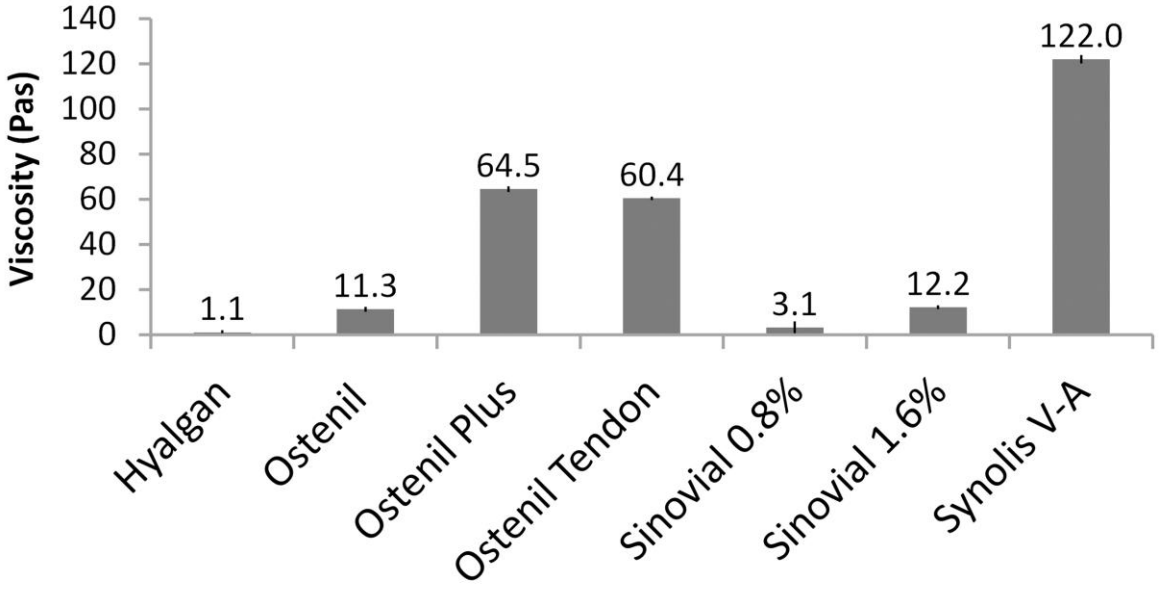

Figure 4: Dynamic viscosity of HA gels.
The dynamic viscosity of the different commercial HA gels represents the mean of the 11 measures made in 20 seconds (based on triplicates) and with the standard deviations shown as error bars.

Comparison of Cell Survival as a Function of Viscosity

Table 2 summarizes the survival of hFPTs directly after resuspension in their respective gels and after 3 days of storage at 4°C alongside their degrees of viscosity.

Table 2: Summary of the cell survival and of the rheology results for the seven commercial HA gels.

Gel	Ostenil	Ostenil Plus	Ostenil Tendon	Sinovial 0.8%	Sinovial 1.6%	Synolis V-A	Hyalgan
Cell survival (resuspension)	+++	+	++	+++	++	+	+++
Cell survival (storage 3 d 4°C)	+++	+	++	++	++	+	+++
Viscosity	++	+++	+++	+	++	+++	+
+++: >85% live cells (survival), >50 Pas (viscosity) ++: 60−85% live cells (survival), 10−50 Pas (viscosity) +: <60% live cells (survival), <10 Pas (viscosity)							

Figure 5 presents hFPT survival after 24 hours at 4°C as a function of the viscosity of the gel in which they were resuspended. The data follow a visible trend and the coefficient of determination of the linear regression line is 0.84. The slope of the regression line equals -0.003, meaning that 0.3% of survival is lost for each supplementary unit of Pas.

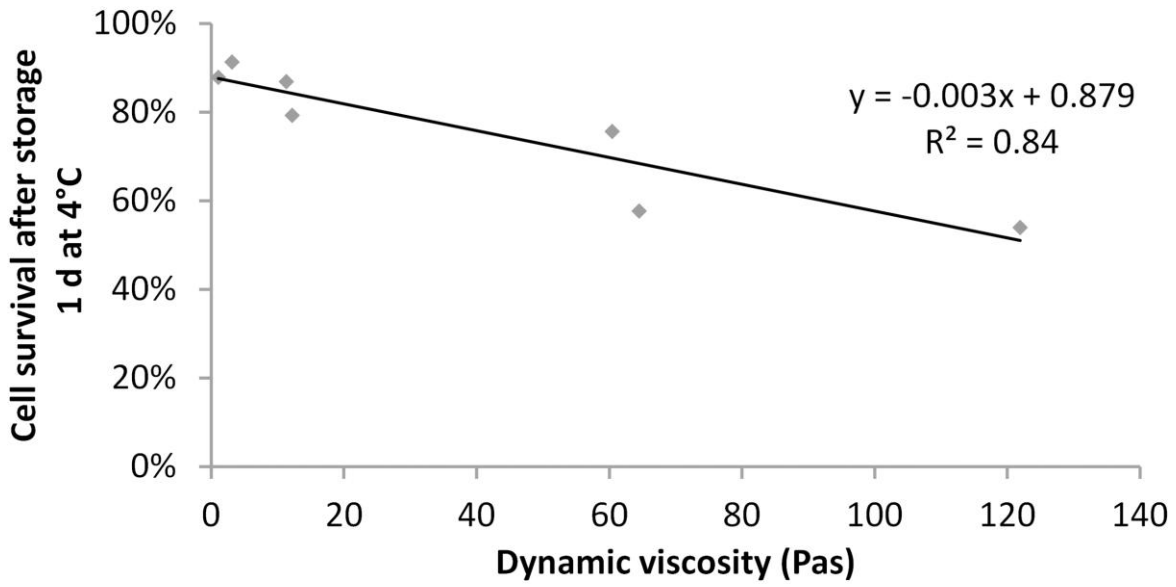

Figure 5: Linear correlation between dynamic viscosity and hFPT survival after 1 day at 4°C.
The linear correlation between the dynamic viscosity of the gel and the cell survival after 1 day at 4°C was very high with a coefficient of determination (R^2) of 0.84. With a slope of -0.003, for each supplementary unit of Pas in one gel, 0.3% of survival was lost for hFPTs.

Cell Survival and Recovery after 72 Hours at 4°C with Higher Cell Concentration and with Packaging in Syringes

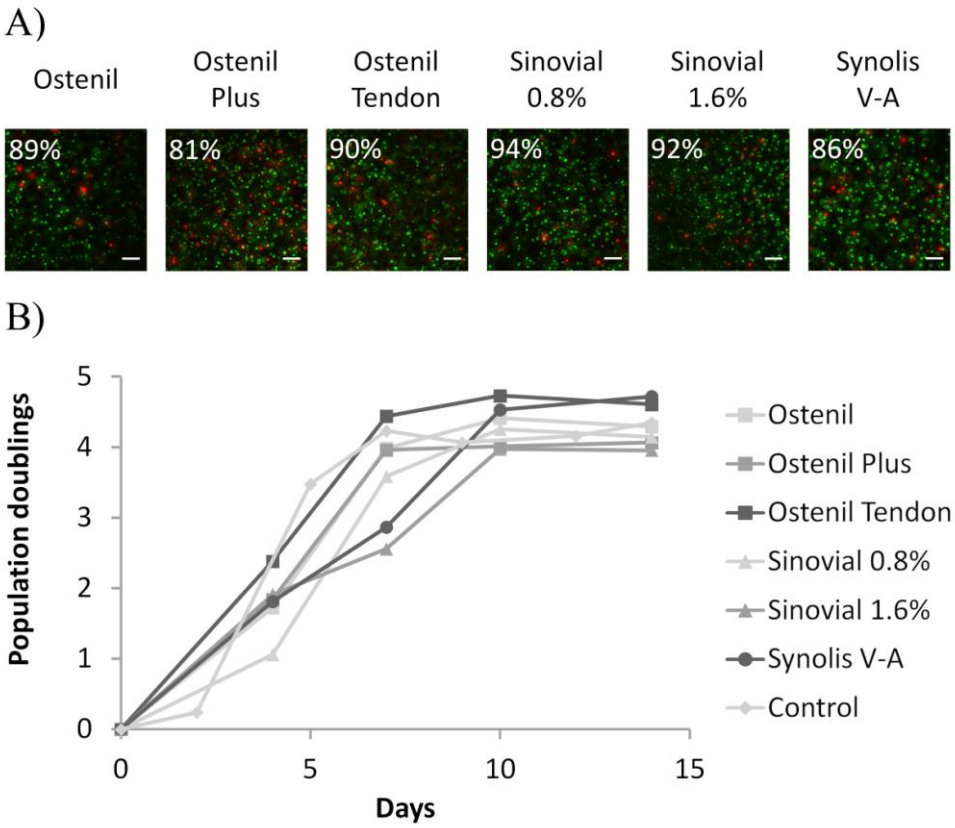

Figure 6: Cell survival (A) and recovery (B) of hFPTs at a concentration of 500,000 cells per 100 µl in syringe after 72h of storage at 4°C.

A) The live cells appear in green, while the dead cells appear in red. Each image was obtained by projection of 3 slices covering a depth of 120 µm into one final image. The percentage indicates the ratio of live cells among the total number of cells for each image. Scale bars: 100 µm. B) The recovery of the cells extruded from the syringe is shown for 2 weeks of monolayer culture. The points represent the cumulative population doublings up to confluency. The results obtained for hFPTs that have never been in contact with gel and grown at the same passage were added for comparison (control).

After 72 hours of storage at 4°C, hFPT survival at higher concentrations in syringes ranged from 81% for Ostenil Plus to 94% for Sinovial 0.8% as seen with the LIVE/DEAD experiment (Fig. 6A). Due to the high concentration, the number of cells for determining survival was higher than in the overall gel screening experiment (mean of 287 cells per final image for the higher concentration instead of 128 cells for the lower concentration), despite a recording on a depth 5 times lower. The recovery assay (Fig. 6B) highlighted the fact that the cells extruded from all syringes were able to attach and proliferate in monolayer culture and 90 to 100% confluency was reached after 7 days for Ostenil, Ostenil Plus, Ostenil Tendon and Sinovial 0.8% and after 10 days for Sinovial 1.6% and Synolis V-A. The proliferation with confluency reached after 7 days was really close to what was found for cells from the same passage that were grown in monolayer culture and that had never been in contact with any gel (Fig. 6B, control).

Discussion

hFPTs present very interesting characteristics in vitro and could be an optimal cell source for regeneration of tendons (15). Nevertheless, a scaffold is required to deliver the cells to the wounded site and the use of an HA gel could be feasible if it allows hFPTs to survive when seeded inside and retains flexibility for manipulation. Furthermore, with regard to clinical application in patients, it is necessary to determine the stability of the cells within the gel. It is advantageous if the formulation can be stored for some time, as the delivery to operating sites can take time and delays in surgery schedules can occur.

First of all, it is important to anticipate regulatory requirements and to be in accordance with the laws and recommendations of the relevant government bodies and institutions. The HA gels tested all received a market authorization with the possibility to be injected. The use of animal-derived products such as fetal bovine serum (FBS) has been challenged lately and such products were omitted in order to retain the potential of being injected in a clinical setting; hFPTs were rinsed and resuspended only with PBS before being mixed with an HA gel, without any medium or any other growth supplements. This introduces more difficulties for maintaining living cells, but it is compulsory for the potential use of hFPTs for therapeutic intervention in the future.

The different experiments were conducted in an incremental process, which means the techniques were adapted experiment after experiment. For example, once primary results were obtained, we changed the concentrations to be closer to what could be used in clinics. This explains why the results appear as a single replicate for an overview of multiple gels and why no further statistical information is added. However, each well was resuspended individually and the trend for a specific gel remained the same throughout the time points with the same gels consistently presenting good survival and always the other gels consistently presenting lower survival. By increasing the concentrations, we obtained better survival, but the trend was the same among the different gels. Thus, our discussion and conclusion are based on several experimental evaluations for each and every gel. After 24 hours of storage, either at RT or at 4°C, the results were clearly favoring the refrigerated preservation in terms of cell survival and this observation could be seen for all of the gels except Ostenil Plus. Tendons are known to have a relatively slow metabolism (6, 18), but tenocytes still require nutrients for their survival. By diminishing the temperature, the metabolic activity of the cells is also diminished and so are their requirements. The absence of nutrients may then have less impact under such conditions and this could explain a better survival at refrigerated temperatures up to a certain limit. Between day 2 and day 3, there was a drop in survival of more than 15% for Ostenil Plus and Sinovial 0.8%. The other gels underwent a similar drop for cell survival between day 3 and day 7. It is a major advantage to have a cellular preparation which can be stored for some time before its clinical application. It was the case in this study with live cells representing still 65% to even 89% in 5 gels after 3 days of conservation when refrigerated. This storage duration allows flexibility and corresponds for example to what is found in our hospital for other therapies, such as platelet rich plasma (PRP) injections (19).

The suspension of hFPTs within Ostenil Plus and Synolis V-A led to negative results in terms of cell survival. This yield was found to already be decreased just after homogenization of the cells within gels and without any storage phase. These two gels presented the weakest proportion of live cells for each time point as expected. This effect was not translated into one single initial preparation, as the cells were resuspended for each time point in their individual wells. These gels were the most viscous of the selection and this could probably explain the low overall survival even with the delicate use of displacement pipettes. With increased viscosity, the shear stress applied on the cell surface during resuspension and homogenization is increased and probably leads to more membrane ruptures. Indeed, there was a strong linear correlation between cell survival after 24 hours at 4°C and viscosity as seen by the coefficient of determination of 0.84 and gels with a high viscosity should be carefully selected for resuspension of hFPTs. The negative survival of cells within Ostenil Plus can nevertheless not only be explained by the viscosity of the gel as the survival was much better within Ostenil Tendon which had a very similar viscosity. This implies that viscosity is probably not the only factor favoring cell death and indicates that these effects may also be due to the gel composition.

Ostenil, Sinovial 0.8% and Hyalgan showed the best cell survival rates throughout the time points studied. These three gels presented the lowest viscosity and it is probable that only a limited number of cells were damaged during resuspension. The survival rates remained high for Hyalgan and Ostenil up to 3 days at 4°C which shows a good tolerance of hFPTs for these two gels. With Sinovial 0.8%, there was a greater decrease in cell survival after 3 days of storage at 4°C, which potentially indicates a weaker tolerance in the long term. Despite excellent survival results, some other characteristics of these three gels, such as the fact that Hyalgan frequently presented less visible cells within the observed layers than in the other gels and that Sinovial 0.8% also did after 3 days should be explored. The observation of the bottom of the wells after one week of storage at 4°C highlighted the presence of many more sedimented cells in these two gels, which demonstrates that a dynamic viscosity of less than 5 Pas is insufficient to maintain the cells in suspension in the long term. Ostenil was subject to a lower sedimentation and the other gels did not seem to be affected. This indicates that the preparation with a gel of low viscosity could become non-homogenous over time and as the final preparations are destined to be packaged in syringes, it would not be easy to resuspend the cells prior to injection. Thus, such gels could be appropriate for preparations that would be used rapidly, but not for preparations that would be stored. Moreover, even if the poor viscosity would not cause any problems for intratendinous injection, it could be responsible for leakage in the case of peritendinous application, for example at the surface of a wounded site. Among these three gels, Ostenil seems the least affected by sedimentation and could be a more desirable candidate.

Ostenil Tendon and Sinovial 1.6% presented intermediate survival results, which were very close to each other. Sinovial 1.6%, even with a viscosity similar to standard Ostenil, presented a lower survival compared to this gel. It is likely that the negative impact on the cells is thus not due to shear stress during resuspension, but simply to a weaker tolerance of the cells towards this gel and thus it would not be an ideal candidate. Despite a much higher viscosity, Ostenil Tendon

presented a similar survival to Sinovial 1.6%. The survival in Ostenil Tendon was also much better than for Ostenil Plus, which shared a similar viscosity. It is probable that some cells died during the resuspension due to shear stress, but Ostenil Tendon did not seem to negatively impact the cells and seemed well tolerated. This gel presents an ideal compromise between a high rate of cell survival and an adapted viscosity for long-term storage and for delivery in zones where leakage would be a problem.

Tendinopathies are already treated with different types of injections, notably PRP (at a volume of 500 µl per site) is frequently used in our hospital. Many cell types have been used for tendon treatment, but the optimal number of cells required remains to be found (20). Based on our experience, we estimate that 2.5 million cells per treatment would be adequate. Concentrations ranging from 1 to 10 million cells are frequently found with stem cells in animal (21-23) and in human (24) models through in vivo experimentation. For these reasons, we prepared syringes with 2.5 million cells in 500 µl of gel in the second part of the study to mimic what could be used in clinics for the treatment of tendons. When aspirating the cell/gel formulations with a syringe we used only the syringe without a cannula or needle to allow the best protection of the cell preparation. However, when extruding the cells, we used a needle of 22 G to be similar to what could be used in the clinic. We did preliminary testing of needles from 20 G to 30 G with very good results up to 23 G and could see a drop in survival with 30 G, with the highest impact on more viscous gel preparations. With other cell therapies for tendinopathies using PRP, we generally used 20-G needles for direct injection into the tendon (19).

Hyalgan was not available for this experiment (due to supply problems), but hFPTs were tested with all of the other gels and the results were very encouraging. After 72 hours at 4°C, the survival rates presented the same trend to what was found in wells with a lower concentration. Synolis V-A and Ostenil Plus presented the lowest survival, Sinovial 0.8% had the highest survival rates and the other gels were in the middle. Furthermore, the survival for each gel after 72 hours of storage was much higher compared to the previous results with lower cell concentrations and the differences between gels were lower. It is probable that a higher cell concentration leads to a better preservation during the preparation. The fact to use larger volumes for the preparation also allowed easier manipulation for the resuspension of the cells.

The LIVE/DEAD assay showed that many cells maintained the integrity of their membrane and that they were still metabolically active, but it is important to note that they did not lose their capacity to migrate, attach and proliferate. The recovery assay highlighted this fact. After 3 days of storage at 4°C, the cells extruded from all the gels were able to attach to culture material and to proliferate. The cells from Ostenil, Ostenil Plus, Ostenil Tendon and Sinovial 0.8% reached 90 to 100% confluency after 7 days and presented a proliferation profile very close to what was found with cells at the same passage which had always been grown in monolayer culture and that had not been in contact with any gel. There was a slight delay for the cells from Sinovial 1.6% and Synolis V-A, but they were still able to reach 90 to 100% confluency after 10 days. These results are excellent and do not suggest further criteria for a discriminative choice between the different gels.

Based on the global results, Ostenil Tendon HA gel seems the best candidate for further development and for the formulation of one preparation that could be used therapeutically. Indeed, this gel presents a very good compromise between cell survival and sufficient viscosity. Moreover, the recovery of cells after 3 days of storage at 4°C is excellent. With the off-the-shelf availability of hFPTs, it would be possible to prepare cell suspensions in this gel on demand from the clinic. The final cell preparations could be prepared under Good Manufacturing Practice (GMP) along with the associated simple logistics to deliver them to the patient. Importantly, they can be stored at 4°C at least up to 72 hours, allowing good flexibility for clinical usage.

Conclusion

With the view of potentially using hFPT preparations clinically, the use of medium, serum, or any other growth supplement was avoided in the preparations with HA gels. With such conditions, the preservation of the cell suspensions in gels was improved by a refrigerated temperature and it was possible to store multiple preparations up to 3 days.

hFPTs had low survival in Synolis V-A and Ostenil Plus, even without storage and these gels are thus not adapted for a formulation containing hFPTs. Hyalgan, Sinovial 0.8%, and Ostenil allowed a high degree of hFPT survival and could be good candidates for the preparation of hFPT suspensions. Due to their low viscosity, they are not adapted for storage and would be more suitable for intratendinous injection than peritendinous injection into a defect, where the risk of leakage would be higher. Ostenil Tendon and Sinovial 1.6% presented intermediate results in terms of cell survival, but as Ostenil Tendon viscosity is approximately 5 times higher than Sinovial 1.6%, it is a more valuable choice for a formulation that could also be used on the surface of a wound, where a higher viscosity should allow a better maintenance of the cellular preparation. It therefore presents the best compromise between cell survival and adapted characteristics for conservation and delivery. Moreover, it is possible to package hFPT suspensions in syringes with 2.5 million cells in 500 µl of gel, which represents an adapted formulation for a clinical use. With such a formulation, it is now possible to imagine an in vivo delivery of hFPTs to further evaluate and characterize this cell source.

Acknowledgments

These studies were funded in part by the Orthopaedic Hospital Foundation of Lausanne and particularly for the support of the doctoral project (AG). We thank the Sandoz Family Foundation and the S.A.N.T.E Foundation for their continued support in our Transplantation Program. We thank Dr. Philippe Abdel-Sayed for his help in rheological measurements and Prof. Brigitte Jolles-Haeberli for helping with obtaining samples of all HA gels to be used in this study. The authors declare no conflicts of interest.

References

1. United States Bone and Joint Initiative. Musculoskeletal Injuries. The Burden of Musculoskeletal Diseases in the United States. Rosemont, IL: American Academy of Orthopaedic Surgeons; 2011. p. 129-79.

2. Tempelhof S, Rupp S, Seil R. Age-related prevalence of rotator cuff tears in asymptomatic shoulders. J Shoulder Elbow Surg. 1999;8(4):296-9.

3. Hess GW. Achilles tendon rupture: a review of etiology, population, anatomy, risk factors, and injury prevention. Foot Ankle Spec. 2010;3(1):29-32.

4. Gaida JE, Alfredson H, Kiss ZS, Bass SL, Cook JL. Asymptomatic Achilles tendon pathology is associated with a central fat distribution in men and a peripheral fat distribution in women: a cross sectional study of 298 individuals. BMC Musculoskelet Disord. 2010;11:41.

5. Paavola M, Kannus P, Järvinen M. Epidemiology of Tendon Problems in Sport. In: Maffulli N, Renström P, Leadbetter W, editors. Tendon Injuries. London: Springer; 2005. p. 32-9.

6. Kannus P. Structure of the tendon connective tissue. Scand J Med Sci Sports. 2000;10(6):312-20.

7. Magnusson SP, Langberg H, Kjaer M. The pathogenesis of tendinopathy: balancing the response to loading. Nat Rev Rheumatol. 2010;6(5):262-8.

8. Maffulli N, Khan KM, Puddu G. Overuse tendon conditions: time to change a confusing terminology. Arthroscopy. 1998;14(8):840-3.

9. Kannus P, Jozsa L. Histopathological changes preceding spontaneous rupture of a tendon. A controlled study of 891 patients. J Bone Joint Surg Am. 1991;73(10):1507-25.

10. James R, Kesturu G, Balian G, Chhabra AB. Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. J Hand Surg Am. 2008;33(1):102-12.

11. Sharma P, Maffulli N. Tendon injury and tendinopathy: healing and repair. J Bone Joint Surg Am. 2005;87(1):187-202.

12. Voleti PB, Buckley MR, Soslowsky LJ. Tendon healing: repair and regeneration. Annu Rev Biomed Eng. 2012;14:47-71.

13. De Buys Roessingh AS, Hohlfeld J, Scaletta C, Hirt-Burri N, Gerber S, Hohlfeld P, et al. Development, characterization, and use of a fetal skin cell bank for tissue engineering in wound healing. Cell Transplant. 2006;15(8-9):823-34.

14. Hohlfeld J, de Buys Roessingh A, Hirt-Burri N, Chaubert P, Gerber S, Scaletta C, et al. Tissue engineered fetal skin constructs for paediatric burns. Lancet. 2005;366(9488):840-2.

15. Grognuz A, Scaletta C, Farron A, Raffoul W, Applegate LA. Human Fetal Progenitor Tenocytes for Regenerative Medicine. Cell Transplant. 2015; http://dx.doi.org/10.3727/096368915X688515.

16. Tezel A, Fredrickson GH. The science of hyaluronic acid dermal fillers. J Cosmet Laser Ther. 2008;10(1):35-42.

17. Quintin A, Hirt-Burri N, Scaletta C, Schizas C, Pioletti DP, Applegate LA. Consistency and safety of cell banks for research and clinical use: preliminary analysis of fetal skin banks. Cell Transplant. 2007;16(7):675-84.

18. O'Brien M. Structure and metabolism of tendons. Scand J Med Sci Sports. 1997;7(2):55-61.

19. Akhundov K, Pietramaggiori G, Waselle L, Darwiche S, Guerid S, Scaletta C, et al. Development of a cost-effective method for platelet-rich plasma (PRP) preparation for topical wound healing. Ann Burns Fire Disasters. 2012;25(4):207-13.

20. Docheva D, Muller SA, Majewski M, Evans CH. Biologics for tendon repair. Adv Drug Del Rev. 2015;84:222-39.

21. Guest DJ, Smith MR, Allen WR. Equine embryonic stem-like cells and mesenchymal stromal cells have different survival rates and migration patterns following their injection into damaged superficial digital flexor tendon. Equine Vet J. 2010;42(7):636-42.

22. Hoffmann A, Pelled G, Turgeman G, Eberle P, Zilberman Y, Shinar H, et al. Neotendon formation induced by manipulation of the Smad8 signalling pathway in mesenchymal stem cells. J Clin Invest. 2006;116(4):940-52.

23. Nourissat G, Diop A, Maurel N, Salvat C, Dumont S, Pigenet A, et al. Mesenchymal stem cell therapy regenerates the native bone-tendon junction after surgical repair in a degenerative rat model. PLoS One. 2010;5(8):e12248.

24. Lee SY, Kim W, Lim C, Chung SG. Treatment of Lateral Epicondylosis by Using Allogeneic Adipose-Derived Mesenchymal Stem Cells: A Pilot Study. Stem Cells. 2015;33(10):2995-3005.

Chapter V

–

Horse Decellularized Extracellular Matrix as a Scaffold for Human Fetal Progenitor Tenocytes

Foreword

In the last chapter, it was possible to obtain a formulation of hFPTs within a gel for injectable delivery. In the present chapter, we evaluate equine superficial digital flexor tendon (SDFT) as a potential source for the creation of extracellular matrix (ECM) scaffolds, which would be more adapted for extended injuries. Different solutions are used for decellularization of the tissue and analysis is based on histology, DNA dosage and biomechanical assessment.

Horse Decellularized Extracellular Matrix as a Scaffold for Human Fetal Progenitor Tenocytes

This chapter was not submitted for publication. It will be combined with a review on biomaterials for the treatment of rotator cuff injuries performed in collaboration with Daniele Angelella for his Master project in Medicine.

A. Grognuz[1], T.M. Thébault[1], A. Farron[2], D.P. Pioletti[3], W. Raffoul[1], C. Scaletta[1], D. Angelella[1] and L.A. Applegate[1]*

1) Unit of Regenerative Therapy, Service of Plastic, Reconstructive and Hand Surgery, Department of Musculoskeletal Medicine, University Hospital of Lausanne, Switzerland

2) Service of Orthopaedics and Traumatology, Department of Musculoskeletal Medicine, University Hospital of Lausanne, Switzerland

3) Laboratory of Biomechanical Orthopedics, Institute of Bioengineering, EPFL, Lausanne, Switzerland

***Address for Correspondence:**
Professor Lee Ann Laurent-Applegate
University Hospital of Lausanne (CHUV)
Service of Plastic, Reconstructive and Hand Surgery
Unit of Regenerative Therapy (UTR)
EPCR/Croisettes 22
1066 Epalinges
SWITZERLAND
E-mail: Lee.Laurent-Applegate@chuv.ch
Telephone: + 41 21 314 35 10
Fax: + 41 21 887 84 14

Abstract

Chronic (tendinopathies) and acute injuries are frequent afflictions. The use of biologics and cellular therapies could help to improve tendon healing, which is often long and unsatisfactory. When facing extended injuries, material with sufficient biomechanical properties is required and could permit delivery of cellular sources. In this study, we evaluate different detergent solutions with the aim of producing a decellularized extracellular matrix (ECM) scaffold beginning with equine superficial digital flexor tendon (SDFT) from accredited food industry with traceable sources. Different analytical methods are used to assess the quality of the decellularization including DNA content as well as the conservation of the structure and of the biomechanical properties. With one of the tested treatments, it was possible to have a resulting scaffold with interesting biomechanical properties and good biocompatibility with human Fetal Progenitor Tenocytes (hFPTs) incorporated. This lays the foundation for a future bioengineered human tendon replacement.

