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Journal

Journal of Tissue Engineering and Regenerative Medicine, 9(5)

ISSN

1932-6254

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Publication Date

2015-05-01

DOI

10.1002/term.1870

Peer reviewed

JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med (2014)
Published online in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/term.1870

REVIEW

Advances in tissue engineering through stem cell-based co-culture

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Abstract

Stem cells are the future in tissue engineering and regeneration. In a co-culture, stem cells not only provide a target cell source with multipotent differentiation capacity, but can also act as assisting cells that promote tissue homeostasis, metabolism, growth and repair. Their incorporation into co-culture systems seems to be important in the creation of complex tissues or organs. In this review, critical aspects of stem cell use in co-culture systems are discussed. Direct and indirect co-culture methodologies used in tissue engineering are described, along with various characteristics of cellular interactions in these systems. Direct cell–cell contact, cell–extracellular matrix interaction and signalling via soluble factors are presented. The advantages of stem cell co-culture strategies and their applications in tissue engineering and regenerative medicine are portrayed through specific examples for several tissues, including orthopaedic soft tissues, bone, heart, vasculature, lung, kidney, liver and nerve. A concise review of the progress and the lessons learned are provided, with a focus on recent developments and their implications. It is hoped that knowledge developed from one tissue can be translated to other tissues. Finally, we address challenges in tissue engineering and regenerative medicine that can potentially be overcome via employing strategies for stem cell co-culture use.

Received 23 September 2013; Revised 19 December 2013; Accepted 3 January 2014

Keywords **stem cells; co-culture; tissue engineering; regenerative medicine; cellular interaction; repair; direct co-culture; indirect co-culture**

1. Introduction: the goals of co-culture

Tissue engineering is a multidisciplinary, translational science that aims to produce new tissues for restoring the functionality of organs and tissues impaired or damaged by disease and trauma (Reddi, 1994). Tissue engineering employs three main tools: scaffolds, signalling factors and cells. Scaffolds serve as substitute cellular microenvironments to support tissue formation by exerting biophysical influences, thus allowing cell attachment, migration and organization, while delivering both soluble and bound biochemical factors. Signalling factors influence and direct cell phenotype, metabolism, migration and organization; methods to use them can be gleaned from signals observed during native tissue formation (Reddi and Huggins, 1972). Cells can be used for a variety of functions; these include synthesizing the bulk of the tissue matrices, integrating with existing native tissues, maintaining tissue homeostasis in general and providing various metabolic services to other tissues and organs. Although terminally differentiated cells are commonly used for synthesizing the matrices that compose the bulk of tissues, stem cells, specifically adult stem cells, are quickly gaining popularity for their favourable properties. With a plethora of competencies, either terminally differentiated or stem cells can be harnessed to drive the tissue-engineering process.

Co-culture is the culture of multiple, distinct cell types, directly or indirectly, within the same culture environment. In direct co-cultures, cells are mixed together within the culture environment and allowed to make direct contact. In indirect co-cultures, cell types are separated within the culture environment and cell interaction occurs

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via soluble factors. Co-culture methods are used in tissue engineering for two purposes. The first and most common application of co-culture in tissue engineering is to drive tissue formation with the direct or indirect interaction of multiple cell types. The second is to maintain the potency of stem cells during their expansion. This is commonly implemented as a feeder layer, in which one cell population, usually of differentiated cells, secretes signalling factors and cytokines to condition the medium for the expansion of stem cells. This can involve either direct or indirect co-culture techniques. Co-culture can effectively recapitulate the relationships among cell types within native tissue, processes that are often inefficient when relying solely on scaffolds and soluble factors.

Co-culture systems control the behaviour and actions of cells through the interaction of the multiple cell types. The types of cells within a co-culture are termed target cells and assisting cells. In general, target cells are those that will eventually compose the engineered tissue and are responsible for the tissue's function (e.g. metabolic, mechanical). When multiple target cell types are cultured together, each can also serve as assisting cells to the other. Assisting cells guide the target cells to display a range of desired behaviours. These include proliferation or differentiation, matrix production or organization by direct cell–cell contact, adhesion of cells to extracellular matrices (produced by the assisting cells) and/or secretion of signalling molecules. The intimate interactions between assisting and target cells are often too complex to implement through exogenous control. Assisting cells constantly monitor and respond to the target cells' needs, effectively serving as a feedback control system that is constantly on and immediately responsive, thus creating an ideally controlled culture environment.

The role of stem cells in co-culture reaches beyond that of simply providing a favourable cell source with multipotent differentiation capacity. In traditional monoculture the use of stem cells is desirable, due to their ability to undergo expansion while maintaining an undifferentiated state, their capacity to differentiate into cells of multiple tissue types and their immune modulatory properties. The use of stem cells can limit or eliminate cell-sourcing issues, such as donor site morbidity and limited primary cell availability, as they may be isolated from embryonic and many types of adult tissue. Stem cells are arguably the most potent tool at the tissue engineer's disposal (Zhang *et al.*, 2009). Frequently in co-culture systems, stem cells are the target cells that differentiate and eventually synthesize the extracellular matrix (ECM) or metabolites that confer function to a tissue. When acting as assisting cells, stem cells can also make up the appropriate milieu toward the homeostasis of engineered tissues (Figure 1). For instance, stem cells have the ability to promote tissue repair, not only directly (Orlic *et al.*, 2001) but also indirectly. Their indirect role consists of local suppression of the immune system, inhibition of apoptosis and promotion of proliferation and differentiation, currently described as trophic mediator effects (Caplan and Dennis, 2006; Scadden, 2006).

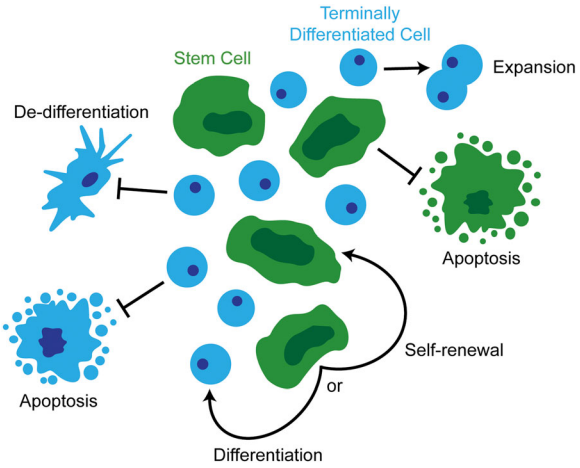

Figure 1. Co-culture systems contribute in a number of ways to tissue engineering. Depending on the design parameters, co-cultures can inhibit cell apoptosis and de-differentiation and encourage cell expansion/self-renewal or differentiation

Whether serving in target or assisting roles, stem cells are pivotal in tissue growth, metabolism, maturation and repair (Kalinina *et al.*, 2011; Sato *et al.*, 2004).

Significant advances in tissue engineering have already been made by employing co-cultures of stem cells together with terminally differentiated cells. Over the past four decades, methods to control and employ stem cells have been of paramount interest in tissue engineering and regenerative medicine (Fisher and Mauck, 2013). A variety of tissues have been engineered in recent years, including cartilage (Bigdeli *et al.*, 2009; Hendriks *et al.*, 2007; Hoben *et al.*, 2009), tendon (Canseco *et al.*, 2012; Schneider *et al.*, 2011), bone (Amini *et al.*, 2012), cardiovascular tissue (Gallo and Condorelli, 2006; Mummery *et al.*, 2007; Rangappa *et al.*, 2003), liver (Bhandari *et al.*, 2001) and kidney (Vanikar *et al.*, 2007; Yokoo *et al.*, 2005). Differentiating stem cells toward specific cell lineages is a common goal in tissue engineering. These examples all utilize co-cultures of stem cells and terminally differentiated cells to achieve that goal. Often tissue function may only be attained by mimicking the tissue's native cellular interactions and architecture. Stem cell co-cultures achieve this recapitulation and therefore show promising advantages for tissue engineering.

