Mesenchymal stem cells: a promising tool for targeted gene therapy of Endometriosis

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Abstract

Endometriosis is a leading, benign gynaecological disorder around the world. Last few years have witnessed tremendous growth in the field of endometriosis and endometrial stem cell (EnSC) research. Despite advancements in the biology and pathology of endometriosis, disease recurrence is still an enigma. Gene therapy holds promise in treating many pathologic conditions including endometriosis. Mesenchymal stem cells (MSCs) serve as ideal candidates for regenerative medicine and cell based therapies. Owing to their specificity to the endometrium, residing endometrial MSC populations could be utilized as ideal candidates for targeting endometrial disorders. Recently, we demonstrated their flexibility for gene transduction using adenoviral vectors. The review highlights the potential of endometrial MSCs in devising targeted gene therapies for endometriosis.

Key words: Mesenchymal stem cells, gene therapy, endometriosis, angiogenesis, endometrial stem cells

Endometriosis: a brief overview

Endometriosis is a growing gynaecological concern around the world with an alarming impact on the reproductive function and social life of the affected women [1]. Symptoms of the disease include chronic pelvic pain, heavy menstrual bleeding, fatigue, dysmenorrhoea and dyspareunia [2]. The disease is also associated with female infertility with a prevalence rate of 30 to 50% worldwide. Although most of the identified lesions are perpetually benign, reports suggest that in some rare instances, atypical lesions can lead to certain types of ovarian cancers [3]. Long-term treatment for endometriosis includes pain management, regulation of estrogen levels, removal of ectopic endometrial tissue by laparoscopy/ laparotomy, and hormonal therapies. Some of the hormonal therapies are associated with serious hypoestrogenic side effects, such as bone mineral density loss, weight gain and high rates of recurrence [4, 5]. They are also ineffective in treating endometriosis-associated infertility and there is often recurrence of pelvic pain after the discontinuation of therapy [4, 6]. Poor understanding of the disease pathogenesis poses technical limitations in designing appropriate treatment strategies for endometriosis. The pathogenesis of endometriosis is attributed to a number of factors such as retrograde menstruation, coelomic metaplasia, embryonic cell rest and lymphatic/vascular dissemination of endometrial cells [7, 8]. Other potential contributing factors include dysfunctional immune response, genetic predisposition, and aberrant peritoneal environment that favour establishment and progression of endometriotic lesions [7]. Recent evidences support a possible involvement of endometrial stem/progenitor cells in the development of endometriosis [8-11].

Regenerating mesenchymal stem cell (MSC) populations are identified both in the healthy endometrial (eutopic MSCs) and endometriotic tissues (ectopic MSCs) [9, 10]. Eutopic MSCs reside in the endometrial lining of the uterus, whereas ectopic MSCs are isolated from the growing endometriotic implants. Comparative studies have shown that ectopic MSCs are

more proliferative, migratory and angiogenic than eutopic MSCs obtained from the endometrial lining of same patient or control MSCs from healthy individuals without endometriosis [9, 10]. Hence, endometriosis has been described as an end result of an ectopic stem cell differentiation process. On the other hand, eutopic MSCs could be considered as the best suited vehicle for treating endometrial disorders, as they are specific to the endometrium. It is advantageous to use the same tissue stem cells as they would survive and function better than MSCs from any other sources. In addition, it is possible that eutopic MSCs may take a lesser time-lag in adjusting to the endometrium microenvironment than MSCs of other origins, and therefore, enhancing its therapeutic efficacy.

MSCs have been used in a number of trials for corrective therapies using gene modification techniques [12, 13]. MSC-mediated gene therapies possess certain unique advantages over direct gene transfers into the body; these include homing towards the site of injury, ease of handling in culture conditions, weak immunogenicity and suitability for gene transduction [14]. A recent study from our group has indicated that MSC mediated gene therapy could be applied to endometriosis [15]. The focus of this review is to highlight the efficacy and benefits of using residing eutopic MSC populations as novel gene therapy tools for endometriosis.

