

# **PROJECT COMPLETION REPORT**

## **ALTERNATE ADULT STEM CELLS FOR OCULAR SURFACE REGENERATION.**

**DST No SR/SO/HS-0042/2012**

### **Principle Investigator**

Dr. T. V. Kumary  
Scientist 'G'  
Tissue Culture Laboratory  
Biomedical Technology Wing  
SreeChitraTirunal Institute for Medical  
Sciences and Technology  
Poojappura, Thiruvananthapuram-695012  
Kerala

### **Co-Investigator**

Dr. Anil Kumar PR  
Scientist 'E'  
Tissue Culture Laboratory  
Biomedical Technology Wing  
SreeChitraTirunal Institute for Medical  
Sciences and Technology  
Poojappura, Thiruvananthapuram-695012  
Kerala

Date Of Commencement: October 16,2014

**1 Title of the Project: Alternate Adult Stem cells :**  
DST No:SR/SO/HS-0042/20

**2 Principle investigator (s) & Co-Investigator (s):**

Principle Investigator:

Dr. T. V. Kumary  
Scientist 'G'  
Tissue Culture Laboratory  
Biomedical Technology Wing  
SreeChitraTirunal Institute for Medical Sciences &  
Technology  
Poojappura,Thiruvananthapuram-695012  
Kerala

Co-Principal Investigator:

Dr. P. R. Anil Kumar  
Scientist 'E'  
Tissue Culture Laboratory  
Biomedical Technology Wing  
SreeChitraTirunal Institute for Medical Sciences &  
Technology  
Poojappura,Thiruvananthapuram-695012  
Kerala

**3 Implementing institution (s) and other collaborating institution(s):**

SreeChitraTirunal Institute for Medical Sciences &  
Technology  
Poojapurra,Thiruvananthapuram-695012  
Kerala

**4 Date Of Commencement : October 16,2014**

**5 Planned Date of Completion : October 16,2017**

**6 Actual Date of Completion : October 16,2017 ? 31 as per UC&SE**

**7 Objectives as stated in the project proposal:**

- i.** Isolation of adult stem cells from different mammalian tissues such as –Bone Marrow, Epidermis, Hair follicle, Oral mucosal epithelium, Adipose and circulating blood progenitor cells.
- ii.** Optimization of culture conditions of stem cells.
- iii.** Characterization of stem cells from different sources.
- iv.** Screening of the different somatic stem cells for the potential to differentiate into corneal cell lineage.
- v.** Characterization of the differentiated cells.
- vi.** Synthesis of a new batch of NGMA and its characterization.
- vii.** The culture of the stem cells and the semi differentiated or differentiated cells onto an inhouse developed thermoresponsive polymer for generation of carrier-free cell sheets.
- viii.** In vitro evaluation of cell, sheet construct for feasibility towards ocular surface regeneration

**8 Deviations made from original objectives if any while implementing the project and reasons thereof:**

Nil.

**9 Executive Summary**

The ability of adult tissues to regenerate is primarily due to the presence of stem cells residing in specific locations known as stem cell niches. Ocular surface regeneration is possible by the action of stem cells residing in the limbus located at the corneoscleral junction. Partial or complete destruction of these stem cells leads to the clinical condition of Limbal Stem Cell Deficiency (LSCD), which is manifested as the inability for proper surface regeneration resulting in impaired vision. Unilateral complete LSCD entail the use of a limbalautograft. The only mode of treatment for bilateral complete LSCD is transplantation of allogenic tissue grafts. Allogeneic transplantation and their long-termoutcomes are not very favorable with repeated instances of tissue rejection and immune suppression treatments.

Another alternative is the use of stem cells expanded ex vivo reduced chances of immune rejection by selective removal of immunogenic cells. The impasse of donor site morbidity and conditions of complete bilateral LSCD limits this treatment and highlight the present clinical need for alternative cell sources. The use of stem cells and living tissue-engineered substitutes as a treatment mode in various pathologies is being attempted with the ultimate aim of restoration of the function of the diseased organ. This project aims to find a suitable alternative cell source for currently practiced limbal stem cells for ocular surface regeneration. Adult stem cells residing in somatic tissues are an attractive source for tissue engineering because of their unique biological properties. This project proposes to screen alternate cell sources such as bone marrow mesenchymal stem cells, epidermal stem cells, hair follicular stem cells, oral mucosal progenitor cells and circulating blood progenitor cells. Few or all of the above cells will be screened for their potential to transdifferentiate into the required corneal cell lineage and most suitable source would be selected. The cells at progenitor and/or the semi-differentiated stage will be expanded in vitro on an in-house developed thermoresponsive polymer and functional carrier free cell sheets would be generated. The generation of a carrier-free cell sheet and use of autologous alternate stem cells, would obviate the need for any immunosuppressive therapy and hence divest the downstream complications associated with the use of such therapies.