In general, to use stem cells in tissue engineering one must expand, differentiate and coax them to synthesize ECM; each of these steps can benefit from co-culture methods, including using other cells to assist the maintenance of stemness or to promote stem cell differentiation toward target tissues. This review provides a description of the different co-culture techniques used in tissue engineering, and highlights the modes of cellular interaction occurring in these systems. It describes the advantages of stem cell co-culture strategies and their applications in tissue engineering and regenerative medicine. By using specific examples of several tissue types, co-culture systems are discussed in relation to the great potential they hold and the challenges that current methods face.

DOI: 10.1002/term

2. Co-culture methodologies used in tissue engineering

Co-cultures are used in various fields of biological research and have moved to the forefront within recent history. Co-culture systems were introduced in the early 1980s as a system for studying cell–cell communication (Lawrence *et al*., 1978). Thereafter, the use of co-culture systems in the field of embryonic development and in regenerative medicine led rapidly to their use with heterogeneous cell populations (Chan and Haschke, 1982; Hendriks *et al*., 2007; White *et al*., 1989). Co-culture systems were then further adapted to differentiate stem cells toward specific cell types (Scheven *et al*., 1986). Co-cultures have evolved during their use, gaining a leading role among culture methodologies and becoming crucial in tissue engineering.

In the context of using heterogeneous mixtures of cells in co-culture systems for tissue engineering, it is necessary to first establish the proper nomenclature. A first and simple classification of the cells used in co-culture is that *terminally differentiated cells* come from a functionally mature tissue or organ, and *stem cells* are cells with potential to differentiate into distinct cell types and the capacity to proliferate without losing 'stemness' (self-renewal). Stem cells are further categorized by their potency, or degree of commitment, and subsequent ability to differentiate into different cell types. A totipotent cell, i.e. a zygote, has the ability to differentiate into all of the cells of an organism (Mitalipov and Wolf, 2009). Pluripotent cells, e.g. embryonic stem cells (ESCs), have the potential to differentiate into cells of any of the germ layers. More limited are the multipotent cells, which are commonly known by the tissues toward which they have the ability to differentiate. Fully mature/terminally differentiated cells are fully committed to a specific cell type. However, from these, induced pluripotent stem cells (iPSCs) may be formed (Takahashi and Yamanaka, 2006). Within co-culture systems, cells are categorized based on their function toward achieving the goals of tissue engineering. Tissue engineering intends to generate tissues that can be used for implantation *in vivo*. As mentioned in the Introduction, there is always a cell type that is considered a *target cell*, meaning that the co-culture is focused on the creation or maintenance of this cell type. Additionally, *assisting cells* are also present in co-culture. These cells provide means to achieve the goal related to the target cell. The terminology used for the different cell types is based on their characteristics and their roles within the co-culture system.

Although the classification of the different cell types is important within a co-culture system, this does not mean that the target cell is receiving all the benefits. As recently shown, there are mutually advantageous effects on both the target and assisting cells (Bian *et al*., 2011). For example, mesenchymal stem cells (MSCs) enhance chondrocyte proliferation, while simultaneously chondrocytes promote the differentiation of MSCs (Acharya *et al*., 2012). *In vivo* implantation of heterogeneous mixtures of cells can be considered a form of co-culture. However, there are distinct differences from direct and indirect co-cultures. First, prior to introduction to the *in vivo* environment, cell mixtures for implantation are typically cultured independently, not in direct or indirect co-culture configurations. Their typical expansion as monocultures may present limitations in terms of phenotype maintenance, speed of proliferation, etc. Thus, the strategy of implanting heterogeneous cell mixtures can likewise benefit from a co-culture period *in vitro*. The concept where mixtures of cells are cultured together prior to implantation will be covered in this review. Second, once implanted, the *in vivo* environment imparts multi-level stimuli upon the implanted cells that are due to both direct and indirect interactions with the body's cells. These interactions are too complex to enumerate and are beyond the scope of this review. Beyond the classifications discussed, co-culture systems in general are divided into two groups, based on the culture conditions of the cells: (a) direct co-culture; and (b) indirect co-culture.

2.1. Direct co-culture

Direct co-culture systems are cultures in which two or more distinct cell types are mixed and cultured together (Figure 2). This can be done in two-dimensional (2D) or

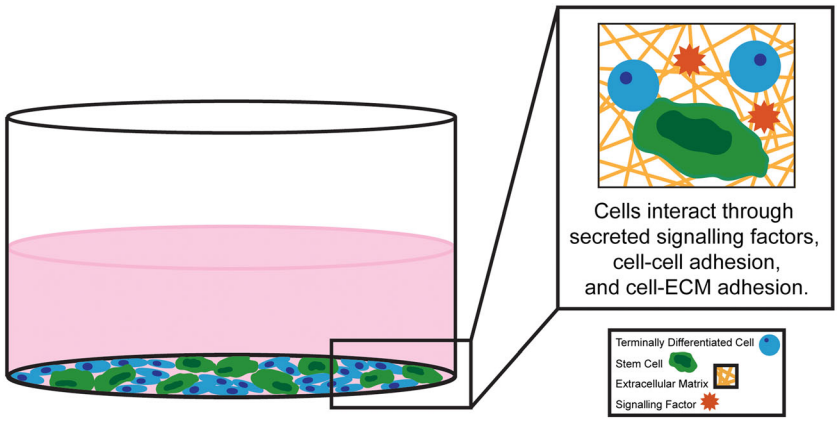

Figure 2. An example of direct co-culture system, where stem cells and terminally differentiated cells are mixed and cultured together. In this system, cellular interactions occur through direct cell–cell contact, cell–ECM adhesion and soluble factors

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three-dimensional (3D) culture. 2D culture environments typically include mixed-population monolayers and feeder layers on slides and in flasks and dishes (Nishiofuku *et al.*, 2011; Sugiyama *et al.*, 2007). For example, neonatal rat cardiomyocytes were co-cultured in monolayer with human amniotic fluid-derived stem cells to determine their *in vitro* differentiation capacities (Guan *et al.*, 2011). Due to their simplicity and ease of control, 2D systems may be most useful to study specific aspects of cellular interactions and behaviours. In contrast, 3D co-culture environments are used to mimic the architecture of native tissues. This is done by culturing mixed populations of cells in synthetic and natural materials, including fibrin, agarose, alginate or collagen. For example, endothelial cells (ECs) were cultured on top of human MSCs (hMSCs) on a collagen gel to monitor vascular network formation (Traphagen *et al.*, 2013). Direct co-culture methods are not isolated to the single purpose of elucidating regenerative mechanisms or driving stem cell differentiation, but have many applications in the field of tissue engineering and regenerative medicine.

Cell interactions in direct co-culture systems take place through paracrine signalling with soluble factors, cell–ECM adhesion and also through direct adhesion between distinct cell types, as the system allows intimate cell contact. It was observed that in a direct co-culture of murine stromal cells and human cord blood progenitor cells in the presence of thrombopoietin, the cord blood cells expanded very rapidly. However when the cord blood progenitor cells were physically separated from the stromal cells within the same culture environment, the proliferative effect of thrombopoietin was abolished (Kawada *et al.*, 1999). Cell–cell contact-dependent changes in cell behaviour have also been observed by others (Yamamoto *et al.*, 2004). This suggests that contact among cells plays a significant role in their behaviour. Therefore, depending on the goals of the co-culture system and the cells involved, cell–cell contact and signalling may be unique and crucial features of direct co-culture that motivates its use for tissue engineering.