Endometrial stem cells: a promising therapeutic tool

The last decade has witnessed great progress in the endometrial stem cell research. Their presence in the human uterine tissue was first reported by Chan *et al* in 2004 [16]. Subsequently, a number of studies reported the presence of resident MSC populations in both human and mouse uteri [17 – 19]. An ever increasing number of FDA-approved clinical trials that aim to treat a number of pathologies reflect the rise in demand for an ideal source of adult stem cells for therapeutic purposes. Endometrium, which undergoes nearly 400

cycles of complete regeneration and differentiation during each menstrual cycle, is thus an attractive and convenient source for regenerating adult stem cell populations [20].

Human endometrium regenerates on a cyclic basis from underlying candidate endometrial stem/progenitors [21]. *In vivo* reports suggest the ability of endometrial stem/progenitor cells to develop human endometrium on subcutaneous injection in NOD-SCID mice [22, 23]. It has been reported that the morphological and functional features of human endometrium could be reproduced in murine models using xenotransplantation techniques [24]. When human endometrial cells (epithelial, stromal, endothelial and immune cells) were xenotransplanted under the kidney capsule in severely immunodeficient mice, regeneration of a functional endometrial tissue that mimics normal endometrium was observed. The engrafted human tissue formed chimeric functional blood vessels within the mouse endothelium validating the idea that human endothelial cells/progenitors derived from the endometrium can migrate, invade and form vasculature in host tissues of different species [24]. Endometrial Stem Cells (EnSCs) are broadly classified into epithelial progenitor cells, MSCs, endothelial progenitor cells and endometrial side population (SP) cells [21]. These stem cells are the likely precursors of endometrial epithelial/stromal fibroblast cells [21]. The epithelial and stromal stem cell-like precursors have high proliferative potential and they undergo up to 30–32 population doublings before senescence, whereas endometrial non-stem epithelial and stromal cells undergo approximately 12 population doublings [9, 17]. Like their counterparts, bone marrow stem cells (BMSCs) [25], EnSCs could be identified using specific set of markers, such as CD9, CD13, CD14, CD29, CD31, CD44, CD73, CD90, CD105, CD117, CD133 and CD146. They lack in expression for STRO-1, CD31 and CD34. Among the different populations that are identified as endometrial stem/progenitor cells, endometrial side population (SP) cells [26] and MSCs [20] have drawn much attention in recent years. A number of studies have suggested that EnSCs possess side population

phenotype and are characterized by their ability to exclude the DNA-binding dye Hoechst 33343 owing to presence of ATP-binding cassette transporter proteins [22, 27, 28]. Endometrial SP cells are identified both in the epithelial and stromal compartment of the endometrium and endometriotic tissues [26, 28, 29]. Freshly isolated and *in vitro* cultures of EnSCs have been reported to comprise up to 5% of SP cells [28, 30, 31]. Endometrial SP cells, both from the stromal and epithelial compartments, displayed genotypic, phenotypic and functional features of somatic stem cells [22]. However, their therapeutic potential is yet to be explored further.

Gargett *et al* identified the presence of regenerating MSC populations in the culture of freshly isolated endometrial stromal cells [17]. The study demonstrated that endometrial stromal cells exhibit MSC properties and co-express perivascular cell markers, such as CD146 and platelet-derived growth factor-receptor β (PDGF-Rβ). $CD146^+$ $PDGFR^+$ EnSCs can differentiate into mesenchymal lineages, such as adipocytes, smooth muscle cells, chondrocytes and osteoblasts *in vitro* [17]. EnSCs can be grown extensively and maintained in culture for up to 40 passages [17]. Under specific conditions, these cells can differentiate into various lineages such as chondrogenic, osteogenic, adipogenic, angiogenic and myogenic [9, 10, 17]. In addition to the above reports, a couple of studies showed that bone marrow-derived stem cells (BMDSCs) contribute to the repair and regeneration of endometrial tissues [32, 33]. CD133(+) BMDSCs, when injected into a murine model of Asherman syndrome (AS), showed cell engraftment and proliferation around endometrial vessels [33]. The studies suggest that the residing endometrial stem cell populations could be BMDSCs that had migrated under stimulating conditions and colonised in the endometrial tissue.

The therapeutic usefulness of EnSCs has been described by a number of *in vitro* [34, 35] and *in vivo* studies [36, 37], and a few clinical trials have also been reported [38 - 40]. *In vivo*

studies reported their successful use in the treatment of Duchenne muscular dystrophy (DMD), cardiac, neural and bone regeneration, pancreatic differentiation, tissue engineering and glioma models [21]. Successful clinical trials include their use in multiple sclerosis, ischemic cardiomyopathy and DMD [21].