Key words: Cell therapy; Human fetal progenitor tenocytes; Extracellular matrix scaffold; Decellularization; Tendon healing.

Introduction

Treatments for acute or chronic tendon injury range from conservative care such as physical therapy up to surgical interventions. Regardless, the final outcome obtained is often of disappointing and poor quality (1-3). During the last decade, biologics and cellular therapies have become of interest as potential treatments for tendon injuries (4). We previously evaluated the characteristics of hFPTs *in vitro* and have obtained promising results (5). For the case of tendinopathies (chronic injuries) or simple lacerations, injectable cell preparations could be interesting and easily delivered to accelerate the healing process. Based on these premises, we resuspended hFPTs in hyaluronic acid gels to create a formulation which would be adapted for small defects (article accepted for Cell Medicine). As for the cases of more serious injuries, surgical intervention is normally necessary and material with stronger mechanical properties must often be used to fill a defect such as that of rotator cuff or to replace a tendon in the hand.

The example of the hand tendon injuries highlights the need to develop new strategies. In case of deep-tissue injury to the hand, at least one flexor or extensor tendon is injured in 92.5% of the cases and in 28% of these cases, multiple tendons are concerned (6). If an extended injury is present, an autograft is frequently needed and it remains almost the only choice, as no suitable alternative exists to date. The harvest of the palmaris longus or plantaris tendon is the prime choice as other graft sites potentially lead to disability. Nevertheless, these two sources are not always present in individuals (7, 8) and in many cases are not optimal for a surgery (8, 9). Thus, autograft remains the ideal choice, but donor site morbidity and potential lack of material are drawbacks of this method and alternatives could be helpful to complete the limited choices available until today.

Various types of scaffolds can be used in tendon healing. A good biocompatibility and sufficient mechanical properties are criteria of high importance among others. Synthetic scaffolds have been tested for tendon treatment (10). They present some advantages such as a well controlled process in formulation and manufacturing and the possibility to adapt the structural, physical and mechanical properties. However, the main problem with such medical devices is their poor biocompatibility and limited integration. Even polymers which have been widely used for tendon repair such as poly-glycolic acid (PGA) and poly-lactid glycolic acid (PLGA) have presented varying results (11, 12). Biological scaffolds are an alternative to synthetic scaffolds. Among them, decellularized extracellular matrix (ECM) scaffolds have presented encouraging results. Such scaffolds are prepared from natural tissue and are processed to make them usable for allografts or xenografts. As cell-associated immunogenic antigens could drive an immune reaction, it is important within the process to have an entire decellularization of the tissue (12). The treatment must be strong enough to eliminate the cell material, but balanced to conserve as well as possible the initial structure, composition and mechanical properties of the tissue.

The process of decellularization has been reviewed and follows generally the following steps (13, 14). First of all, the undesired tissues need to be removed to improve access to the desired structure. Thereafter, the decellularization process can begin and the cell membrane is disrupted

with the help of physical or chemical treatments. Enzymatic treatments are sometimes used to eliminate remaining materials. It is then necessary to rinse the ECM to eliminate the products employed to lyse cells as they would have deleterious effect on the seeded cells or on the host tissue and would compromise the therapeutic effect. Sterilization is finally required to avoid a risk of contamination of the final preparation.

The matrix obtained can be employed alone or it can serve as a support for the delivery of cells or biologics. If processed correctly, the ECM conserves its proteins (15) and even growth factors (16-18) and could sustain healing somewhat on its own. The tridimensional structure and biochemical composition provide an adapted environment for cell adhesion and migration and the interaction with host's tissue is rapid (15). Once grafted, they can permit a temporary support and host tissue will progressively replace the grafted ECM over time. (12).

Many biological sources have been tested in the past. Small intestinal submucosa (SIS) from pigs has been widely used and it was tested for ligament and tendon repair already for more than 20 years (19, 20). Some sources have lead to commercial products with full regulatory approval by the FDA and other regulatory agencies worldwide. Among them, some are indicated for tendon augmentation in rotator cuff tendinopathies, such as porcine SIS (Restore, Orthobiologic Implant, CuffPatch), dermis of human (Graftjacket, Allopatch HD, ArthroFlex), bovine (TissueMend, Bioblanket) or porcine (Zimmer, Conexa) origin, equine pericardium (OrthoADAPT) and human fascia lata (AlloPatch) and different groups have evaluated their characteristics and efficacy (21-25). Among these products, none is directly obtained from tendon and except for AlloPatch from facia lata, the biomechanical characteristics are far from those found in native tendon (22).

Starting with tendon as a source could lead to an ideal scaffold with better overall mechanical results. Indeed, tendon structure is very specific and optimally adapted to its role to transmit forces from muscle to bone. The tenocytes are dispersed in a well-organized extracellular matrix (ECM), mainly composed of fibrillar collagen I arranged in fibers and fascicles for excellent tensile resistance. Moreover, other molecules such as proteoglycans, glycoproteins and other collagens play a role in the development, homeostasis and repair of tendon and their preservation in the final product could have a positive impact (1, 26, 27). Strategies have been tested to decellularize tendon of various species and included rodent (28), porcine (29, 30), canine (31), rabbit (32-35), equine (36, 37) and even human (38-40). Many species have been tested as a proof-of-concept, but tendons of small animals would not be well adapted to humans. Human cadaveric tendons are interesting for the obvious reason of similar characteristics to the tendon to be replaced, but their availability is limited. The equine superficial digital flexor tendon (SDFT) presents an interesting source due to its large dimension. Moreover, equine tendons are pieces discarded from the food industry, available in high numbers and obtained from accredited and traceable sources and thus could be valorized. As it is possible to have an excellent traceability with tendons which are obtained from a source of quality, this would increase security for disease transmission.

As previously mentioned, the tissue can be decellularized with physical or chemical treatments. Among the latter group, detergents have the capacity to solubilize the cell membrane and we have tested six different methods with products alone or in combination. Sodium dodecyl sulfate (SDS) and t-octyl-phenoxypolyethoxyethanol (Triton X-100, later referred as Triton) are respectively ionic and non-ionic detergents. They have been the detergents the most widely reported for decellularization of different tissues and organs (13, 14). Tri-n-butyl phosphate (TBP) is a zwitterionic detergent which has permitted good results on tendon in different studies (28, 41). Finally, Tween 20 is a polysorbate surfactant that is largely employed in pharmacological applications and which could prove to be effective for tissue decellularization.

The effectiveness of the decellularization must be controlled. In parallel, it is important to evaluate the impact of treatment on the structure. Histological staining and DNA measurements can answer these questions directly. Hematoxylin-Eosin (HE) is a simple and very common stain allowing the detection of the cell nuclei which are stained in purple and the extracellular matrix in pink. DAPI, with its specific binding to DNA permits a second control with bright fluorescence where nuclear material is present, but it does not show the structure of tissue. To evaluate the efficiency of decellularization, it is also possible to analyze the specific dosage of DNA in the tissue. Concerning the impact on structure (mainly composed of aligned collagen), the evaluation is made easier by Sirius Red which interacts relatively specifically with collagen to augment its birefringence (optical anisotropy) and therefore allows observation of the fibers in colors varying from green to red depending on the depth of the section and the size of the collagen fibers (42, 43).

Despite giving information on the structure, histological sections do not permit assessment on the final impact of mechanical properties. Biomechanical properties are nevertheless also very important as these materials are destined to be grafted and will need to sustain and transmit forces while avoiding reruptures. It is possible to use a tensile testing machine to obtain several biomechanical parameters. The sample is fixed between two clamps and it is put under tension by lengthening the distance between the two clamps and the elongation (mm) can be followed over time. As the distance increases, the force (N) applied by the tissue on the clamp linked to a sensor can be recorded. The conversion of elongation to strain (%) by dividing elongation by the initial length and the conversion of force to strain (MPa) by dividing force with the cross-section permits a normalization of the results which do not depend on the initial dimensions of the sample anymore. Usually, the results are presented on a graph showing the stress as a function of strain. The slope of the plotted curve in its linear region corresponds to the Young's modulus (or elastic modulus, MPa) and the higher the value, the more rigid is the sample. This parameter represents the capacity to transmit forces as a function of elongation of the tissue, but avoids reference to the dimension of sample. Results are sometimes presented as stiffness (N/mm), but the value is then influenced by the dimensions of the tested sample.

Materials and Methods

Tissue Harvesting and Conservation

The tendons were obtained from Profil Export (Chavrieu Chavagneux, France) with traceability for each source animal. At the slaughterhouse, the horse superficial digital flexor tendons were harvested, put into hermetic bags labelled with identification of each source animal and transferred to the production keeping a constant refrigerated temperature. At the production site, the tendons were rinsed 3 times for a few seconds with 70% ethanol and transferred into a conservation buffer consisting of a 50 mM Tris buffer solution at pH 8.0 (Sigma, St. Louis, MO, USA, code No. T6066) with 5 mM sodium ethylenediaminetetraacetic acid (EDTA: Sigma, code No. 03680), 0.4 mM phenylmethylsulfonyl fluoride (PMSF: AppliChem, Darmstadt, Germany, code No. A0999) and 1% (v/v) penicillin-streptomycin (Pen-Strep: Gibco, Life Technologies Ltd., Paisley, UK, code No. 15140-122). The conservation buffer was chosen based on previous studies aiming to produce decellularized tendons (28, 29). The basic pH inhibits many proteases functioning in acidic conditions while EDTA inhibits metalloproteases and PMSF inhibits serine proteases. The tendons were finally transferred to the laboratory in Lausanne University Hospital where they were drained and conserved in hermetic bags at -80°C until preparation.

Tissue Preparation (Dissection to Desired Dimensions)

The tendons were thawed at room temperature in order to facilitate the removal of the surrounding tissue with a scalpel. They were subsequently frozen at -20°C to obtain a sufficient rigidity and were chopped into pieces of 1.2 mm depth with the aid of a dermatome (Aesculap GA630; Aesculap AG, Tuttlingen, Germany). The pieces were frozen at -20°C again and dissected to a size of 150 mm in length and 10 mm in width with a guide and a scalpel. They were conserved at -80°C until usage and the same pieces also served as control samples.

Decellularization

The tendon pieces were further cut to obtain pieces of 20 mm in length. They were placed in 6-well plates and decellularized with 6 ml of decellularizing solution under constant circular agitation at 300 RPM. The different treatments tested were 1% and 2% SDS (Ambion, Thermo Fischer Scientific Inc, Waltham, MA, USA, code No. AM9820), 1% Triton (Applichem, code No. A1388), 1% SDS - 1% Triton, 1% TBP (Sigma, code No. 158615) or 1% Tween 20 (Applichem, code No. A1389). Treatment periods of 24 h, 48 h and 72 h (with replenishment of solutions every 24h) were tested and images of the pieces were taken with a compact camera and with a Leica M205 FA stereomicroscope equipped with a Leica DFC 295 color camera. After the decellularization process, the pieces were rinsed with deionised water 3 times rapidly and 3 times for 20 min.

Histology

Following the decellularization and rinsing processes, the tendons were fixed in 4% (w/v) neutralized formalin solution (J.T. Baker, Deventer, The Netherlands, code No. 3933) for 24 h at 4°C, washed 3 times with phosphate-buffered saline (PBS: NaCl 6.8 g/l, Na_2HPO_4 1.5 g/l, KH_2PO_4 0.4 g/l, Bichsel, Interlaken, Switzerland, code No. 100 0 324) and subsequently dehydrated and embedded in paraffin (Merck Millipore, Darmstadt, Germany, code No. 111609). Sections of 5 μm were performed in the length direction of tendon and sections corresponding to the middle depth were taken for staining. Each tendon was stained with HE, SR and DAPI.

Images of HE sections were obtained with a Leica DMR bright field microscope equipped with a Leica DFC 295 color camera. SR sections were observed and recorded under polarized light with an Olympus AX70 microscope. The two filters were at right angles to each other in order to have the background as dark as possible. The images were taken with an Olympus DP70 color camera. DAPI sections were recorded with an inverted Olympus IX81 microscope equipped with fluorescence and a digital black and white Andor iXon camera. For DAPI, all the sections were processed on the same day and recorded with the same exposition parameters in one color channel coded with 16 bits. The images were then treated within Image J and the same display range limits were applied to all the pictures. Finally, the images were converted to 8 bits RGB with the signal shown in blue.

DNA Content

To better evaluate the quality of the decellularization process, extraction, purification and quantitative dosage of DNA were realized. Prior to decellularization, one adjacent piece was taken as the control and conserved at -80°C for every condition tested. For each of the 18 samples (six solutions and three time points) and of their 18 controls, 25 to 35 mg of tissue were minced in small pieces and dehydrated at 80°C overnight. The tissues were then digested with proteinase K and purified according to the instructions of the kit "Genomic DNA from tissue" (Macherey-Nagel, Düren, Germany, code no 740952). The determination of DNA quantity was realized in a black 96-well plate (to avoid cross emission) with the "Quant-it PicoGreen" kit (Molecular Probes, Life Technologies Ltd, Paisley, UK code No. 7589) which relies on the binding of a fluorophore to DNA. The emission was recorded during 180 s and the DNA quantity was determined based on a standard curve and converted in relation to the initial dry mass for allowing comparisons.

Biomechanical Assessment

Biomechanical tests were performed on pieces of tendons measuring 150 mm in length, 10 mm in width and 1.2 mm in depth which were prepared according to the protocol described above. One group consisted of seven pieces obtained from the same tendon which were not subject to decellularization to control the intra-variability within a tendon (control intra.). One group

consisted of 10 pieces obtained from different tendons which were not decellularized either to control the inter-variability between tendons (control inter.). As the inter-variability was not significantly higher than the intra-variability, the two other groups consisted of 10 pieces each from different tendons with one treated for 72 h with 1% SDS and the other one with 1% Triton for the same period. The measures were realized with an Electropuls Dynamic Test System (Instron E3000; Instron, Norwood, MA, USA). The pieces were fixed with 2 clamps especially designed in collaboration with the workshop of the Institute of Mechanical Engineering at EPFL (Fig. 1). A gap of 80 mm was left between the clamps and a precharge of 5N was applied to the tissue. The new position was defined as the origin, the gap was considered as the initial length and the elongation and force were set to zero. A rate of 25 mm/min was then used until tendon rupture. The force and elongation were recorded approximately every 20 ms all along the process and subsequently normalized to stress and strain. The maximal stress and strain were recorded. The Young's modulus was determined by calculating the slope of the stress strain curve in its linear region. The process was standardized by calculating the slope of the regression line for the values comprised between 4% and 2% prior to failure. The energy to failure was calculated by integration with the rectangle method for each measure of force and elongation.

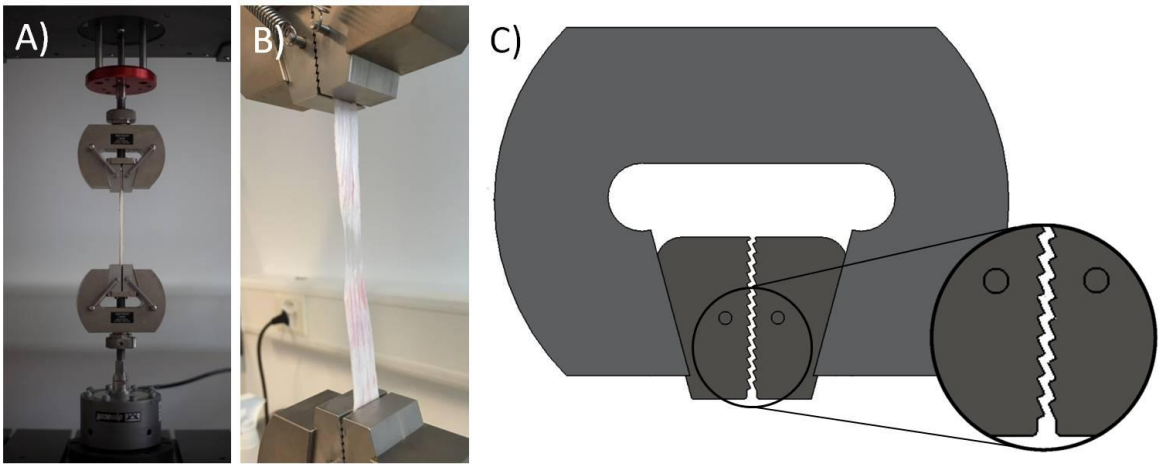

Figure 1: Electropuls Dynamic Test System used for biomechanical assessment.
A: The tendon band is fixed between the two clamps with a sufficient length in between. One of the two clamps is attached to a force captor (visible at the bottom). B: The tested specimen is stretched up to failure. C: Detailed pattern of the clamps specifically designed for the evaluation of tendon tissue.

Matrix Reseeding with hFPTs

Pieces of tendons decellularized with 1% SDS or 1% Triton for 72 h were used as matrix for cell reseeding. They were sterilized for 4 h with 70% ethanol and subsequently rinsed 3 times for 20 min with sterile PBS. They were then cut in sterile conditions to obtain pieces of 10 mm x 10 mm x 1.2 mm. The pieces were transferred into a 24-well plate and they were seeded with hFPTs from our cell bank (FE002-Ten). These cells were isolated from the Achilles tendon of a male 14-week gestation organ donation according to a protocol approved by the State Ethics Committee (University Hospital of Lausanne - CHUV, Ethics Committee Protocol No. 62/07:

14-week gestation organ donation, registered under the Federal Transplantation Program and its Biobank complying with the laws and regulations) and details about the creation of the cell bank and the culture conditions have already been published (5, 44). For this experiment, they were detached at the end of passage 5 to be seeded on the matrix at passage 6 with either 10^6 cells, 10^5 cells or without cells (decellularized controls). Cell suspensions at concentrations of $20x10^6$ cells/ml and $2x10^6$ were prepared with standard growth medium [Dulbecco's modified Eagle medium (DMEM: Gibco, code No. 41966-029) supplemented to 5.97 mM L-glutamine (L-glut: Gibco, code No. 25030-024) and with 10% fetal bovine serum (FBS: Sigma, code No. F7524)]. Suspensions of 50 µl were distributed on the ECM to seed either 10^6 cells or 10^5 cells on the ECM pieces and 50 µl of medium was distributed on the decellularized controls. After 30 min (to allow cell attachment), 500 µl of medium were added in every well. The medium was changed after 24 h and after 72 h, the samples were rinsed twice with PBS and then processed. Some of the samples were fixed in formalin solution for 4 h at 37°C, washed 3 times with PBS, dehydrated and embedded in paraffin with orientation to obtain sections of the depth. Sections of 4 µm were performed and an HE staining was realized. The other samples were tested for DNA content by following exactly the same protocol as for DNA quantification on decellularized samples.

Results

Decellularization

The control tendons were processed to obtain the same size of pieces as the tested items, but were not further processed with detergent solutions. Due to cycles with temperature changes for preparation and storage, a light alteration of the tissue was visible, but the effect was very limited. Tenocytes were still present between collagen fibers and the tissue still appeared much aligned, although some small gaps were present probably due to the processing (Fig. 2).

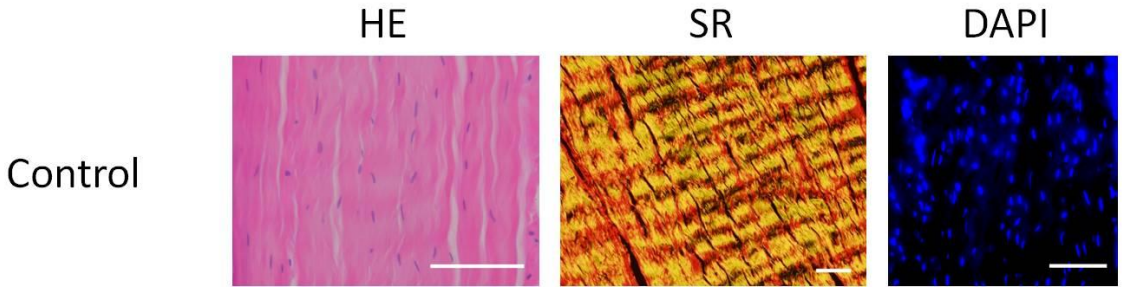

Figure 2: Histology of control horse tendon.
The images present the structure of a control horse tendon piece (cut to the desired dimensions, but not treated). HE staining shows elongated tenocytes in purple dispersed within the extracellular matrix in pink. The SR stains collagen and it allows a better observation of the structure under polarized light. The DAPI staining allows a better detection of the nuclei in blue. Sections of 4 µm. Scale bars: 100 µm.

The global aspect of the treated pieces was observed after the decellularization process. The pieces treated with 1% Triton or 1% Tween 20 all presented a white color and a regular overall

aspect. The pieces treated with 1% TBP presented the same aspect, but seemed to have lost elasticity. The pieces treated with SDS at 1% or 2% had shrunk and presented a jelly-like degraded aspect on the borders. The pieces treated with a mix of 1% SDS - 1% Triton also presented shrinkage and had changed in the global aspect, but to a lower extent than the latter. Figure 3 shows these observations for 72 h of treatment and the global aspect was similar also after 24-h and 48-h treatments.

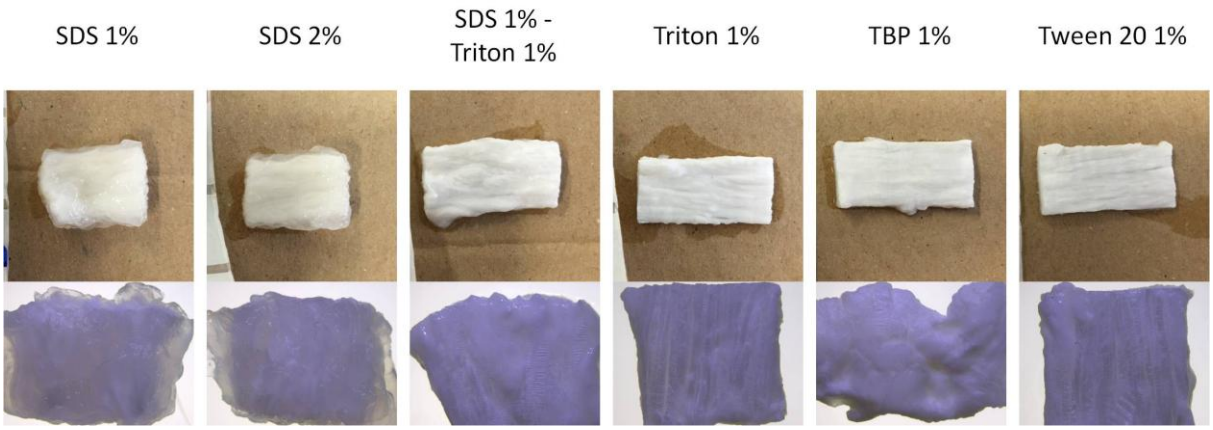

Figure 3: Pieces of tendon after 72 h of decellularization with various treatments.
Top line presents the morphology of tendon after a decellularization treatment of 72 h. Bottom line presents the same pieces observed with a stereomicroscope.

The following images present horse tendon after the decellularization process and subsequent staining with HE (Fig. 4) SR (Fig. 5) and DAPI (Fig. 6).

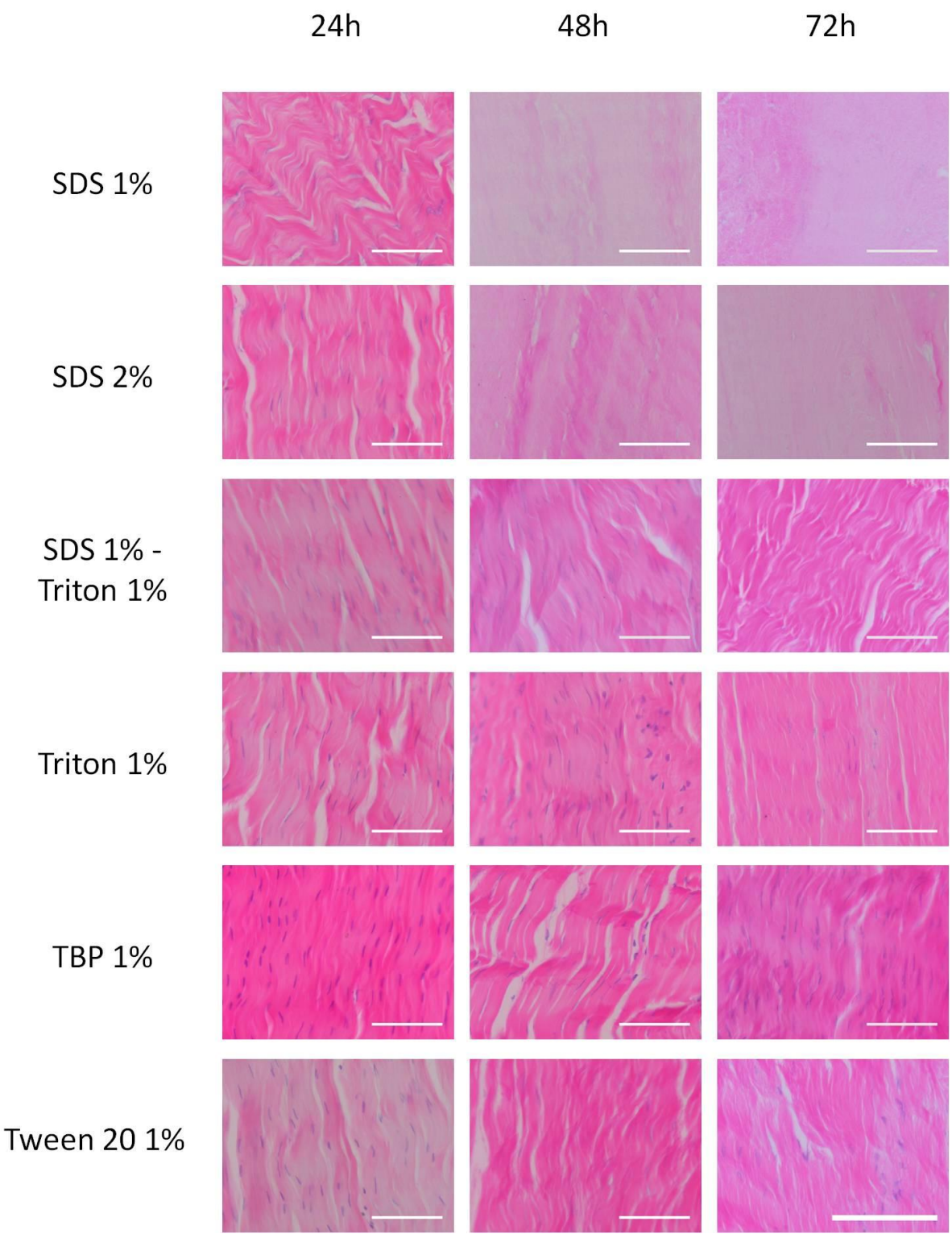

Figure 4: HE staining of horse tendon after decellularization processing.
Histological images of tendon tissues that were processed with various decellularizing solutions and for various time points. When present, nuclei appear in purple within the pink extracellular matrix. The zones presented were chosen in regions without apparent vascularization. Sections of 4 µm stained with HE and observed in bright field microscopy. Scale bars: 100 µm.

