Aptamer-Based Strategies for Stem Cell Research

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Abstract: Aptamers have been introduced to analytical applications, target validation, and drug discovery processes and, recently, applied directly as therapeutic agents. Aptamers can be generated by a method called SELEX (Systematic Evolution of Ligands by Exponential Enrichment). This is quite remarkable for such a young technology, which is only created in the early 1990s. This paper reviews recent new applications of aptamers in stem cell research and tissue engineering.

Key Words: Mesenchymal stem cells, aptamer, FACS analysis.

INTRODUCTION

Aptamers are ssDNA or RNA that can bind with a variety of targets with high binding affinity and specificity. Given their small size, ease of synthesis, low cost, and high specificity, aptamers provide versatile tools for validation of intracellular and extracellular targets. With a number of additional aptamers expected to enter into clinical trials over the next years, aptamers appear to make a significant contribution to the treatment of acute and chronic diseases. Mesenchymal Stem Cells (MSC) are one of the stem cell populations that are being introduced in the clinic for treatment of several degenerative diseases. MSCs have several advantages including the differentiation potential and the stability of their phenotype *in vitro*. The use of these cells in therapy showed also promising results in phase I clinical trials. It is hoped that using stem cells in the clinic will bring major advances in the therapy of several chronic and degenerative diseases. But, due to the lack of specific phoenotype, the isolation of pure MSCs is an obstacle on the application ways. The traditional method of the isolation is based on their selective adherence to plastic surfaces; other methods are based on the characteristics of MSC, using antibodies against MSCs or positive depletion of other cells, but the specificity of the antibodies are still under research. Thus, the cell populations obtained by current methods are essentially heterogeneous. But, when aptamers are introduced to the field and act as molecules to fish out MSCs from bone marrow, the isolation method of MSC is put forward greatly.

1. APTAMERS

1.1. Concept of Aptamers

Aptamers are nucleic acid molecules that bind to molecular targets, including proteins, with high affinity and specificity. Aptamers are typically from 15 to 50 nucleotides in length and can be composed of DNA, RNA, peptide(PNA) or nucleotides with a chemically modified sugar backbone to increase their stability in biological environments (i.e., 2′-fluoro, 2′-O-methyl, phosphorothioate). The secondary structure of aptamers consists primarily of short helical arms and single-stranded loops which are the effective part to bind the target *via* van der Waals, hydrogen bonding, and electrostatic interactions. Aptamers can recognize their targets such as most small molecules, peptides, or protein targets, with KD values ranging from 10 pM to 10 nM for proteins with great specificity (for antibodies, the KD value is from pM to uM). For instance, an aptamer to bFGF (FGF-2) binds with up to 20,000-fold greater affinity to bFGF than it does to its closely related fibroblast growth factor (FGF) -1, -4, -5, -6, and -7 homologues [1]. Aptamers can also distinguish between closely related members of a protein family, or between different functional or conformational states of the same protein [2].

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1.2. SELEX

Aptamers can be generated by a method called SELEX (Systematic Evolution of Ligands by Exponential Enrichment) [3]. This method starts with a large library of random sequence single-stranded oligonucleotides, RNA or DNA. This library pool normally has up to 10^{14}-10^{15} variants. The complexity of the library provides a source for a variety of the targets. The further method involves an iterative process of binding, partitioning, and amplifying novel nucleic acids (Fig. **1**). The applications of this technology extend from basic research reagents to the identification of novel diagnostic and therapeutic agents. Examples of these applications are described along with a discussion of underlying principles and future developments expected to the utility of SELEX. Meanwhile the development of SELEX has driven from the bench top to fully automated systems [4-6].