Endometrial Stem Cells: are they flexible tools for gene therapy?

Gene therapy holds an exciting promise for the treatment of numerous disorders; a couple of animal studies and *in vitro* experiments have indicated its application in endometriosis [5, 15, 41, 42]. Potential targets for gene therapy of endometriosis include genes related to angiogenesis, hormonal balance and inflammatory mediators [43]. The advantages of using gene therapy for endometriosis include site-specific action of therapeutic agents and avoidance of adverse side effects of conventional estrogen therapy and disease recurrence. Gene therapy trials for endometriosis are mentioned later in the article. Globally, nearly 600 clinical trials have been registered that use MSCs for the treatment of a wide range of immune and degenerative disorders [44]. The clonogenic, immunogenic and differentiation potentials of MSCs offer the flexibility of these cells for use in such interventions [9, 18]. Endometrial (eutopic) MSCs serve as a powerful gene therapy tool due to their characteristic anti-inflammatory, immunosuppressive and tissue reparative properties [9]. Like MSCs from other sources, eutopic MSCs have also been extensively characterized for their immunogenic properties [9, 10, 18]. We have earlier explored the immunosuppressive and immunomodulatory features of eutopic and ectopic MSCs using peripheral blood mononuclear cells (PBMCs) [9]. *In vitro* co-culture experiments involving human eutopic MSCs and mitogen-activated PBMCs resulted in approximately 50% reduction in the proliferation of PBMCs, whereas the same was observed to be compromised in ectopic MSCs. We reported the immunosuppressive properties of eutopic MSCs and their feasibility in utilizing for cell transplantation studies [9]. Recently, we explored the therapeutic potential

of eutopic MSCs in devising a targeted anti-angiogenic therapy for endometriosis [15]. Women with endometriosis exhibit enhanced angiogenesis at the ectopic lesion sites [10, 15]. Hence, we focused on targeting angiogenesis in order to curb disease progression in endometriosis. Eutopic MSCs isolated from human endometrial biopsy specimens were transduced with adenoviral vectors expressing the anti-angiogenic factor, soluble truncated VEGF receptor-1, sFLT-1(Ad-*sflt-1*). In Ad-*sflt-1* vector, sFlt-1 (soluble fms related tyrosine kinase 1) expression was under the control of cytomegalovirus (CMV) promoter. The vector was propagated using human embryonic kidney 293 (HEK 293) cell lines and purified before transducing to eutopic MSCs. These experiments, for the first time, reported the flexibility of eutopic MSCs to undergo any vector mediated transduction. The transduced cells were able to express MSC markers confirming their phenotypic stability following transduction procedures. The study demonstrated that eutopic MSCs could be genetically manipulated to express soluble Flt-1. Genetically manipulated eutopic MSCs expressed and secreted sFlt-1, and their therapeutic anti-angiogenic ability was validated in a SCID mouse model of endometriosis. Endometriosis was created subcutaneously in SCID mouse models and therapeutic MSCs were administered intravenously. We were able to identify the presence of sFlt-1 secreting MSCs at target sites in the treated group [15]. Secreted sFlt-1 arrested lesion growth and angiogenesis and impaired expression of VEGF (vascular endothelial growth factor) and MMPs (matrix metallo proteinases). This study demonstrated the success of a novel strategy employing genetically manipulated endometrial MSCs for the treatment of endometriosis (Fig. 1).

An effective anti-angiogenic agent should be able to inhibit angiogenesis both in the newly developing/nascent lesions as well as established lesions. Usually, in experimental endometriosis, anti-angiogenic reagents were administered in three different ways; (1) pre-treatment of the study models before endometrial implantation; (2) at the time of

transplantation of endometrial fragments; and (3) following lesion formation as a treatment strategy [45]. Nascent microvessels establish in the endometrioic lesion sites usually between 5 and 8 days of endometrial implantation [45]. In our study, anti-angiogenic therapy was administered two weeks following endometrial implantation [15]. This time period is sufficient for vascularisation necessary for lesion establishment in the animal. Administration of four doses of 10^6 MSC-Ads*flt1* following two weeks of lesion establishment resulted in a highly efficient anti-angiogenesis response; the disease progression was inhibited effectively without any notable sign of new lesion growth. The study envisaged that employing MSC-Ads*flt-1* gene therapy would be most suitable for the abrogation of nascent ectopic lesions that may eventually help in reducing the chances of recurrence of the disease. Further studies in higher animal models employing new improved target specific vectors are required to fully assess this promising strategy in endometriosis care.

