Biomaterials Translational

Mesenchymal stem cell differentiation and usage for biotechnology applications: tissue engineering and food manufacturing

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Key Words:
biotechnology, chromatin remodelling, differentiation, gene expression, mesenchymal cells

From the Contents

ABSTRACT

Recent advances in the field of stem cell research now enable their utilisation for biotechnology applications in regenerative medicine and food tech. The first use of stem cells as biomedical devices employed a combination of cells and scaffold to restore, improve, or replace damaged tissues and to grow new viable tissue for replacement organs. This approach has also been adopted to replace meat production in the food industry. Mesenchymal stem cells are the source material used to induce cells to differentiate into the desired lineage. These technologies require mass propagation and rely on supplying the regulatory factors that direct differentiation. Mesenchymal stem cells can differentiate into fibroblastic and skeletal cells; fibroblastic/chondrogenic/osteogenic/myogenic and adipogenic lineages. Each differentiation fate requires specific key molecular regulators and appropriate activation conditions. Stem cell commitment determination involves a concerted effort of coordinated activation and silencing of lineage-specific genes. Transcription factors which bind gene promoters and chromatin-remodelling proteins are key players in the control process of lineage commitment and differentiation from embryogenesis through adulthood. Consequently, a major research challenge is to characterise such molecular pathways that coordinate lineage-specific differentiation and function. Revealing the mechanisms of action and the main factors will provide the knowledge necessary to control activation and regulation to achieve a specific lineage. Growing cells on a scaffold is a support system that mimics natural tissue and transduces the appropriate signals of the tissue niche for appropriate cellular function. The outcome of such research will deepen the understanding of cell differentiation to promote and advance the biotech, allowing the cell expansion required for their usage in therapy or the development of food tech.

http://doi.org/10.12336/biomatertransl.2022.01.003

How to cite this article:
Benayahu, D. Mesenchymal stem cell differentiation and usage for biotechnology applications: tissue engineering and food manufacturing. *Biomater Transl.* **2022**, *3*(1), 17-23.

Introduction

Stem cells have the capability to self-renew and maintain a reservoir of cells in the developing embryo and in various tissues throughout adult life. Stem cells differentiate into multiple cell types, tissues, or organs, and are essential for supporting tissue homeostasis and tissue repair. Once the stem cells become committed, they begin to function as specialised cells that are synchronised by the expression of the appropriate genes, a process that is governed by transcription and epigenetic regulation. Specialisation is associated with changes in chromatin that control the binding of master regulatory factors to tissue-specific gene promoters and thereby orchestrate the correct performance of tissue events.[1-5] Mesenchymal stem cells (MSCs) resident in the bone marrow[6-16] or adipose tissue[17-19] can differentiate into adipose tissue, cartilage, bone, and skeletal muscle. MSCs are an attractive source of cells for biotechnological applications such as in regenerative medicine and recently, the cells have also been used as a source of alternative protein for the food tech industry.[20] This review focuses on the current understanding, including the transcriptional and epigenetic mechanisms governing differentiation of MSCs, as well as their application in biomedical and food industries.

Mesenchymal Stem Cells: Lineage Fate

The proliferation of stem cells and commitment of their descendants to specific lineages rely on regulation of key genes. It is important to identify the stem cells and their proliferating clones progressing towards their final fate in the tissue. The approach used to follow a cell lineage relies on a unique and heritable DNA barcode assigned to a single cell. Although cells can be labelled by various techniques, the natural barcodes used are microsatellite markers identified by sequencing. Cells are identified as the same lineage if they share the same DNA barcode and therefore originated from the same founder cell. Alternatively, labelling a single cell with a marker can serve as it will be faithfully transmitted to all daughters of the initial cell allowing identification of the members of a particular clone.[21, 22] Naturally, most of the dividing cells labelled in a lineage experiment are non-stem cells, known as transient clones. These cells tend to create variable populations and clonal sizes because they may be caught at any point along the lineage. Transient clones are short-lived, and ultimately mature to contain only differentiated cells. However, when the initial marked cell is a stem cell, the clone will display different properties. Stem cells and their clones tend to be larger and to be uniform in size because they all commenced at a single upstream position in the lineage. In addition, stem cell clones persist longer than transient clones and always contain undifferentiated, intermediate, and differentiated progeny. The profile of a clonal population reveals information about the state of differentiation, and thorough analysis of all clonal types can resolve the complexity of these cells.[21, 22] Such characteristics are important for the understanding of pathologies related to tissue specificity or for the use of stem cells for propagation for use in biotechnology applications for tissue regeneration.