1.3. Advantage of Aptamers

When aptamers are considered as binding molecules, they are inevitably compared to antibodies, which are still the general reagents in the field of diagnostics. Monoclonal antibodies (MABs) became widespread in the 1970s [7] and have since then made a great contribution to many diagnostic applications. Soon after the discovery of aptamers, they started to demonstrate their potential and versatility in diagnostic assay formats where they substituted for MABs [8]. Recently, Somalogic Inc. (Boulder, USA) reported the development of an aptamer chip that can assess approximately

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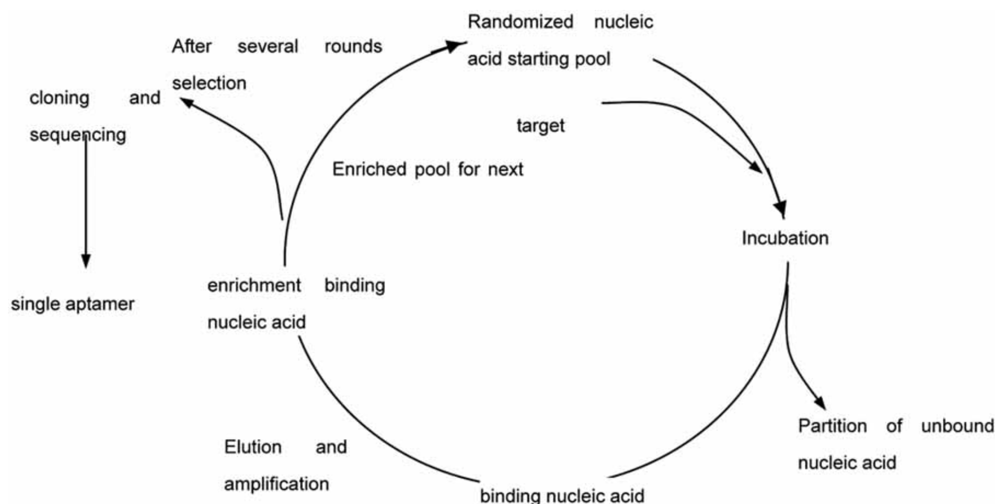

Fig. (1). Overview of the SELEX technique (Systematic Evolution of Ligands by Exponential Enrichment). This method starts with a huge library of random sequence single-stranded oligonucleotides with up to 10^{15} variants. After 10-15 rounds of selections and counter selections high specific aptamers against nearly all kind of target structures can be generated.

50 different analytes in patient samples [9]. These new data underline the potential of aptamers as powerful diagnostic reagents in sophisticated assay formats. Aptamer-based assays reach very low detection limits and can be performed in solution by simple one-tube reactions without the need for washes and separations. These "mix and measure" assays benefit from the fact that aptamers share certain properties with both MABs and nucleic acids: aptamers combine specific recognition of their corresponding target's 3-D shape with the broad diversity of nucleic acids in terms of enzymatic and synthetic chemistry [10].

In addition, aptamers have a number of advantages that make them very promising in analytical and diagnostic applications. The selection of aptamers starts from a big ssDNA library which normally includes 10^{14-15} different sequences. During the selection procedure, a counter-SELEX can be made to get rid of the unspecific binding of other targets to guarantee the specificity of aptamers to the designed target. Another advantage is that there is no need of animals for their production. Thus it shows low costs and high efficiency. Antibody production mainly starts in biological systems by inducing an immune response to the target, but the immune response can fail when the target molecule, i.e. protein, has a structure similar to endogenous proteins or when the antigen consists of toxic compounds. On the contrary, aptamers are isolated by *in vitro* methods that are independent from animals: an *in vitro* combinatorial library can be generated against any target. In addition, generation of antibodies *in vivo* means that the animal immune system selects the sites on the target protein to which the antibodies bind. The *in vivo* parameters restrict the identification of antibodies that can recognize targets only under physiological conditions limiting the extension to which the antibodies can be functionalized and applied. Moreover, the aptamer selection process can be manipulated to obtain aptamers that bind to a specific region of the target and with specific binding properties in different binding conditions. After selection, aptamers are produced by chemical synthesis and purified to a very high degree by eliminating the batch-to-batch variation found when using antibodies. Additionally, by chemical synthesis, modifications in the aptamer can be introduced enhancing the stability, affinity and specificity of the molecules. Finally, because of their simple structure, sensor layers based on aptamers can be regenerated more easily than antibody-based layers, are more resistant to denaturation and have a much longer half life. Moreover, the selection process itself, with the amplification steps, gives some advantages to aptamers in respect to other "non-natural" receptors, such as oligonucleic acids, peptides, which cannot be amplified during their selection procedure.