Vectors used in gene therapy of endometriosis: success stories

In order to maximize the therapeutic effects of gene therapy and minimize toxicity on non-target tissues, specific vector designing/targeting strategies need to be implemented [46]. This can be achieved by using two major approaches; transductional targeting and/or transcriptional targeting. Transductional targeting enables selective delivery of the therapeutic gene at the target of interest. Transductional targeting of adenoviruses involves specific modifications in the adenovirus fibers, which includes attachment of targeting peptides, serotype knob switching or fiber replacements [47]. These modifications in the capsid proteins of adenovirus would help in routing its cell entry through receptors that are specifically expressed on pathological tissues [5]. In the transcriptional targeting approach, the vector may initiate non-specific gene transfer to a large number of cells; however, its transgene expression is restricted specifically to the target tissue [47]. This strategy uses

tissue specific promoters that display preferential activity in the pathological tissues. An ideal tissue-specific promoter for transcriptional targeting could be defined by the selective display of "pathological tissue on" and "non-target tissue off" phenotype [5].

Adenoviral and adeno-associated vectors are being widely used for gene therapy studies [46]. The advantages of recombinant adenoviruses include their ease of propagation, limited pathogenicity and low mutagenesis potential in humans [5]. They are able to transfer the genetic material effectively in a wide spectrum of dividing and non-dividing cells [47]. However, the wide tropism of adenoviruses is a major limitation, since it could lead to non-specific gene transfer affecting non-target cells. This is due to the recognition of the coxsackie-adenovirus receptor (CAR), the cell surface receptor, by the C-terminal part of an adenovirus fiber protein, termed the knob [48]. CAR receptor is distributed widely over many cells and this poses a major drawback wherein the vector identifies even the non-target cells/tissues [48]. Hence, there is a need for a more specific viral vector in gene therapy based transplantation studies. Othman *et al* developed an advanced, conditionally replicative adenovirus expressing tissue-specific promoters such as heparanase (Ad-heparanase-luc) [5]. These targeted adenoviruses encode luciferase reporter gene in the E1 region under the transcriptional control of the heparanase promoter. Heparanase is a heparan sulfate-specific glucuronidase that plays an important role in tumor cell metastasis. They cleave extracellular matrices and are found to be present in endometriosis tissues [49]. Use of Ad-heparanase-luc resulted in an efficient removal of primary endometriotic cells obtained from ovarian endometrioma lesions *in vitro*. These viral vectors were target-specific and did not impact on liver tissues adversely [5].

Another tissue specific promoter that has been used for targeting endometriotic cells is secretory leukocyte protease inhibitor (SLPI), which is a potent inhibitor of leukocyte serine proteases that protects mucosal surfaces against injury associated with inflammation [5].

The presence of transcripts of SLPI was detected in ovarian endometrioma, peritoneal endometriosis, and deep recto-vaginal endometriosis; it was absent in normal ovarian tissue or eutopic endometrium of healthy females [50], suggesting that SLPI could be the likely promoter for transcriptional targeting. A comparative study, however, reported a higher activity for Ad-heparanse-luc vectors over Ad-SLPI-luc in endometriosis cells owing to its relatively lower activity in the liver tissues [5]. Ad-heparanse-luc exhibited an "endometriosis on, liver off" phenotype, hence, it was considered to be a promising vector for future endometriosis gene therapy trials [5].

A few reports have shown therapeutic benefits of the recombinant adenoviral and adeno-associated viral vectors in the murine models of endometriosis. Adenoviral and adeno-associated viral vectors carrying human angiostatin [41] and endostatin [51] genes respectively could inhibit endometriosis associated angiogenesis at lesion sites. Adenoviral vectors overexpressing angiostatin gene, a natural angiogenesis inhibitor, when delivered to the peritoneal cavities of mice, resulted in eradication of all the endometriotic lesions in the mouse model [41]. Although an effective anti-angiogenesis was observed, the study reported a lack of target specificity. The vector infected a wide range of cells within the peritoneal cavity.