2.2. Indirect co-culture

Indirect co-culture systems are systems in which two or more distinct types of cells are cultured within the same environment but are physically separated (Figure 3). As with direct co-culture, this may also be done in 2D or 3D. Physical separation is often achieved with a Transwell™ well insert/Boyden chamber. In 2D, well inserts may be used to separate the monolayers of distinct cell populations. For example, co-cultures of hESCs and chondrocytes were established by growing hESCs in wells with chondrocytes on porous membrane inserts to promote hESC differentiation (Vats *et al.*, 2006). Like monolayer cultures, 2D indirect co-cultures are simple and easy to control, and also allow for the separation of modes of cellular communication. Therefore, they are often used to study specific aspects and mechanisms of cellular interaction and behaviour. In 3D, cell types may be separated by hydrogel encapsulation. For example, embryoid bodies (EBs) were cultured in a collagen matrix indirectly with cardiac fibroblasts, using well inserts to enhance cardiomyocyte differentiation of the EBs (Ou *et al.*, 2011). Indirect co-culture allows cells to reside in environments with similar architecture to native tissue, specifically tailored to each cell type, while still receiving the benefits of communication with other cell types.

Signalling in indirect co-culture takes place between cell types through paracrine signalling using soluble factors. The physical separation of cell types does not allow for cell communication via direct contact. However, depending on the culture parameters, this direct cell contact may not be necessary. It was found that the percentage of beating EBs in indirect 3D co-culture with cardiac fibroblasts was significantly higher than that without co-culture (Ou *et al.*, 2011). This suggests that paracrine signalling was sufficient to promote ESC differentiation. Others have achieved similar success in directing cell behaviour and fate through non-contact signalling between cell types (Liu and Chan-Park, 2010). Indirect co-cultures have the ability to achieve the goals of tissue engineering, thereby depending on the goals and cells involved in the culture, eliminating the need for direct cell–cell contact of distinct cell types.

3. Characteristics of cellular interactions in co-culture

Cell–cell interactions are a key element in the microenvironment present in co-culture. Three types of cell–cell interactions take place in co-culture: cell–cell adhesion,

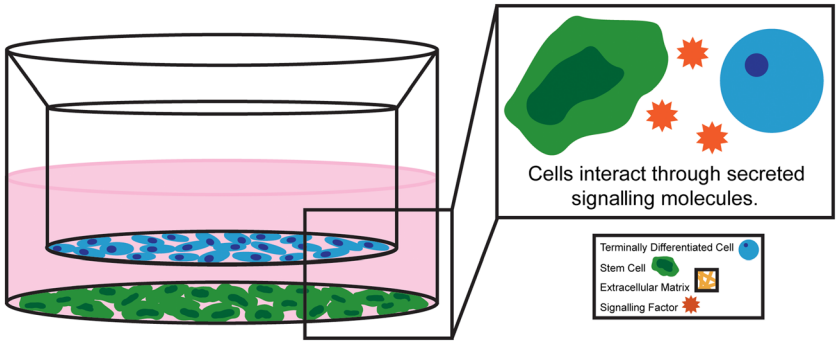

Figure 3. An example of indirect co-culture system. In this type, cells are not in direct contact, therefore, cell interaction occurs only through soluble factor signalling

DOI: 10.1002/term

cell–ECM adhesion, and paracrine signalling through soluble factors. All three types of interactions may take place simultaneously within the same co-culture, or each type can exist separately. The types of interactions that exist in a co-culture system have profound effects on the outcome of the system. For example, when MSCs were co-cultured with ECs in a direct co-culture model where all three types of signalling take place, MSCs differentiated into smooth muscle cells with well-organized actin filaments (Ball *et al*., 2004). In contrast, when MSCs and ECs were co-cultured indirectly, there was a minimal effect on MSC phenotype, and actin filaments remained poorly organized (Ball *et al*., 2004). Thus, co-culture environments and signalling elements may be manipulated to benefit tissue-engineering applications (Huang *et al*., 2009).

3.1. Signalling via cell–cell adhesion

Direct cell–cell contact is generally achieved through cell junctions of three main categories: adherens junctions, gap junctions and tight junctions. These interactions were initially studied in terminally differentiated cells. Recently, the presence and role of cell junctions between stem cells and differentiated cells were also clarified. Gap junctions existing between stem cells and terminally differentiated cells were demonstrated to influence stem cell differentiation (Beeres *et al*., 2005; Guillotin *et al*., 2004). In addition, MSCs demonstrated a remarkable ability to generate tight junctions that could abolish other cell–cell junctions (Schmidt *et al*., 2006). In a co-culture system including human amniotic fluid-derived stem cells (hAFSCs) with neonatal cardiomyocytes, a redistribution of connexin43 and N-cadherin proteins that contribute to gap junction formation occurred (Guan *et al*., 2011). These properties were mediated only through physical cell–cell contact and not when indirect co-cultures were used (Guan *et al*., 2011). Via cell junctions, stem cells and terminally differentiated cells continuously exchange signals that are associated with stem cell fate and differentiation.

3.2. Signalling via cell–ECM adhesion

The ECM acts as an environment that provides the necessary stimuli that contribute to the control of stem cell activity and fate (Guilak *et al*., 2009). Properties of the ECM, such as geometry, elasticity and the presence of mechanical signals, are important for the behaviour of stem cells. The continuous remodelling of the ECM influences the shape and migration of stem cells (Daley *et al*., 2008). Assembly and degradation of the ECM plays an important role in stem cell proliferation, self-renewal and differentiation, possibly through integrins (Daley *et al*., 2008). Furthermore, mechanical forces originating from the ECM and subsequent alterations in intracellular tension are able to control stem cell differentiation via cytoskeletal tension and RhoA–ROCK pathway activation (Cohen and Chen, 2008). In tissue engineering, modifying the properties of the ECM changes the behaviour of stem cells in co-culture systems (Hoben *et al*., 2008; McBride and Knothe Tate, 2008). Cell–ECM interactions are critical factors that continuously influence stem cell behaviour and their modification represents a powerful tool in tissue regeneration.

For stems cells specifically, a microenvironment or 'niche' is present that is responsible for maintaining and regulating stem cell properties (Fuchs *et al*., 2004; Morrison *et al*., 1997). Within the niche, several factors can influence stem cells to self-renew or differentiate (Xie and Spradling, 2000). The most important factors appear to be the types of interaction within stem cell populations, the interactions between stem cells and neighbouring differentiated cells, and the interactions between stem cells and the ECM (Morrison *et al*., 1997; Scadden, 2006). Other factors, such as oxygen level, ion concentration and the presence of growth factors and cytokines, are also important (Drueke, 2006; Eliasson and Jonsson, 2010; Hsu and Drummond-Barbosa, 2009; Kawase *et al*., 2004; Sato *et al*., 2004; Scadden, 2006). Novel co-culture strategies that can control the niche hold great promise for successful tissue engineering.