Mesenchymal Lineage Fate Relies on Transcription Regulation and Chromatin Remodelling

To follow the lineage fate of a stem cell, we need to identify the distinct sets of genes that are switched on or off to achieve lineage-specific activation or repression. Such regulation is attained through concerted interactions between transcription factors (TFs) and chromatin remodellers. A chromatin remodeller makes the promoter available to TFs binding to the promoter of a target gene and results in the transcription of functional genes that control stem cell fate. It is not completely clear how chromatin-remodelling complexes are recruited to specific promoters to allow tissue-selective gene transcription. Therefore, it is a challenge to characterise the molecular pathways that coordinate transcription with chromatin-modifying factors in order to achieve a proper understanding of lineage-specific gene expression.[1-4]

Genes known to serve as key regulators of differentiation for skeletal lineages from MSCs and progenitors have been intensively studied and here we will mention some examples. The commitment of MSCs towards the chondro-osteogenic phenotype relies on Runt-related transcription factor 2/Cbfa1, a key downstream regulator of Osterix/Osx.[23-31] Commitment and differentiation to the myogenic lineage are governed by the myogenic regulatory factors (MRFs): Myf5, MyoD, myogenin, and MRF4.[1, 2, 32-38] The earliest step in the progress of differentiation from the stem cell is the binding of TFs, which are responsible for orchestrating the multiple molecular mechanisms in crosstalk among TFs, signalling pathways, and epigenetic regulators that together control cell differentiation.[1, 2] Identifying these control steps would facilitate determination of the aetiological and pathogenic causes of diseases, and a deeper understanding will direct the development of new diagnostic and therapeutic strategies. The ability to direct cells to differentiate into a desired lineage will greatly contribute to biotech approaches relying on these cells for therapeutic applications and tissue repair.

In general, the self-renewal of stem cells is controlled by specialised niche signals, whereas epigenetic regulation of gene expression by chromatin remodelling factors underlies cell fate determination. Lineage-specific activation or repression is regulated through interactions between TFs, chromatin remodellers and regulatory elements on target genes. Regulation of the fate decision of stem cells and progenitors via a complex of TFs is affected by local growth factors and hormones that induce changes in chromatin structure. The role of chromatin remodelling in mesenchymal lineage fate commitment is to regulate chromatin condensation and the outcome of gene expression.[1-3, 28-31] Research has shown that when cells are diverted from their intended skeletal lineage fate, they may become fibrotic and/or adipogenic.[39-41]

Thus, the regulation of stem cells in the cell niche is known to depend on local growth factors and hormones and is also mediated through the dynamic extracellular matrix (ECM) which presents biochemical cues, as well as mechanical signals to the cells. Chromatin regulation in the cell nucleus is also affected by mechano-sensing from the tissue niche that is translated into molecular signals and the resultant chromatin condensation or remodelling provides access for the transcription machinery to gene activation, or denies access so that other genes remain silenced.[2, 3, 42]

Osteoblasts and chondrocytes, which are responsible for skeletal bone formation and remodelling, are derived from a common mesenchymal progenitor. Stem cell commitment towards the chondro-osteogenic lineage depends on multiple signalling pathways involving tissue-specific master regulators that induce sequential expression of transcription and downstream cascades that activate specific structural and functional genes. Runx2 is a master regulator that governs the commitment of MSCs to the chondro-osteogenic phenotype.[23-25] Major TFs that play a role in the chondro-osteogenic commitment include the Sox genes, which are required for commitment to the chondrogenic differentiation path, and Runx2/Cbfa1 with