Rein *et al* used conditionally replicative adenoviruses (CRADs), which replicate within and destroy target cells, but not normal cells, in the endometriosis models [52]. The viral replication was maintained under the control of VEGF promoter and *in vitro* studies showed induction of apoptosis in endometriotic cells. Intra-peritoneal administration of the vector, however, resulted in a reduced VEGF promoter activity in the liver as well as endometrium. Subsequently, Paupoo *et al* demonstrated the promise of a modified CRAD, CRAD-S-pK7**,** with dual advantages of infection enhancement and promoter specificity [53]. Polylysine pK7 promoted the best infection enhancement of the adenoviruses whereas survivin promoter

exhibited the highest activity in endometriotic cell lines. The CRAD-S-pK7 vector exhibited higher replication rates and cell-killing efficiencies *in vitro* [53].

A suicide gene therapy approach, where an enzyme transforms a pro-drug into a toxic metabolite, has been used to target endometrial cells using adenoviral vectors [43]. Herpes simplex virus thymidine kinase (HSV-tk) transforms ganciclovir (GCV) into ganciclovir triphosphate, which is toxic for endometrial cells. Adenoviral vectors encoding the HSV-tk gene (AdTK) were delivered into human endometrial cells, which were further treated with GCV. This resulted in a significant level of induction of cell death. When administered into mouse models of endometriosis, AdTK significantly reduced the size of the endometriotic lesions [43].

The role of P27 protein, an important cell cycle regulatory factor has been implicated in endometriosis pathogenesis [54]. P27 regulates cell cycle checkpoint at the G1 to S transition state in normal cells. Absence of P27 results in exaggerated proliferation of cells and their down-regulation has been reported in ovarian, endometrial and breast neoplasia [54]. Endometriotic tissues have a dysregulated expression for cell cycle and inflammatory proteins and lower levels of $p27^{kip1}$ protein [55]. Cells isolated from endometriotic tissues also have lower levels of $p27^{kip1}$ compared to healthy endometrial cells [54]. A recent study demonstrated that gene therapy using Adp27 carrying the $p27^{kip1}$ coding gene (Adp27EGFP), restored the $p27^{kip1}$ expression, promoting a G1 cell cycle arrest and reduced cellular proliferation [55]. Similar studies targeting estrogen receptors, earlier described by Othman *et al,* yielded successful results using adenoviral vectors. In the *in vitro* system, transfer of the dominant negative estrogen receptor gene into human endometriotic cells resulted in the initiation of apoptosis [56].

Concerns yet to be addressed

Despite a number of promising reports highlighting the success of gene therapy approaches for endometriosis, clinical trials remain overdue. Although expanded MSCs have great proliferation and differentiation potential *in vitro*, their loss of *in vivo* self-renewal capacity poses a major clinical concern. This is attributed to the extensive expansion of MSCs carried out during *in vitro* culturing, leading to the loss of their native properties when administered *in vivo*. Thus, novel approaches are required that would allow MSCs to maintain their stem cell function *in vivo.* Likewise, recent reports on endometrial SP cells open up a new arena in understanding endometrial regeneration and its implications in several endometrial disorders. While encouraging *in vitro* and *in vivo* results are reported, further studies are warranted to elucidate the full therapeutic effects of endometrial SP cells for regenerative gene therapies. Another important concern is the lack of a specific set of markers in order to identify and isolate putative EnSC populations. Another issue is concerning the similarity of MSCs to other stromal cells such as fibroblasts. Thus, greater understanding of the MSC biology is required in order to redefine the complex and heterogeneous family of stromal cell populations. Safety and regulatory concerns surrounding the long term effects and fate of the genetically engineered MSCs at target sites should also be addressed.

Conclusion and future perspectives

Addressing the above mentioned concerns would unleash the vast clinical potential of stem cells for treating a range of diseases. With specific advantages associated with EnSCs, they are promising vehicles for cell and gene therapy-based approaches for endometriosis and other disorders and are worthy of further exploration. We envisage that the next 5–10 years is crucial in developing regulatory models for clinical investigation of cell and gene therapy protocols targeting endometriosis globally.