Due to all these characteristics, aptamers have been used in numerous investigations, as therapeutic or diagnostic tools and for the development of new drugs. Moreover, aptamers have been recently used in analytical chemistry applications, as immobilized ligands or in homogeneous assays.

2. MESENCHYMAL STEM CELLS (MSC)

Mesenchymal stem cells (MSCs) are clonogenic, non-haematopoietic stem cells present in various tissues including bone marrow and are able to differentiate into multiple mesoderm-type cell lineages e.g. osteoblasts, chondrocytes, myocytes, endothelial-cells and also non-mesoderm-type lineages e.g. neuronal-like cells.

2.1. Genesis of MSCs

The existence of MSCs in bone marrow has been suspected for many years. However, Freidenstein *et al.* [11] were the first to develop *in vitro* culture methods for their isolation and for testing their differentiation potential in 1991. They termed this group of cells bone marrow fibroblasts. These cells were named by later investigators: bone marrow stromal cells, mesenchymal stem cells or skeletal stem cells. The stem cell characteristics of mesenchymal stem cells are based on their ability to differentiate into multiple mesoderm- and non-mesoderm-type lineages including osteoblasts, chondrocytes, myocytes, endothelial cells and even neuronal-like cells.

2.2. Isolation Methods of MSCs

No prospective markers exclusively defining MSC are known at the moment. Currently, there are several methods for isolation of MSCs from bone marrow established. Traditionally, they are isolated based on their adherence to plastic surfaces [12-14]. One disadvantage of this method is the unavoidable haematopoietic cell contamination and the cellular heterogeneity of the cultures. Other investigators tried different methods to isolated homogenous cell populations. Simmons *et al*. [15] developed a monoclonal antibody: Stro-1 that has been used to isolate a pure population of cells with mesenchymal stem cells characteristics. Reyes *et al.* [16] isolated a pluripotent mesenchymal stem cell population (termed Multipotent Adult Progenitor Cells: MAPC) from CD45a/GlycoproteinA- depleted bone marrow-derived mononuclear cell fraction that selectively adhered to laminin-coated plates under low serum conditions. And, marrow-isolated adult multilineage inducible (MIAMI) cells were isolated from the whole bone marrow cells by selective adhesion to fibronectin-coated plates in presence of reduced serum conditions and under low oxygen tension. Interestingly, side by side comparison of these different cell populations did show major differences [17]. Recently, CD271 (LNGFR, low affinity nerve growth factor receptor) has also been shown to be the best known marker for the enrichment of nonhematopoietic stem cells from bone marrow aspirates [18,19], termed marrow stromal cells (MSCs): colony forming unit - fibroblast (CFU-F) activity was found to occur only in isolated CD271+ cells, but not CD271– cells [20]. MSCs selected for CD271 expression were shown to have a 10- to 1000-fold higher proliferative capacity in comparison to MSCs isolated by plastic adherence. Newer studies have also demonstrated the possibility of isolating MSC-like cells from the "stroma" of a number of organs including synovial membranes [21] and deciduous teeth [22]. MSCs were detected circulating also in peripheral blood [23] and in umbilical cord blood [24].

2.3. Multilineage differentiation potential of MSCs

The multilineage differentiation potential of MSC populations has been extensively studied *in vitro* since their first discovery in 1960s [25]. These studies demonstrate that populations of bone marrow derived MSCs from human, canine, rabbit, rat, porcine and mouse show the capacity to develop into terminally differentiated mesenchymal phenotypes both *in vitro* and *in vivo*, including bone [26], cartilage [27], tendon [28,29], muscle [30,31] adipose tissue [32,33], and hematopoietic-supporting stroma [33]. The ability of MSCs to differentiate into a variety of connective tissue cell types has rendered them an ideal candidate cell source for clinical tissue regeneration strategies, including the augmentation and local repair and regeneration of bone [34], cartilage [35] and tendon. Individual colonies derived from single MSC population which derived from single MSC precursors have also been reported to be heterogeneous in terms of their multilineage differentiation potential. For instance, Pittenger *et al*. [36] reported that only one-third of the initial adherent bone marrow-derived MSC clones are pluripotent (osteo/chondro/adipo). Furthermore, non-immortalized cell clones examined by Muraglia *et al*. [37] demonstrated that 30% of the *in vitro* derived MSC clones exhibited a tri-lineage (osteo/chondro/adipo) differentiation potential, while the remainder displayed a bi-lineage (osteo/chondro) or uni-lineage potential (osteo). These observations are consistent with other *in vitro* studies using conditionally immortalized clones [38-40]. Additionally, Kuznetsov *et al.* [41] demonstrated that only 58.8% of the single colony-derived clones had the ability to form bone within hydroxyapatite-tricalcium phosphate ceramic scaffolds after implantation in immunodeficient mice. Similar results were reported by using purer populations of MSCs maintained *in vitro* [42]. Taken together; these results suggest that clonally-derived MSCs are heterogeneous with respect to their developmental potential.