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its downstream Osterix/Osx protein, which plays a pivotal role in hypertrophic chondrocyte maturation and osteoblast differentiation. Bone morphogenetic protein 2, and the osteogenesis pathway activated by fibroblast growth factors, activate Runx2. In addition to Osterix/Osx, the homeodomain proteins, Msh homeobox 2, delafloxacin 3, and delafloxacin 5 also associate with Runx2 to act on target ECM proteins such as collagen, osteopontin, alkaline phosphatase, bone sialoprotein, and osteocalcin.[26, 27]

The chromatin-related mesenchymal modulator, CReMM/chromodomain helicase DNA binding protein 9 (CHD9), which interacts with nuclear receptors and the glucocorticoid receptor[31] has been identified in skeletal tissue and proliferating cells, namely osteoprogenitors and newly-formed bone.[28-31] CHD9 interacts with various receptors such as the retinoic acid receptor,[43] and retinoid acid signalling significantly affects the expression of the skeletogenic master regulatory factors Sox9 and Runx2. Retinoid acid and oestrogen signalling come into play at distinct stages of skeletogenesis, highlighting their fundamental role in formation of the skeleton. Further distinguishing between the different regulatory factors will provide insights into how chromatin remodelling and gene activation come together to regulate osteogenesis. CHD9 binds to gene promoters in cells at various stages of osteogenesis both *in vivo* and *in vitro*.[28-31] The binding of CHD9 *in vivo* to specific promoters at distinct stages during osteogenic differentiation has been analysed by chromatin immunoprecipitation of bone/cartilage micro-dissected tissues. The results indicate that CHD9 binds Runx2, myosin, and collagen-II promoters in the periosteum, a region that contains multipotential cells.[30] In osteogenic cells, CHD9 is associated with biglycan and osteocalcin, but when osteogenic cells in culture are treated with factors such as transforming growth factor β and bone morphogenetic protein 4 or with 17β-estradiol, alternate binding sites are involved.[31]

The commitment of MSCs to the alternative, myogenic lineage is orchestrated by MRFs (such as Myf5, MyoD, myogenin, and MRF4), which cooperate with myocyte enhancer factor 2.[32, 33] Both MyoD and myocyte enhancer factor 2 recruit enzymes that introduce post-translational histone modifications and chromatin remodelling leading to activation or repression of muscle-specific genes.[1, 2, 34-37] Interestingly, growth factors such as transforming growth factor β and basic fibroblast growth factor impede myogenesis. Myogenic differentiation and maturation induced by MyoD are associated with a switch from repressive to activating factors that allow the transcription and expression of new genes.[44, 45]

Differentiation relies on MyoD and together with chromatin-remodelling enzymes switch/sucrose-non fermentable (SWI/SNF) is phosphorylated by the p38 kinase to allow targeting of the complete SWI/SNF complex to muscle promoters. Interestingly, Brm or brahma-related gene-1, which are members of the SWI/SNF family, block the MyoD-mediated chromatin remodelling and differentiation of myogenic cell lines but do not prevent cell-cycle arrest. This highlights the differences in the chromatin structure of cell cycle regulatory *vs.* muscle differentiation genes.[1, 2, 38] For terminal differentiation, myoblasts must irreversibly exit the cell cycle and lose the ability to proliferate.[32] The regulation of and cross-talk between MyoD, cyclin-dependent kinase (cdk) inhibitors (such as p21 and p57) and the phosphorylated-retinoblastoma tumour suppressor protein (pRb), is a key element in the control of terminal differentiation. MyoD promotes transcription of the cyclin-dependent kinase inhibitor p21,[46] which in turn activates pRb. Activated pRb then sequesters histone deacetylase 1 away from MyoD allowing initiation of MyoD-dependent transcription.[47] Direct interaction between pRb and MyoD I occur *in vitro* and *in vivo* where the absence or malfunction of any of these proteins induces apoptosis.[48] The importance of pRb for skeletal muscle development in animal studies demonstrated that new-born mice lacking Rb exhibit severe skeletal muscle defects. This indicates the importance of pRb in the terminal differentiation and post-mitotic state of myotubes.[49] pRb promotes epigeneic modofocation via histon hypoacetylation and methylation, leading to chromatin condensation which eventually persist in absence of pRb.[50] Such complexity illustrates the role of chromatin remodelling in the regulation of muscle-specific gene expression and a deeper understanding highlights the differentiation process, while pathological situations also reveal novel and potentially druggable targets which could aid in restoring or maintaining the skeletal muscle functional phenotype.

