【10】

氏　　名	SHIPLU ROY CHOWDHURY（シプル ロイ チョウジュリ）
博士の専攻分野の名称	博　士（工　学）
学位記番号	第　２３３５２　号
学位授与年月日	平成21年９月25日
学位授与の要件	学位規則第４条第１項該当 工学研究科生命先端工学専攻
学位論文名	Culture Process Design to Expand Myoblasts for Clinical Applications （医療応用を目指した筋芽細胞の増幅培養プロセス設計）
論文審査委員	（主査） 教　授　田谷　正仁 （副査） 教　授　大竹　久夫　　教　授　紀ノ岡正博

論文内容の要旨

Autologous transplantation of human cells is an extremely sophisticated process where the steps, including cell harvest from a patient and implant to the patient through cell expansion and tissue formation, are critical and interconnected. The starter cells harvested from the patients are limited in quantities, which require multiple passages to yield the sufficient number of cells for subsequent transplantation. In this aspect, the *in vitro* culture raises several issues, such as scheduling of culture to manufacture final products and risk of biological contaminations. Thus, in manufacturing consideration, the culture process for cell expansion should be designed to enhance the cell productivity concerning growth achievement and growth potential. For the treatment of cardiovascular disease, the patient's skeletal muscle myoblasts has been applied to transplant. However, *in vitro* expansion of myoblasts is still challenging because of their low growth ability. It is well known that myoblasts upon making contacts with surrounding cells initiate the process of the cell differentiation toward myotube formation, which is associated with the active fusion among mononuclear proliferate cells to be multinuclear myotube that permanently loose the proliferative ability. Therefore, in the expansion culture, the myotube formation should be prevented. This thesis focused on the culture process design for myoblast expansion to promote the apparent growth ability in cultures for obtaining the desired quantity of cells, and consists of three chapters describing the characterization of myoblast growth properties (chapter 1), the promotion of growth ability by improvement of culture environment (chapter 2), and the improvement of culture operation for myoblast expansion and the design for culture performance in serial cultures using automation systems (chapter 3).

In chapter 1, the growth properties of myoblasts in the culture were characterized in terms of proliferation and differentiation. With elapsed time, the total concentration of myoblasts, based on the nucleus number, increased exponentially accompanied with the increment in confluence degree (C_d). The growth potential as a ratio of proliferative myoblasts (R_p) in culture decreased steeply with elapsed time, being $R_p = 0.11$ at $t = 672$ h, which was one-eighth of that at $t = 48$ h. The reduction of R_p coincided with the induction of the myoblast differentiation, being concurrent with the reduction of efficiency of cell attachment after reseeding. These results suggested that myoblast differentiation could affect the cell expansion process by reducing apparent growth rate of cells as well as by increasing cell loss on repeated seeding. From this viewpoint, the modification of culture environment is considered to improve the cell productivity during serial subcultures with suppressing myoblast differentiation. In addition, during prolongation of cultures, the achievement of higher confluence degree enhances the frequency of cell-cell contacts that induce the process of myoblast differentiation, indicating that the confluence degree of culture could be critical for the culture operation of passaging to maintain higher proliferative populations during serial cultures.

In chapter 2, the culture conditions were designed to promote the migration of myoblasts for enhancement of expansion, considering the reduction of cell-cell contacts to hinder the myoblast differentiation. The dynamic behaviors of human skeletal muscle myoblasts were investigated in the culture on a laminin-coated surface in the presence of 100 ng/ml epidermal growth factor (EGF) in medium. The coexistence of laminin and EGF caused the enhancement of myoblast migration, giving an average migration rate of 62.0 ・m/h, which was 2.7 times that on a plain surface. This encouraged migration could be a driving force to separate the dividing cells each other, accompanied by shortened disjunction time (t_{dis}) of daughter cells to complete cytokinesis. Moreover, some of the daughter cells did not complete their cytokinesis within 7.0 h after the furrow formation, while keeping in contact with each other. The lower locomoting activity of cells resulted the higher frequency of incompleted cells with $t_{dis} \geq 7.0$ h ($f_{c,incom}$). The $f_{c,incom}$ on the plain surface was estimated to be 0.31, which was 16 times higher than that on the laminin-coated surface with EGF supplementation. The synergic effect of laminin and EGF led to the promotion of myoblast growth with keeping a relatively high fraction of proliferative cells during the culture for 150 h, which is considered to arise from the reduced frequency of cell-cell contacts during cytokinesis and thereby suppressing the process towards myotube formation after cell division. Thus, the encouragement of migration can be an effective way to improve the cell expansion of myoblasts with reducing the process of myotube formation.