Executive summary

Endometriosis: a brief overview

- Currently available therapeutic options for endometriosis are often associated with disease recurrence. Thus, there is a compelling argument for developing novel therapeutic approaches for the management of endometriosis.

Endometrial stem cells (EnSCs)**: a promising therapeutic tool**

- EnSCs have been isolated and characterized by a number of research groups and their promising therapeutic potential has been investigated by several *in vitro* and *in vivo* studies.

Endometrial Stem Cells: are they flexible tools for gene therapy?

- A recent study from our laboratory explored the gene transduction potential of EnSCs. Genetically modified EnSCs expressed and secreted human anti-angiogenic factor, sFlt-1.

Vectors used in gene therapy for endometriosis: success stories

- Recombinant adenoviral and adeno-associated vectors were successful in the gene therapy of endometriosis.

Concerns yet to be addressed

- Clinical trials involving gene therapy of endometriosis are overdue
- Several concerns regarding clinical use of the *in vitro* expanded MSCs need to be addressed
- Specific set of markers for identifying and separating pure populations of EnSCs is essential

- Long term effects and fate of the genetically engineered MSCs at the target sites need to be ascertained

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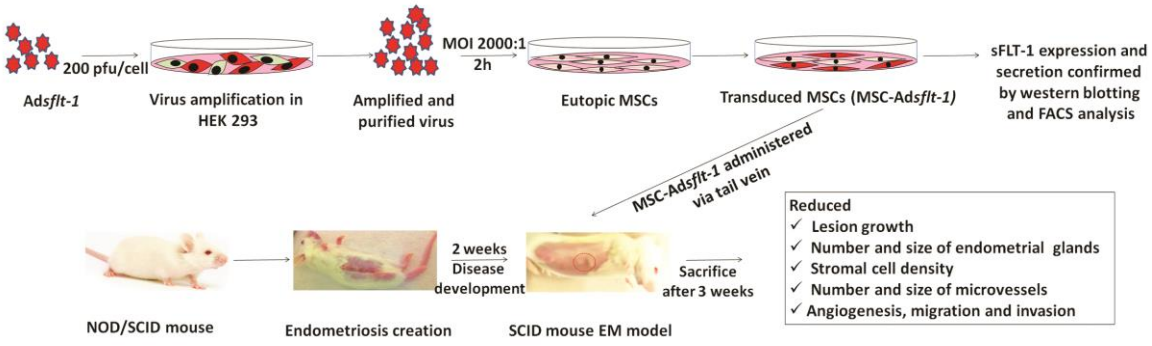
Figure legend

Figure 1: Anti-angiogenic effects of Ad*sflt-1* mediated genetically modified eutopic endometrial mesenchymal stem cells in SCID mouse model of endometriosis (EM). Ad*sflt-1* viral vectors were amplified in HEK 293 cell lines at 200 plaque forming unit (pfu)/cell. The virions were later released from host HEK cells by repeated freeze thaw technique. Eutopic MSCs were infected with purified Ad*sflt-1* viral particles at a multiplicity of infection (MOI) of 2000:1. Expression and release of sFlt-1 by transduced eutopic MSCs were confirmed by flow cytometry and western blotting analysis. SCID mouse model of endometriosis was created by subcutaneous endometrial implantation. Following two weeks of endometrial implantation, four doses of 10^6 MSC-Ad*sflt-1* were administered via tail vein. Animals were sacrificed after three weeks of initiation of the study and monitored for signs of lesion development, invasion, migration and angiogenesis.


200 pfu/cell
MOI 2000:1
2h
sFLT-1 expression and secretion confirmed by western blotting and FACS analysis
Adsflt-1
Virus amplification in HEK 293
Amplified and purified virus
Eutopic MSCs
Transduced MSCs (MSC-Adsflt-1)
MSC-Adsflt-1 administered via tail vein
2 weeks
Disease development
Sacrifice after 3 weeks
Reduced
✓ Lesion growth
✓ Number and size of endometrial glands
✓ Stromal cell density
✓ Number and size of microvessels
✓ Angiogenesis, migration and invasion
NOD/SCID mouse
Endometriosis creation
SCID mouse EM model