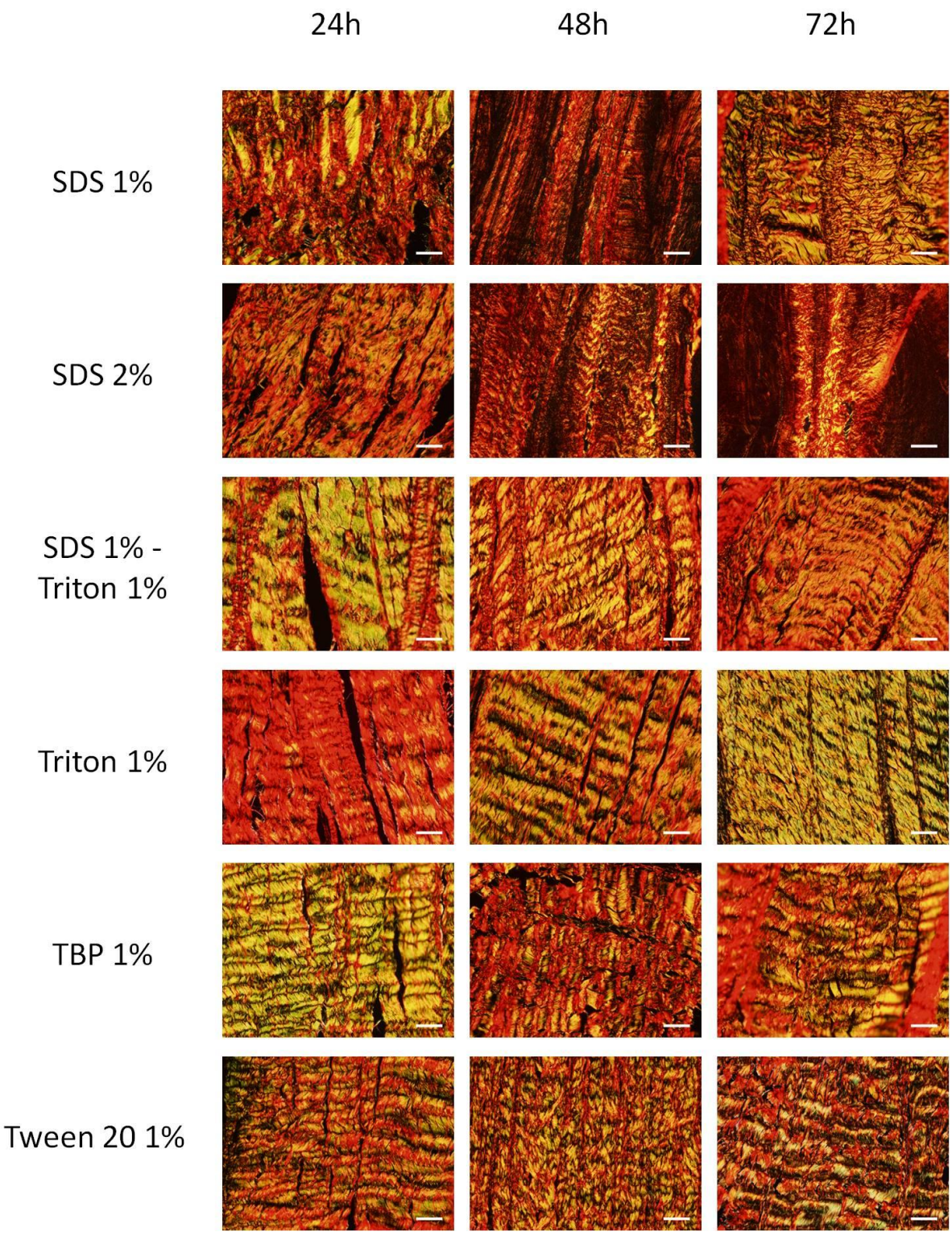

Figure 5: SR staining of horse tendon after decellularization processing.
Histological images of tendon tissues that were processed with various decellularizing solutions and for various time points. The collagen fibers appear in colors varying from green to red and it is possible to detect fiber orientation and to better evaluate the impact of the treatment on the structure than with an HE staining. Sections of 4 µm stained with SR and observed under polarized light. Scale bars: 100 µm.

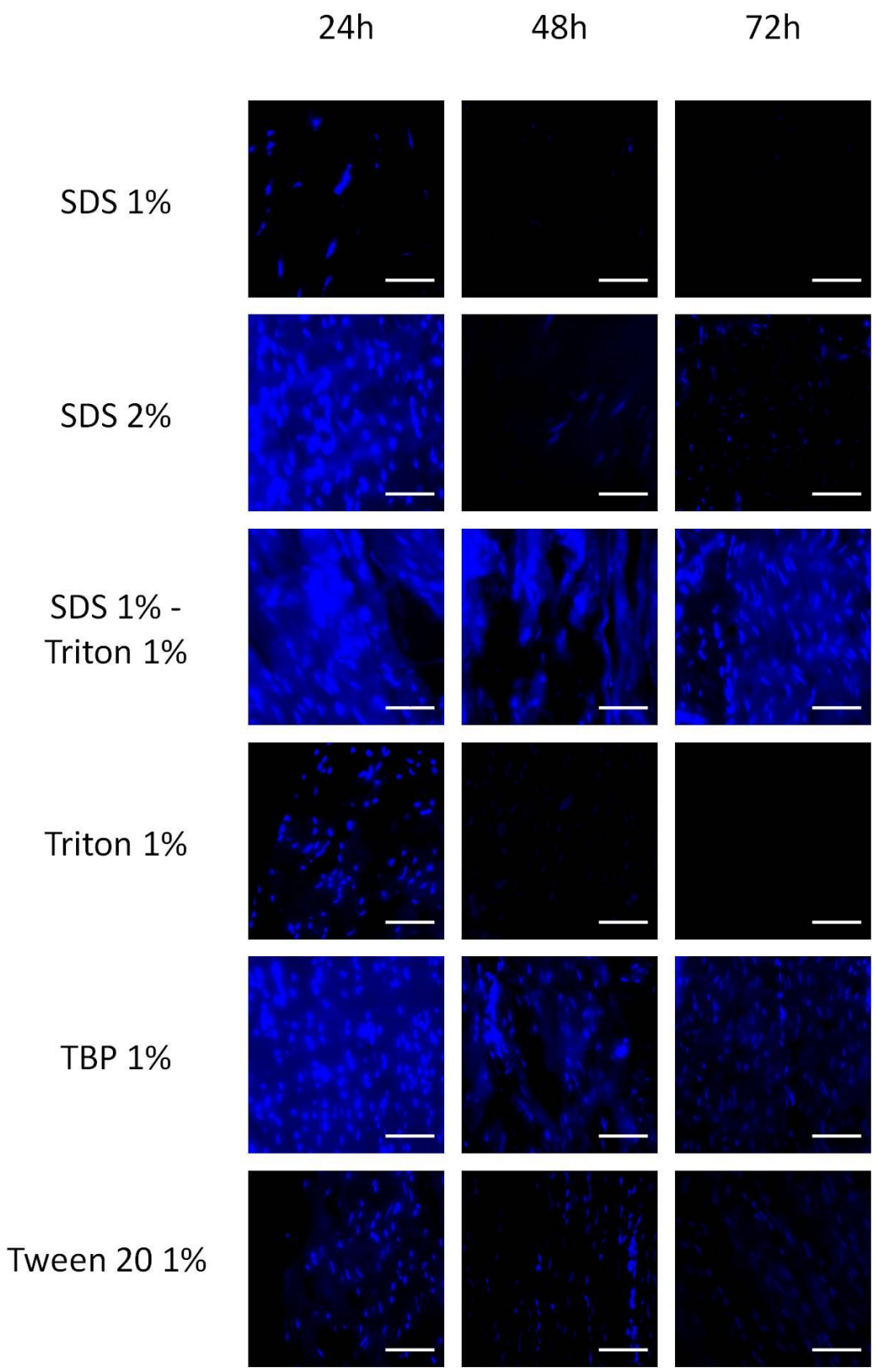

Figure 6: DAPI staining of horse tendon after decellularization processing.
Images of tendon tissues that were processed with various decellularizing solutions and for various time points. The DAPI allows detection of remaining DNA in blue and it is possible to better evaluate the efficiency of cell material removal than with an HE staining. The zones presented were chosen in regions without apparent vascularization. Sections of 4 µm treated with DAPI and observed in fluorescence microscopy. Scale bars: 100 µm.

Treatment with 1% SDS allowed a large diminution in cell number already after 24 h. With HE staining, the cells seemed lysed, but purple coloration was still detectable. The DAPI confirmed a large diminution in nuclear material, but some nuclei were still visible. A disorganization within the structure of the tissue was already visible at this time point. After 48 h and 72 h, it was not possible to detect the purple coloration anymore with HE, but a few very light blue areas of coloration were still detectable with DAPI. The tissue seemed to have lost its structure and appeared less dense both with HE and SR.

After 24 h, the treatment with 2% SDS seemed less efficient than the one with SDS at 1%. A higher proportion of cells were present and the structure was better conserved. After 48 h and 72 h, there were many similarities between the two treatments. The structure appeared less dense and disorganized alongside a large diminution in cell material. No cells could be detected with HE, but diffuse blue staining could still be detected with DAPI, even after 72 h.

Triton at 1% permitted to well conserve the structure throughout the treatment. Some gaps appeared between fibers, but the collagen was well aligned and seemed more intact than for other treatments. Nuclei could be easily detected after 24 h. After 48 h and 72 h, the cells were still visible but seemed lysed and the intensity of DAPI was strongly diminished. Nevertheless, the treatment was less effective on vessels and cellular material was clearly visible in these regions both with HE and DAPI.

The mix between SDS at 1% and Triton at 1% gave surprising results. As for 1% Triton, there were some gaps between fibers, but the structure seemed well conserved. With HE, cells seemed lysed and the purple color was diffuse, but nuclei were clearly visible with DAPI. As with 1% Triton, the treatment was ineffective for decellularizing vessels. Globally, these results were closer to Triton than to SDS treatments and decellularization was not achieved.

Treatment with 1% TBP permitted a good preservation of the tissue structure. Collagen alignment seemed well conserved with SR, although tissue appeared denser with HE. Anyway, the decellularization efficiency was extremely limited and most nuclei seemed completely intact, either for tenocytes or in vessels (no diffuse staining).

The results obtained with 1% Tween 20 were close to those obtained with 1% TBP. The impact on structure was limited, but the efficiency was very low concerning decellularization even though cells appeared more diffuse than for TBP at 48 h and 72 h.

DNA Content

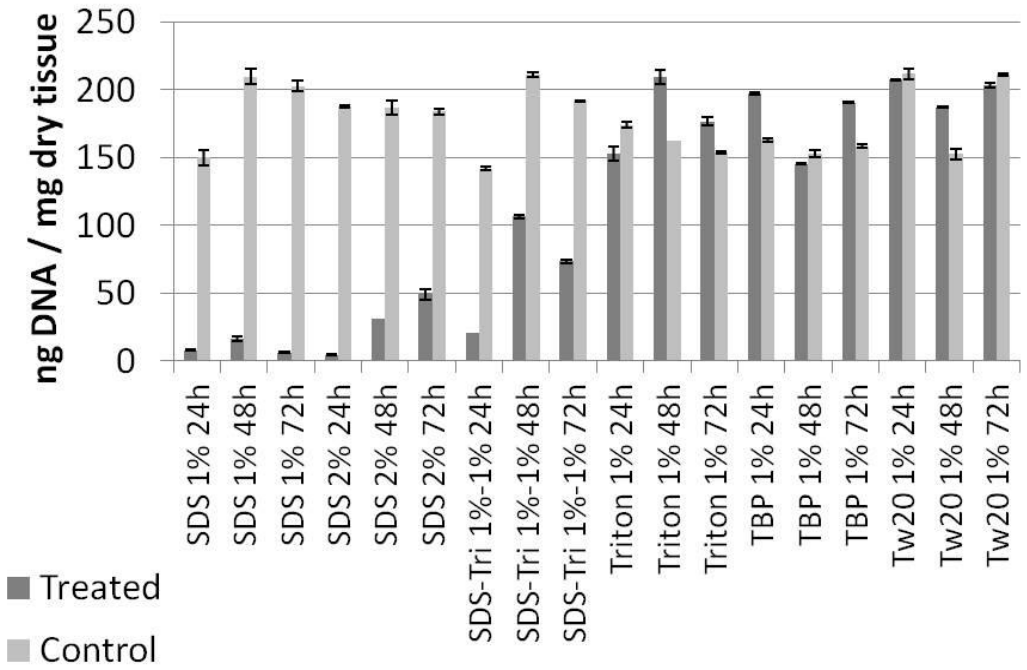

Figure 7: DNA content after decellularization processing.
DNA content is shown for each treatment and time point (dark grey) in comparison to an adjacent control tissue not decellularized (light grey). The results are presented as mean of 3 measures with standard deviations indicated as scale bars.

Some preliminary tests had shown variability in DNA content between the small tissues tested. To avoid a comparison to a mean value of great variability, it was preferred to compare each sample to a control from the neighbouring tissue but not decellularized (Fig. 7). SDS at 1% was very efficient in cell removal already at 24 h and the 3 time points presented an amount of DNA inferior to 20 ng per mg of dry tissue. SDS at 2% was slightly less efficient, but DNA was never superior to 50 ng per mg of dry tissue. The mix of 1% SDS - 1% Triton presented intermediate decellularization with DNA values comprised between 20 and 110 ng per mg of dry tissue. 1% Triton, 1% TBP and 1% Tween 20 were all inefficient for cell material removal and the DNA contents were close to non-decellularized controls with values comprising between 140 and 210 ng per mg of dry tissue. Interestingly, prolongation of the treatment period did not seem to favor cell removal for any treatment.

Biomechanical Assessment

The specially designed clamps permitted the measurement of biomechanical parameters without any tissue slipping. The tested samples and controls all presented a stress strain curve relatively similar in shape to the ones found for tendon with one toe region, one linear region and a rupture region (Fig. 8A).

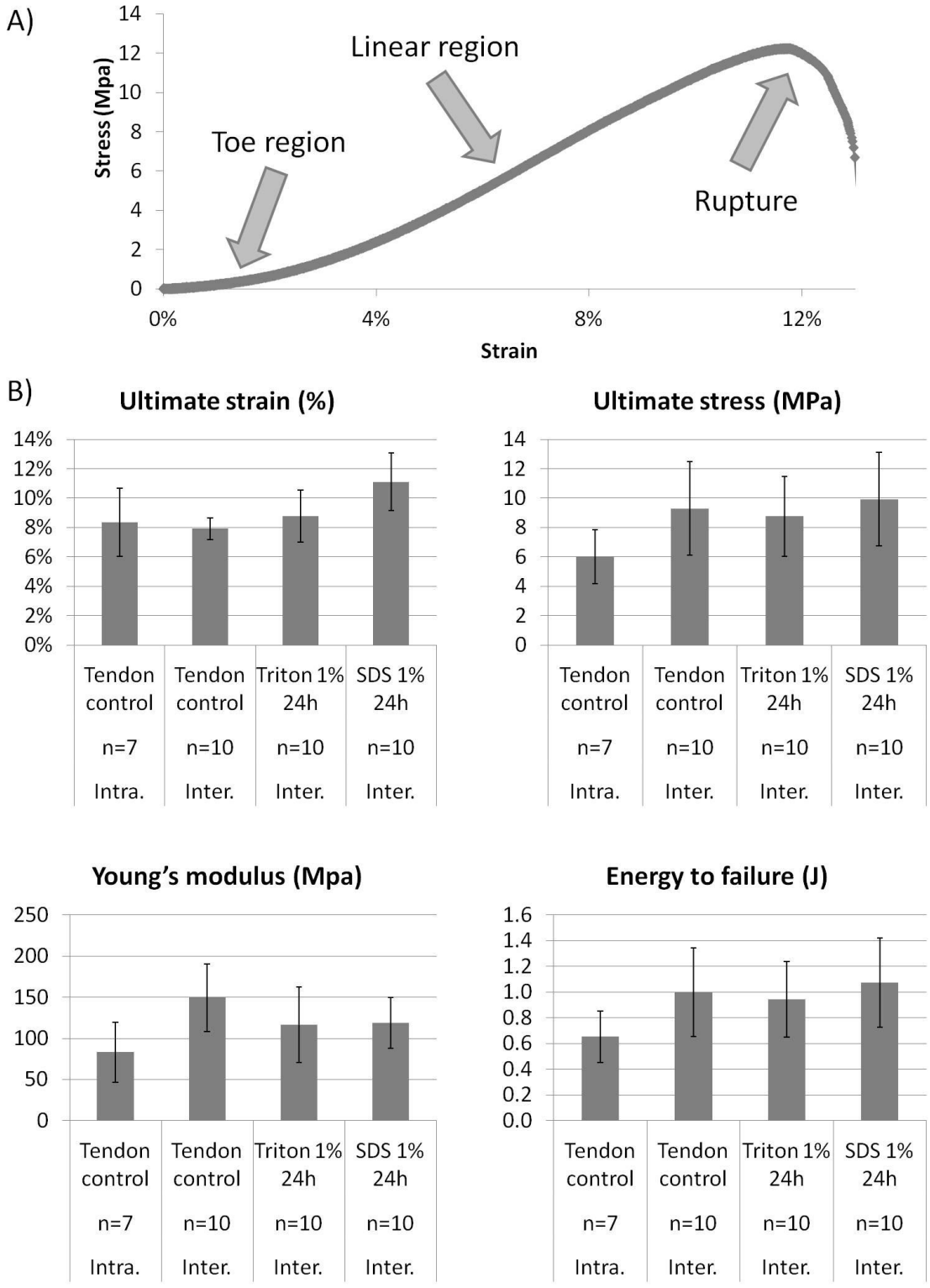

Figure 8: Biomechanical parameters obtained with control and decellularized bands of tendon.

A: The tested control and decellularized tendons all presented a stress-strain curve relatively similar in shape to natural tendon with a toe region, followed by a linear region and finally a rupture. B: The four tested groups are all constituted of samples measuring 150 mm x 10 mm x 1.2 mm. The first group is composed of sample obtained from the same tendon (intra.) while the others is composed of samples obtained from different animals (inter.). The results present the means with standard deviations indicated as error bars.

A summary of the biomechanical results is presented in Figure 8B. The intervariability between pieces coming from different tendons was compared to the intravariability of pieces obtained from the same tendon. The F-tests performed showed that the variances for the measure of ultimate stress, Young's modulus and energy to failure were not significantly different between the two groups ($p>0.05$). For ultimate strain, the variance was surprisingly significantly higher for the pieces obtained from the same original tissue as for pieces obtained from different tendons. Globally, these results indicate that the variances obtained are not due to the fact that the pieces come from different tendons.

For each parameter, the mean of the control group (inter), 1% Triton group and 1% SDS group were compared with t-tests. The mean ultimate strain of 11.1% obtained after treatment with SDS at 1% was significantly higher than for control (7.9%) and for Triton at 1% (8.8%). The difference was not significant between the two others. The mean ultimate stress for the control tissues was 9.3 MPa. It was also 9.3 MPa for 1% SDS and it was decreased to 8.8 MPa for 1% Triton, but this difference was not significant. The rigidity of the tissue was slightly diminished by the decellularization treatments, but not in a significant proportion. The control tissues had an average Young's Modulus of 150 MPa, while tissues treated with 1% SDS obtained 119 MPa and the ones treated with 1% Triton obtained 116 MPa. The total energy required to break the tissue was not significantly affected by the treatments. A mean of 0.94 J was required to break control tendons and tissues treated with Triton at 1%. The value for tendons treated with SDS at 1% was 1.07 J.

Reseeding

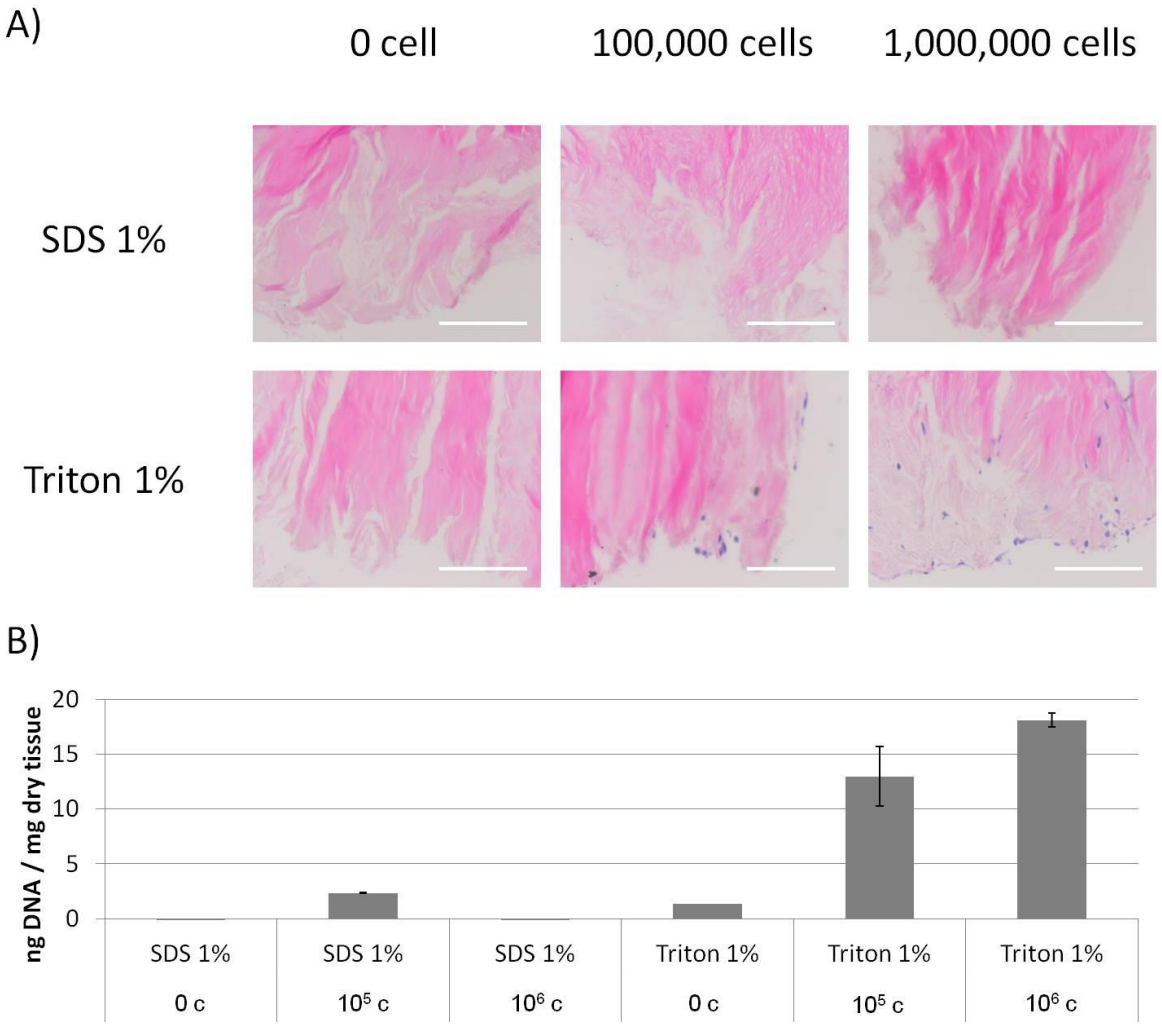

Figure 9: ECM reseeded with hFPTs.

A: ECM previously decellularized with 1% SDS or 1% Triton for 72 h were reseeded with hFPTs. The left images show that the ECM are correctly decellularized prior to reseeding with hFPTs. No cell can attach on the ECM obtained with 1% SDS. In opposition, the cells can attach to the surface of the matrix treated with 1% Triton and even infiltrate inside in regions where the tissue appears looser or gaps are present. Scale bars: 100 µm. B: The DNA measurements confirm the histological observations. The matrixes were correctly decellularized prior to seeding and only traces of DNA can be detected. hFPTs can attach to matrix treated with Triton, although the final DNA to matrix ratio is low. No DNA is detected in the matrix seeded with 10^6 cells, but traces of DNA are found in the one seeded with 10^5 cells. Results present the average of three measures one the same sample with standard deviations indicated.

hFPTs were not able to recellularize the ECM obtained after decellularization with SDS at 1% for 72 h (although DNA in trace amount was found for seeding with 10^5 cells). The observations done at the bottom of the wells showed that cells seemed to have adhered to the plastic, but then died. The biocompatibility with this matrix was not optimal and the cells in the well not only avoided the ECM but died which indicated the presence of soluble toxic factors. The rinsing step should be increased to assure a total elimination of the remaining detergent.

In contrast, hFPTs presented a standard morphology at the bottom of the wells and around the ECM. They had the capacity to attach to the ECM as indicated by histology and DNA quantification. The observation of histological sections done after 72 h indicated that hFPTs could adhere on all the faces of the ECM. Some cells were able to infiltrate the matrix up to several hundreds of microns where the tissues presented gaps or apparently looser tissue. Anyway, they could not reach deeper regions into the tissue within this period and the DNA measured in matrix was relatively low compared to original amount of equine DNA before decellularization (140−210 ng, Fig. 7). The matrix seeded with 10^5 and 10^6 cells presented respectively 13 ng and 18 ng of DNA per mg of dry tissue. The ratio of cells that were able to adhere was thus better for the lower concentration, but the higher concentration permitted an increase of the total amount of cells attached to the matrix.

Interestingly, the decellularized controls presented extremely low DNA content for the two treatments. DNA was not detectable with 1% SDS and it was in trace amount (1 ng per mg of dry tissue) with 1% Triton. Concentrations lower than 50 ng per mg of tissue were already easily achieved in precedent experiments with SDS (Fig. 7) but it was not the case with 1% Triton. The supplementary steps consisted in sterilization with 70% ethanol for 4 h, subsequent rinsing with PBS 3 times for 20 min, incubation at 37° with standard growth medium and finally two rapid PBS rinses.

Discussion

hFPTs have previously been tested *in vitro* and showed potential for tendon treatment (5). However, for the treatment of extended defects, they should be delivered in combination with a solid matrix that possesses sufficient mechanical properties, along with good biocompatibility. ECM scaffolds are especially interesting from this point of view.

Due to its dimensions, the horse superficial digital flexor tendon represents an attractive source to create ECM scaffolds. Indeed, its length and width are large enough to dissect pieces with the desired dimensions to mimic human tendon. Moreover, its native rigidity is very close to the one found in human flexor and extensor tendons and also to palmaris longus and plantaris tendons which are the primary choice for autografts in the hand. Carlson *et al.* (45) determined that the Young's modulus (elastic modulus) of these four tendons ranged from 1161 to 1673 MPa without significant differences between them. Thus, the horse superficial digital flexor tendon is perfectly in this range with a mean Young's modulus of approximately 1200 MPa based on two distinct studies (46, 47). Another advantage to work with horse tendon is its availability and the quality of the source. In the food industry, even if nearly all the pieces of horse can be reused, it is not the case of tendons which are discarded. We decided to work with Profil Export in France as this company respects the International Food Standard (IFS, approbation by Loyd's Register Quality Assurance) and the traceability of each tendon can be linked to the source animal. Such traceability is a big advantage in modern processing with the idea to potentially use this material clinically.

Concerning the size of ECM scaffolds, the ultimate target would be to obtain tissue pieces tailored for grafts. To replace hand flexor or extensor tendons, an adapted size would be a minimum of 3 mm in diameter and 15 cm in length (8, 9). For rotator cuff injuries, the tears are more diffuse and pieces of 5 mm in depth 20 mm in width and 50 mm in length would be more adapted and could be further dissected by the surgeon at the moment of use. Unfortunately, such dimensions are not well adapted for preliminary screening of treatments. To compare the impact of a detergent on the cell content and on the structure, it is more reliable to have pieces reproducible and thin enough to be sure that the treatment can reach all the tissue in the same manner. The use of a dermatome permitted the creation of pieces of 1.2 mm in depth which were well adapted for screening with the different tests. This method permitted also increasing the number of samples from one tendon. The upscaling to a graft size can be done at a later time only with the most promising treatments and a repetition of the tests would be necessary.