3.3. Signalling via soluble factors

Paracrine signalling is an important factor for regulating the behaviour of stem cells and terminally differentiated cells within a co-culture. Although it is present in all co-culture systems, it is well studied in indirect co-culture systems. This type of remote signalling occurs via soluble factors. Traditionally, co-cultures were used to promote the differentiation of stem cells. Soluble signals from the local environment promoted differentiation of MSCs toward the vascular cell lineage when co-cultured with ECs (Lozito *et al*., 2009). In a 3D indirect co-culture system, ligament fibroblasts were shown to induce the differentiation of MSCs toward fibroblasts (Fan *et al*., 2008). The differentiation of stem cells is dependent on the origin of the terminally differentiated assisting cells in the co-culture and the matrix biochemistry (Philp *et al*., 2005; Wagers *et al*., 2002). Additionally, the secretion of soluble factors by stem cells affects the behaviour of terminally differentiated cells. Specifically, bone marrow-derived MSCs regulated dermal fibroblast proliferation, migration and gene expression in an indirect co-culture system (Smith *et al*., 2010). Adipose-derived stem cells (ADSCs) enhanced the wound-healing potential of human dermal fibroblasts, mainly with soluble factors, such as secreted growth factors and ECM proteins (Kim *et al*., 2007). In addition, MSCs were shown to exhibit an immunomodulatory effect via inhibition of cytokines secreted from T cells, and also directly through prostaglandin E secretion by the MSCs themselves (Aggarwal and Pittenger, 2005). Remote cell signalling renders stem cells capable of supporting and regulating target cell populations in a co-culture system.

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Cellular interactions that take place in co-cultures mutually benefit both cell populations (Bhandari *et al*., 2001; Bilko *et al*., 2005; Wang *et al*., 2006). For example, a co-culture system of MSCs with chondrocytes enhanced MSC differentiation toward the chondrogenic lineage, while simultaneously decreased MSC hypertrophy (Bian *et al*., 2011). Also, the co-culture of MSCs with meniscus cells enhanced meniscus ECM production as well as reducing hypertrophy of the MSCs (Cui *et al*., 2012). For tissue-engineering applications, it is important not to ignore that cell interactions are seldom unidirectional, with both cell populations being affected in a co-culture system.

4. Application and advantages of co-culture in tissue engineering

Tissues are complex, 3D structures comprised of cells and their surrounding ECM. ECM structure and cell organization are essential to a tissue's development, homeostasis and repair, and are highly related to the tissue's function (Hendriks *et al*., 2007). To maintain tissue form and functionality, cells interact constantly with each other, with the ECM and with the cells in surrounding tissues. Co-culture techniques, with the ability to control target cells through feedback from assisting cells, are advantageous over traditional culture conditions in tissue engineering. The goal of tissue engineering is to create tissues that are functional and, as such, it is essential to establish structure–function relationships in engineered tissues by recapitulating the interactions that take place *in vivo* (Hendriks *et al*., 2007). Through the differential adhesion and differential tension hypotheses (Brodland, 2002; Foty and Steinberg, 2005), co-cultured cells can spontaneously mix or segregate to give rise to physiologically relevant structures (Athanasiou *et al*., 2013).

The following are examples of how co-culture is applied to achieve the goals of tissue engineering and overcome challenges associated with traditional culture methods in different tissues.

4.1. Orthopaedic soft tissues

Traditionally, it was believed that cartilage would be among the first tissues to be regenerated, due to its perceived monocellular nature and its relatively simple histological appearance that is void of vessels and nerves; the same characteristics that contribute to the tissues' inability to self-repair (Huey *et al*., 2012). Cartilage's relatively acellular nature provides few differentiated cells to utilize as a cell source for tissue engineering. The dense ECM, responsible for the mechanical properties of cartilage, bears large loads that frequently exceed body weight many-fold and, thus, presents a difficult task for regeneration. To an extent, these challenges are tackled by soluble factors that assist in cell expansion (Appel *et al*., 2009; Huey *et al*., 2013) and mechanical stimuli to increase biomechanical properties (Kock *et al*., 2012; Lima *et al*., 2007; Makris *et al*., 2013; Responte *et al*., 2012). In terms of stem cells, the use of various factors, such as dynamic compressive loading, resulted in higher expression of chondrogenic genes and improved the mechanical properties of MSC constructs (Huang *et al*., 2010). For ESCs, hypoxia enhanced the ability of human ESCs (hESCs) to produce collagen types I and II and glycosaminoglycans and to achieve better biomechanical functionality in engineered cartilage (Koay and Athanasiou, 2008). However, these factors can be highly time-dependent and even counterproductive. For instance, transforming growth factor-β1 (TGFβ1), which is required for functional chondrogenic development of ESCs, also suppressed ESC chondrogenic induction in a manner dependent on the stage of differentiation of the ESCs (Yang *et al*., 2009). Nonetheless, cartilage regeneration remains elusive (Huey *et al*., 2012), and the same issues of cell source and tissue biomechanics similarly plague other orthopaedic soft tissues, such as the meniscus, ligament and tendon.

Stem cell co-culture has shown itself as a useful addition to the arsenal against the intractable problem of cartilage injuries and, from it, lessons can be derived for application to other soft tissues. The idea of using co-cultures in the regeneration process of cartilage, which has been thought to only contain one cell type, may have appeared counterintuitive until bone marrow-derived MSCs were shown to have chondrogenic potential, i.e. the ability to form cartilage (Yoo *et al*., 1998). A direct co-culture of MSCs with chondrocytes resulted in increased growth of chondrocytes and chondrogenic differentiation of the MSCs (Tsuchiya *et al*., 2004). MSCs decreased in number when co-cultured with chondrocytes, but at the same time increased collagen type II and decreased collagen type X expression. Recently, in an indirect co-culture of MSCs with synovial cells, the synovial fluid was shown to induce chondrogenic differentiation of MSCs, showing that MSCs may not require contact with its assisting cells to differentiate chondrogenically (Chen *et al*., 2005). MSCs may thus act as target cells to mitigate the problem of sparse donor chondrocytes.

The fact that both the target MSCs and assisting chondrocytes were enhanced in their ability to form cartilage serves as an example in which both the target and assisting cells mutually benefit each other, and it naturally follows that MSCs can also act as assisting cells in cartilage tissue engineering. Chondrocytes co-cultured indirectly with MSCs increased in number and maintained a differentiated phenotype (Acharya *et al*., 2012), while MSC chondrogenesis was also enhanced (Acharya *et al*., 2012). In this case, MSCs assisted *in vitro* chondrogenesis by playing a trophic role (Wu *et al*., 2012), while chondrocytes protected MSCs from hypertrophy (Fischer *et al*., 2010). These mutually beneficial interactions seem to be maintained in osteoarthritic disorders, suggesting a promising role for MSCs in the treatment of osteoarthritis (Diao *et al*., 2013). In a 3D co-culture of MSCs with osteoarthritic chondrocytes, MSCs acted as target cells, demonstrating enhancement of the chondrogenic profile,

DOI: 10.1002/term

while simultaneously assisting osteoarthritic chondrocytes to partially recover their phenotype (Diao *et al*., 2013). Thus, whether using MSCs as target or assisting cells, co-culture is a promising tool for decreasing the amount of donor cells needed in cartilage tissue engineering.

ESCs can likewise serve as target cells. Due to the large variety of cells into which ESCs may differentiate, methods are needed to increase the efficiency by which ESCs differentiate into the cell type of interest. Co-cultures have demonstrated promising results toward this goal. For instance, human ESCs were induced in a co-culture with primary chondrocytes to differentiate toward cells of the chondrocyte lineage (Vats *et al*., 2006). The microenvironment in these co-cultures enhanced the efficiency of differentiation by decreasing the ESCs' osteogenic potential (Bigdeli *et al*., 2009; Vats *et al*., 2006). Primary chondrocytes enhanced cartilage tissue formation when co-cultured directly with ESCs (Hendriks *et al*., 2010). Co-culture systems represent a favourable tool for successful differentiation of ESCs toward cartilaginous tissue.