Adipose-derived stromal/stem cells (ASCs) are stem and multipotent cells of adipose tissue with the ability to differentiate into various lineages. ASCs are an attractive source of cells for regenerative applications and their advantages over MSCs are that they are easier to harvest and more efficient than those from bone marrow.[16-19] In addition, ASCs expand faster *in vitro* and can differentiate reproducibly into various lineages. Both MSCs and ASCs are immune-privileged, and can therefore be used as autologous or allogeneic transplants. The differentiation of ASCs into adipocytes is noted by the accumulation of fatty acids or glycated products. The chromatin accessibility and consequent interactions with TFs that promote adipogenesis lead to fat accumulation and tissue expansion resulting in the development of obesity. Dynamic chromatin accessibility during adipogenesis plays a role where less chromatin regulation is necessary in undifferentiated fibroblasts in order to allow greater chromatin accessibility for the enhanced binding of TFs. The well documented activator protein 1 TF subfamily plays an important role in the expression of genes governing adipocyte differentiation (*PPARγ*, *CEBPα*, *AGPAT2*), and function (*ADIPOQ, FABP4*, *LPL*, *PLIN1*, *SLC2A4*) as well as genes involved in triacylglycerol synthesis, which is important for the accumulation of lipids. An interesting possibility is the observation that the differential gene expression associated with triacylglycerol synthesis, lipid oxidation, free fatty acid beta-oxidation, and oxidative phosphorylation is downregulated by a growth factor such as transforming growth factor β1.[17-19, 51]

The Cell Niche, Extracellular Matrix, and Scaffold Proteins

The function of cells in a niche is strongly influenced by the proteins in the ECM, which is a three-dimensional network

of macromolecules. The predominant protein is collagen (fibrillary/non-fibrillary) although glycoproteins also contribute to the biomechanical nature of the niche and provide a structural support framework or "natural scaffold".[52, 53] The ECM includes anchoring proteins that function via integrins and cadherins and play a role in regulating cell-ECM binding. ECM proteins provide signals as a scaffold that binds cells, but also serve as a storage depot for growth factors that modulate cell fate during tissue remodelling. The main proteins in a tissue ECM are the collagen family of proteins that give the tissue structure and shape. Collagen is a native scaffold material, which makes it a target molecule which has been applied in a variety of tissue-engineering approaches as a natural scaffold.

Collagen is highly conserved across evolution from invertebrates to mammals. Collagen used as a scaffold material from mammalian sources requires tedious processing to remove all vestiges of cells or pathogens in order to avoid an immunological response when used for transplantation as a medical device for tissue regeneration. Bovine or porcine-derived collagen needs extensive chemical processing for extraction, isolation, and purification to avoid the risk of pathogens. Another problem lies in the isolation of mammalian collagen which can be structurally damaged and even destroyed during the process which then reduces its strength compared to the natural protein.[54-58] These difficulties have prompted efforts to produce human-derived collagen by biotechnology and genetic engineering approaches. One option has used the expression of human genes in tobacco plants for mass production of collagen, although this is a very expensive process and also does not provide material with high enough mechanical properties needed for some applications.[59]