When transforming initial tissue into an ECM scaffold, the primary target is to achieve complete decellularization of the tissue to diminish the risk of host immune reaction. In case of incomplete decellularization, macrophages tend to shift to the M1 phenotype *in vitro* (48) and massive lymphocyte infiltration can be observed *in vivo* (49). Among immunogenic antigens, MHC class I proteins an alpha-gal have been implicated in severe reactions, but they can be removed with the decellularization treatment (40, 50). Even if DNA is not directly responsible for an immune reaction, it is a good marker to detect the remaining presence of initial cell material and therefore, its diminution is desired. It has been suggested that a decellularized ECM should not present nuclear material on histology sections and should present an amount of DNA inferior to 50 ng per mg ECM dry weight (13). In parallel, the impact on structure and mechanical properties should be as limited as possible.

In this study, we decided not to use physical treatments for the decellularization process, as changes in temperature were already done to dissect the tissue pieces and for storage. The observations of control sections indicated that the cell morphology and tissue structure were not much affected by these preparation steps. Instead we concentrated on chemical decellularization with detergents.

The different analysis techniques did not always give the same information, but were complementary. HE staining allowed the detection of cells and their distribution within the tissue while already giving information on the global structure. It also permitted the detection for the lysis of cells as a function of time illustrated when the purple coloration became more diffuse. Anyway, some samples which seemed decellularized with HE still presented a bright DAPI fluorescence and this second technique was more sensitive to detect remaining DNA although not giving any information on the structure. As for HE, DAPI fading could also indicate cell lysis without removal of nuclei material. Concerning the structure evaluation, staining with SR permitted a more precise observation of the impact on structure than with HE, especially on collagen fibers. Taken together, these results show that HE seems less efficient than the two other staining procedures, but it is nonetheless valuable to perform as it is simple, inexpensive

and permits a first evaluation of both decellularization and structure. It must be accompanied by the two other stains for confirmation of the overall observations.

DNA measurements with PicoGreen revealed an even more sensitive technique for detection of remaining cell material within tissue and permitted detection of the presence of DNA in situations where cells were not detectable with HE or DAPI. Histological sections seemed to indicate that extension of the treatment period was positive for all the treatments as the nuclei stainings appeared more and more diffuse over time. Anyway, the PicoGreen assay did not indicate a trend towards reduction of DNA over time. DNA dosage should always be performed to assess the quality of decellularization, but this technique cannot replace histology as information on spatial distribution and presence of intact cells is lacking, as well as information on the impact of structure.

Concerning the DNA dosages, we had previously measured the concentrations with a Nanodrop, but some treated samples presented higher amounts of DNA than controls (data not shown). Therefore, we decided to test the samples with another method based on a different technique. We opted for PicoGreen which relies on the emission of fluorescence of a dye when binding to DNA, while the Nanodrop was a spectrophotometric assay with absorption of DNA at 260 nm. Anyway, the results were very similar between the two distinct methods indicating that the measures with the Nanodrop were not incorrect, but that there was a variation between the tested samples. To diminish the bias we hypothesized that a comparison of a treated sample to a control obtained in the same region instead of a general control would be more adapted. The evaluation of all controls confirmed differences in initial DNA contents and some treated samples still presented higher values than their control, but the difference was reduced compared to an average value of controls.

Globally, our results are based on a degree of uncertainty of the initial DNA content in the tissues tested. Anyway the results clearly showed that DNA was dramatically reduced in tissues treated with SDS at 1% and 2%, far under the amount found in the control with the lowest value. In opposition, the SDS-Triton mix presented intermediate results and the three other solutions seemed ineffective for DNA removal.

The technique could be improved for further evaluations. The tests were realized on 25 to 35 mg of wet tissue as recommended to be in accordance with the column binding capacity. However, vascular zones showed high cellularity both with HE and DAPI and it is possible that with the small quantities used for the test, bias occurred with selection of zones more vascularised for some specimens than for others which could explain the differences observed in controls. To optimize the technique, digestion should be done on larger pieces (to increase homogeneity between specimens by not involuntarily selecting only a specific zone), the solution should be homogenized and a quantity corresponding to approximately 25 mg should be taken for purification and dosage.

SDS at both 1% and 2% were clearly the best solutions for cell material removal. A great majority of cells were lysed after 24 h and DNA content was reduced to less than 50 ng per mg of dry

tissue already at this moment. On the other hand, SDS treatments were the worst solutions in terms of structure preservation. The effect was even visible with the naked eye where it was seen that tissue pieces shrunk and presented a jelly-like aspect on their borders. The doubling of the concentration did not add value. Surprisingly, the addition of Triton to SDS diminished its efficiency in cell removal, but the structure was better preserved than for SDS alone.

The three other treatments did not permit a decellularization at all, even after 72 h, despite the reduction of the cells on HE and DAPI sections with time. Thus, it was probable that these treatments achieved cell lyses at least to a certain extent, but were unable to retrieve DNA from the tissue. With the three solutions, the structure was better conserved and the final tissues presented an aspect close to controls without shrinkage or jelly-like formation. Tween 20 is not frequently used in decellularization protocols and we did not have expectations for its efficacy. Triton is widely used for the decellularization of various tissues. In the case of tendon, some studies obtained positive results (32), while others failed to remove cells (28) as we have seen. We had more expectations with TBP, but the results were disappointing with a DNA presence comparable to untreated controls. This result is in contradiction with two precedent studies performed on rat and porcine tendon where a treatment with TBP for 48 h was considered as the best option (although SDS was also able to decellularize the tissue) (28, 29). However, the evaluations made in these studies were performed after a supplementary rinsing step with 70% ethanol. Pridgen *et al.* concluded that a weaker decellularization of TBP compared to SDS, but like us, they did not use ethanol after TBP processing (39).

It is possible that the supplementary steps played an important role. Indeed, in the present study, the tissues treated with Triton for 72 h were further processed and that permitted the reduction of DNA to trace quantities. The supplementary steps consisted in sterilization with 70% ethanol for 4 h, subsequent rinsing with PBS 3 times for 20 min and immersion in standard growth medium for 72 h. It is possible that the further processing would have had beneficial effects for the other treatments as well. Therefore, TBP and Tween should not be totally discarded as decellularizing agents and they should be re-evaluated after treatment with supplementary steps. Modification of the concentrations, lengths of treatment and mixing between solutions could also help.

Histological sections with HE and SR allowed the observation of the structure, but they could not provide information on the final impact of a treatment on the mechanical properties. Tension assays were performed to address these concerns with evaluation of tissues treated with 1% SDS or 1% Triton. As tendons are both extremely resistant and very slippery, the risk of slippage before rupture is high and it was necessary to develop a system permitting a robust fixation. Many groups faced this problem of clamping and developed strategies such as customized clamps (51), nitrogen-frozen clamps (52, 53), dry-ice-frozen clamps (54, 55), thermoelectrically-cooled tissue clamp (56), sandpaper (57, 58), glue at the ends of the tendon (59) or on extension hull technique (60). The stiffening of the extremities by freezing was the most adopted method. Anyway, if cold is conducted further, the biomechanical properties of the specimen could be modified. We decided to work with the workshop of the Institute of Mechanical Engineering in

EPFL on the development of specially adapted clamps with a zigzag pattern to increase fixation while preventing weakening of the structure around clamps. With these designed clamps, we did not face any slippage during the measurements.

The length of controls and samples was 150 mm, which was suitable for a distance of approximately 80 mm between the clamps and more than 30 mm of grip on each site for good fixation. A sufficient length between the clamps is important for analysis of soft tissues to diminish the impact of end-effects found in the characteristic decay length (61-63). The depth of the tissue pieces is extremely important for the biomechanical assays. If the piece possesses a portion with a smaller transverse area, the stress is increased in this portion which could lead to an anticipated rupture. Theoretically, it would be possible to measure the cross-section in that location and convert force to strain to compare pieces with different thickness between them. Unfortunately, it is complicated to determine precisely and in a simple manner the transverse area over its entire length. Thus, it is preferable to work with pieces prepared in the same manner and with a constant depth throughout the sample. The use of a dermatome allowed us to obtain this regularity with a constant depth of 1.2 mm.

We decided to evaluate and compare the bands treated with 1% SDS for 72 h, 1% Triton for 72 h and the control non-decellularized bands. SDS at 1% was chosen because it permitted the best decellularization and Triton at 1% because it presented a good preservation of the structure along its ability to lyse cells which seemed better than for TBP or Tween 20.

A precharge was necessary to avoid the differences that could be present in tension at the moment of clamping the material. We had noticed shrinkage of approximately 10% of the length for the bands of tendon treated with 1% SDS. Their rigidity was extremely weak during the precharge and the preload of 5N required a longer elongation of these bands compared to the two other groups and permitted the recovery from approximately 5 of the 10% shrinkage. The results obtained showed a slight but significant increase in maximum strain for the 1% SDS group in comparison to the two others. This difference would have been even more pronounced if no precharge had been applied. The ultimate stress and the energy to failure were close between the three groups and no significant differences were detected. The Young's modulus was diminished by approximately 30% in the two treated groups, but this difference was not statistically significant. It is important to mention high variations between samples and thus difficulties to demonstrates differences if present.

The samples treated with SDS at 1% or Triton at 1% presented mean Young's modulus between 115 and 120 MPa. With a Young's modulus of 150 MPa, the rigidity of the control tendon was already far beyond the 1200 MPa found in native horse superficial digital flexor tendon (46, 47). Different suggestions can explain these low values for our controls and scaffolds. Before any decellularization treatment, it was necessary to vary the temperature of the tissue for the different step of the preparation of material (storage at -80°C, removal of surrounding tissues at RT, chopping in pieces at -20°C, storage at -80°C) and it is possible that the freezing and thawing already affected the mechanical properties, despite the fact that the structure of controls seemed relatively well conserved. Also, the morphology of the band was different and characterized by a

smaller area/perimeter ratio compared to native tendon. If the fibers on the perimeter are more affected by the cutting than the ones in the centre, this could have an influence and lead to lower values. Anyway, what is of primary importance is the fact that all the pieces of tissue from the different groups were cut in the same manner in order to compare them without bias.

Despite what we considered as low biomechanical values, the decellularized ECM scaffolds we obtained still presented stronger features than many commercial products indicated for tendon treatment. Indeed, Aurora *et al.* (22) reviewed mean Young's modulus between 15.2 and 40.1 MPa for 4 out of 5 tested products (Restore, CuffPatch, GraftJacket and TissueMend). Only AlloPatch presented a higher rigidity with a Young's modulus of 304 ± 52 MPa. Our scaffolds were also more rigid and resistant than those obtained by Youngstrom *et al.* also from horse superficial digital flexor tendons. Their pieces of 400 um in depth presented Young's modulus of 76.23 ± 4.12 and 70.31 ± 5.91 MPa and maximum stress of 6.029 ± 0.414 MPa and 6.045 ± 0.759 MPa for the treated and untreated samples respectively (37). In comparison, our scaffolds presented maximum stress of 9.3 MPa for 1% Triton and 8.8 MPa for 1% SDS and thus were slightly more resistant at equal corrected cross-sections.

The histological and biomechanical assessments were realized prior to sterilization to specifically screen and compare the effect of the decellularizion treatments. Decellularization is nevertheless not the last step and supplementary tests should be done also after the sterilization which is required for cell reseeding or transplantation.

In this study, an immersion of 4 h in 70% ethanol was effective to obtain sterile scaffolds and no contamination was seen with culture medium after reseeding. It could be imagined that techniques without immersion such as ethylene oxide, γ irradiation or electron beam irradiation would be more desirable, but they all impact the structure and mechanical properties of the ECM (64). The scaffolds treated with 1% Triton showed a good biocompatibility with hFPTs and the cells could adhere over the entire surface, but could not infiltrate deeply into the scaffold. The period of 72 h between seeding and fixation is probably short for the cells to migrate and the tests should be done at a later time points to evaluate if cells penetrate into the matrix or not. The tendon extracellular matrix is densely packed and it is possible that cells face difficulty in migration. The penetration is slightly deeper in regions where ECM is more disorganized. During the decellularization, it is desired to maintain the structure of ECM as close as possible to the original tissue, but a moderate impact on the structure could help in migration of the re-seeded cells. Injection within the midsubstance of the scaffolds or creation of small holes with a needle could also help for recolonization, but the impact on the mechanical properties should be controlled.

Conclusion

Due to its availability, dimension and possibility of good traceability, the horse superficial digital flexor tendon represents an attractive source to create ECM scaffolds. The detergent solutions

tested did not permit to obtain an optimal solution, but this study gave overall information for further development and analysis. SDS treatments were very efficient in cell removal but lead to scaffolds that had shrinkage and which presented a jelly-like aspect. The other detergents were less efficient in cell removal, but allowed a better preservation of the structure. The addition of supplementary steps after detergent treatment could also improve the efficiency as observed for the tissue pieces treated for 72 h with 1% Triton followed by steps with 70% ethanol rinsing and immersion in growth medium. The best treatment remains to be found before upscaling the specimens to a graftable size.

The biomechanical results were interesting as scaffolds treated with SDS at 1% or Triton at 1% for 72 h could achieve biomechanical features that were stronger than the ones of many commercial ECM already employed for rotator cuff augmentation. The rigidity and resistance seem sufficient to fill gaps in tendinopathic rotator cuffs, but the values seem still far too low to replace autografts in the hand.

Finally, the scaffolds treated with 1% Triton for 72 h and subsequently sterilized were adequate for hFPTs to adhere to the ECM scaffold. Adaptation in time for penetration and technique to deliver them deeper into the tissue would be necessary.

Globally, the results are promising but many steps could be optimized, upscaled in size and re-evaluated to obtain a graftable biomaterial for tendon repair.

Acknowledgments

These studies were funded in part by the Orthopaedic Hospital Foundation of Lausanne. We thank the Sandoz Family Foundation and the S.A.N.T.E Foundation for their continued support in our Transplantation Program. We thank Profil Export for Providing horse tendons, the Histology Core Facility at EPFL for the HE and SR staining and the workshop of the Institute of Mechanical Engineering at EPFL for helping to design and produce the clamps. The authors declare no conflicts of interest.

References

1. Sharma P, Maffulli N. Tendon injury and tendinopathy: healing and repair. J Bone Joint Surg Am. 2005;87(1):187-202.

2. Voleti PB, Buckley MR, Soslowsky LJ. Tendon healing: repair and regeneration. Annu Rev Biomed Eng. 2012;14:47-71.

3. James R, Kesturu G, Balian G, Chhabra AB. Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. J Hand Surg Am. 2008;33(1):102-12.

4. Docheva D, Muller SA, Majewski M, Evans CH. Biologics for tendon repair. Adv Drug Del Rev. 2015;84:222-39.

5. Grognuz A, Scaletta C, Farron A, Raffoul W, Applegate LA. Human Fetal Progenitor Tenocytes for Regenerative Medicine. Cell Transplant. 2015; http://dx.doi.org/10.3727/096368915X688515.

6. Tuncali D, Yavuz N, Terzioglu A, Aslan G. The rate of upper-extremity deep-structure injuries through small penetrating lacerations. Ann Plast Surg. 2005;55(2):146-8.

7. Wehbe MA. Tendon graft donor sites. J Hand Surg Am. 1992;17(6):1130-2.

8. Jakubietz MG, Jakubietz DF, Gruenert JG, Zahn R, Meffert RH, Jakubietz RG. Adequacy of palmaris longus and plantaris tendons for tendon grafting. J Hand Surg Am. 2011;36(4):695-8.

9. Harvey FJ, Chu G, Harvey PM. Surgical availability of the plantaris tendon. J Hand Surg Am. 1983;8(3):243-7.

10. Longo UG, Lamberti A, Petrillo S, Maffulli N, Denaro V. Scaffolds in tendon tissue engineering. Stem Cells Int. 2012;2012:517165.

11. Lomas AJ, Ryan CN, Sorushanova A, Shologu N, Sideri AI, Tsioli V, et al. The past, present and future in scaffold-based tendon treatments. Adv Drug Deliv Rev. 2015;84:257-77.

12. Schulze-Tanzil G, Al-Sadi O, Ertel W, Lohan A. Decellularized tendon extracellular matrix-a valuable approach for tendon reconstruction? Cells. 2012;1(4):1010-28.

13. Crapo PM, Gilbert TW, Badylak SF. An overview of tissue and whole organ decellularization processes. Biomaterials. 2011;32(12):3233-43.

14. Gilbert TW, Sellaro TL, Badylak SF. Decellularization of tissues and organs. Biomaterials. 2006;27(19):3675-83.

15. Hodde J. Naturally occurring scaffolds for soft tissue repair and regeneration. Tissue Eng. 2002;8(2):295-308.

16. Hoganson DM, O'Doherty EM, Owens GE, Harilal DO, Goldman SM, Bowley CM, et al. The retention of extracellular matrix proteins and angiogenic and mitogenic cytokines in a decellularized porcine dermis. Biomaterials. 2010;31(26):6730-7.

17. Voytik-Harbin SL, Brightman AO, Kraine MR, Waisner B, Badylak SF. Identification of extractable growth factors from small intestinal submucosa. J Cell Biochem. 1997;67(4):478-91.

18. Koizumi NJ, Inatomi TJ, Sotozono CJ, Fullwood NJ, Quantock AJ, Kinoshita S. Growth factor mRNA and protein in preserved human amniotic membrane. Curr Eye Res. 2000;20(3):173-7.

19. Badylak S, Toombs J, Shelbourne K, Hiles M, Lantz G, Van Sickle D. Small intestinal submucosa as an intra-articular ligamentous graft material: a pilot study in dogs. Vet Comp Orthop Traumatol. 1994;7(3):36-40.

20. Badylak SF, Tullius R, Kokini K, Shelbourne KD, Klootwyk T, Voytik SL, et al. The use of xenogeneic small intestinal submucosa as a biomaterial for Achilles tendon repair in a dog model. J Biomed Mater Res. 1995;29(8):977-85.

21. Derwin KA, Baker AR, Spragg RK, Leigh DR, Iannotti JP. Commercial extracellular matrix scaffolds for rotator cuff tendon repair. Biomechanical, biochemical, and cellular properties. J Bone Joint Surg Am. 2006;88(12):2665-72.

22. Aurora A, McCarron J, Iannotti JP, Derwin K. Commercially available extracellular matrix materials for rotator cuff repairs: state of the art and future trends. J Shoulder Elbow Surg. 2007;16(5 Suppl):S171-8.

23. Longo UG, Lamberti A, Maffulli N, Denaro V. Tendon augmentation grafts: a systematic review. Br Med Bull. 2010;94:165-88.

24. Barber FA, Herbert MA, Coons DA. Tendon augmentation grafts: biomechanical failure loads and failure patterns. Arthroscopy. 2006;22(5):534-8.

25. Valentin JE, Badylak JS, McCabe GP, Badylak SF. Extracellular matrix bioscaffolds for orthopaedic applications. A comparative histologic study. J Bone Joint Surg Am. 2006;88(12):2673-86.

26. Mienaltowski MJ, Birk DE. Structure, physiology, and biochemistry of collagens. Adv Exp Med Biol. 2014;802:5-29.

27. Kannus P. Structure of the tendon connective tissue. Scand J Med Sci Sports. 2000;10(6):312-20.

28. Cartmell JS, Dunn MG. Effect of chemical treatments on tendon cellularity and mechanical properties. J Biomed Mater Res. 2000;49(1):134-40.

29. Deeken CR, White AK, Bachman SL, Ramshaw BJ, Cleveland DS, Loy TS, et al. Method of preparing a decellularized porcine tendon using tributyl phosphate. J Biomed Mater Res B Appl Biomater. 2011;96(2):199-206.

30. Ingram JH, Korossis S, Howling G, Fisher J, Ingham E. The use of ultrasonication to aid recellularization of acellular natural tissue scaffolds for use in anterior cruciate ligament reconstruction. Tissue Eng. 2007;13(7):1561-72.

31. Omae H, Zhao C, Sun YL, An KN, Amadio PC. Multilayer tendon slices seeded with bone marrow stromal cells: a novel composite for tendon engineering. J Orthop Res. 2009;27(7):937-42.

32. Zhang AY, Bates SJ, Morrow E, Pham H, Pham B, Chang J. Tissue-engineered intrasynovial tendons: optimization of acellularization and seeding. J Rehabil Res Dev. 2009;46(4):489-98.

33. Kryger GS, Chong AK, Costa M, Pham H, Bates SJ, Chang J. A comparison of tenocytes and mesenchymal stem cells for use in flexor tendon tissue engineering. J Hand Surg Am. 2007;32(5):597-605.

34. Xing S, Liu C, Xu B, Chen J, Yin D, Zhang C. Effects of various decellularization methods on histological and biomechanical properties of rabbit tendons. Exp Ther Med. 2014;8(2):628-34.

35. Tischer T, Vogt S, Aryee S, Steinhauser E, Adamczyk C, Milz S, et al. Tissue engineering of the anterior cruciate ligament: a new method using acellularized tendon allografts and autologous fibroblasts. Arch Orthop Trauma Surg. 2007;127(9):735-41.

36. Burk J, Erbe I, Berner D, Kacza J, Kasper C, Pfeiffer B, et al. Freeze-thaw cycles enhance decellularization of large tendons. Tissue Eng Part C Methods. 2014;20(4):276-84.

37. Youngstrom DW, Barrett JG, Jose RR, Kaplan DL. Functional characterization of detergent-decellularized equine tendon extracellular matrix for tissue engineering applications. PLoS One. 2013;8(5):e64151.

38. Huang Q, Ingham E, Rooney P, Kearney JN. Production of a sterilised decellularised tendon allograft for clinical use. Cell Tissue Bank. 2013;14(4):645-54.

39. Pridgen BC, Woon CY, Kim M, Thorfinn J, Lindsey D, Pham H, et al. Flexor tendon tissue engineering: acellularization of human flexor tendons with preservation of biomechanical properties and biocompatibility. Tissue Eng Part C Methods. 2011;17(8):819-28.

40. Raghavan SS, Woon CY, Kraus A, Megerle K, Choi MS, Pridgen BC, et al. Human flexor tendon tissue engineering: decellularization of human flexor tendons reduces immunogenicity in vivo. Tissue Eng Part A. 2012;18(7-8):796-805.

41. Chen J, Xu J, Wang A, Zheng M. Scaffolds for tendon and ligament repair: review of the efficacy of commercial products. Expert Rev Med Devices. 2009;6(1):61-73.

42. Junqueira LCU, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. The Histochemical Journal. 1979;11(4):447-55.

43. Junqueira LCU, Montes GS, Sanchez EM. The influence of tissue section thickness on the study of collagen by the Picrosirius-polarization method. Histochemistry. 1982;74(1):153-6.

44. Quintin A, Hirt-Burri N, Scaletta C, Schizas C, Pioletti DP, Applegate LA. Consistency and safety of cell banks for research and clinical use: preliminary analysis of fetal skin banks. Cell Transplant. 2007;16(7):675-84.

45. Carlson GD, Botte MJ, Josephs MS, Newton PO, Davis JL, Woo SL. Morphologic and biomechanical comparison of tendons used as free grafts. J Hand Surg Am. 1993;18(1):76-82.

46. Crevier N, Pourcelot P, Denoix JM, Geiger D, Bortolussi C, Ribot X, et al. Segmental variations of in vitro mechanical properties in equine superficial digital flexor tendons. Am J Vet Res. 1996;57(8):1111-7.

47. Thorpe CT, Stark RJ, Goodship AE, Birch HL. Mechanical properties of the equine superficial digital flexor tendon relate to specific collagen cross-link levels. Equine Vet J Suppl. 2010(38):538-43.

48. Keane TJ, Londono R, Turner NJ, Badylak SF. Consequences of ineffective decellularization of biologic scaffolds on the host response. Biomaterials. 2012;33(6):1771-81.

49. Zheng MH, Chen J, Kirilak Y, Willers C, Xu J, Wood D. Porcine small intestine submucosa (SIS) is not an acellular collagenous matrix and contains porcine DNA: possible implications in human implantation. J Biomed Mater Res B Appl Biomater. 2005;73(1):61-7.

50. Yoshida R, Vavken P, Murray MM. Decellularization of bovine anterior cruciate ligament tissues minimizes immunogenic reactions to alpha-gal epitopes by human peripheral blood mononuclear cells. Knee. 2012;19(5):672-5.

51. Shi D, Wang D, Wang C, Liu A. A novel, inexpensive and easy to use tendon clamp for in vitro biomechanical testing. Med Eng Phys. 2012;34(4):516-20.

52. Wieloch P, Buchmann G, Roth W, Rickert M. A cryo-jaw designed for in vitro tensile testing of the healing Achilles tendons in rats. J Biomech. 2004;37(11):1719-22.

53. Wren TAL, Lindsey DP, Beaupré GS, Carter DR. Effects of Creep and Cyclic Loading on the Mechanical Properties and Failure of Human Achilles Tendons. Ann Biomed Eng. 2003;31(6):710-7.

54. Pearsall AWt, Hollis JM, Russell GV, Jr., Scheer Z. A biomechanical comparison of three lower extremity tendons for ligamentous reconstruction about the knee. Arthroscopy. 2003;19(10):1091-6.

55. Giannini S, Buda R, Di Caprio F, Agati P, Bigi A, De Pasquale V, et al. Effects of freezing on the biomechanical and structural properties of human posterior tibial tendons. Int Orthop. 2008;32(2):145-51.

56. Kiss MO, Hagemeister N, Levasseur A, Fernandes J, Lussier B, Petit Y. A low-cost thermoelectrically cooled tissue clamp for in vitro cyclic loading and load-to-failure testing of muscles and tendons. Med Eng Phys. 2009;31(9):1182-6.

57. Beredjiklian PK, Favata M, Cartmell JS, Flanagan CL, Crombleholme TM, Soslowsky LJ. Regenerative Versus Reparative Healing in Tendon: A Study of Biomechanical and Histological Properties in Fetal Sheep. Ann Biomed Eng. 2003;31(10):1143-52.

58. Hirpara KM, Sullivan PJ, O'Sullivan ME. The effects of freezing on the tensile properties of repaired porcine flexor tendon. J Hand Surg Am. 2008;33(3):353-8.

59. Haraldsson BT, Aagaard P, Krogsgaard M, Alkjaer T, Kjaer M, Magnusson SP. Region-specific mechanical properties of the human patella tendon. Journal of applied physiology (Bethesda, Md : 1985). 2005;98(3):1006-12.

60. Schottle P, Goudakos I, Rosenstiel N, Hoffmann JE, Taylor WR, Duda GN, et al. A comparison of techniques for fixation of the quadriceps muscle-tendon complex for in vitro biomechanical testing of the knee joint in sheep. Med Eng Phys. 2009;31(1):69-75.

61. Horgan CO. End effects in mechanical testing of biomaterials. J Biomech. 2013;46(5):1040-1.

62. Anssari-Benam A, Legerlotz K, Bader DL, Screen HR. On the specimen length dependency of tensile mechanical properties in soft tissues: gripping effects and the characteristic decay length. J Biomech. 2012;45(14):2481-2.

63. Legerlotz K, Riley GP, Screen HR. Specimen dimensions influence the measurement of material properties in tendon fascicles. J Biomech. 2010;43(12):2274-80.

64. Freytes DO, Stoner RM, Badylak SF. Uniaxial and biaxial properties of terminally sterilized porcine urinary bladder matrix scaffolds. J Biomed Mater Res B Appl Biomater. 2008;84(2):408-14.

CHAPTER VI

–

TRANSPLANTATION OF HUMAN FETAL PROGENITOR TENOCYTES INTO A RABBIT MODEL FOR TREATMENT OF TENDON INJURIES

Foreword

The chapter II has shown interesting properties of human Fetal Progenitor Tenocytes (hFPTs) *in vitro* and the chapter III has permitted the resuspension of these cells in hyaluronic acid gels. In the present chapter, we evaluate a similar formulation (with adapted concentration) within a newly developed *in vivo* rabbit model. A special focus is placed on the security of these cells and the quality of healing.

Transplantation of Human Fetal Progenitor Tenocytes into a Rabbit Model for Treatment of Tendon Injuries

This chapter represents the Phase 1 of a two-phase *in vivo* study. The submission of an article will be done only after the completion of the whole study.