In chapter 3, to realize a desired performance of myoblast expansion, the effects of seeding density and confluence degree on growth were investigated quantitatively in terms of cell attachment and division as well as proliferative cell population in the culture on a laminin-coated surface. Myoblasts cultured to high confluence degree induced a high frequency of myotube formation, which caused a poor attachment of cells on the surface in subsequent culture. Moreover, myoblast cultured at low cell density ($<1.0\times10^3$ cells/cm^2) also caused the similar phenomena. The quantitative analyses of these cell behaviors helped to determine the appropriate seeding density and attainable confluence degree during one passage, which were 1.0×10^3 cells/cm^2 and 0.5 as the initial and boundary conditions, respectively. In addition, culture operation by passage in serial cultures led to the achievement of adequate amount of cells with moderate proportion of proliferative cells at the end of culture. An automated culture system that could manage two serial cultures by monitoring the confluence degree was constructed. The performance of serial cultures was conducted successfully by monitoring the time-dependent variation in confluence degree, and the expansion index on a nucleus basis and populational ratio of proliferative cells at the end of culture reached $E_c = 201$ and $R_p = 0.32$, respectively, which were equivalent to those obtained in the manual culture performances. The intelligent culture system accompanied with autonomous passage operation is considered to enhance stabilities not only of culture performance but also of cell populational quality. These results indicate that the monitoring of confluence degree is effective to perform the culture passage of myoblasts, being contributable to automating the cell expansion process.

The present work demonstrated that the cell-cell contacts had a significant impact in the reduction of myoblast growth potential and the induction of differentiation during the *in vitro* culture. The environmental modification for enhancing of myoblast migration was an effective way to reduce the cell-cell contacts, and subsequently the differentiation, resulting in the promotion of cellular expansion. Moreover, the boundary condition of confluence degree at the end of culture and the initial condition of seeding density were important operational parameters for the achievement of higher population of proliferative cells. The culture system developed enabled to determine the time for autonomous passage to perform serial cultures of myoblasts, and provided stable performance for the expansion of myoblasts in the culture. It was concluded that the sophisticated process management regarding the optimization of culture conditions and automation of culture operations assists the *in vitro* expansion of myoblasts for clinical applications.

論文審査の結果の要旨

患者自身から得た細胞を増幅し組織化した後、疾患部へ移植する再生医療を目指した臨床研究が盛んである。特に、ヒト骨格筋由来筋芽細胞は拡張型心筋症などの細胞移植に用いられており、その増幅手法の確立が望まれている。筋芽細胞は、単核細胞の際には増殖能を示すが、分化の過程で細胞融合を起こした筋管形成後は多核細胞となり、増殖能を失うことが知られていることから、増幅培養において、単核の筋芽細胞の維持が必要とされている。

第1章「筋芽細胞の増殖特性の評価」においては、筋芽細胞の増幅培養における細胞分裂および分化に関する解析を行った。倍加時間および分裂可能細胞率は、細胞増幅を定量的に表現できるパラメータとして有効であることを示した。また、倍加時間は培養中一定であるが、培養時間とともに、培養面上の細胞占有率が上昇すると細胞間接触が多くなり、その結果、分化が進行することで分裂可能細胞率が減少し、細胞増殖が低下することを明らかとした。

第2章「培養環境の改善による筋芽細胞の増殖促進」においては、上皮増殖因子（EGF）の添加および培養面上へのラミニン塗布を検討し、種々の培養条件下での細胞の遊走性、増殖能について細胞観察に基づき評価した。ラミニン塗布面では、細胞の遊走性が向上し、分裂後の細胞―細胞間離脱時間が短縮され、その結果、単核細胞が維持され、増殖速度が向上することが分かった。EGF添加とラミニン塗布面の条件では、細胞の遊走性と増殖速度がさらに向上し、両者の共同効果が認められた。

第3章「培養操作の改善による筋芽細胞の増殖促進ならびに自動継代培養システムの構築」においては、ラミニン塗布面上での継代培養における細胞播種量（初期条件）および継代時の細胞占有率（境界条件）を検討した。筋芽細胞の分化が進むと細胞接着性の低下が観察され、さらに、長期の細胞増幅培養では、増殖性細胞の割合の低下がみられた。以上の結果に基づき、細胞播種量が 1.0×10^3 cells/cm^2 以上、継代時の細胞占有率が0.5以下のとき、適正な増殖結果を達成できることを見出した。そこで、本培養操作条件のもと、細胞観察ツールにて自律的に継代のタイミングを判断できる培養システムを構築し、上記培養条件のもと、自動的な継代操作を伴う増幅培養が可能であることを示した。

以上のように、本論文は、筋芽細胞継体培養プロセスについて、接触、遊走性などの細胞挙動を考慮した設計指針を提示しその有効性を実証したものである。本論文を博士（工学）の学位論文として価値あるものと認める。