Similar to cartilage, stem cells have been used as a cell source in fibrocartilage tissue engineering. When studying co-cultures of MSCs with meniscus cells from different regions of the meniscus, it was shown that cells from the outer region could be supplemented with MSCs in order to generate grafts for the inner region (Saliken *et al*., 2012). For instance, co-culture of meniscus cells with bone marrow-derived MSCs resulted in enhanced matrix formation (Matthies *et al*., 2012). Toward engineering the knee meniscus, indirect co-culture of ESCs with fibrochondrocytes resulted in a close to 10-fold increase in collagen type II production by ESCs (Hoben *et al*., 2009). Co-culture systems have also been used successfully in therapeutic approaches for the intervertebral disc. For example, a direct co-culture system using bone marrow-derived stromal cells together with nucleus pulposus cells demonstrated beneficial outcomes with increased nucleus pulposus cell viability (Yamamoto *et al*., 2004). Thus, co-cultures of stem cells should be considered as a useful treatment option for the management of fibrocartilage disorders.

The need for ligament tissue engineering exists due to the poor healing potential of ligamentous tissues, e.g. the anterior cruciate ligament, as well as problems, such as donor site morbidity and limited tissue availability. Co-cultures using stem cells with ligament cells have been used either to regenerate ligaments or to engineer the ligament–bone interface. In an indirect co-culture system using MSCs together with ligament cells, signals released from the ligament cells promoted the selective differentiation of MSCs toward ligamentous cells (Fan *et al*., 2008; Lee *et al*., 2007). In a similar setting, stem cell co-culture allowed simultaneous application of mechanical stress that promoted the secretion of ligament ECM (Lee *et al*., 2007). The introduction of a co-culture model containing fibroblasts, bone marrow-derived MSCs and osteoblasts showed evidence of MSCs' differentiation toward a bone–ligament interface-relevant phenotype, i.e. fibrocartilage (Wang and Lu, 2006). Using the same co-culture model on a hybrid silk scaffold, MSCs' differentiation into cells of the fibrocartilaginous lineage resulted in the formation of a gradual transition from an uncalcified to a calcified region, mimicking the ligament–bone interface transition (He *et al*., 2012). Co-cultures may also play a significant role in improving the repair of ligamentous injuries. The use of a co-culture of anterior cruciate ligament cells together with MSCs resulted in improved regeneration potential of the ligament compared to the one observed when utilizing either cell source independently (Canseco *et al*., 2012). Stem cell co-cultures can play an important role in engineering ligament tissue, promoting ligament–bone integration and improving ligament healing potential.

Recently, more advanced co-culture systems were introduced, aiming to achieve a more structurally organized engineered tissue (Allon *et al*., 2012). For example, a bilaminar cell pellet system was introduced for fibrocartilage tissue engineering, where a sphere of MSCs was enclosed in a shell of nucleus pulposus cells. This system showed increased MSC proliferation and differentiation compared to randomly organized co-cultures (Allon *et al*., 2012). Aside from this, however, it appears that few other studies have examined co-culture's utility in forming organized orthopaedic soft tissues, despite the fact that collagen alignment confers specialized biomechanical function to cartilage, meniscus, tendons and ligaments. These issues serve as continual challenges in using co-culture for engineering orthopaedic soft tissues.

4.2. Bone

Bone has a complex structure with distinct mechanical functions and containing different cell types, such as osteoblasts, osteoclasts and ECs. However, bone tissue engineering should not focus only on restoring the mechanical properties of bone, but also on generating a tissue that is fully functional in terms of, for example, calcium metabolism, haematopoiesis and lymphocyte production. For this tissue, robust protocols exist to differentiate MSCs into bone-forming cells, and a plethora of materials are on the market to supplement lost bone volume. However, in engineering large, biomechanically functional bone, a persistent challenge has been the development of stable vascular network within the tissue. When engineering bone, it is critical to consider all the different properties of the osseous tissue that contribute to its manifold functionality.

Scaffolds play a major role in providing mechanical strength and structural integrity for bone tissue engineering. Additionally, they create a 3D environment for co-culture systems that mimic the *in vivo* condition (Fuchs *et al*., 2007). For instance, co-cultures of MSCs and MSC-derived ECs seeded in porous β-tricalcium phosphate ceramic resulted in successful repair of bone defects (Zhou *et al*., 2010). Regarding ESCs, direct co-culture of ESCs with primary cells isolated from bone explants seeded in a poly (lactic-co-glycolic) acid (PLGA)–hydroxyapatite 3D scaffold

DOI: 10.1002/term

induced osteogenic differentiation of ESCs and new bone formation *in vivo* (Kim *et al.*, 2008). Progress has also been made toward engineering mineralized structures without the use of scaffolds. For example, ESCs co-cultured with osteoblasts promoted differentiation of the ESCs to osteoblasts and enhanced the formation of mineralized nodules (Buttery *et al.*, 2001). These results suggest that stem cell co-culture systems, especially those in conjunction with scaffold use, are a promising strategy for bone tissue engineering.

Vascularization is a crucial step in bone development. Considering the diversity of cell types within bone, co-cultures of multiple cells have naturally been applied in developing vascularized bone grafts. It has been reported that there is a mutual cell–cell interaction between ECs and osteoblast-like cells during osteogenesis (Grellier *et al.*, 2009; Santos *et al.*, 2009). Direct co-culture of MSC-derived ECs with MSCs in a 3D β-tricalcium phosphate ceramic biomaterial resulted in prevascularized tissue-engineered bone (Zhou *et al.*, 2010). In a co-culture of epithelial cells with MSCs, osteogenesis occurred only under direct co-culture; monolayer culture of MSCs in epithelial cell-conditioned media exhibited no osteogenic effect (Kaigler *et al.*, 2005). In another direct co-culture system, crosstalk between human umbilical vein endothelial cells (hUVECs) and MSCs not only enhanced the osteogenic differentiation of MSCs but also increased the proliferation of differentiated MSCs (Bidarra *et al.*, 2011). The use of human progenitor-derived endothelial cells (PDECs) in a 3D co-culture with MSCs showed new osteoid formation (Guerrero *et al.*, 2013). The parallel formation of a vascular network, surrounding the tissue when implanted *in vivo*, represents a promising approach for achieving vascularization (Guerrero *et al.*, 2013). In addition, mechanical stimuli from a bioreactor in a co-culture system of umbilical cord-blood endothelial progenitor cells with MSCs have shown improved mineralization and calcium deposition and signs of early vessel infiltration when implanted *in vivo* (Liu *et al.*, 2013). These results suggest that co-culture has potential to resolve the vascularization issue and foster biomimetic engineered bone constructs.

4.3. Heart

A new era for cardiac regeneration started with the identification of a subpopulation of myocytes that were not entirely differentiated (Beltrami *et al.*, 2001). Traditionally, heart, a highly specialized organ, was considered to have only terminally differentiated cells (Chien and Olson, 2002). However, some cells have the ability to re-enter the mitotic cycle and proliferate when stimulated by mechanical stress (Urbanek *et al.*, 2003). The identification of pools of these cells, known as cardiac progenitor cells, in animals and humans revolutionized the field of cardiac muscle regeneration (Laflamme *et al.*, 2007; Messina *et al.*, 2004; Mummery *et al.*, 2002). Multilevel cell–cell interactions are responsible for the activation of anti-apoptotic and angiogenic pathways that are involved in stem cell activation (Beltrami *et al.*, 2003; Urbich *et al.*, 2005). Even though there are numerous questions regarding the mechanisms involved in this process and the exact role of stem cells in tissue response to stress or injury that need to be addressed, growing evidence suggests that cardiac progenitor cells play a leading role in the regeneration process.