A. Grognuz[1], C. Scaletta[1], W. Raffoul[1], A. Farron[2], B. Von Rechenberg[4], K. Klein[4], K. Nuss[4], P. Kronen[4], S. Darwiche[4], L.A. Applegate[1]*

1) Unit of Regenerative Therapy, Service of Plastic, Reconstructive and Hand Surgery, Department of Musculoskeletal Medicine, University Hospital of Lausanne, Switzerland

2) Service of Orthopaedics and Traumatology, Department of Musculoskeletal Medicine, University Hospital of Lausanne, Switzerland

3) Laboratory of Biomechanical Orthopedics, Institute of Bioengineering, EPFL, Lausanne, Switzerland

4) Musculskeletal Research Unit, Tierspital, University of Zurich, Switzrerland

***Address for Correspondence:**
Professor Lee Ann Laurent-Applegate
University Hospital of Lausanne (CHUV)
Service of Plastic, Reconstructive and Hand Surgery
Unit of Regenerative Therapy (UTR)
EPCR/Croisettes 22
1066 Epalinges
SWITZERLAND
E-mail: Lee.Laurent-Applegate@chuv.ch
Telephone: + 41 21 314 35 10
Fax: + 41 21 887 84 14

Abstract

Tendon injuries are common and represent a non-negligible burden for society. The healing process is long and the quality of the initial tissue is never attained. Biological and cell therapies have emerged as promising alternatives or complements to the standard therapeutic care. We have previously demonstrated interesting characteristics of human Fetal Progenitor Tenocytes (hFPTs) *in vitro*. In this phase of the study, we evaluate these cells in a newly developed partial-thickness defect of rabbit patellar tendon. The results presented are based on the phase 1 of a two-step *in vivo* study. The phase 1 relied on two groups of three animals each with defects treated either with a treatment item [TI: hFPTs, PBS and hyaluronic acid (HA) gel] or with a reference item (RI: PBS and HA gel). After 6 weeks, the repaired matrix presented a tendon-like direction orientation in several tendons, but the healing process was not achieved and no significant differences could be detected between the two groups. Interestingly, both items were well tolerated and hFPTs did not induce deleterious immune reactions. The phase 2 will be accomplished with the same surgical model, but with an extension of the survival time to judge the quality of the final repair.

Key words: Cell therapy; Human fetal progenitor tenocytes; Hyaluronic acid gel; *In vivo* study; Rabbit model; Patellar tendon; Tendon healing.

Introduction

Tendon afflictions are very common and their healing outcomes remain often unsatisfactory which is mainly due to the creation of scar tissue during the repair. Aiming to improve these modest outcomes, the field of biologics and cellular therapies has encountered much interest and many cell types have been tested *in vivo* for tendon healing with various final results (1, 2). Although encouraging, none of these therapies to date have demonstrated supremacy and the search of new treatment modalities continues.

We previously evaluated human Fetal Progenitor Tenocytes (hFPTs) *in vitro* as we thought they could be an interesting cell source for the treatment of tendon injuries for animals and humans alike. Our reflection was based on the fact that fetal tissue heals in a regenerative scarless manner (3), that this process seems driven by the cells (4) and that it is seen in different tissues including tendon (5). Due to the high proliferation capacity of hFPTs, it was possible to create large consistent cell bank permitting an off-the-shelf availability for the creation of treatments. During our *in vitro* experiments, we found that these cells presented a strong and stable tenogenic profile (even under induction towards other cell types) and that they could enhance adult tenocyte activity (6).

To deliver the cells, we then investigated different hyaluronan (HA) based hydrogels and we could develop a formulation with good cell survival and sufficient viscosity for a potential application and retention within a wound. The formulation was conditioned in syringes easy to handle and could be stored for up to 72 hours at refrigerated temperatures (article accepted for Cell Medicine).

The aim of the present research is to evaluate the tolerance of hFPTs and their impact on wound healing when delivered in an *in vivo* environment. For this purpose, we developed a new model consisting of a partial-thickness defect in rabbit patellar tendon. This model allows the spatial containment of the delivered item within the defect and aims to give a strong preliminary indication of the safety of hFPTs and their capacity to heal a tendon injury. The treatment item (TI) is composed of hFPTs within HA gel. A specific highly concentrated hFPT formulation had to be prepared to deliver a sufficient amount of cells in the relatively small defect. A reference item (RI) composed of HA gel without cell was used for comparison.

The complete study has been divided in two phases and will be based on a final number of six animals in each group (TI and RI). We present here the first phase of the study which is based on three animals in each group and which was conducted to validate the model for the second phase.

Materials and Methods

Cell Culture

Vials of hFPTs were taken from our cell bank stored in the vapor phase of liquid nitrogen for which the detailed production was described previously (6, 7). hFPTs constituting this cell bank were isolated from the Achilles tendon of a male 14-week gestation organ donation according to a protocol approved by the State Ethics Committee (University Hospital of Lausanne - CHUV, Ethics Committee Protocol No. 62/07: 14-week gestation organ donation, registered under the Federal Transplantation Program and its associated Biobank and complying with their associated laws and regulations). The cells were seeded at passage 5 in monolayer culture with a density of 3,000 cells/cm^2 (75-cm^2 flask with filter screw cap, TPP, Trasadingen, Switzerland). During proliferation, the cells were placed in cell culture incubators at 37°C in a humidified atmosphere containing 5% CO_2. The medium was composed of Dulbecco's modified Eagle medium (DMEM: Gibco, Life Technologies Ltd., Paisley, UK, code No. 41966-029) containing 25 mM dextrose, 1 mM sodium pyruvate, supplemented to 5.97 mM L-glutamine (L-glut: Gibco, code No. 25030-024) and 10% fetal bovine serum (FBS: Sigma, St. Louis, MO, USA, code No. F7524), without antibiotic supplementation and it was changed twice a week.

Formulation of Treatment Items (TI) and Reference Items (RI)

hFPTs were rinsed with phosphate-buffered saline (PBS: NaCl 6.8 g/l, Na_2HPO_4 1.5 g/l, KH_2PO_4 0.4 g/l; Bichsel, Interlaken, Switzerland, code No. 100 0 324), detached with TrypleE (Gibco, code No. 12605-010) and transferred into tubes. TrypleE was then inactivated by the addition of an equal volume of medium. The cells were counted with a hemocytometer and were transferred in as many tubes as desired, with 28 million cells per tube. They were then centrifuged at 250 x g, the supernatants were discarded and they were rinsed twice with PBS to eliminate all residue of medium. The processing allowed the cells to be ready for use at passage 6.

The syringes were prepared aseptically under the same laminar flow hood as employed for cell manipulations. To prepare one TI syringe, the 28 million cells were resuspended with PBS to obtain a volume of 175 µl. The suspension was transferred into one syringe (Omnifix-F 1ml; B. Braun, Meslungen, Germany) with a micropipette. With the help of a female-female Luer adapter (Ark Plas, Flippin, AR, USA, code No. AP18FLXFEP), 175 µl of Ostenil Tendon (TRB Chemedica, Haar, Germany) gel was added into the syringe. A new Luer adapter was mounted on the syringe with a new syringe on the other side and the suspension was homogenized through passing from one syringe to another. The cell suspension was kept in one of the two syringes, the air bubbles were eliminated, the volume was corrected to 200 µl and a cap (Fresenius Kabi, Oberdorf, Switzerland, code No. 8501552) was placed on the syringe. The final concentration was of 2 million cells per 25 µl. The preparation was then transferred in a sterile bag which was subsequently taped and labelled. Multiple TI syringes could be prepared by following the same protocol with the cells in the other tubes. All the syringes prepared from the same cell lot were

packaged together in a secondary sterile bag. The syringes could be stored at 4°C up to 72 hours; they were transferred to the operating room in a box refrigerated with cooling blocs. At the moment of injection (48h after syringe preparation), a sterile catheter (BD Venflon 22 G 0.8 x 25 mm; BD, Franklin Lakes, NJ, USA) was placed on the syringe for precise delivery into the defect.

The reference items (RI) are prepared exactly following the same process as for the treatment items, except that the 175 µl of cell suspension in PBS are replaced by 175 µl of PBS only before mixing with Ostenil Tendon.

Control of the Viability of Cells from TI Syringes

After injection, the syringes were left at room temperature for up to 8 hours and then taken back to refrigerated temperature. On the day following the operations, the remaining contents were used for cell survival analysis. For each syringe, 50 µl was extruded in a tube and diluted with 8 ml PBS. 100 µl of cell suspension were then transferred into a well of a 96-well plate and mixed with 100 µl of LIVE/DEAD solution (Molecular Probes, Life Technologies Ltd., code No. L-3224) prepared according to the manufacturer's instructions. After 30 minutes of incubation at room temperature and with protection from light, images were taken with an inverted microscope equipped for fluorescence (IX81; Olympus, Tokyo, Japan) and a digital camera (iXon; Andor Technology Ltd., Belfast, UK). Green and red channels were recorded separately and the images were then analyzed with Cell Profiler to obtain the number of live and dead cells and a ratio representing the cell survival could be calculated for each image. The identification of primary objects (live and dead cells) within the 2 color layers was done for a diameter of 10 to 120 pixels with automatic threshold strategy and distinction of clumped objects based on the shape with dividing lines based on intensity. Each identification was controlled manually to check the accuracy of the method.

Animal Model and Experimental Design

Six female New Zealand white rabbits were used in this *in vivo* study and the experiments followed a protocol approved by the state ethical committee under the license No. ZH034/15. At the moment of operation, the six animals had an average age (and standard deviation) of 5.7 ± 0.7 months (ranging from 5.2 to 6.6 months) and an average weight of 4.3 ± 0.7 kg (ranging from 3.950 kg to 5.005 kg). The six tested animals were separated in two groups of three animals. One group was operated with the TI (hFPTs + HA gel) and the other one with the RI (PBS + HA gel). All the rabbits had only one hindlimb operated (left or right according to randomization seen in Table 1) and the other hindlimb served as untreated control. The rabbits were housed individually during the recovery period for 24−72 hours and in group the rest of the time. They were sacrificed 6 weeks after surgery.

Table 1: Allocation table.

Animal	Group	Operated Hindlimb
1	TI	Left
2	TI	Right
3	TI	Left
4	RI	Right
5	RI	Left
6	RI	Right

Surgical Technique (Operative Technique)

The patellar tendon was used to create the defect. The rabbits were first sedated with alfaxan [3−5 mg/kg body weight (BW), intranasal (in)] and midazolam [0.1 mg/kg BW, intramuscular (im)] could also be given depending on the ease of intubation. After sedation, an intravenous catheter was placed. A blood sample was collected at that moment. Standard general anaesthesia, with loco-regional anaesthesia, was then performed according to routine and aseptic conditions were established for the surgeries.

An approximate 3-cm-long incision was performed from the lateral aspect of the knee extending to medial from 1 cm above to 2 cm below the knee joint space. A mid-tendon scalpel incision was made at the uppermost layer of the patellar tendon, creating a flap (ca. 6 mm long and 2−2.5 mm wide). The flap was lifted carefully and a hollow space was created with a microscalpel blade within the underlying tendon tissue, preserving the outer wall of the tendon. The flap was then partially sutured closed with absorbable Vicryl 6/0 (FS-3 needle and Polyglactin 910; Ethicon, Cincinatti, OH, USA, code No. V387H) and the TI or RI was delivered into the defect cavity up to filling it completely (approximately 30 μl). The tendon flap was sutured completely closed in a continuous manner with Vicryl 6/0 with the test items contained within the defect securely. The fascia was closed in layers with Biosyn 4-0 absorbable sutures (V-20 needle and monofilament Glycomer 631; Syneture, Medtronic, Minneapolis, MN, USA, code No. GM-121) in a simple continuous fashion. The skin was finally closed with Biosyn 4-0. Images of the different steps are presented in Figure 1.

Buprenorphine [0.01 mg/kg BW, subcutaneous (sc)] was given every 6–8 hours on the day of surgery and twice daily the following days. Additionally, metacam (1 mg/kg BW, sc) was given once daily for five days, starting with the surgery day. Prophylactic antiobiotic therapy was administered by giving enrofloxacin (7.5 mg/kg BW, sc) once daily on the day of surgery and for 4 days thereafter. Ranitidine was given as an antacid therapy twice perioperatively [before and after surgery, 1–2 mg/kg BW, sc or intravenous (iv)]. The rabbit status was controlled twice daily until sacrifice.

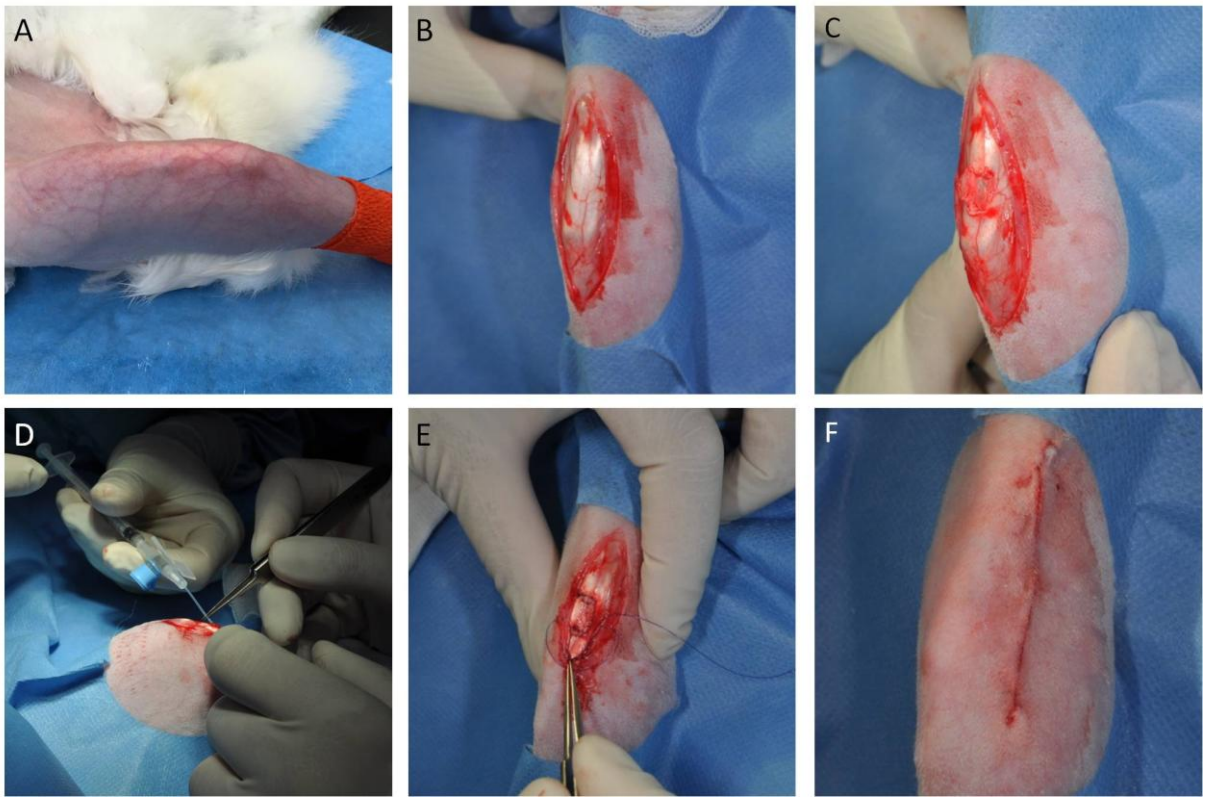

Figure 1: Surgery procedure.
One hindlimb is shaved and the patellar tendon can be localized (A). An approximate 3-cm-long incision is performed to access the patellar tendon (B). A mid-thickness defect of approximately 6 mm long and 2−2.5 mm wide is created (C). The flap is partially sutured closed and the TI or RI is injected into defect (approximately 30 µl) (D). The tendon flap was sutured completely closed in a continuous manner (E) and the fascia and skin are closed with absorbable sutures (F).

Sample Harvesting and Macroscopic Observations

Six weeks after the operations, the rabbits were sacrificed. They were first sedated with alfaxan (3−5 mg/kg BW, in) and midazolam (0.1 mg/kg BW, im) matching the pre-operative doses. After sedation, an intravenous catheter was placed. A blood sample was collected at that moment. The animals were then euthanized with pentobarbital (0.5 mL/kg BW, iv). Sacrifice was confirmed by absence of heartbeat and pupillary reflex.

After sacrifices, the treated limbs were dissected and a macroscopic evaluation was realized. The treated patellar tendons were cut at the insertion of the tuberositas tibiae and patella, inspected macroscopically and documented with digital photography. Special focus was placed on important indicators such as inflammation, tissue adhesion, fibrosis and global tissue quality. A customized scoring was performed to semi-quantitatively evaluate the tissues. The scoring scale was based on Table 2 and the lower the score, the more normal the tendon.

Table 2: Scoring for macroscopic evaluation of the operated tendon 6 weeks after intervention.

Task	Grade
Inflammation	☐ 0 None ☐ 1 Mild ☐ 2 Severe
Tissue Adhesion	☐ 0 Normal ☐ 1 Mild ☐ 2 Severe
Fibrosis	☐ 0 None ☐ 1 Mild ☐ 2 Severe
Defect filling extent and quality	☐ 0 Healthy tendon tissue with aligned fibers ☐ 1 Disorganized tendon tissue ☐ 2 Scar tissue, partial filling or no filling

The treated tendon was then transferred into 4% formalin, before being treated for histology analysis. The contralateral untreated patellar tendon was also harvested and placed in 4% formalin to serve as an untreated control.

Samples from the spleen, liver, lung, heart, quadriceps muscle, skin tissue adjacent to the surgical site, left and right inguinal and popliteal lymph nodes were harvested and stored in an RNA Stabilizing Solution for up to 24 hours at room temperature on a shaker. Afterwards, they were placed in a refrigerator at 0−8°C for up to 6 days. Subsequently, they were placed in a freezer at -20°C. The biopsies will be analyzed for presence of human cells at a later time when all the study results are collected for the final report.

Histological Analysis (Histology and Immunohistochemistry)

Both operated and contralateral patellar tendons were fixed in 4% formalin for up to 2 days, then placed in 75% Ethanol for up to 3 days before embedding in paraffin. The operated tendon and the contralateral patellar tendon were embedded within the same block along with a reference lung tissue to recognize the operated tendon within the block, as well as the proximal-distal orientation of the tendons. Sections of 5 μm were performed in the sagital plan in direction of tendon fibers and stained with Hematoxylin-Eosin (HE), Alcian Blue (AB), Picrosirius Red (SR) and von Kossa (VK).

Tendon healing was assessed by studying tendon morphology at the original defect site with a microscope (Leica DMR; Leica Microsystems, Wetzlar, Germany). A gross evaluation was done

with a 5x objective to evaluate the overall quality of the repair site on its whole surface. The defect location, size and edges were observed as well as the reactivity of the surface (graded 0 for no reaction, 1 for mild reaction and 2 for severe reaction). The presence of zones with structural abnormalities (disorganization, open spaces, foreign material, hypervascularity, hypercellularity, calcifications) was also recorded. Two zones were then selected within the defect area for a micro evaluation. When biomaterial was distinguishable, one zone was chosen around it and the other in a region where it was not seen. The regions with suture material were not selected as zones of evaluation. Tendon tissue was evaluated with a 10x objective and a scoring was made based on Table 3. The score for each zone could range between 0 (no abnormalities) and 14. A camera (Leica DFC320; Leica Microsystems) was used for acquisition of images of interest.

Table 3: Scoring for microscopic evaluation of the sections of the operated tendon 6 weeks after intervention.

Micro Evaluation (10x) 2 zones	Grade		
Hypercellularity (excl. inflammatory and vascular cells) (0 - 2)	☐ 0 None ☐ 1 Mild ☐ 2 High		
Vascularity (0 - 3)	Hypervascularity ☐ 0 No ☐ 1 Yes	Predominant Size ☐ 0 Small ☐ 1 Large	Transverse vessels ☐ 0 None, Rare ☐ 1 Some, Many
Inflammatory Reaction (0 - 3)	☐ 0 None ☐ 1 Mild (Some macrophages) ☐ 2 Moderate (More macrophages, some foreign body cells) ☐ 3 Severe (Many foreign body cells, granulocytes)		
Foreign Material (0 - 2)	☐ 0 None ☐ 1 Some, scattered ☐ 2 More, clustered		
New matrix quality (0 - 2)	☐ 0 Tendon-like matrix ☐ 1 Pre-differentiated, immature matrix ☐ 2 Scar tissue, fibrosis		
New matrix directionality (0 - 2)	☐ 0 Aligned fibers ☐ 1 Mildly disorganized fibers ☐ 2 No discernable directionality		

Blood Analysis

Blood samples of approximately 0.5 ml in an EDTA K tube and 0.5 ml in a Li Heparin tube were collected from the central ear artery of each rabbit prior to operation and 6 weeks later, prior to sacrifice. The routine parameters were measured and there was a special focus on leukocyte concentrations.

Statistical Analysis

SPSS Statistics Software (Version 22) was used to conduct a Levene's Test for Equality of Variances and a two-tailed independent t-test in order to detect statistical differences between RI and TI for individual score parameters as well as the total score. The significance was set on a p value below 0.05.

Results

Evaluation of the Remaining TI Material.

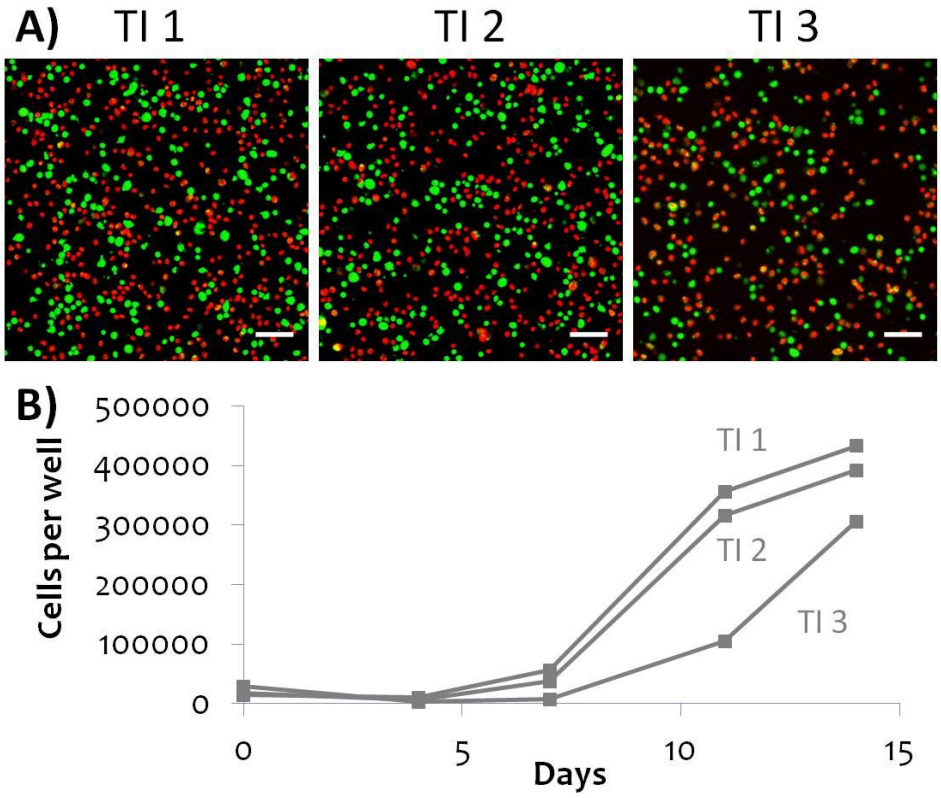

Figure 2: Survival and recovery of cells from the treatment items (TI).
The TI syringes were prepared 48h prior to injection. After injection, they were left at room temperature for up to 8h, before being placed back at refrigerated temperatures. On the day following the operations, the formulations were tested. There were still alive cells as demonstrated by the presence of green dots in the LIVE/DEAD assay (A) and they had conserved the ability to attach and proliferate as seen with the recovery assay (B). Scale bars: 100 μm.

The syringes used for injection were not discarded. The LIVE/DEAD assay performed on the day following the injection assured that a proportion of alive cells were injected as seen by the green dots on the Figure 2 (39%, 45% and 41% cells alive respectively). The recovery assay demonstrated that the cells extruded from the gels had conserved their ability to attach and proliferate. The preparation did not show any sign of contamination during the recovery assay performed in absence of antibiotics.

Blood Composition

There was no important change in the blood routine parameters. A special focus was placed on the leukocyte concentrations. There was a global mean concentration of $10.5x10^3$ leukocytes per ml (SD: 2.3, n = 6) prior to operations and exactly the same mean concentration of $10.5x10^3$ leukocytes per ml (SD: 1.7, n = 6) 6 weeks later, prior to sacrifice. The rabbits which received the treatment item had a mean concentration of $10.2x10^3$ leukocytes per ml (SD: 2.2, n = 3) at the moment of sacrifice, while the ones which received the reference item had a mean concentration of $10.9x10^3$ leukocytes per ml (SD: 1.3, n = 3). No rabbit experienced major change.

Macroscopic Evaluation

Five rabbits were quoted 0 on the 8-grade scale and one rabbit was quoted 1 on 8. The surgeon who performed the macroscopic evaluation did not notice any inflammation, tissue adhesion, fibrosis or defect in filling, except for one tendon injected with the TI which presented a mild inflammation. There was a very slight change in color seen in five of the six tendons (3x TI, 2x RI).

Microscopic Evaluation

The contralateral untreated tendons presented all a normal structure, without any special observations to notice and they would all have received a 0 grade with the quotation employed for microscopic evaluation. The operated tendons, either with TI or RI, were all in the healing process and they were thicker than their contralateral untreated tendon.

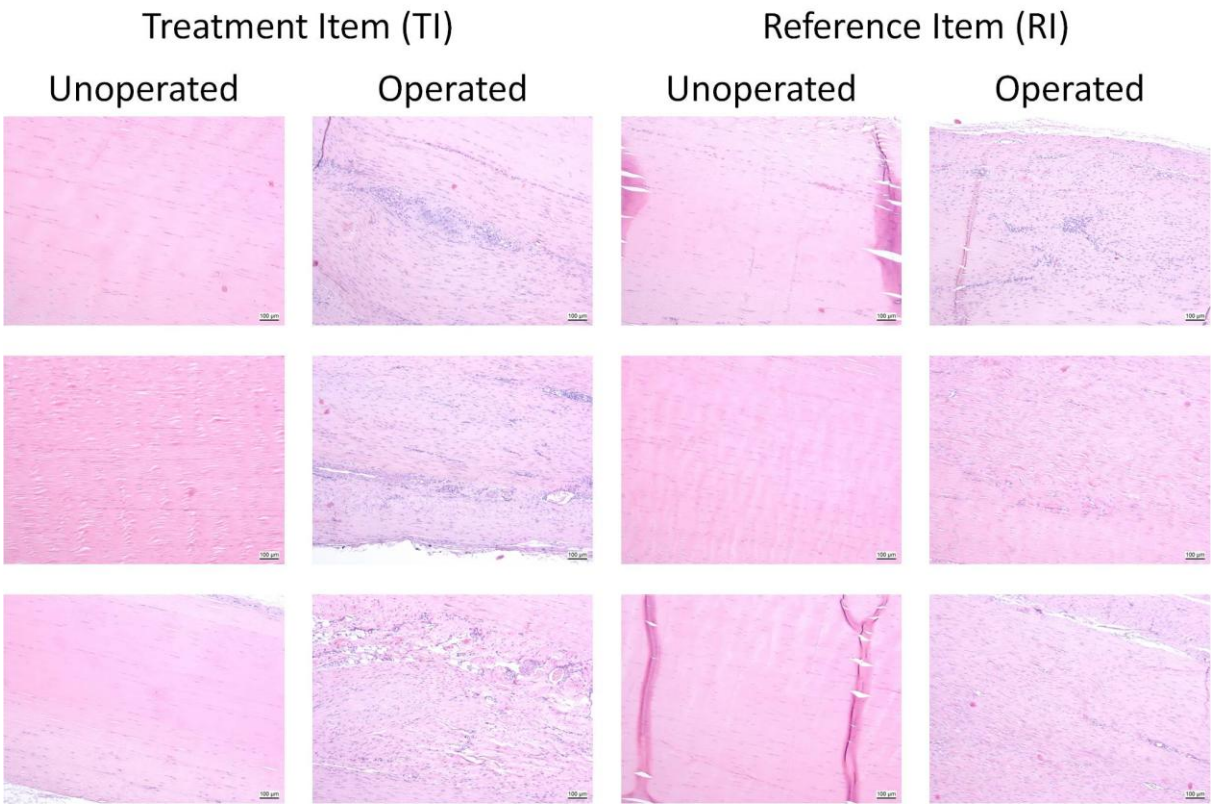

Figure 3: Structure of the operated and contralateral untreated tendons.