2.4. Self-Renewal Potential of MSCs

The self-renewal potential of MSCs is the ability to generate identical copies of themselves through mitotic division over extended time periods (even the entire lifetime of an organism). The absolute self-renewal potential of MSCs remains an open question, in large part due to the different methods employed to derive populations of MSCs and the varying approaches used to evaluate their self-renewal capacity. As a population, bone marrow derived MSCs have been demonstrated to have a significant but highly variable self-renewal potential during *in vitro* serial propagation [43]. Continuous labeling of fresh bone marrow cell harvests with tritiated thymidine reveals that CFU-Fs are not cycling *in vivo* [44], and their entry into cell cycle and subsequent development into colonies depends on serum growth factors [45]. Cell seeding density also plays a role in the expansion capacity of MSC. For example, Colter *et al.* [46] demonstrated that higher expansion profiles of MSC can be attained when plated at low density (1.5-3 cells/cm^2) but not at high density (12 cells/cm^2), which resulted a dramatic increase in the fold expansion of total cells (2,000-fold *vs*. 60-fold expansion, respectively). This work and other similarly reported work [47] strongly suggest that MSC and isolated MSC clones are heterogeneous with respect to their self-renewal capacity.

3. APPLICATIONS OF MSCS

Adult MSCs are ideal candidates for regenerative medicine in cell and gene therapy applications, because of their multipotentiality and capacity for extensive self-renewal. Four areas for potential clinical use of mesenchymal stem cells have been explored: (1) local implantation for localized diseases, (2) systemic transplantation, (3) combining stem cell therapy with gene therapy, (4) tissue engineering protocols [48]. Locally injected expanded autologus mesenchymal stem cells for treatment of large bone defects in patients with defective fracture healing has been reported successful [49]. Systemic transplantation of allogenic normal mesenchymal stem cells has been tried in children with severe osteogenesis imperfecta. Homing of mesenchymal stem cells in bone as well as the production of normal collagen by the transplanted mesenchymal stem cells has been demonstrated [50]. However, the contribution of the transplanted mesenchymal stem cells to the clinical improvement observed in these patients is not clear. And one of the challenges is to improve the engraftment efficiency of mesenchymal stem cells to bone marrow and bone, but what is encouraging is that neither autolo-

gous nor allogeneic MSCs induce any immuno-reactivity in the host [51-53], thus rendering MSCs an ideal carrier to deliver genes into the tissues of interest for gene therapy applications. Genetic modification of stem cells is an attractive target for gene therapy because of their higher proliferative capacity and long-term survival compared with other somatic cells. Mesenchymal stem cells have been demonstrated to be able to express exogenous proteins (e.g. factor VIII and IL-3) for an extended period of time and to maintain this ability after transplantation *in vitro* [54]. Several approaches have been examined and used to introduce exogenous DNA into MSCs to render them useful in tissue regeneration therapies. Viral transduction, particularly using adenovirus mediated gene transfer, can generate stable cell clones with high efficiency and low cell mortality, thus making it a popular option in gene therapy. For example, MSCs infected with an adenovirus vector containing dominant-negative mutant collagen type I gene have been used successfully to repair the bone in individuals with the brittle bone disorder, osteogenesis imperfecta etc. [55]. However, the safety concerns associated with viral transduction have prompted researchers to look for alternative non-viral gene delivery approaches.