Co-cultures are one of the most important tools used in the attempt to engineer functional cardiac muscle and to improve the heart muscle repair process. Three different types of stem cells are commonly used in cardiac muscle tissue engineering: MSCs, ESCs and cardiac progenitor cells. Recently, various mechanisms of action have been proposed for MSCs, highlighting the fact that the MSCs could act as both target and assisting cells. As target cells, MSCs can differentiate to cardiomyocytes, whereas, as assisting cells, they stimulate the proliferation and differentiation of endogenous cardiac stem cells (Hatzistergos *et al.*, 2010). hESCs co-cultured with visceral endoderm-like cells demonstrated cardiomyocyte differentiation, with characteristics of beating cardiac muscle proven electrophysiologically (Mummery *et al.*, 2003). In addition, human ESCs were induced to differentiate to cardiomyocytes in co-culture with endoderm-like cells (Mummery *et al.*, 2002). Also, cardiac cell sheets were formed in a co-culture of ESC-derived cardiomyocytes and fibroblasts cultured in a bioreactor (Matsuura *et al.*, 2012). Cell sheets were not created when ESC-derived cardiomyocytes were seeded alone, whereas co-culturing them with cardiac fibroblasts promoted cell sheet formation (Matsuura *et al.*, 2011). Finally, cardiac progenitor cells have shown promise toward their use as target stem cells. Co-cultures of neonatal myocytes with cardiac progenitor cells expressing transcription factor islet-1 (isl1) demonstrated that these progenitor cells had a great potential to differentiate to mature cardiac myocytes (Laugwitz *et al.*, 2005). The presence of a co-culture with cardiomyocytes was necessary for human 'cardiosphere' progenitor cells to be able to beat. Co-cultures of different types of stem cells, used either as assisting or as target cells, appear to be an irreplaceable tool not only for developing functional cardiac tissue but also for enhancing tissue repair processes in heart tissue regeneration.

To be able to engineer heart tissue, it is important to understand the sequence of events during heart muscle development. Because of the major role that cell–cell interactions play in the developmental process, co-cultures are one of the most important tools for studying cardiac muscle development and regeneration. A 3D co-culture system of embryonic cardiomyocytes and MSCs was used as a model for evaluating the interactions leading to cardiomyogenic differentiation of these cell populations (Valarmathi *et al.*, 2010). Additionally, in co-cultures using myocytes with progenitor cardiac cells, the cardioblasts were entering myocytic differentiation (Laugwitz *et al.*, 2005). However, in the absence of myocytes, the cardiac progenitors did not exhibit differentiation, indicating that this is a cell-mediated process via secreted or membrane-bound factors (Laugwitz *et al.*, 2005). Even

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though the exact process of cardiac tissue development is yet unknown, co-culture techniques are successfully mimicking certain parts of this complex process.

4.4. Vascularization

Tissue engineering and regenerative processes are often faced with the obstacle of overcoming ischaemia caused by injury or the challenge associated with engineering vasculature into engineered tissues. Almost every tissue requires vasculature, as it provides oxygen and nutrients while removing toxic waste products. Stem cells play a key role in angiogenesis and, therefore, are often used in co-culture systems to engineer vasculature (Beckner *et al*., 2002; Nicosia and Villaschi, 1995). The challenge of engineering vasculature needs to be overcome to translate the success of tissue engineering efforts into clinical applications.

Stem cell co-culture systems are beneficial in vascular engineering, as they provide the required signalling factors and cellular interactions to promote angiogenesis and reduce the effects of ischaemia (Griffith *et al*., 2005; Griffith and Naughton, 2002). Both MSCs and ESCs have shown promising findings toward this approach. Bone marrow-derived MSCs assisted the survival of cardiomyocytes in a hypoxic co-culture, and released significantly more vascular endothelial growth factor (VEGF) than under monoculture conditions, promoting angiogenesis (Dai *et al*., 2007). Additionally, co-culture systems have shown promise for the regeneration of vascularized complex tissues that maintain their functionality with the use of ESCs. For example, synchronously contracting cardiac tissue was engineered with a co-culture of cardiomyocytes and hESCs containing endothelial vascular networks (Caspi *et al*., 2007). Scaffold-free, vascularized heart tissue that survives implantation and integrates with host coronary circulation *in vivo* has also been produced from direct co-culture of human cardiomyocytes, hESC-derived ECs, hUVECs and fibroblasts (Stevens *et al*., 2009). Stem cells play an important role in promoting angiogenesis in early tissue ischaemia, and their use in co-cultures serves as a promising tool for vascular tissue engineering and regeneration.

The ultimate goal of tissue engineering is to create structured tissues that can be implanted *in vivo*, mature and become fully functional. However, the thickness of tissues limits the capacity of the implanted tissue to become vascularized (Ko *et al*., 2007). Although oxygen diffusion *in vitro* is often not a limiting factor, capillary networks are required for tissues *in vivo* that are thicker than 2 mm (Griffith *et al*., 2005). Creating a preformed vascular network *in vitro* may be useful to overcome ischaemia in engineered tissues. For example, a co-culture of myoblasts, EBs and ECs seeded on a polymer scaffold formed endothelial vessel networks in engineered skeletal muscle tissue, a tissue that has traditionally not been engineered successfully due to its thickness and high level of vascularity (Levenberg *et al*., 2005). Furthermore, *in vitro* prevascularization improved the *in vivo* vascularization, blood perfusion and survival of the muscle tissue constructs after transplantation (Levenberg *et al*., 2005). Importantly, the addition of embryonic fibroblasts increased the levels of VEGF expression in the constructs and promoted the formation and stabilization of the vessels (Levenberg *et al*., 2005). Blood-derived endothelial progenitor cells, combined with human saphenous vein smooth muscle cells in Matrigel™ and implanted subcutaneously *in vivo*, exhibited a vasculogenic activity that led to the creation of vascular structures (Melero-Martin *et al*., 2007). These examples highlight the critical assisting role that stem cells can fulfil in co-culture systems. Stem cell co-cultures provide an environment supportive of cell interactions that induce vascularization, even in thick tissues.

4.5. Lung

Major challenges in lung tissue engineering are not only the differentiation of stem cells into functional pulmonary cells, but also the maintenance of these differentiated phenotypes of pulmonary cells while in culture. For example, conventional 2D culture of type II airway epithelial cells causes them to rapidly dedifferentiate and lose many of their specialized features, including their ability to produce surfactant. Although these cells cover significantly less surface area than type I airway epithelial cells, they comprise 30% of the total cells in the entire lung (Mondrinos *et al*., 2006). They serve crucial roles, such as synthesizing, storing and secreting the surfactant that is responsible for stabilizing alveoli and decreasing surface tension, and therefore facilitating gas exchange. For this reason, co-culture systems have been frequently used to address these issues.

Many groups attempt to achieve stem cell differentiation into pulmonary cells and to maintain differentiated phenotypes by co-culturing MSCs with fully differentiated type II airway epithelial cells (Mondrinos *et al*., 2006). Fetal pulmonary cells, comprised of epithelial and endothelial cells and MSCs, formed ring-like structures resembling alveolar forming units when 3D co-cultured inside Matrigel™. Numerous alveolus-forming units with structures resembling the morphology of adult mouse lung tissue were observed. Ultrastructural analysis showed the presence of gap junctions, indicating direct cell–cell communication of epithelial cells within the alveolus-forming units. When fetal pulmonary cells were seeded at a higher density, branching morphogenesis and sacculation were observed. After 1 week of culture, the branching lumen structures were histologically similar to native fetal lung tissue (Mondrinos *et al*., 2006). Epithelial–mesenchymal interactions provide differentiation cues, most likely through paracrine signalling, that are essential to lung development (Shannon and Hyatt, 2004).