The contralateral untreated tendons all present a normal structure. The healing process is not achieved after 6 weeks for the operated tendons injected either with RI or TI. The defect zone is recognizable due to its hypercellularity.

Figure 4 presents the different remarkable structures that could be seen in normal and operated tendons 6 weeks after operations.

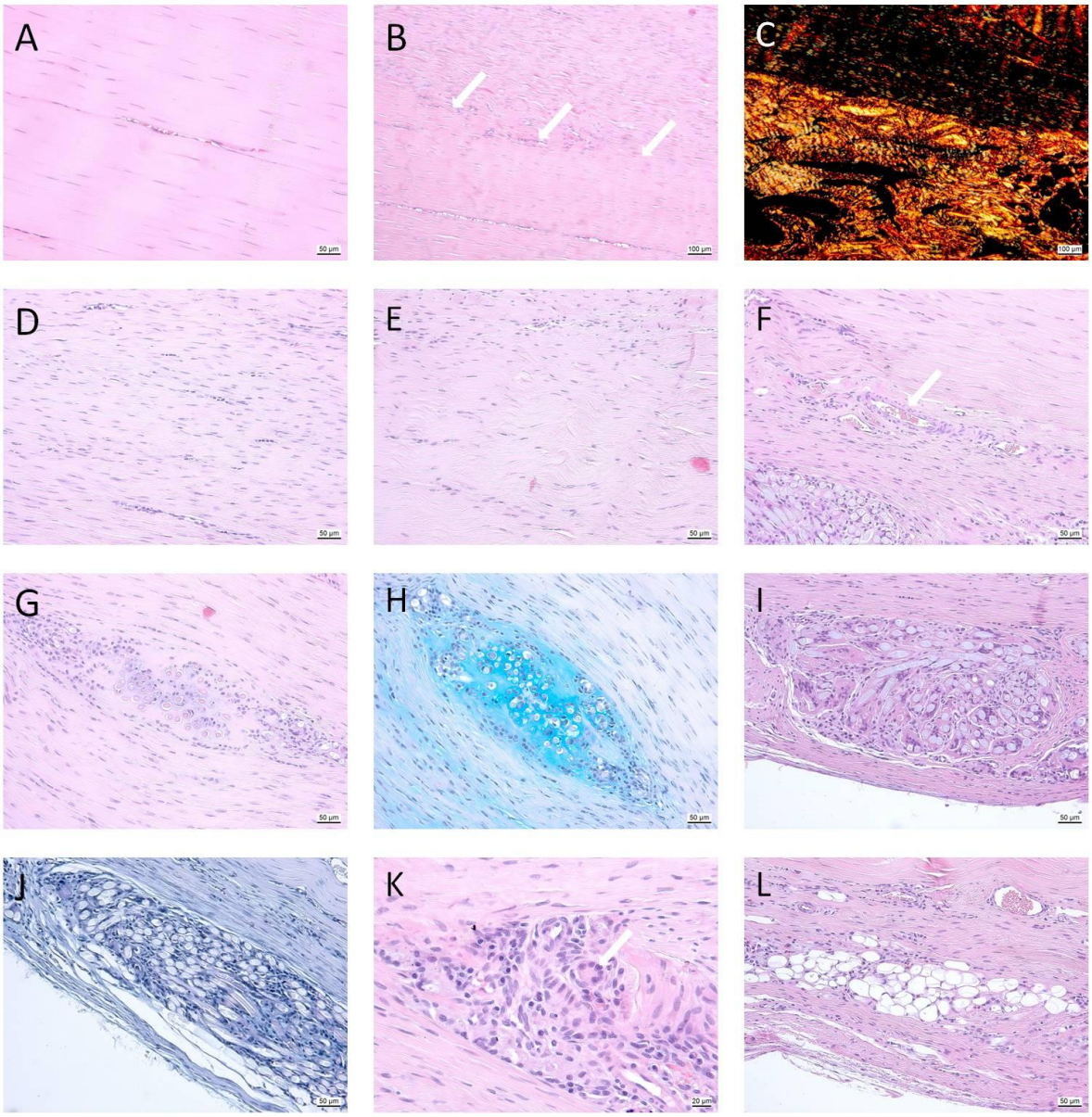

Figure 4: Microscopic pattern that can be observed on the tendon sections with Hematoxylin-Eosin (HE), Picrosirius Red (SR) or Alcian Blue stains (AB) and which are taken into account during the quoting process [Treatment Item (TI) and Reference Item (RI)].

A: Normal structure of an untreated tendon with tenocytes dispersed in the matrix and with presence of a naturally aligned blood vessel (HE). B: Delimitation of a defect (arrows) in an operated tendon (HE). The upper part shows the repaired defect and the bottom part shows normal structure with a standard vessel. C: The delimitation of one defect can be shown more clearly by an observation with SR stain under polarized light. The upper part shows a natural structure with good alignment and little diffraction of light, while the bottom part shows more light reflection due to misalignment of collagen in the matrix. D: Hypercellularity (HE). E: Disorganization of the structure with misalignment (HE). F: Hypervascularity and presence of transverse vessels (arrow) (HE). G-H: Presence of remaining biomaterial. It is possible to detect its presence with HE stain (G), but the AB stain allows an easier detection with bright blue (H). I-J: Presence of suture material recognizable with HE stain (I). The AB stain excludes the presence of biomaterial as there is no bright blue (J). K: Immune cell reaction and presence of a foreign body giant cells close to suture material (arrow) (HE). L: Presence of open spaces (HE).

The gross evaluations permitted the localization of the defect and estimation of its size and apparent quality of repair. The defect extended from one third to two thirds of the tendon in depth and the bottom edge was detectable in all tendons. The distal edge of the defect was visible in only one tendon (TI) and the proximal edge was visible in two tendons (TI and RI), but it was diffused in all other tendons. It was difficult to distinguish between the flap and the repaired tissue. The surface tissue was mildly reactive for all tendons, except one from the RI group where it was highly reactive. The suture material was not absorbed 6 weeks after operations and was discernible in 4 tendons (2xTI and 2xRI). There were inflammatory cells within and around suture material. Residual biomaterial could be detected in one tendon of each group (and in a third tendon from RI group during microscopic evaluation at higher magnification), the areas were larger for the tendon from TI group and were more cellular. One tendon of each group presented open spaces close to the upper border, probably in the flap. One tendon injected with the treatment item presented open spaces in the midsubstance. Hypercellularity and hypervascularity was visible in all the repaired regions. The tissue was generally oriented longitudinally, although not perfectly aligned and with zones of higher disorganizations. Necrosis was seen in none of the tendons, and except around foreign material, there were no signs of immune reactions. In addition, calcification (von Kossa) was never seen (data not shown).

The higher magnification microscopic evaluation permitted to score the repaired tissue semi-quantitatively. A summary of the results is presented for each group in the Table 4. The results of the two zones were averaged for each rabbit and the results are presented as a mean and standard deviation of the three rabbits within each group.

Table 4: Summary of the microscopic evaluation under high magnification (presented as a mean (SD) of the three rabbits within each group).

	Cell. (0−2)	Vasc. (0−3)	Inflam. (0−3)	Biomat. (0−2)	Quality (0−2)	Align. (0−2)	Total (0−14)
TI	2.0 (0.0)	1.8 (1.6)	1.5 (0.5)	0.3 (0.6)	0.3 (0.6)	1.3 (0.6)	7.3 (2.4)
RI	1.8 (0.3)	1.5 (0.5)	1.2 (0.8)	0.5 (0.5)	0.3 (0.6)	1.0 (0.9)	6.3 (2.3)

There was a high hypercellularity in all the evaluated zones, except in one from the RI group where it was more moderate. There were signs of hypervascularization in all the evaluated zones except for one tendon from the TI group. Macrophages were noticeable in the repaired tissues, but there were never groups of foreign body cells or granulocytes in the observed zones (although the presence of foreign body cells was visible around suture material, not included in evaluation zones). The presence of remaining biomaterial was seen in one tendon from the TI group where it was large, clustered and highly cellular. It was seen in two tendons from the RI group, but with smaller areas, more scattered between fibers and with hypercellularity, but not to the extent seen with TI. The repaired extracellular matrix was tendon-like in two tendons of each group and appeared pre-differentiated and immature in one tendon of each group. The

directionality was evident in five tendons, although slightly misaligned compared to normal tendon. In the last tendon from the TI group, the directionality was not discernible in the focused zones.

Basing significance on a p value below 0.05, the two-tailed independent t-test realized to compare the two groups showed no statistical difference for any individual parameter nor the total score.

Discussion

In this study, we wished to evaluate the tolerance and effect of hFPTs (previously tested *in vitro* (6) when injected in a tendon defect *in vivo*.

The creation of a defect within the midsubstance of a tendon was intended to evaluate the quality of the repaired tissue. The size of the tendon was important to obtain a defect large enough to inject a sufficient quantity of cells. We decided to choose rabbit as the animal type for the development of the model. Rabbits are considered by some as a small animal model and by others as a large animal, due to its characteristics found between the two groups. Compared to the rat or mouse, the tendon size of rabbit and larger animals is advantageous when surgical intervention is intended (8). Anyway, the complexity of management and the costs are linked with the size of animals, thus sample size must usually be reduced for *in vivo* studies with large animals in comparison to smaller animals (8). The rabbit, with its position at the interface between small and large animals presents a very good compromise with an adequate tendon size and the possibility to have a sufficient sample of animals for statistical analysis.

The patellar tendon was chosen for intervention as it is easily accessible and large enough for the creation of the intended surgery procedure. The creation of a partial-thickness tendon defect was desired to minimise the risk of a complete failure. After injection, the flap was sutured to further reduce the risk of tendon rupture and such an event was not observed. The surgeries performed well and the rabbits recovered rapidly and were able to stand on their hindlimbs already a few hours after surgery. Taken altogether, the characteristics of the rabbit patellar tendon are interesting for the creation of a surgical defect.

However, even if the rabbit patellar tendon is larger than tendons in smaller animals, the volume of the defect which can be created remains low and the preparation of formulations containing cells have to be specifically adapted. Preliminary studies on cadaveric rabbits had shown that the volume of injection that would fit in the defect would approximate 25−40 µl (data not shown). The desired number of cells had thus to fit in a volume of 25 µl and it was decided to prepare very concentrated TI formulations containing 80 million cells per ml to inject at least 2 million cells into the limited volume of the defect. With such a high concentration, the cell represent approximately one third of the final volume (the 28 million hFPT pellet measured 115 µl and had to be resuspended in a final formulation of 350 µl). They were diluted in a very small amount of PBS (60 µl) to facilitate the manipulations, while maintaining a final concentration of 50% HA

hydrogel in the formulation. The presence of at least 50% hydrogel was important to conserve a sufficient viscosity in order to prevent leakage of the formulation once within the defect.

As a comparison to TIs, we chose to work with RIs composed of 50% of HA hydrogel and 50% of PBS (cellular volume replaced by PBS). It was necessary to have the same proportion of HA as for the TI to prevent a potential difference of impact due to HA. With another RI, or with defect but without injection, it would not have been possible to discriminate if the gel or if the cells were responsible of a potential difference in healing. A third group with defect, but absence of injection would have been interesting. Although such a group was not essential in order to see the tolerance of the host towards hFPTs, it would be useful for complete validation of the model and it would allow to compare RI and TI to natural healing of tendon.

As the cells are more viscous than PBS, we noticed a small difference of viscosity between TI and RI, with RI being slightly more liquid. Anyway, the viscosity of RI seemed sufficient for a good maintenance within the defect.

The preparation of the TI syringes was characterized by a low yield. From the initial 350 µl of formulation, it was only possible to fill 200 µl in a syringe and only 150 µl are dispensable due to dead volumes in the tip and catheter. This represents only 45% of the initial cells that can be dispensed. Moreover, a margin is present to allow more flexibility at the moment of injection and from the 150 µl, only 25 to 40 µl finish in the defect. Thus, finally only approximately 2 to 3 million cells were injected in the defect from the 28 million used to prepare one syringe. This represents a yield of less than 10% which is really low, but which is mainly due to the specific conditions of the very small volume injected. In opposition, the volume that would be used for humans or larger animals would be higher and one could use lower concentrations to inject the same amount of cells. We estimate that for human preparations, a concentration of 2−2.5 million cells in a volume of 500 µl would be adequate. We previously prepared such formulations and the yield was of more than 60% of cells dispensable (article accepted for Cell Medicine) and would probably attain 70% with the double syringe technique used herein. The yield could further be improved by preparation of the formulation in higher volumes and division into multiple syringes instead of processing syringes one by one.

The minimal number of animals treated in each group should be six to permit a stronger comparison between the two groups. However in this phase, the model was newly developed and there were some concerns about the healing time frame and the quality of the model. During the preparation of the experimental study, we chose to proceed in two phases. The present study with three animals in each group represented the phase 1 of the full study and was intended for a preliminary evaluation. If healing would occur as rapid as expected and the surgical technique would require no further adaptation, six additional rabbits would be operated in Phase 2, with 3 in each group and with the same survival time of 6 weeks as in Phase 1. If adaptation would be necessary, the second phase would consist in the operation of six new rabbits in each group with an adapted procedure and/or survival time. The results obtained on the six animals would then be sufficient to decide of the scenario of phase 2.

As expected, the contralateral untreated tendons all presented a normal structure, without any special observations noticeable. Six weeks post injury, the operated tendons presented many similarities between the TI and RI groups and the t-test realized could not detect any significant differences for any individual parameter nor for the total score. Thus, with such a small number of animals, it is obviously difficult to detect significant differences and the higher number of animals in the whole study could help to discriminate potential differences between the two groups.

Tendons from both TI and RI were at a similar stage of healing and the repair was not completed 6 weeks post-injury. The healing process in tendon is clearly described and relies on 3 phases. There is first an inflammatory stage which occurs very rapidly after the wound. Multiple cell types infiltrate the wound, particularly erythrocytes and inflammatory cells and the numerous factors released begin to induce angiogenesis and to attract tenocytes. During the proliferative stage (second step), the repair site is highly cellular, there is an extensive blood vessel network and a matrix is deposited under control of tenocytes. Macrophages are still present but in lower amounts and they help in the repair process. That is only during the remodelling stage that cellularity decreases and that the tissue is organized longitudinally with an evolution from cellular to fibrous and the final creation of a scar tissue in adult (9-12). Based on these remarks, we can estimate that the operated tendons were between the proliferative and the remodelling phase. Indeed, a high tenocyte hypercellularity and an increased vascularization were visible in the two groups. The vascularity was increased with augmentation in number and in size and there was also the presence of transverse vessels (not seen in normal tendon). There were signs although that the remodelling stage had begun as the matrix was tendon-like in two thirds of the tendons and directionality of the fibers could be seen in five of the six tendons. Supplementary evaluations could potentially permit finding differences between the two groups. For example, imunostaining of collagen I and III could be interesting to evaluate if the two groups depose matrix in the same manner or with different sequences. In adult wound healing, the first phases are characterized by deposition of large amounts of collagen III, while collagen I peaks only later in the process (11). In fetal healing, the tissue heals rapidly and the structure is indiscernible from unwounded tendons (mainly composed of collagen I) (5). Anyway, as the appearance of scar tissue takes place in the last stages of healing, it would be interesting to repeat the experiment with later evaluation to see the final quality of the repaired tendon and compare the results obtained in presence and in absence of hFPTs.

One of the main aims of this study was to evaluate if hFPTs were well tolerated when transferred into a host. Precedent studies with skin fetal progenitor cells had shown an absence of inflammation when topically applied, even in a repeated manner (13-15). Thus, we wanted to know if hFPTs would also be well accepted upon transplant. The observations concerning inflammation highlighted the presence of macrophages within the operated tendons, but they are integral to the normal healing process. In opposition, we did not detect the presence of giant foreign body cells or granulocytes, except around suture material which had not been absorbed at the moment of sacrifice. The blood samples did not indicate elevation of leukocytes. These

results are very promising and tend to show a good tolerance of the host for hFPTs. It is nevertheless important to keep in mind that the immunological system of animals is different from the one of humans and further evaluation in other species would be desired to increase the safety prerequisite before a potential application to human.

The fate of hFPTs within host is not known yet. The sections should be performed for an in situ hybridization with Alu which would permit highlighting human DNA if present in the defect. It would also respond if the cells detected in the remaining biomaterial are of human or host origin. Samples from different tissues were harvested but have not been processed yet. Their analysis could give some insight about the diffusion of the injected cells in the body and tell if the cells migrate to other organs. It is sure that a proportion of cells were alive and had the capacity to attach and proliferate as demonstrated by the survival and recovery tests. These tests were done to obtain qualitative information and not quantitative information. Indeed, after the injections, the material remaining in TI syringes had been left for up to 8 hours at room temperature instead of 4°C and was tested only on the day following the operations. A high proportion of cells probably died between the injection and the test, but it was possible to be sure that cells alive and with the capacity to attach and proliferate were extruded from the syringes.

Conclusion

The developed surgical model of partial-thickness defect in rabbit patellar tendon was well adapted and the phase I of the study permitted highlighting some interesting aspects. Among them, the most important is indeed the fact that there is no induced immune reaction in the operated tendons, which shows a good tolerance of the host for HA as expected and more importantly for hFPTs. However, at this early time point, it was not possible to detect significant differences between the two groups. This can be due to the small amount of animals, but also to observations accomplished too rapidly within the healing process. The appearance of scar tissue appears only in the latest stages of healing and it would thus be more interesting to evaluate the two groups at a later time point where differences could be increased. Indeed, fetal cells are known to have an impact on the scarless regeneration and a therapeutic influence could be detected only at a later stage. Based on these observations, the phase 2 of the study should be done on 12 animals and with a longer healing time after injury. A subset of three animals with creation of defect but without injection of any item should be added to evaluate natural healing and compare TI and RI to it. With these adaptations, the presented model could set a basis for future studies in tendon

Acknowledgments

These studies were funded in part by the Orthopaedic Hospital Foundation of Lausanne. We thank the Sandoz Family Foundation and the S.A.N.T.E Foundation for their continued support in our Transplantation Program. We thank Silvana Resegeatti who took car of the rabbits,

Dr. Dragmar Verdino who performed anesthesia, Aymone Lenisa who performed the histological sections and stainings and Dr. Sabine Koch who assessed quality assurance. The authors declare no conflicts of interest.

References

1. Petrou IG, Grognuz A, Hirt-Burri N, Raffoul W, Applegate LA. Cell therapies for tendons: old cell choice for modern innovation. Swiss Med Wkly. 2014;144:w13989.

2. Docheva D, Muller SA, Majewski M, Evans CH. Biologics for tendon repair. Adv Drug Del Rev. 2015;84:222-39.

3. Adzick NS, Longaker MT. Scarless fetal healing. Therapeutic implications. Ann Surg. 1992;215(1):3-7.

4. Lorenz HP, Lin RY, Longaker MT, Whitby DJ, Adzick NS. The fetal fibroblast: the effector cell of scarless fetal skin repair. Plast Reconstr Surg. 1995;96(6):1251-61.

5. Beredjiklian PK, Favata M, Cartmell JS, Flanagan CL, Crombleholme TM, Soslowsky LJ. Regenerative versus reparative healing in tendon: a study of biomechanical and histological properties in fetal sheep. Ann Biomed Eng. 2003;31(10):1143-52.

6. Grognuz A, Scaletta C, Farron A, Raffoul W, Applegate LA. Human Fetal Progenitor Tenocytes for Regenerative Medicine. Cell Transplant. 2015; http://dx.doi.org/10.3727/096368915X688515.

7. Quintin A, Hirt-Burri N, Scaletta C, Schizas C, Pioletti DP, Applegate LA. Consistency and safety of cell banks for research and clinical use: preliminary analysis of fetal skin banks. Cell Transplant. 2007;16(7):675-84.

8. Derwin KA, Baker AR, Iannotti JP, McCarron JA. Preclinical models for translating regenerative medicine therapies for rotator cuff repair. Tissue Eng Part B Rev. 2010;16(1):21-30.

9. James R, Kesturu G, Balian G, Chhabra AB. Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. J Hand Surg Am. 2008;33(1):102-12.

10. Voleti PB, Buckley MR, Soslowsky LJ. Tendon healing: repair and regeneration. Annu Rev Biomed Eng. 2012;14:47-71.

11. Sharma P, Maffulli N. Tendon injury and tendinopathy: healing and repair. J Bone Joint Surg Am. 2005;87(1):187-202.

12. Lucas T, Waisman A, Ranjan R, Roes J, Krieg T, Muller W, et al. Differential roles of macrophages in diverse phases of skin repair. J Immunol. 2010;184(7):3964-77.

13. De Buys Roessingh AS, Hohlfeld J, Scaletta C, Hirt-Burri N, Gerber S, Hohlfeld P, et al. Development, characterization, and use of a fetal skin cell bank for tissue engineering in wound healing. Cell Transplant. 2006;15(8-9):823-34.

14. Hohlfeld J, de Buys Roessingh A, Hirt-Burri N, Chaubert P, Gerber S, Scaletta C, et al. Tissue engineered fetal skin constructs for paediatric burns. Lancet. 2005;366(9488):840-2.

15. Ramelet AA, Hirt-Burri N, Raffoul W, Scaletta C, Pioletti DP, Offord E, et al. Chronic wound healing by fetal cell therapy may be explained by differential gene profiling observed in fetal versus old skin cells. Exp Gerontol. 2009;44(3):208-18.

Chapter VII

–

Conclusion and Perspectives

Foreword

This chapter summarizes the principal results obtained throughout the thesis and draws some conclusions on the work performed as well as some perspectives on the work that could be done to improve and complete the actual results and bring hFPTs closer to a clinical application.

Conclusion and Perspectives

For translational medicine, it is of utmost importance to think from the beginning of a project about the potential clinical use and to always remember that the aim is to create a product which ultimately is beneficial for the patient. Security and efficacy are the basis to be maintained and the final product must be associated with the evolving regulatory aspects. To be useful, the product must be available when the medical doctor needs it and it should be designed for simple handling. Moreover, in the context of cost reduction, the strategy required to improve the therapeutic benefit/economic investment ratio should be emphasized to have a chance to be used one day clinically. It is with all these points taken into consideration that this project was elaborated.

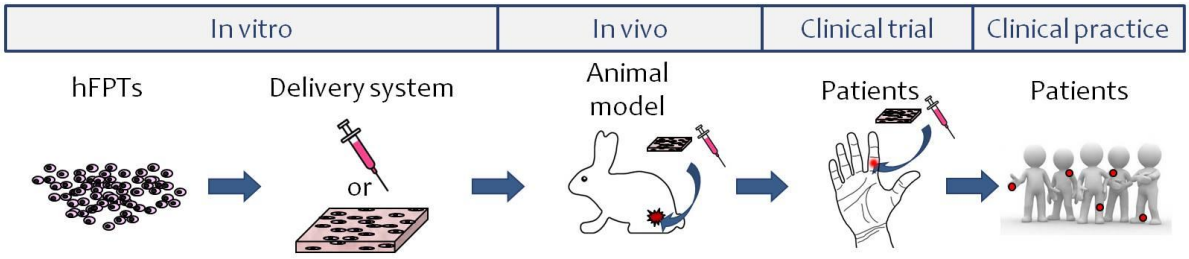

Figure 1: Whole process, step by step, before a potential therapeutic use of hFPTs.
First of all, it is very important to assess the cell source *in vitro* to know the characteristics and associated potential advantages and limitations of the cells. This is presented in Chapter II of the thesis. If the results are promising, it is necessary to find systems to deliver the cells to the afflicted site. The delivery system can function as a vehicle to deliver the cell or it can have a direct implication by creating an artificial tendon substrate with sufficient mechanical properties within the defect. Chapter III presents results obtained in the development of an injectable product, while Chapter IV presents results obtained in the development of a solid scaffold. Once an adequate formulation is developed, the delivery can be done *in vitro* in an animal model to evaluate the security of the therapy as well as its efficacy. Chapter V evaluates this aspect by first choosing the appropriate animal model and then the specific defect to be examined. Only if all these steps have been performed with promising results, it is possible to imagine the use of advanced therapies in humans. Anyway, before a routine clinical practice on patients, it is necessary to perform clinical trials with patients respecting inclusion criteria for a defined type of affection and the respective treatment.

As pointed out throughout this thesis, the treatment of tendon afflictions remains a challenge. Many unsatisfactory outcomes result from the development of scar tissue with properties incomparable to the tissue found prior to injury. The idea to use human fetal progenitor tenocytes (hFPTs) was brought to the foreground based on the knowledge that fetal tissue heals in a regenerative manner without scar formation and perhaps this cell source, since only one organ donation would be necessary, could be developed into advanced cellular products for the clinic.

Globally, hFPTs presented very promising results when tested *in vitro*. Starting from one organ donation, it was possible to create a cell bank functioning as a stock of 50 vials of 10 million cells each at the end of passage 1. The storage of the cells was possible with excellent survivals in the vapor phase of liquid nitrogen and the cells could proliferate rapidly when seeded in monolayer culture. The results did not indicate any sign of aging up to passage 9 and if each vial was

expanded up to the two third (passage 6), this would lead to an approximate quantity of $2x10^{14}$ cells and thus multiple millions of possible patient treatments. The tenocyte phenotype was confirmed by the presence of collagen I, scleraxis and tenomodulin, three different markers with important roles in tendon. The cell stability was excellent both genetically and phenotypically. The karyotype was considered as normal up to elevated passages (passage 12). The cells did not present any mineral or lipid accumulations even when confronted with conditions inducing differentiation of mesenchymal stem cells. Finally, hFPTs had the capacity to increase the activity of adult tenocytes when placed in a coculture system. All of these positive results lead to the aim to find solutions to deliver these specific banked cells into an afflicted site.

The evaluation of different hyaluronic acid hydrogels was intended for development of injectable cell preparations that would be useful in case of tendinopathies or simple lacerations. Such formulations would permit delivery of the cells to the wounded site in absence of surgery or around the repair site in the presence of surgery. Our observations highlighted two diverging objectives. On one hand, the carrier had to be sufficiently fluid to prevent important cell death during resuspension. On the other hand it had to be viscous enough to maintain homogeneity within the formulation and to prevent leakage if applied around a repair site. It was possible to obtain a formulation with a good compromise between these two prerequisites and hFPTs could be handled and delivered easily with such a final preparation and maintaining cellular quality.

Unfortunately, if the medical doctor is confronted with an extended injury, injection would most likely be insufficient and an artificial substrate with sufficient mechanical properties would be required to fill the gap. The use of equine superficial digital flexor tendon (SDFT) seems a good starting material to create ECM scaffold. It was possible to achieve a scaffold with intermediate biomechanical properties and was biocompatible with the re-seeding of hFPTs. These studies prepared the foundation for a future bioengineered human tendon replacement.