As collagen is a highly-conserved protein, a potential alternative source is marine-derived collagen that has the advantages of reduced immunogenicity, lower cost, and a lack of ethical issues associated with its application. Collagen isolated from jellyfish is gel-like and has been proven to be biocompatible and to support cell viability but has poor mechanical properties and is thus of limited applicative use.[60-66] An additional source of collagen is a soft coral that contains fibrillar collagen which can be isolated by a mechanical process and thus, unlike collagen harvested from other sources, which loses its natural structure following extraction from the tissues, coral collagen retains the natural spring and physical properties of the fibres. Coral collagen fibres have been identified biochemically and by imaging methods (transmission electron microscopy, X-ray diffraction) which confirmed the fibrillary structure of triple helical collagen.[54-56, 58] This collagen has proven biocompatibility as a scaffold that promotes oriented cell growth *in vitro*;[54] and also allows provision of a three-dimensional structure similar to the natural tissue used as a scaffold material *in vivo* for tissue repair and regeneration.[67, 68] The three-dimensional structure created as a biocomposite of collagen fibres embedded in a hydrogel matrix serves as a support for tissue growth and can also encapsulate materials for drug delivery. The benefits of using a natural collagen protein scaffold lie in the ability to provide a fully-functional biological support for cell growth both *in vitro* and *in vivo*.[54, 67, 68] Coral collagen has the advantage that it allows the design of a hybrid scaffold with biomimetic properties, is biocompatible, and can be designed with a range of mechanical properties according to the tissue strength needed. A collagen alginate hybrid scaffold may have superior mechanical compatibility (e.g., strength and elasticity) in addition to biocompatibility.[54, 67, 68] Other natural biopolymers used in scaffolds include natural fibrin, hyaluronic acid, alginate, and chitosan. An alternative is to use synthetic polyamide, polyethene glycol, or poly-lactic-co-glycolic acid[60, 66] but these materials lack the cell attachment properties and therefore their biomimetic ability is lower than that of natural proteins. It is possible to add smart surface coatings that promote cell attachment, but such materials are expensive. A possible alternative could be a good and highly porous plant-based scaffold made from decellularised plant tissue which has the potential to mitigate the issues of cost and sustainability.[20]

Matrix as A Scaffold for Use in Biomedical and Biotechnology Applications

Two important areas that have found practical applications for the differentiation of stem cells in biotechnology are cell therapy and food tech. i) Mesenchymal cells loaded onto a scaffold are a medical device used in both tissue regeneration and the pharmaceutical industry. ii) Another use of mesenchymal cells has been in the search for an alternative source of protein in the new era of food tech.

i) Cell therapy with a scaffold has been used to create medical devices and in tissue regeneration. Mesenchymal cells when introduced into a site of damage are required to grow and to activate the resident cells, and these processes are controlled by transcription regulation as detailed above. The ability to use cells together with appropriate scaffolds for biomedical applications lies in the ability to biomimic the cell attachment and mechanical properties of a tissue. The cells attached to the collagen activate the cytoskeleton in response to the cells' tensional forces, leading to tissue-like formation. The collagen with additional gel-like proteins such as alginate forms a three-dimensional structure which also allows cell growth and differentiation (*in vitro*), and can be used for tissue regeneration (*in vivo*).[54, 67, 68] A successful transplanted scaffold enables the formation of tissue three-dimensional structure, facilitates cell migration and new blood vessel formation. The advantages of a biocomposite material are that the mechanical and biological properties are designed to mimic the structure of natural tissue. Many of the known soft tissues in humans and other animals exhibit a variable collagen-fibre density that is appropriate for their specific function, for example for cartilage or abdominal wall repair, blood vessels, or cardiac tissue. The ability to tailor the properties of a biocomposite can produce a series of scaffolds suitable for a wide variety of tissue engineering applications.[56-58]

ii) Biotechnology in food tech is geared towards the preparation of cultured meat. New and exciting use of stem cell biology lies in the area of laboratory-grown cultured meat.[20, 69, 70] This field is attracting increasing interest from industry and the public, but faces significant impediments. The main problems are due to fundamental gaps in the knowledge of how to produce realistic meat tissues via conventional tissue-engineering approaches, even before the translational challenges in scaling up in an