Determining the requirement for paracrine signalling versus direct cell–cell contact for MSC differentiation into lung cell types is a crucial step in lung tissue engineering

DOI: 10.1002/term

(Popov *et al.*, 2007). An indirect co-culture of bone marrow-derived MSCs and ECs resulted in an increase in mRNA expression for several lung epithelial markers in the MSCs (cytokeratin-5, -8, -14, -18, -19, pro-surfactant protein C). MSCs in short-term co-culture expressed XO-1, an epithelial-specific marker for tight junctions, but MSCs in long-term co-culture did not. This suggests that ECs induce differentiation of MSCs indeed through paracrine mechanisms (Popov *et al.*, 2007). This was further supported by the findings that bone marrow-derived MSCs developed phenotypic and ultrastructural characteristics (lamellar bodies) of type II airway epithelial cells and expressed surfactant protein C mRNA when indirectly co-cultured with lung tissue (van Haaften *et al.*, 2009).

Co-culture for lung tissue engineering is not exclusive to the use of adult stem cells. Direct co-culture of EBs with lung mesenchymal cultures gave rise to the formation of endodermal tissue. EBs co-cultured indirectly with dissected mesenchyme expressed surfactant protein C mRNA. These results indicate that co-culture of murine EBs with pulmonary mesenchyme promotes the formation of type II airway epithelial cells from ESCs (Van Vranken *et al.*, 2005). Given this information, co-cultures of multiple stem cell and primary cell types may be specifically tailored to emphasize the appropriate signalling methods for lung tissue engineering.

There are promising applications of tissue engineering in the pulmonary system, especially as it pertains to cystic fibrosis, a lethal disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. This, in turn, results in increased risk for chronic bacterial infections and respiratory failure. MSCs in co-culture are investigated as a therapy for cystic fibrosis. MSCs from healthy individuals only expressed CFTR under co-culture with airway epithelial cells, but MSCs cultured alone did not (Wang *et al.*, 2005). MSCs from cystic fibrosis patients that were transduced with a viral vector to express CFTR continued to express this gene after an initial culture period with airway epithelial cells. Furthermore, these gene-corrected MSCs maintained their pluripotency and contributed to apical chloride secretion (Wang *et al.*, 2005). Similarly, it was shown that MSCs with low CFTR expression have increased expression upon direct co-culture with airway epithelial cells (Paracchini *et al.*, 2012). Indirect co-cultures, however, did not cause an increase in MSC CFTR expression, demonstrating that contact between the cells is necessary. Expanding the successes of co-culture in cystic fibrosis research is a desirable avenue for tissue-engineering pursuits, as cystic fibrosis poses a significant unsolved problem in the medical community (Paracchini *et al.*, 2012).

4.6. Kidney

The use of stem cell co-cultures for kidney applications aims to understand and to promote kidney cell repair. MSCs have been shown to have a critical role in the renal tissue repair process (Poulsom *et al.*, 2001). MSCs can be mobilized from the bone marrow into the circulation and move to tissues injured by ischaemia, using a mechanism similar to chemotaxis (Kale *et al.*, 2003). Additionally, MSCs are able to restore kidney function even in cases of chronic renal insufficiency (Kirpatovskii *et al.*, 2006). All these observations lead to the extensive use of MSCs as part of co-culture systems to promote both renal tissue repair and regeneration.

In stem cell co-culture systems, MSCs are proven to contribute significantly to normal renal tissue turnover and tissue repair (Poulsom *et al.*, 2001). For example, when introducing cisplatin into a co-culture of proximal tubular epithelial cells together with MSCs, the MSCs exhibited a protective effect toward the proximal tubular epithelial cells in terms of cell viability and promoting cell proliferation. This protective effect of MSCs was mediated through expression of insulin-like growth factor mRNA and protein, which is known for its mitogenic activity (Imberti *et al.*, 2007). MSCs and rat renal tubular cells, when cultured together, established direct intercellular contact in the form of tunnelling nanotubes. Following the formation of these nanotubes, cytosol, mitochondria and other organelles were transferred from one cell to the other. MSCs appear to be guided to differentiate through this process, while renal tubular cell proliferation is promoted (Plotnikov *et al.*, 2010). Interestingly, transport can occur in both directions, confirming the mutually beneficial interaction between target and assisting cells in co-cultures.

Aside of MSCs, ESCs have also been used to differentiate toward renal tissue. ESCs have been successfully differentiated into an epithelial cell type found in kidney tissue with the use of retinoic acid, activin A and bone morphogenetic protein 7 (BMP-7), which are known nephrogenic factors (Kim and Dressler, 2005). While it is yet unclear whether co-cultures with cells from the kidney would enhance ESC differentiation, ESCs have been observed to incorporate into kidney microenvironment and tissue, which implies a great potential for these cells in tissue engineering (Steenhard *et al.*, 2005).

4.7. Liver

Due to the limited clinical success of liver transplantations, tissue engineering-based hepatocyte cell therapies represent a promising alternative treatment method. The major challenge in liver tissue engineering is the maintenance of phenotype and hepatocyte-specific functions of cells in culture. Cell–cell interactions are essential to the function of a liver. The adult liver provides a scaffold for many cell–cell interactions that allow for effective and coordinated organ function. In addition, cell–cell interactions also modulate hepatocyte phenotype maintenance. Therefore, direct cell–cell contact and the 3D microenvironment are crucial for the maintenance of hepatocytes.

ESC co-cultures have been investigated for liver tissue engineering and exemplify the importance of direct cell–cell contact in these efforts. Indirect co-culture of ESCs with hepatocytes has no effect on ESCs' differentiation

DOI: 10.1002/term

(Moore *et al*., 2008). In contrast, direct co-culture systems promote ESCs' differentiation toward the hepatocyte lineage (Moore *et al*., 2008). Additionally, co-culture systems with direct cell–cell contact maintain the cuboidal, cobblestone morphology of hepatocytes.

Adult stem cells are also commonly used in liver co-cultures. Direct co-culture of bone marrow-derived MSCs with hepatocytes increased MSC proliferation and differentiation (Mizuguchi *et al*., 2001). Recently, direct co-culture of ADSCs with human primary hepatocytes led to the formation of 3D liver spheroids. ADSCs act as assisting cells to improve hepatocyte functions, such as enzymatic activity and secretion of albumin and urea (No da *et al*., 2012). This early success warrants further exploration of co-culture systems in liver tissue engineering.

It has been hypothesized that stem cells in the liver have the potential to differentiate into liver-specific lineages, such as hepatocytes or biliary epithelial cells (Thorgeirsson, 1996). Various studies have reported the isolation and differentiation of adult liver stem cells (Nagai *et al*., 2002). For example, an epithelial cell line derived from the liver of adult rats showed potential to differentiate into hepatocyte-like cells in direct co-culture with mature hepatocytes. Direct co-culture of hepatocytes with liver adult stem cells may show promise in phenotype maintenance, proliferation of hepatocytes and differentiation of these stem cells into functional hepatic tissues.

Recently, the utility of co-cultures has been extended to the treatment of hepatic disorders. A co-culture system of MSCs and hepatocytes exhibited inhibition of hepatocyte apoptosis, improvement in hepatic functionality and decrease of inflammatory serum cytokines (Yagi *et al*., 2009). In addition, interactions between stem cells and hepatocytes have been recognized as key for preserving hepatic morphology and functionality (Gu *et al*., 2009). A recent co-culture model highlighted the importance of both static and dynamic interactions between cells and their microenvironment in tissue engineering (Wright *et al*., 2007). A co-culture of vascular mesenchymal cells with ECs resulted in the formation of structures in a coordinated manner, mimicking the structure of liver lobules (Chen *et al*., 2012). These finding imply that advances in liver tissue engineering may be useful to treat acute liver failure (Yagi *et al*., 2009).

4.8. Nerve

Neural tissue co-cultures also exemplify the role of stem cells as both target cells, with the potential to differentiate toward neural cells, and as assisting cells, providing neurotrophic and neuroprotective stimuli. MSCs, ADSCs, ESCs, and adult neural stem cells are commonly used for neural tissue engineering.