The *in vitro* evaluations are necessary to describe and anticipate many characteristics of the cells particularly necessary for regulatory compliance. Anyway, the cell final formulation must be evaluated *in vivo*, in presence of an environment as close as possible to the final conditions and with the implication of the immune system. Therefore, we developed a partial-thickness defect model within rabbit patellar tendon to evaluate the characteristics of hFPTs when placed *in vivo*, in collaboration with the Unit of Musculoskeletal Research in the Zurich University (MSRU). The formulation which was considered the most adequate from the testing of the different HA gels could be adapted in concentration and delivered within the defects. The Phase I safety testing with evaluation performed 6 weeks post-injury did not permit evaluation of the final quality of the repair process, as the healing was still ongoing; however, no deleterious immune rejections could be detected in the treated locations at 6 weeks post-injury.

The next step should be the adaptation of the *in vivo* study protocol with a longer recovery period for the Phase II. In parallel, the addition of three control animals receiving the surgery but no treatment (hFPTs within HA gel) or reference (HA gel without cells) item would be very useful to have some indication of the natural healing process of the model developed. If the results are positive, a new *in vivo* study on another species (such as goat, sheep or horse) could be performed

to confirm the first results in a larger animal model to be able to approach an evaluation in humans with a clinical trial.

To imagine using these cells in humans, many steps would be required to upscale the whole hFPT cell banking and processing to good manufacturing practice (GMP). Until now, it was possible to integrate current good manufacturing practice (GMP) for cell processing for the creation of the parental cell bank, which is of primary importance. The amplification of cells for the different phases of the studies to date was not performed under this stringent regulation and but was adapted to good laboratory practice (GLP) standards.

The development of clinical-grade cells implicates that the whole process must be performed according to GMP. This means the cells must be stored and handled by accredited personnel within an accredited structure and with full monitoring of all the processes. These conditions are extremely stringent and can only be performed within dedicated structures. In the past, our group has worked with Bioreliance in Scotland for the processing of other cell types in GMP conditions. However, our hospital has recently developed a cell production center (CPC) which received the accreditations for GMP practice and collaboration with this infrastructure would permit the processing of the cell bank under GMP conditions directly in our hospital. In addition to GMP processing, several more security tests would need to be performed. The serology of the mother donor was already assessed 1 month and 3 months after organ donation to assure no seroconversion, but the GMP banked cells should be further tested to assure the absence of pathogens such as bacteria (including mycoplasms), yeast, many different viruses (both animal and human depending on the cell culture additives) or prions. As the cells are all derived from the same organ donation, it would be possible to include within the process an extensive screening phase. Additional aspects concerning the security would include that tumorigenicity testing be performed on the specifically banked cells (hFPTs) regardless of the fact that other fetal progenitor cells have been fully tested.

To obtain a final product ready for use clinically, not only the cells would have to be clinical grade, but also the final formulation. The formulation of hFPTs within HA gel would permit having syringes ready to use for the surgeon. The preparation yield was characterized by loss of a high proportion of product during conditioning. An adaptation in the material to reduce dead volumes would diminish this loss. Moreover, if the number of patients is sufficient, it would be possible to increase the number of preparations produced at one time and thus also diminish the loss. The whole process could be easily integrated into GMP processing.

The development of a "replacement tendon" would however require more development and optimization. Several important aspects evolved during this phase of experimentation. The search of scaffolds with sufficient biomechanical properties should be continued. Indeed, the possibilities available for the surgeons are limited when facing extended injuries. As many synthetic products are linked with poor integration, the perseverance for developing a new biological matrix in the field of ECM scaffold is necessary and reasonable. Advanced decellularization processing is required to remove the cellular material while maintaining the biomechanical characteristics of tendon. The development of techniques to precisely shape

scaffolds to the desired dimensions would also be necessary and the seeding of cells should be improved to distribute cells in the mid-substance of the scaffold as well as on the surface. Finally, the combination with a cyclic tensile system could be desirable as the cells would probably remain relatively latent in absence of stimulation and could lead the project into the interesting field of mechanobiology.

Finally, even if hFPTs have presented some very promising results, fundamental research on these cells should be pursued to improve the knowledge on this cell source and better understand their differences with other cell types and their manner to function during healing.

CHAPTER VIII

–

ANNEXES

Education

2011 – 2016 PhD Candidate
Section of Pharmaceutical Sciences, Universities of Geneva and Lausanne, Switzerland
Unit of Regenerative Therapy, University Hospital of Lausanne, Switzerland
Doctoral Thesis: Human Fetal Progenitor Tenocytes for the Treatment of Tendon Afflictions
Thesis directors and co-directors: Prof. Lee Ann Laurent-Applegate and Prof. Olivier Bugnon

2008 – 2010 Master of Pharmacy
Section of Pharmaceutical Sciences, Universities of Geneva and Lausanne, Switzerland
Master Project: Evaluation de la thérapie cellulaire fœtale à l'aide du modèle CAM et d'un test métabolique cellulaire
Supervisors: Prof. Lee Ann Laurent-Applegate and Dr. Florence Delie

2005 – 2008 Bachelor of Pharmacy
Section of Pharmaceutical Sciences, Universities of Geneva and Lausanne, Switzerland

Publications, Scientific Communications and Parallel Projects

Peer-reviewed Publications (Published or Accepted)

- Petrou, I. G., A. Grognuz, N. Hirt-Burri, W. Raffoul and L. A. Applegate (2014). "Cell therapies for tendons: old cell choice for modern innovation." Swiss Medical Weekly 144: w13989.
- Grognuz, A., C. Scaletta, A. Farron, W. Raffoul and L. A. Applegate (2015). "Human Fetal Progenitor Tenocytes for Regenerative Medicine." Cell Transplantation http://dx.doi.org/10.3727/096368915X688515.
- Grognuz, A., C. Scaletta, A. Farron, D. P. Pioletti, W. Raffoul and L. A. Applegate. "Stability Enhancement Using Hyaluronic Acid Gels for Delivery of Human Fetal Progenitor Tenocytes." Article accepted for Cell Medicine.
- Krähenbühl, S. M., A. Grognuz, M. Michetti, W. Raffoul and L. A. Applegate (2015). "Enhancement of Human Adipose-Derived Stem Cell Expansion and Stability for Clinical Use." International Journal of Stem Cell Research & Therapy 2(1).

Oral Communications

- 11th CBT Research Day, Lausanne, 14.06.2013
    - *Human Progenitor Tenocytes for Artificial Tendon Constructs Designed for Treatment of Tendon Injuries*
- eCM XIV Stem & Progenitor Cells, Davos, 23−25.06.2015
    - *Human Progenitor Tenocytes to Improve Healing in Tendon Injuries*
- SSCM meeting, Bienne, 14.11.2013
    - *Human Progenitor Tenocytes and Extracellular Matrix Scaffold to Treat Hand Flexor Tendon Injuries*
- PhD Day, Archamps, 13.06.2014
    - *Development of an Artificial Tendon*
- SICT meeting, Genève, 12.11.2014
    - *Human Fetal Progenitor Tenocytes as a Perspective for Tendon Treatment*
- 13th TMB Research Day, Lausanne, 19.06.2015
    - *Human Progenitor Tenocytes as a Potential Cell Source for Tendon Healing*

Posters

- SSB Meeting, Zürich, 03.05.2012
    - *Human Progenitor Tenocytes for Re-Cellularization of Matrix for Rotator Cuff Repair*
- FBM Day, Lausanne, 07.06.2012 (Didactic Poster Award)
    - *Human Progenitor Tenocytes for Re-Cellularization of Matrix for Tendon Regeneration*
- ICBS meeting, Tsukuba, Japan, 19−22.03.2013 (Travel Award)
    - *Decellularized Extracellular Matrix of Horse Tendon in Combination with Human Progenitor Tenocytes for Tendinopathies Treatment*
- CHUV Hand Center inauguration, Lausanne, 15.03.2014
    - *Development of an Artificial Tendon for Hand Surgery*

Parallel projects, Teaching, Formation and Seminar Participation

- Supervision of a technician student during a 4-month stage
- Supervision of a technician trainee during a 7-month stage
- Supervision of a medical doctor student during his Master project
- Collaborations with the intern medical doctors of the Service of Plastic, Reconstructive and Hand Surgery during their 1 year stage

- Preparation and teaching of the course « From Clinics to Basics… and Back! » in the M4 Module of the 6th semester of Bachelor in Biology at University of Lausanne
- Preparation and teaching of a lesson on continuing education on the subject « From Laboratory to Clinical Application of Cellular Therapies» in a meeting of the HUG Pharmacy
- Several presentations in research meetings of the Unit of Regenerative Therapy from CHUV, of the Unit of Community Pharmacy from PMU and of the Laboratory of Biomechanical Orthopedics from EPFL
- Preparation and teaching at Expo for Cell Therapy in Museum Verdan
- Technical assistance on histology, photography and film for Swissnex Expos in San Francisco, New Delhi, Bangalore and coming soon in China
- Participation in Burn Prevention TV spots for CHUV Burn Center and Flavie Burn Association

Peer-Reviewed Publications as Co-Author

Krähenbühl et al. Int J Stem Cell Res Ther 2015, 2:1

International Journal of
Stem Cell Research & Therapy

Research Article: Open Access

Enhancement of Human Adipose-Derived Stem Cell Expansion and Stability for Clinical use

Swenn Maxence Krähenbühl, Anthony Grognuz, Murielle Michetti, Wassim Raffoul and Lee Ann Applegate*

Plastic, Reconstructive and Hand surgery, Regenerative Therapy Unit, University Hospital of Lausanne, Switzerland

***Corresponding author:** *Lee Ann Laurent-Applegate, University Hospital of Lausanne (CHUV), Department of Plastic and Hand Surgery, Regenerative Therapy Unit (UTR), EPCR/Croisettes 22, CH-1066 Epalinges, Switzerland, Tel: 41 21 314 3510, E-mail: Lee.Laurent-Applegate@chuv.ch*

Abstract

Co-culture techniques associating both dermal fibroblasts and epidermal keratinocytes have shown to have better clinical outcome than keratinocyte culture alone for the treatment of severe burns. Since fat grafting has been shown to improve scar remodelling, new techniques such as cell-therapy-assisted surgical reconstruction with isolated and expanded autologous adipose-derived stem cells (ASCs) would be of benefit to increase graft acceptation. Therefore, integrating ASCs into standardized procedures for cultured skin grafting could be of benefit for the patient if cell quality and quantity could be maintained. The purpose of this study was to evaluate ASC processing from adult tissue with simple isolation (without enzymatic steps), expansion (low density of 325-3,000 cells/cm^2) and storage conditions to assure methods to enhance the cellular resistance when transferred back to the patient. Co-culture with cell-banked skin progenitor cells (FE002-SK2) showed an increase of 40-50% ASCs yield at high passages alongside with a better preservation of morphology, proper adipogenic and osteogenic differentiation and efficient biocompatibility with 3D collagen scaffolds. ASCs can be considered as a valuable additional cell source to be delivered in biological bandages to the patient in a need of tissue reconstruction such as burn patients.

Keywords

Adipose-derived stem cells, Cell stability, Co-culture, Fat grafting, Lipofilling, Burn treatment, Biological bandages

Introduction

Multipotent stem cells may be beneficial in tissue engineering applications for reconstructive surgery, particularly for soft tissue enhancement and around implants for better integration [1,2] and potentially for burn patients [3-6].

Until recently, the predominant source of stem cells considered for clinical applications was bone marrow-derived mesenchymal stem cells (BM-MSCs). More recent studies using fresh stem cells derived from white adipose tissue, termed adipose-derived stem cells (ASCs) [7], have confirmed them as multipotent mesenchymal stem cells [8-11]. Advantages are high availability and easy accessibility from direct excision or from fresh lipoaspirate [12]. It was shown that their therapeutic potential is equal or even superior compared to BM-MSCs because of their higher number and viability upon transplantation [8,13].

In severely burned patients, it is now accepted that co-culture of both fibroblasts (dermal) and keratinocytes (epidermal) provides a better clinical outcome for burn applications [14]. With such patients, there is also a lack of soft tissue and adipose tissue injection has been proposed to help in scar tissue remodelling [3]. Therefore, with standardized methods for ASC culture using small quantities of tissue (that could be taken during initial debridement and biopsy for keratinocyte culture), these patient cells could be easily integrated into the co-cultured treatment for burns and perhaps provide a better overall treatment for skin and soft tissue reconstruction.

Isolation methods from lipoaspirate digested using collagenase, filtered and finally centrifuged have been used in research [2]. Recent techniques have shown that less aggressive enzymatic treatment using trypsin could be employed for better cellular stability but had lower overall cell yields. Reports have demonstrated that although mechanically isolated ASCs (mincing) revealed lower cell numbers, the viability was higher [15-17]. In addition, Zeng et al. [18] have shown isolation of ASCs without collagenase or trypsin although they treated the tissue for 3 consecutive days with 100% fetal bovine (FBS) serum to "digest" the tissue and allow cells to emerge from the tissue mass. Although many protocols have been tested and allowed to improve stability by decreasing enzymatic treatment, ASCs remain a cell population that loses growth potential already beginning in passage 4 (similar to that seen in BM-MSCs) and has difficult recovery following freezing [19]. Even if ASCs have been proposed as the best stem cell source in terms of quantity, large amounts of tissue are generally not available from burn patients, so we optimized a standard method for rapid ASC isolation revising latest results of protocols without enzymatic digestion and using only very small quantities of tissue. Important culture parameters for clinical use were controlled, including population growth and stability and differentiation potential. The growth of ASCs co-cultured with progenitor skin fibroblasts was evaluated for potential increase in cell number, quality, and overall robustness to better prepare cells for *in vivo* environments.

ClinMed International Library

Citation: Krähenbühl SM, Grognuz A, Michetti M, Raffoul W, Applegate LA (2015) Enhancement of Human Adipose-Derived Stem Cell Expansion and Stability for Clinical use. Int J Stem Cell Res Ther 2:007

Received: March 28, 2015: **Accepted:** April 04, 2015: **Published:** April 06, 2015

Table 1: Adipose tissue from healthy patients undergoing elective reconstructive surgeries

Id n°	Gender	Age	BMI	Technique
G01	F	49	24.2	lipoaspiration
G02	F	47	23	lipoaspiration
G03	F	47	30.1	direct excision
G04	F	51	26.4	lipoaspiration
G05	F	39	29.4	lipoaspiration
G06	F	27	24.5	lipoaspiration
G07	F	61	28.7	lipoaspiration
G08	F	45	21.3	direct excision
G09	F	46	24.2	direct excision
G010	F	49	22.3	lipoaspiration
G011	F	43	22.3	lipoaspiration
G012	F	52	28.7	direct excision
G013	F	41	26.3	direct excision
G014	F	54	21.9	direct excision
G015	F	40	27.7	direct excision

High expectations that these cells may help solve some of the reconstruction problems faced in clinics could be met if the cell source can be shown to be stable, reliable and routinely isolated, cultured and stocked under standardized systems allowing for safe clinical use.

Experimental Methods

Tissue processing, isolation and cell bank preparation

Adipose tissue from healthy patients (n=15, between 27-61 yr - average age and standard deviation of 46.1 ± 2.0 years and mean BMI of 25.4 ± 0.8kg/m^2) (Table 1) undergoing various elective reconstructive surgeries were obtained either by direct excision or manual aspiration (Protocol included in the Department of Musculoskeletal Biobank Regulation: 12/2012 "Conservation of Biological human material for research"). They were given a code (Table 1) and placed in 50ml sterile polypropylene tubes containing transport solution of phosphate buffered saline (PBS) (NaCl 6.8g/l, Na_2HPO_4 1.5g/l, KH_2PO_4 0.4g/l) and 1% penicillin-streptomycin (P/S). Samples were processed within 12 hours and were rinsed twice with 5% P/S in PBS for 10 min until connective tissue and blood were released. Tissue (~10g) was then minced into small pieces (diameter: <5mm) and the washing step was again repeated in 2% P/S in PBS. Approximately 1g of tissue was then directly seeded onto 10cm petri dishes prepared with scalpel incisions to attach tissue fragments at <20% density of the surface (Figure 1). Initial experiments using three patient tissues were accomplished to determine the influence of cell culture media, based on population doubling-time and morphological analysis. Culture media assessed were as follows: A. Dulbecco's modified Eagle medium (DMEM, Gibco) / FBS (Sigma) / l-glutamine (l-glut, Gibco) (89:10:1); B. DMEM / Ham-F12 - GlutaMAX™-I (Gibco) / FBS (45:45:10); C. DMEM / Iscove's Modified Dulbecco's Medium (IMDM, Gibco) - GlutaMAX™-I / FBS (45:45:10); D. DMEM / Minimum essential medium alpha (α-MEM, Gibco) / FBS / l-glut (59:30:10:1). Samples were cultured in an incubator (37°C, 95% humidity, 5% CO_2) and culture media was renewed twice a week. Cells were then passed when reaching 80-90% confluency by removing cells from culture plates with 2ml of TrypLE™ Express (TrypLE: Gibco) for 10 min. Cells were then reseeded into 75cm^2 flasks for further expansion. Cell banks were established for each patient up to passages 3-4, by freezing a minimum of 20 vials of cells at 1 million cells in 1ml freezing media [50% culture media, 40% FBS and 10% dimethyl sulphoxide (DMSO)] and cryopreserved at -80°C until later use. Cells were cryopreserved for a minimum of 2 weeks to measure overall viability. They were then seeded into 6-well culture plates at 1,500 cells/cm^2 density to assess for potential adverse impact on cell growth rate and recovery following freezing (hemocytometer total viable cell counts with trypan blue exclusion, n=3). Cells that were cryopreserved were compared to cells that had not been cryopreserved in the same passage. Morphology of cells was captured in images at 8 days of culture and counting and doubling times were assessed at 14 days of culture.

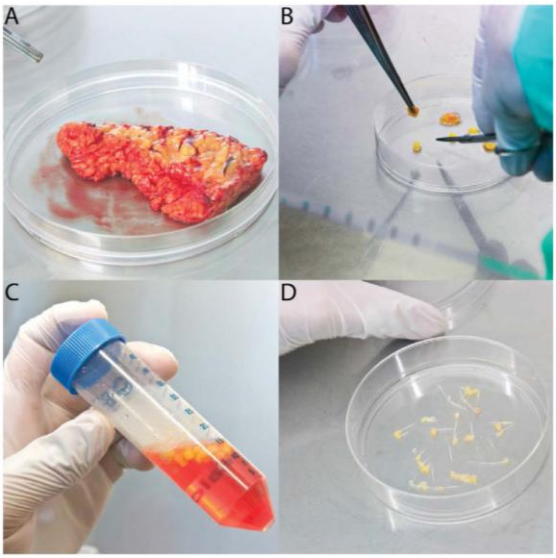

Figure 1: Processing of adipose tissue samples for primary culture

Adipose tissue was transported into the lab following tissue extraction (A) and following a first rinsing procedure in 5% P/S in PBS, adipose tissue was then minced into small pieces (<5mm) (B), rinsed again in 2% P/S (C) and resulting tissue pieces were placed into cell culture dishes that had been deeply scored with a scalpel to attach the tissue into the culture plate for primary culture (D).

Mechanical versus enzymatic isolation of adipose-derived stem cells

ASCs from two patients were isolated and harvested as described above, and the cells from two other patients were isolated with collagenase for either 30 min or 120 min according to the protocol from Buschmann et al. [20]. All four cell lines established were used at the same passage (P2) for experimentation. Cells from each cell line were seeded in triplicate into 96-well culture plates at 3,000 cells/cm^2 in order to assess differences between growths of collagenase- and mechanically-isolated cells up to 19 days. Growth rates were assessed following a metabolic assay based on Cell Titer (Promega) and metabolite absorbance (Tecan Infinite F50 plate reader with 40 min incubation at 37°C and blank subtraction).

Impact of cell seeding density for ASC culture

Mechanically-isolated ASCs were seeded into 96-well cultures plates at various low densities from passages 2 to 5, in order to assess the lowest density for ASC culture and observe the evolution of population doubling time among the passages. Growth rates were assessed at day 12 according to a metabolic assay based on Cell Titer, as described above. Experiments were repeated three times with three different donors.

Differentiation culture assay and staining procedures

Differentiation assays were assessed to confirm that the differentiation potential is maintained with the retained protocols for mechanical mincing of small amounts of tissue, standard medium, low cell density seeding along with freezing of cell stocks. Cells from three patients were seeded at 3,000 cells/cm^2 into 12-well plates and cultured with our standard culture medium (formulation A) for 3-5 days, before the beginning of the differentiation assay, in order to obtain around 50% confluence. Cells were then cultured in 2ml of the proper induction medium and changed twice a week over a period of 21 days from the beginning of the induction (three experiments conducted in triplicate).

Osteogenic potential was induced with alpha-MEM, 10% FBS, 1% l-glut, 50µg/mL l-ascorbic acid, 5mM β-glycerophosphate and 100nM

dexamethasone. Adipogenic potential was induced with DMEM, 1% l-glut, 1x ITS, 1µM dexamethasone, 100µM indomethacin and 100µM 3-isobutyl-1-methylxanthine (IBMX). A part of osteogenically-induced cells in monolayer were fixed in 4% paraformaldehyde for 10 min at room temperature and washed with deionized H_2O and followed standard von Kossa (VK), and Alizarin red (AR) staining procedures in order to show visual mineralization. The other cells were fixed in a citrate/acetone solution for 30 sec and followed alkaline phosphatase (ALP) staining to highlight ALP activity. Similarly, adipogenic-induced cells in monolayer were fixed in 4% paraformaldehyde and washed as described above and stained using Oil Red O (ORO) standard procedure to reveal cytoplasmic lipid deposits.

Cell stability and yield over passages using co-culture (FE-002γ)

In order to assess potential growth stability and stimulation of ASC co-culture with progenitor fibroblasts, ASCs from 4 different patients were cultured between passages 4-6, with and without co-culture prepared with human progenitor skin cells (FE002-SK2) that had been gamma-irradiated with 200Gy (referred as FE002γ) (dose response was performed by using increment doses until no cell growth or colonies were formed). These off-the-shelf frozen cell banks are used for routine skin graft cultures for severely burned patients in our hospital to provide cytokines and growth factors for better quality cell growth (Ethics protocol #62/07 and organ donation registered under the Federal Transplantation Program complying with the laws and Biobank procedures of the University Hospital of Lausanne and SwissMedic. The cells are registered with the European Collection of Cell Cultures with the Health Protection Agency at Porton Down, ECACC-FE-002-SK2#12070301). These cells have been used in clinical trials as a viable fibroblast cell source for patients with burns and wounds [21,22]. Patient ASCs were seeded between 1,500-3,000 cells/cm² into 75cm² culture flasks with FE002γ at a constant dose of 1,000 cells/cm². Cell growth of ASCs was assessed by trypan blue exclusion with total viable cell counts using a Neubauer growth chamber (hemocytometer) at 3-6 days. Control flasks of FE002γ alone were prepared for each experiment and did not show any cell growth or viability (trypan blue) following the irradiation and freezing protocol for preparation of clinical batches.

Biocompatibility assessment of biodegradable scaffold

Aiming for further clinical use on patients and better localized cell delivery, we evaluated the biocompatibility of the ASCs when seeded into a collagen membrane (TissuFleece E®, Baxter). Briefly, cells were seeded at 1,000 cells/cm² into the collagen matrix, and cellular colonisation of the matrix was evaluated by a Live/Dead metabolic assay at 3 different time points (24, 48, 72h in triplicate). The Live/Dead solution was prepared fresh [1ml PBS, 2µl Ethidium homodimer-1 (EthD-1), 0.5µl calcein acetsymethyl ester (calcein-AM)] as described by the manufacturer (Life Technologies, L-3244, Live/Dead Viability/Cytotoxicity). Membranes were placed in 6-well culture plates, rinsed with PBS and 50µl of Live/Dead solution was pipetted onto each membrane. They were incubated at room temperature for 30 min and images were taken with an Olympus IX81 fluorescent microscope with an Andor iXon digital camera.

Results and Discussion

Standardization/simplification of isolation, expansion and stocking of adult ASCs

As regulations have been severely intensified for cell therapies,

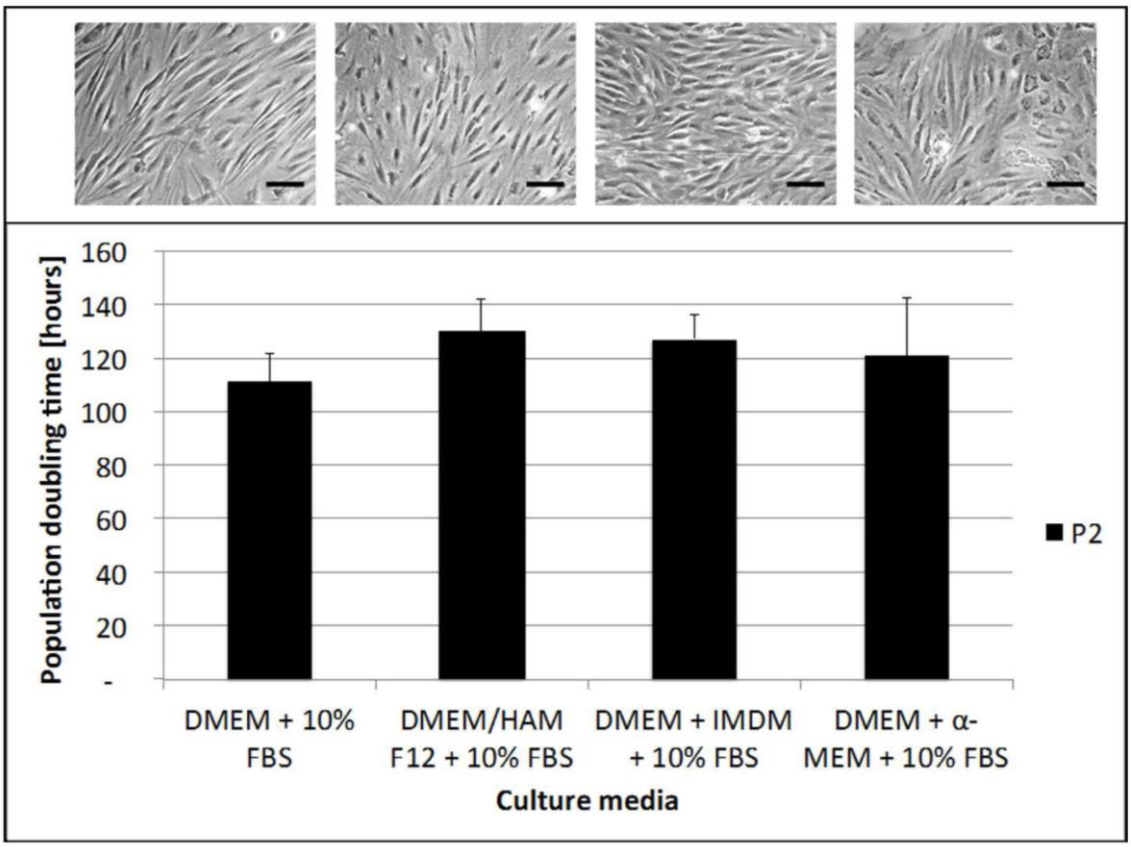

Figure 2: Media effect on ASC morphology and growth

Cell growth and morphology was compared using four different growth media. Resulting cell cultures plated at 3,000 cells/cm² were imaged at 8 days and cell growth for 14 days was assessed with doubling times. Experiments were done in triplicate (mean ± SEM). Scale bar on images represents 100µm.

each step including tissue procurement, cell isolation, cell culture and stocking is very important. Within the technical scope of manipulating stem cells for clinical use, procedures need to be simplified to assure safety and respect manufacturing guidelines. Using simple materials and methods for isolation is of utmost importance in order to treat the tissue and the subsequent cells in the least aggressive manner possible. In this study, the dissociation of small quantities of tissue could be done rapidly and without any aggressive enzymes, centrifugation forces or high serum treatments. Adipose tissue harvested from 15 women patients was minced and placed into cell culture dishes (~1g) that had been scored to allow good attachment and early cell migration (Figure 1). Cells were seen to emerge as early as 3-4 days following this procedure and grown to confluence within 10-14 days (80-90% growth over culture surface) for all patients. By using this mechanical mincing and "direct attachment" procedure, only small amounts of tissue were necessary (Figure 1B,1D), which helped to avoid contamination since tissue could be dissected within small masses and not on the edges. Media optimization was then determined using three separate cell cultures and it was found that the most simple composition [DMEM / FBS / l-glut (89:10:1)] was the most stable between passages. Early passage ASCs showed similar population doubling times with all media used (~110 h) (Figure 2). At later passages, the population doubling time was seen to increase in a linear manner with the DMEM / FBS / l-glut (89:10:1), but the other media combinations portrayed high variability among passages. Once the culture conditions were determined, the use of collagenase treatment of various times (30 and 120 min) was compared to the mechanical isolation procedure we have defined for tissue isolation. Since digestion is currently used for cell separation, it was of interest to see if this step could be eliminated and still have rapid cell growth. We found that 30 min of collagenase treatment showed lower cell growth compared to the less aggressive treatment of tissue without collagenase treatment. If tissue in small quantities (~10g) was subjected to 120 min of collagenase, there was no cell growth observed indicating that this variable may be of interest to eliminate. Other previous studies have shown that cell growth is sensitive to trypsin enzyme treatments also [15]. Freezing the cell gently with temperature increment freezing, and keeping them stored at only -80°C instead of liquid nitrogen (which is normally more difficult to have in the clinical surgical site) allowed very high viability (~97-98%) after 2 weeks or more. More important is the actual recovery of cultured ASCs following freezing protocols to assure their growth stability and potential to stock patient cells until they are needed for therapeutic procedures. With our isolation method, we have seen a slight shift in recovery of ASCs when cryo-preserved for two weeks or more by approximately 3 days, as compared to cells at the same passage without freezing (Figure 3). Even with a slight decrease in cell recovery once ASCs had been cryopreserved, banked cells would however allow to obtain high potential number of cells for patient treatments.