efficient, sustainable, and high-volume manner. There is a need to define the molecular basis for desirable meat attributes, such as taste, texture, and customising food biopolymers to mimic the properties of conventional meat products. This is challenging, since for instance, the chemistry behind the taste of meat is complex and not completely understood as it mainly relies on subjective analysis by taste reviewers. The food industry takes a similar approach to tissue engineering but a considerably larger scale is necessary for food production than the individualised production needed for medical tissue regeneration. In order to feed large populations, the scale of cell and tissue culture and/or conditioning will have to be several orders of magnitude higher than even that needed for medical applications. The focus of tissue engineering for food will therefore shift from individual construct production to mass production using huge bioreactors, biomaterial selection and production, culture medium optimisation, optimisation of tissue conditioning and quality control, for instance, regarding the genetic stability of the cells.

The next generation in biomanufacture of alternative proteins requires isolating cells from animals by minimally-invasive methods, and growing cells by fermentation in bioreactors with nutrients provided by a cell culture medium. The immature cells proliferate with or without a scaffold and can be persuaded to differentiate *in vitro* into tissue with the composition of structured meat (muscle, fat, connective tissues). Such production of different cells/tissues from their pluripotent stem cells resembles the concept used for tissue engineering; however, the animal cells in the food industry for production of cultivated meat from traditional animal sources (beef, poultry, and fish) are less studied than human or rodent cells. Therefore, the food industry will require the generation of new cell lines from various animal species, which based on the experience of the pharma industry will require a considerable outlay of time and money. Preparing a feasible protocol will therefore require understanding, selecting, and engineering cell lines with properties that are suited for large-scale manufacturing and specific products. The challenges in the field of food tech are different as they require reducing the cost of cell culture media, nutrients (basal media with glucose, inorganic salts, vitamins, and amino acids) and supplementation with growth factors needed for cell proliferation and differentiation. Reducing the cost of growing the stem cells to that of food-grade components instead of pharma grade, and scaling up the production of cells by fermentation in mass production bioreactors, are prerequisites for success. The scaffolds, which are key for cell growth and differentiation, need to be made from materials that are abundant, affordable and food-safe. It is necessary to develop techniques to assemble cells on plant-based alternative scaffolds suitable for use in large-scale bioreactors.[20, 69, 70]

This new concept of growing alternative proteins for "meat" production on a three-dimensional scaffold raises a completely new set of questions. For optimal culture, the cells must have the ability to proliferate and to be able to provide the conditions for differentiation to produce a "new tissue" when grown on an appropriate scaffold. The scaffold must be composed of a non-toxic biomaterial, which will not induce an allergic response itself or by any of its degradation products. Eggs, milk, shellfish, and peanuts are recognised as particularly allergenic, but based on the reactions to materials used for tissue engineering, scaffold biocomposites are not expected to augment the risk of food allergy. No less important, the scaffold should support full maturation of the cells to form the required tissue *in vitro*. For this purpose, the interaction of cells with the substrate is important; also, the stiffness of the material is a critical parameter, since the cellular matrix affects cell differentiation and maturation.[42] Cells can become quiescent when placed on a scaffold with low stiffness, which mimics the natural elasticity of fat tissue. This changes when the cells proliferate, differentiate, and develop into adipocytes and adipose tissue.[71] The challenge to obtaining large scale structured cultured meat is an ongoing effort worldwide and there remains a need to optimise the conditions and to improve shelf life and reduce costs.

In summary, much is known about the regulation of stem cell lineage, optimisation of conditions for cell growth and scaffold materials. Yet the need for large-scale fermentation to produce building blocks for tissue for both tissue growth for medical engineering or food tech is the challenge of the field. The goals are clear yet are somewhat different for each application and many variables have not yet been fully characterised.

Author contributions
The review was conceived and written by DB.
Financial support
None.
Acknowledgement
The author thanks Ann Avron for editorial assistance and Adi Dagan for designing the Graphical Abstract using Biorender (https://biorender.com/).
Conflicts of interest statement
There is no conflict of interest.

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Received: November 29, 2021
Revised: February 7, 2022
Accepted: February 23, 2022
Available online: March 28, 2022