MSCs co-cultured with mesencephalic or striated cells were induced to differentiate into neural cells (Sanchez-Ramos *et al*., 2000; Woodbury *et al*., 2000). MSCs were also shown to differentiate into Schwann cell-like cells in a co-culture with neurons; subsequently the MSC-derived Schwann cells exhibited a prominent assisting role by enhancing neurite outgrowth and branching (Brohlin *et al*., 2009; Caddick *et al*., 2006). In a co-culture with dorsal root ganglia neurons, MSCs showed an age-dependent neurotrophic effect, with young donors' MSCs demonstrating favoured properties (Brohlin *et al*., 2012).

ADSCs can similarly act either as assisting or target cells. Their use as assisting cells in a co-culture is supported by their ability to secrete neuroprotective and neurotrophic factors (Wei *et al*., 2009). Co-culture of ADSCs with neurotrophic factor-secreting cells resulted in neuronal differentiation of the ADSCs, indicating that their dual role of both assisting and target cell could be beneficial for neural degenerative disorders (Razavi *et al*., 2013).

ESCs were able to differentiate into neural cells in a co-culture, using either embryonic fibroblasts or glioblastoma cells as a feeder cell type (Ozolek *et al*., 2007). A strong neurogenic effect on ESCs was shown when ESCs were co-cultured with notochords and somites, suggesting a possible mechanism for *in vivo* neuronal induction (Salehi *et al*., 2011). In addition, ESCs have shown potential of retinal differentiation in a co-culture with retinal cells, highlighting their potential use in the treatment of degenerative diseases of the nervous system (Zhao *et al*., 2002).

Another type of stem cells, adult neural stem cells, can differentiate toward active neurons and exhibit functional synaptic transmission in co-cultures with primary neurons or astrocytes (Song *et al*., 2002b). In an indirect co-culture, human neural progenitor cells assisted primary cortical neuron cultures to increase dendritic branching, dendritic length and axonal length (Andres *et al*., 2011). Neural adult stem cells are an excellent example of the importance of cell type and cellular interactions in the fate of stem cells. When co-cultured with mature astrocytes, adult neural stem cells exhibited neurogenesis, while co-culture with neurons promoted differentiation toward oligodendrocytes (Song *et al*., 2002a). Interestingly, the neurogenetic effect was limited to astrocytes originating from certain locations (Song *et al*., 2002a). Co-cultures of different types of stem cells demonstrate a promising dual profile of these cells toward the engineering of functional neural tissues.

Co-culture techniques have been used successfully in reversing neural tissue ischaemia. MSCs have mainly been used for this purpose, where their assisting role was maintained under ischaemic conditions as well. Specifically, MSCs acted as assisting cells that downregulated TGFβ1 and plasminogen activator inhibitor-1 mRNA expression in an indirect co-culture of astrocytes and microglia under experimental stroke conditions (Xin *et al*., 2013). This downregulation was subsequently shown to promote neurite outgrowth and enhance functional recovery in an *in vivo* stroke model (Xin *et al*., 2013). MSCs again acted as assisting cells in an indirect co-culture with primary cortical neurons under experimental stroke conditions to reduce neuronal cell death by 30–35% (Scheibe *et al*., 2012). Future studies should clarify and further take advantage of the neuroprotective and anti-apoptotic role of co-cultures in neural tissue ischaemia.

DOI: 10.1002/term

5. Conclusions and future perspectives

Not surprisingly, the use of stem cells has expanded since their discovery and introduction to research, and their advantages have been exploited in tissue engineering and regeneration to successfully engineer various tissues. As seen with the tissue examples discussed, the use of stem cells in co-culture systems provides key missing elements that are required to overcome the critical limitations faced by tissue engineering and regenerative medicine. Stem cells are advantageous for their self-renewing and potential to differentiate toward multiple cell types. When applied in co-cultures, stem cells can, in addition, promote tissue growth and repair, both directly and indirectly, as target cells that form specialized tissues or as assisting cells that support terminally differentiated cells by enhancing, for example, cell survival, proliferation, phenotype maintenance and organization. Due to their properties and ability to regulate cell functions, stem cells serve a role in a continuous feedback loop over the cells they assist in co-culture. In turn, their intricate needs can be promptly fulfilled by the terminally differentiated cells with which they cohabit. Stem cell co-culture systems are unique and powerful tools, due to their range of design specifications and feedback control properties, and have already shown success in engineering tissues.

The design of a co-culture system often aims to recapitulate cellular interactions that take place *in vivo*. These interactions can be via direct cell–cell contact, cell–ECM adhesion and transfer of signalling molecules. Increasing evidence suggests that these interactions are more complicated than initially believed. Both stem cells and terminally differentiated cells simultaneously interact with each other in a way that has proved difficult to recapitulate via exogenous control schemes, such as growth factor dose and dosing regimens. Also, the ECM secreted by assisting cells contributes significantly to these interactions. ECM influences the behaviour of stem cells, as it acts like a microenvironment that maintains and regulates the anatomical and functional characteristics of all cells within it. It is of note that tissue engineering is also beginning to harness novel interactions that do not naturally occur. For instance, many of the examples discussed in the previous sections utilize co-cultures of ESCs with terminally differentiated cell types, a combination that does not occur *in vivo*. Often, it is desirable to create interactions and environments that do not naturally occur to overcome the limitations that exist in native tissue systems to drive the tissue engineering and regenerative processes. Novel co-culture strategies that manipulate this environment towards a creative collaboration between stem cells and terminally differentiated cells are a promising area for *in vitro* tissue engineering.

Another promising direction for co-culture applications is the use of more than two cell populations in a single system. This can include culturing several terminally differentiated cells or several stem cell types together. As is seen with the examples of cardiac and vasculature tissue engineering, tissue response to ischaemia appears to be mediated by multiple stem cell types. While promising data have already been obtained using MSCs, having different types of stem cells present simultaneously in a co-culture system may yield even better results.

Co-culture systems that can recapitulate the structure of the native tissues are a favourable tool for engineering complex tissues. Novel scaffold designs that allow proper organization of the engineered tissue, such as bio-printing, can incorporate the use of co-cultures. Bioreactors that allow 3D co-culture of multiple cell types together with the ability of simultaneous biomechanical stimulation are promising tools. The supportive role of stem cells to terminally differentiated cells in co-cultures holds great promise for overcoming limitations in the tissue engineering of complex tissues, such as the vascularization and organization of thick tissues.

Stem cell co-culture applications have achieved great success in their development and use thus far, and will continue to achieve great success if we continue to push their evolution by incorporating powerful tools used in other aspects of tissue engineering. One such exciting application of stem cell co-cultures is the use of techniques that mimic the developmental process of tissue growth and maturation, such as the self-assembly process (Hu and Athanasiou, 2006). As we begin to understand and successfully use exogenous mechanical and chemical stimuli in monocellular tissue-engineering processes, we should apply these stimuli in stem cell co-culture systems. The capacity of co-cultures to use xenogeneic cells to drive target cells, without compromising the quality of the generated tissue, needs to be expanded in the future. Also, the immunomodulation characteristics of stem cells in co-cultures have yet to be explored extensively, especially in the arena of how stem cells may aid in the incorporation of engineered tissues into the patient's tissues. In this continuously evolving field, it is crucial to share and apply the knowledge obtained from one tissue to the other, in an attempt to develop functional complex organs and tissues.

Conflict of interest

The authors have declared that there is no conflict of interest.

Acknowledgement

This work was funded in part by the California Institute for Regenerative Medicine (CIRM) grant #TR3-05709.

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