Maintenance of differentiation potential

The ASC culture with our defined protocol assured that patient cells maintained differentiation potential after 21 days of stimulation in respective media for osteogenic or adipogenic cell culture conditions (Figure 4). Osteogenic induction was observed by the presence of mineral deposition and enzymatic activity with three different staining procedures including Alizarin red (Figure 4A,4E), von Kossa (Figure 4B,4F) and alkaline phosphatase (Figure 4C,4G). There was light staining of ALP already in non-induced ASCs and since it has been shown that ALP enzymatic activity varies with the age of the donor, the two other staining procedures were used to evaluate mineralization. Adipogenic potential was seen by the large lipid droplets stained with oil red O in the induced cells (Figure 4D,4H) and good overall differentiation potential was similar to that seen recently by Lin et al. [23] showing a uniform multipotent stem cell source.

Optimization of cell growth by seeding density and co-culture

Low density seeding was accomplished over early passages (seeding densities of 325, 750, 1500 and 3000; passages 2-4) to observe full growth characteristics and all showed good cellular expansion capacity. The densities of 1500 to 3000 cells/cm^2 would have a slight

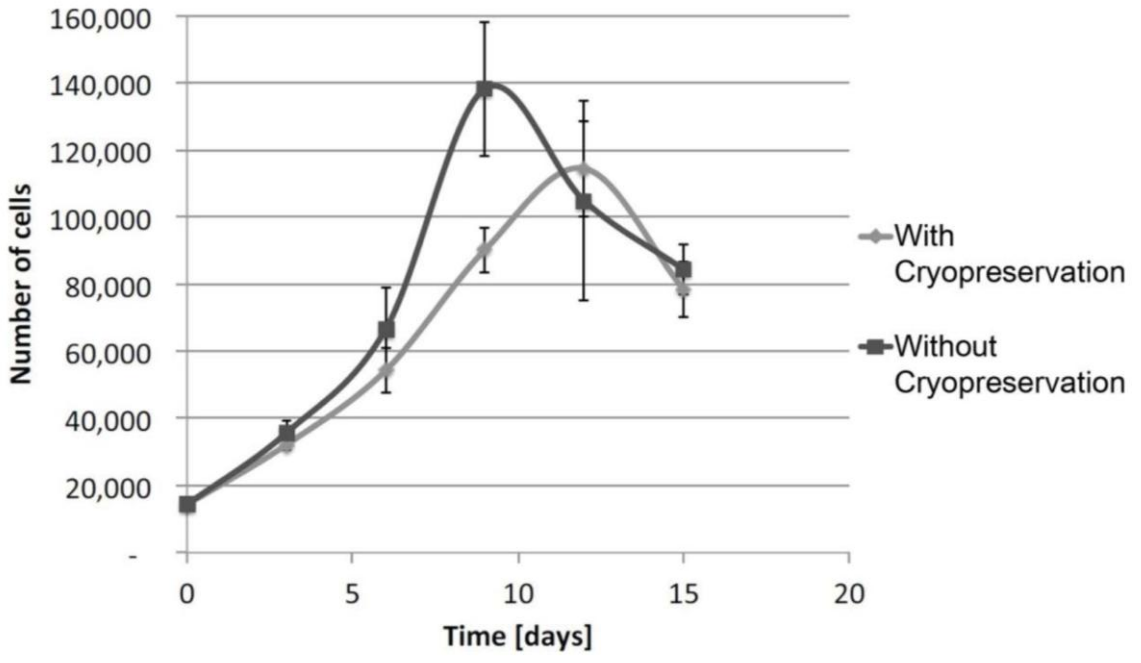

Figure 3: Recovery of ASCs following cryopreservation

Growth rate of cells after 2 weeks or more of cryopreservation (◆) were measured as a function of cell number over time in days. They were compared to cells at the same passages without cryopreservation (■). Results are from experiments in triplicate (mean ± SEM).

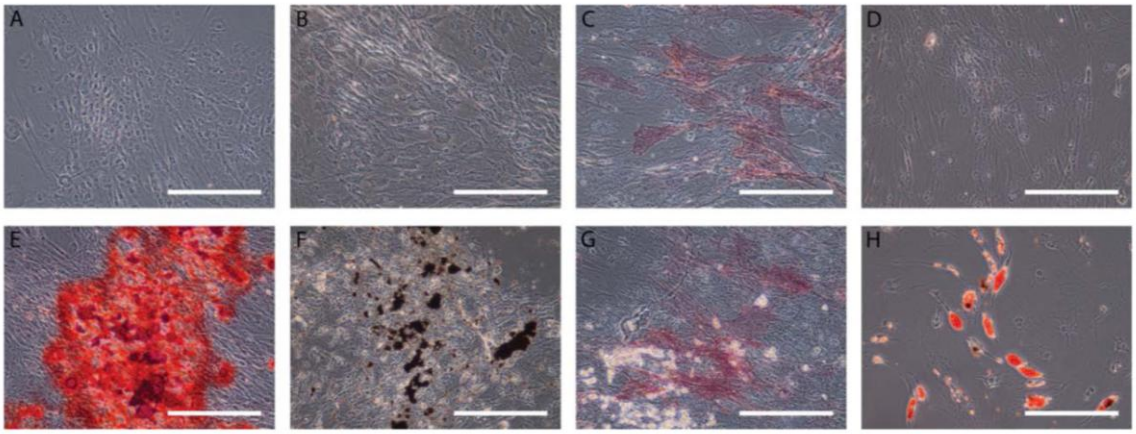

Figure 4: Osteogenic and adipogenic differentiation of ASCs

Patient ASCs were cultured in an osteogenic-inducing medium and stained with Alizarin red (A and E) and von Kossa (B and F) showing mineral deposits and alkaline phosphatase (C and G) showing enzymatic activity. ASCs were cultured in adipogenic-inducing medium and stained with oil red O showing lipid deposits (D and H). Scale bar on images represents 250µm.

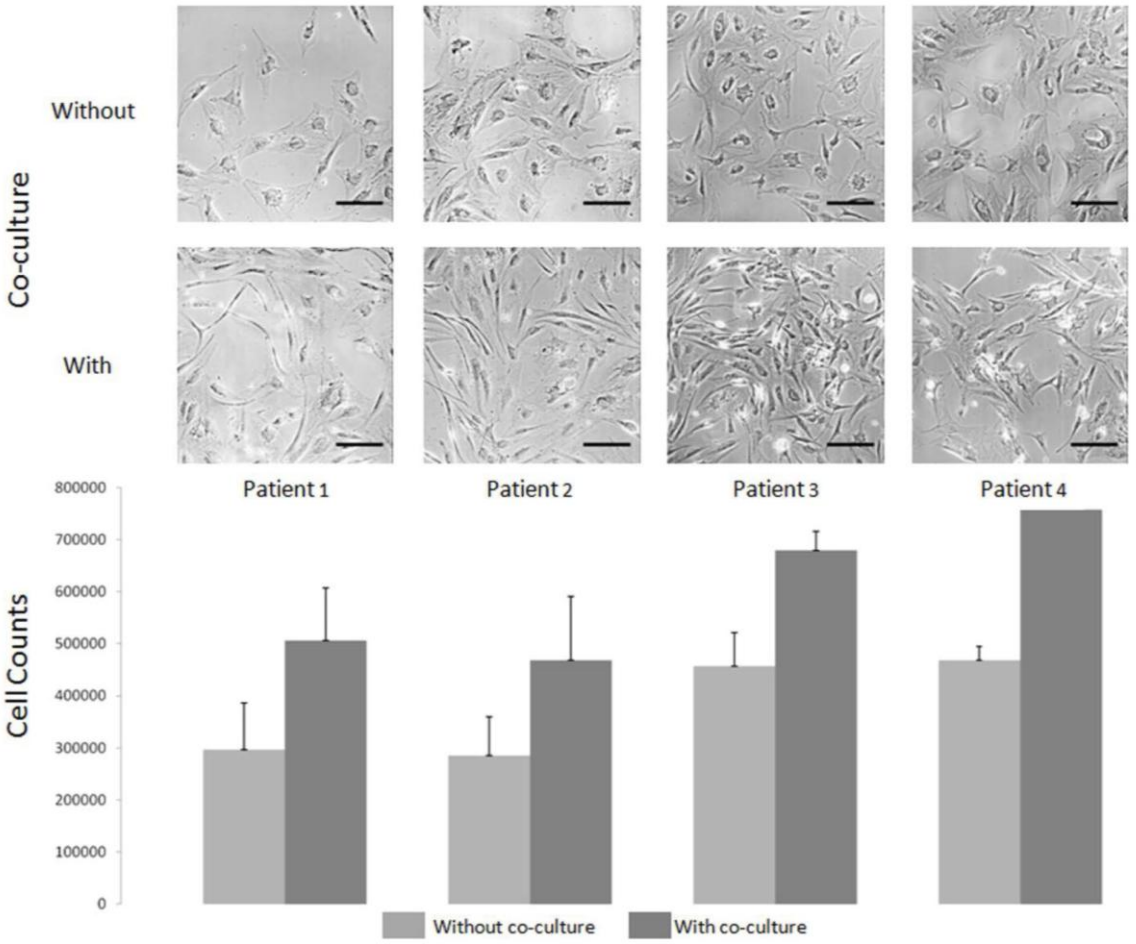

Figure 5: Cell stability and yield over passages using co-culture (FE-002γ)

Cells from passage 6 of four patients are illustrated with growth conditions with and without skin progenitor cells (FE002γ, progenitor skin fibroblasts pre-treated with gamma irradiation of 200Gy and kept as frozen stocks) portraying typical fibroblastic cultures of later passages with many cells larger with wide cytoplasm when under normal culture conditions (without co-culture). ASCs grown in combination with FE002γ showed cell populations more similar to those in passage 2-4 for most patients. Cell numbers were counted for each patient in triplicate without co-culture (dark grey) and with co-culture (light grey). Scale bar on images represents 100µm.

benefit to obtain enough cells rapidly. Of the 15 patients, only two had cell growth that extended to passages 6 and beyond, without the typical cell morphology changes (enlarged cells with wide cytoplasmic spread). This aspect of culturing adult stem cells has also been illustrated by others showing slowing of cell growth and large, spreading cell morphology already at passage 4-6 [19]. Primary cell cultures from patient tissues are known to show heterogeneous growth capacities with overall lower growth over time. Because of this, we evaluated the use of co-culture directly with skin progenitor cells (FE002γ) to culture or to help adult adipocytes to remain stable over longer periods and thus, even higher passages could be used to prepare protocols for clinical batches of cells if needed. As early cells can only be frozen after several amplifications, it is only at passage 2-3 that enough cells could be stocked. Therefore, placing the cells into culture with further expansion would bring them easily to passage 5-6. It would therefore be important, particularly for severely burned patients where multiple grafts are needed over time, to have the possibility to increase cell expansion potential for cells that may be relatively less stable over passages. This variability, when working with adult stem cells, has been difficult to integrate into standardized protocols. Cell growth of ASCs in the presence of FE002γ was found to be 40-50% higher in number and the morphology and stability

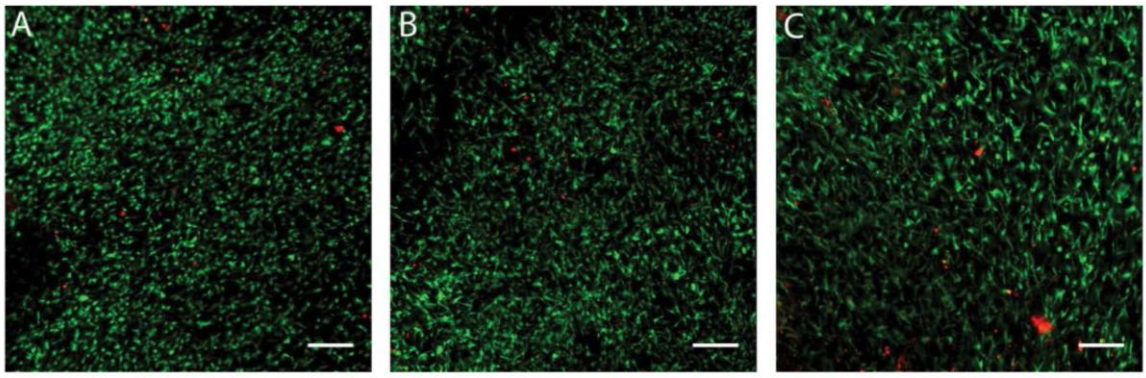

Figure 6: Biocompatibility and delivery potential of ASCs in collagen scaffold

Cells were seeded into collagen scaffold (TissuFleece®, Baxter) and stained with Live/Dead kit after 24h (A) 48h (B) and 72h with live cells stained in green and rare non-viable cells in red. Scale bar on images represents 250µm.

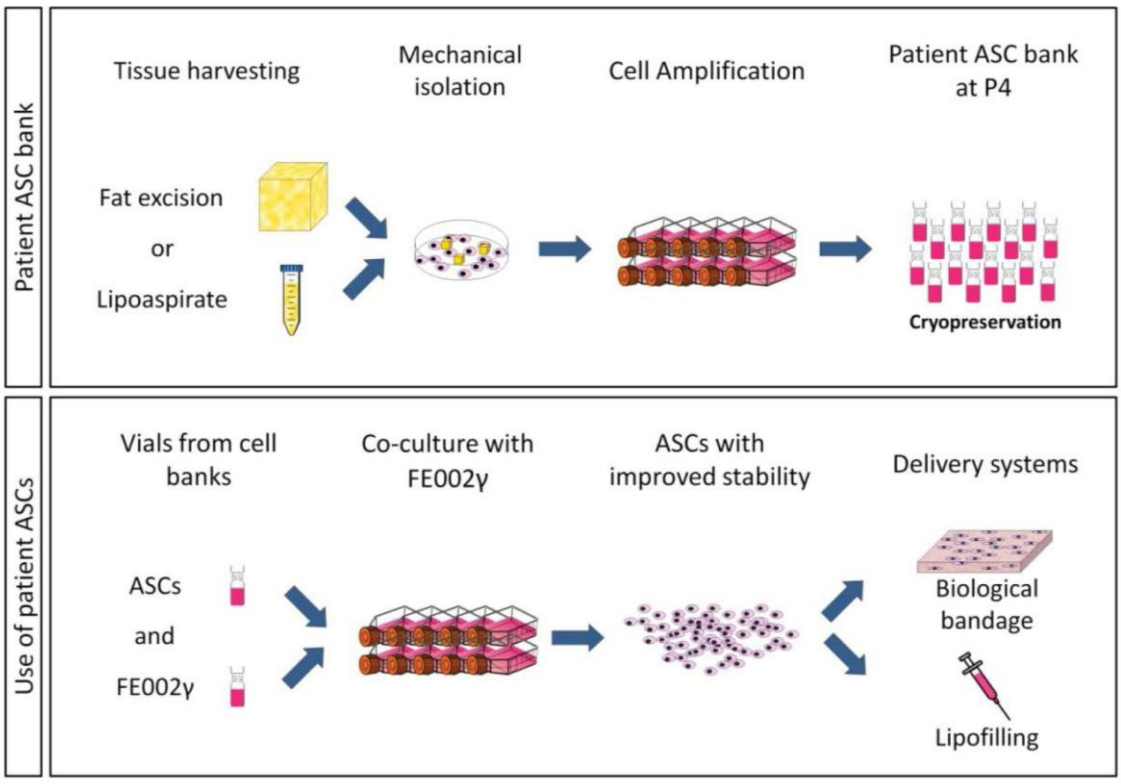

Figure 7: Development of ASC banks and protocol to enhance cell stability through co-culture

Patient adipose tissue is micro-dissected into culture plates to allow rapid cell outgrowth and amplification for cryopreservation. Cells can be either used directly if enough cells are acquired or continued for expansion. Enhancement of cell numbers and quality can be obtained by co-culture with progenitor cells before associating them with matrix materials.

were optimized (cells showing finer spindle-shaped morphology rather than large cytoplasm and large round cell morphology) (Figure 5). Thus, we could increase total cell number through at least two cell passages. Use of FE002γ could prepare a robust adult ASC population for clinical use, as has been shown for skin cells for burn patients [21,22], and could be used in co-culture association for burn therapy.

ASC association with delivery matrix for clinical application

By having more robust cells with the co-culture protocol, we have shown that these cells could be integrated easily with 3D scaffolds to provide a delivery system to the patient. Use of CE marked and clinical-grade hemostatic sponge collagens used routinely in surgery (TissuFleece E®, Baxter) could be an elegant method to deliver ASCs now since they grow well in co-culture with fibroblasts. The ASCs showed integration into the matrix already at 24 h (Figure 6A) and following 48 h adhered strongly and migrated within the scaffold (Figure 6B,6C). Patient ASCs could now be considered to be associated within biological bandages used for burn patients to help in overall skin repair and soft tissue reconstruction [21,22].

The recent study in the Lancet by Kole and colleagues [1] showed that fat grafts supplemented with autologous culture-expanded ASCs were able to increase graft volume retention up to ~81% compared to only ~16% if no cultured cells were associated to the human fat grafts transplanted into the upper arm [1]. Therefore, the specific technique for cultured ASCs has shown to be of importance for cell selection and activity. This technique could prove to be a great advantage for the future of lipofilling and soft tissue reconstruction. More and more patients are in need of lipofilling with delays between operations due to their pathologies (i.e. scleroderma or damage from radiotherapy). There are also situations where multiple injections are needed such as for breast augmentation or scar corrections. There are particular problems around implant surfaces and matrix enriched with stem cells could provide a solution to help integration into the surrounding tissues with improved tolerance.

Importantly, ASCs could be added to grafting procedures routinely used for severely burned patients adding them with co-culture of keratinocytes and fibroblasts. Thus, having stocks of cells from individual patients would be of benefit for a large variety of interventions. The lower amounts of fat tissue from donor sites would increase safety and minimize donor site deformation. Furthermore, it would be possible to obtain sufficient amount of cultured ASCs also from patients with low fat deposit and for severely burned patients and their use could be a great advantage to be associated with fat grafts or skin grafts.

Methods and techniques for cellular therapies have to be performed under full Good Manufacturing Processes (cGMP compliance). As all the techniques for burn management use traditional methods employing basic media, fetal bovine serum and growth factors since 30 years in our hospital, it is of importance to include this new cell type with similar adapted culture conditions as has been positively illustrated in this study and adapt in a step-wise manner for potential improvements.

To this end, there has been much effort world-wide imagining the replacment of animal components in tissue and cell processing including patient serum [19], platelet derivatives [5,24,25] and recombinant growth factors [26,27]. These alternatives could now be analyzed in our long-term clinical setting to see if cellular growth and stability of cell stocks can be maintained whenever instigating changes in clinical protocols. All of these should be done step-by-step to assure the high quality necessary for patient use.

Conclusions

When working with tissue and stem cell isolation procedures that are destined for clinical use, it is important to have the simplest and the least aggressive treatments to allow robust cell populations. With mechanical isolation of ASCs instead of more common (but also more aggressive) enzymatic treatments, it is possible to obtain better cell growth alongside full maintenance of the differentiation potential. The cells could be frozen with a high survival rate (~98%) up to passages 4-6 and then co-cultured with FE002γ to improve the stability. Co-culture can significantly enhance primary ASC culture with higher numbers of patient cells obtained from a low quantity of tissue so that it would be possible to store the cells and thaw them when needed for transplantation. Finally, the fact that ASCs could be dispersed on collagen scaffolds with a good survival opens the possibility to use them in various manners, including filling of larger gaps and for skin grafting techniques routinely used to allow better soft tissue reconstruction with co-culture of keratinocytes and fibroblasts for burn patients (Figure 7). Cell culture assisted surgical techniques will need to be developed with Good Manufacturing Processing in mind since regulations have become more strict in the hospital environment, but taking care at integrating each cell type at each step of the tissue processing will increase benefits towards patient treatments and safety.

Ethical Statement

All of the work conducted in this study has been accept by the State Ethics Board with Progenitor skin cells under Ethics protocol #62/07 and the organ donation registration under the Federal Transplantation Program complying with the laws and regulations of the Biobank procedures of the University Hospital of Lausanne and SwissMedic. Adipose tissue collection is under Protocol #12/2012 "Department of Musculoskeletal Medicine Biobank conservation of biological human material for research and use" following all of the rules and regulations of this program approved by the State Ethics Board.

Acknowledgements

We thank Drs. Valérie Decrouy-Duruz, Pietro di Summa, Nathalie Koch, Giorgio Pietramaggiori, Laure Emilie Rey and Saja Sandra Scherer-Pietramaggiori of the surgical team of the Plastic, Reconstructive and Hand Surgery Service for providing the samples of adipose tissue, and Corinne Scaletta for valuable technical guidance and discussions. This study was supported in part by the Foundations Sandoz Family, S.A.N.T.E and Orthopaedic Hospital and the European Grant FP7-EPIONE.

References

1. Kolle SF, Fischer-Nielsen A, Mathiasen AB, Elberg JJ, Oliveri RS, et al. (2013) Enrichment of autologous fat grafts with ex-vivo expanded adipose tissue-derived stem cells for graft survival: a randomised placebo-controlled trial. Lancet 382: 1113-1120.
2. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, et al. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7: 211-228.
3. Klinger M, Marazzi M, Vigo D, Torre M (2008) Fat injection for cases of severe burn outcomes: a new perspective of scar remodeling and reduction. Aesthetic Plast Surg 32: 465-469.
4. Trottier V, Marceau-Fortier G, Germain L, Vincent C, Fradette J (2008) IFATS collection : Using human adipose-derived stem/stromal cells for the production of new skin substitutes. Stem Cells 26: 2713-2723.
5. Kakudo N, Minakata T, Mitsui T, Kushida S, Notodihardjo FZ, et al. (2008) Proliferation-promoting effect of platelet-rich plasma on human adipose-derived stem cells and human dermal fibroblasts. Plast Reconstr Surg. 122: 1352-1360.
6. Jeong JH (2010) Adipose stem cells and skin repair. Curr Stem Cell Res Ther 5: 137-140.
7. Locke M, Feisst V, Dunbar PR (2011) Concise review: human adipose-derived stem cells: separating promise from clinical need. Stem Cells 29: 404-411.
8. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, et al. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8: 315-317.
9. di Summa PG, Kalbermatten DF, Raffoul W, Terenghi G, Kingham PJ (2013) Extracellular matrix molecules enhance the neurotrophic effect of Schwann cell-like differentiated adipose-derived stem cells and increase cell survival under stress conditions. Tissue Eng Part A 19: 368-379.
10. Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, et al. (2006) Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells 24: 376-385.
11. Gronthos S, Franklin DM, Leddy HA, Robey PG, Storms RW, et al. (2001)

Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol 189: 54-63.

12. Dong Z, Luo L, Liao Y, Zhang Y, Gao J, et al. (2014) In vivo injectable human adipose tissue regeneration by adipose-derived stem cells isolated from the fluid portion of liposuction aspirates. Tissue Cell 46: 178-184.

13. Strioga M, Viswanathan S, Darinskas A, Slaby O, Michalek J (2012) Same or not the same? Comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells. Stem Cells Dev 21: 2724-2752.

14. Koch N, Erba P, Benathan M, Raffoul W (2010) Nouveautes dans la reconstruction cutanee : cultures de cellules et substituts dermiques et revue de la littérature. Ann Burns Fire Disasters 23: 131-136.

15. Markarian CF, Frey GZ, Silveira MD, Chem EM, Milani AR, et al. (2014) Isolation of adipose-derived stem cells: a comparison among different methods. Biotechnol Lett 36: 693-702.

16. Baptista LS, da Silva KR, Pedrosa C, Borojevic R (2011) Processing of lipoaspirate samples for optimal mesenchymal stem cells isolation. In: Advanced Techniques in Liposuction and Fat Transfer. (Ed. Serdev N) ISBN 978-953-307-668-3.

17. Kim JB, Lee HM, Kim SY, Park KS, Bae J, et al. (2011) Cryopreservation of Human Adipose Tissues using Human Plasma. J Biotechnol Biomaterial 1: 4.

18. Zeng G, Lai K, Li J, Zou Y, Huang H, et al. (2013) A rapid a efficient method for primary culture of human adipose-derived stem cells. Organogenesis 9: 287-295.

19. Kocaoemer A, Kern S, Klüter H, Bieback K (2007) Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue. Stem Cells 25: 1270-1278.

20. Buschmann J, Gao S, Harter L, Hemmi S, Welti M, et al. (2013) Yield and proliferation rate of adipose-derived stromal cells as a function of age, body mass index and harvest site-increasing the yield by use of adherent and supernatant fractions? Cytotherapy 15: 1098-1105.

21. Hohlfeld J, de Buys Roessingh AS, Hirt-Burri N, Chaubert P, Gerber S, et al. (2005) Tissue engineered foetal skin constructs to Avoid Autografting for Burns. Lancet 366: 840-842.

22. de Buys Roessingh A, Scaletta C, Hirt-Burri N, Hohlfeld J, Gerber S, et al. (2006) Development and Characterization of a fetal skin bank for tissue engineering and its clinical application for acute and chronic wounds. Cell Transplantation 15: 823-834.

23. Lin CY, Huang C-H, Wu Y-K, Cheng N-C, Yu J (2014) Maintenance of human adipose derived stem cell (hASC) differentiation capabilities using a 3D culture, Biotechnol Lett 36: 1529-1537.

24. Doucet C, Ernou I, Zhang Y, Llense J-R, Begot L, et al. (2005) Platelet lysates promote mesenchemal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications. J Cell Physiol 205: 228-236.

25. Bieback K (2013) Platelet lysate as replacement for fetal bovine serum in mesenchymal stromal cell cultures. Transfus Med Hemother 40: 326-335.

26. van der Valk J, Brunner D, De Smet K, Svenningsen AF, Honegger P, et al. (2010) Optimization of chemically defined cell culture media-replacing fetal bovine serum in mammalian in vitro methods. Toxicol in Vitro 24: 1053-1063.

27. Lund P, Pilgaard L, Duroux M, Fink T, Zachar V (2009) Effect of growth media and serum replacements on the proliferation and differentiation of adipose-derived stem cells. Cytotherapy 11: 189-197.