Tissue engineering provides alternative ways for obtaining tissues and organs needed for transplantation due to lack of sufficient number of organ donors and limitations attributable to immunological rejection and mismatch of physical dimensions. Tissue engineering may allow obtaining the patient's own cells, seeding them on bio-degradable scaffolds that allow formation of a particular tissue. These tissues can be used to repair tissue defects due to disease or trauma. Furthermore, tissue engineering may also allow ex vivo engineering of tissue by the means of 3-dimensional bio-scaffolds seeded with mature cell or stem cells and consecutive cultivation in bioreactors that lead to the formation of tissues or organs like liver, hearts, blood vessels, cartilage or kidney. MSCs are good cell types for use in tissue engineering protocols because of the relative ease for establishing the cells *in vitro* cultures and their good proliferation and differentiation potential [56,57]. Several scaffolds are currently available and may be classified as either biologically derived polymers isolated from extracellular matrix, plants and sea-weed, e.g. collagen type I or fibronectin, alginate from brown algae or synthetic e.g. hydroxyapatite, tri-calcium phosphate ceramics, polylactide and polyglycolide and a combination of these in the form of poly DL-lactic-co-glycolic acid. There are several animal experiments that show the success of using this approach e.g. for treatment of large bone defects in animal models [58] and it is expected that transplantation of tissues based on these methods to human beings will be achieved within the next years.

4. COMBINATION OF APTAMER TECHNOLOGY TO STEM CELL RESEARCH

The potential of MSC to replicate undifferentiated and to mature into different various mesenchymal tissue cells suggests that it is an attractive source for tissue engineering. However, due to the lack of specific phenotype of MSCs, the separation of MSCs from bone marrow is a barrier for its application. Though, many research groups look for new antibodies to isolate MSCs, for example, w8b2 and 57D2 [59], the binding specificity of these antibodies is still under investigation. Guo *et al*. [60] tried to find other ways than antibodies to separate MSCs from bone marrow. Aptamers may come out to realize this task. Aptamers are ssDNA or ssRNA which can fold into second structure to bind to targets with high affinity and specificity. With its advantage such as easy synthesis, being a small molecule, etc, the application of aptamers has been greatly investigated. In our studies, we made new use of aptamers as capture molecules to MSCs. The term capture molecule means aptamers could play the role of a specific antibody to MSCs, to separate MSCs from bone marrow; on the other hand, they could be used as a coating material to enhance the MSCs adhesion and enrichment. To prove this principle, Guo *et al*. [61] first used an osteoblast cell line from sarcoma as a target to generate aptamers and tested our design. They started from an ssDNA library which includes about 10^{14} random sequences. 10^5 osteoblasts were used for each round of selection. After 10 rounds selection, the binding sequences were cloned and sequenced. The binding affinity of individual aptamer was identified by FACS. The aptamer with the best affinity against osteoblasts was synthesized and coated on solid surfaces to attach osteoblasts out of a solution. The adhesion looks quite good and further scanning electron microscopy shows that the binding between the aptamer coated surfaces with osteoblasts is much stronger than the binding between the non-coated surfaces with osteoblasts [62]. When this principle was fully approved, we used MSCs from adult pigs to perform the experiments. Targeting porcine MSCs, an aptamer that binds to porcine MSCs with high affinity and specificity was identified. To identify the binding ability and specifinity of the aptamers generated, FACS assays were used to test. Then we used the streptavidin magnetic microbeads to bind with biotin labeled aptamers and then use this beads-aptamer to separate MSCs out of bone marrow blood. This new application of aptamers can facilitate MSCs isolation and enrichment greatly. By using this aptamer, MSCs could be separated by FACS sorting or by magnetic beads (MACS, magnet associated cell separation), thereby enhancing the rate of aMSC-derived cells after *in vitro* differentiation for various applications in regenerative medicine. Additionally, the analysis of freshly isolated MSCs by aptamers from bone marrow reveals novel insights to the MSC subpopulations and their antigenic profile in their natural environment. The potential clinical applications will not only reveal unknown aspects of isolation and characteristics of MSCs but also provide a new tool for the fascinating field of tissue engineering and regenerative medicine.

CONCLUSION

This paper gives an overview on the applicability of immobilized aptamers as capture molecules to fish out definite target cell populations from a biological mixture and introduces the selection method for generating cell specific aptamers. This new application of aptamers will bring novel aspects of MSCs to the field of regenerative medicine and tissue engineering.

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