## 9.1 Objectives i-iii

### 9.1.1 *Isolation of adult stem cells from different mammalian tissues, optimization of culture conditions and Characterization.*

- i. Isolation of adult stem cells from different mammalian tissues such as –Bone Marrow, Epidermis, Hair follicle, Oral mucosal epithelium, Adipose and circulating blood progenitor cells.
- ii. Optimization of culture conditions of stem cells.
- iii. Characterization of stem cells from different sources.

All required permissions for dissecting tissues and subsequent isolation of cells from rabbit models were obtained from the Institute Animal Ethics Committee, SCTIMST

**Hair follicle stem cells:** Hair follicle stem cells (HFSC) were isolated from rabbit vibrissae using the explant method. The adult stem cells were isolated from the dermal papillae and hair follicle bulge region. After 14 days of in vitro culture, epidermal cells started growing as sphere cells exhibiting the characteristic germinative morphology of epidermal cells. The cells obtained were characterized and found positive for the putative stem cell marker alpha-6-

integrin. The stemness of these cells was also confirmed by multilineage differentiation of these stem cells to adipogenic and osteogenic lineage. Adipogenesis was confirmed by oil red o staining and osteogenesis by alizarin red, vonkossa, and Masson's Trichrome. The Flow cytometry (BD, FACS ARIA) profile of rabbit Hair follicle-derived stem cells showed 1.8% positivity for CD90, 5.7% for CD105 and were also negative for CD34 expression.

**Adipose-derived mesenchymal stem cells (ASCs):** ASCs were isolated from the dorsal fat pad of New Zealand White rabbit by collagenase digestion. Isolated cells were cultured in Dulbecco's modified eagles medium. After 24-36h the cells started adhering on to the cell culture dishes and the adherent cells showed spindle-shaped morphology, proliferated and were passaged at the confluence. ASCs characterized for their stemness using flow cytometry (BD, FACS ARIA) and the cells showed more than 85% positivity for CD 105, CD 90, and CD 44 when incubated with mesenchymal stem cell markers and were negative for CD 34. ASCs also showed multilineage differentiation potential to adipogenic and osteogenic lineage. Adipogenesis was confirmed by oil red o staining and osteogenesis by alizarin red and vonkossa.

**Peripheral blood mononuclear cells (PBMNCs):** Around 10 ml of blood was collected from the marginal ear vein of healthy New Zealand White rabbits. PBMNCs were isolated by density gradient centrifugation using Histopaque – 1077 (Sigma- Aldrich), following the manufacturing protocol. The cells after 7 -10 days of culture in alpha-MEM with 10% FBS small colonies of cells were observed. Cells depicted both fibroblastic and cobblestone shaped morphology. Cobblestone shaped cell colonies were lost during passaging and were outgrown by fibroblast-like cells. PBMNCs when characterized using MSC cascade of markers and were found to be positive for CD34 and CD 105 while negative for CD 90. PBMNCs also showed differentiation potential towards adipogenic and osteogenic lineage. Adipogenesis was confirmed by oil red o staining and osteogenesis by alizarin red and vonkossa.

**Bone marrow Mesenchymal Stem Cells (BMSCs):** Rabbit bone marrow MSC cells were isolated from the femur using alpha-MEM supplemented with Fetal Bovine Serum as the culture medium. The isolated cells showed spindle-shaped morphology and single cells were observed from day two or three. The cells were in flow cytometry analysis was positive for CD90, CD105 and was negative for CD34. They also displayed multilineage differentiation potential. This implies that isolated cells were of mesenchymal origin.

**Rabbit limbal explant culture:** Limbal explants were obtained from limbal tissue of rabbit models. The limbal ring was cut into small pieces when cultured *in vitro* in alpha-MEM media. In less than a week, the cells started migrating out from the explant to the culture dish. These cells included a diverse population of fibroblastic and polygonal shaped cells. Limbal explant cells were stained differentially for the cytokeratin marker CK 3/12. The medium used to culture a confluent monolayer of limbalexplant-derived cells were collected, filtered and stored. This medium in 3:1 ratio with normal growth medium was called conditioned medium, to be used for differentiation of other adult stem cell population to corneal epithelial-like cells

**Oral mucosal epithelial cells:** Rabbit oral mucosal progenitor cells obtained from explant cultures of oral mucosal tissue were cultured directly in condition medium for 7 days in conditioned media from rabbit limbal epithelial culture. The cells after induction showed positive immune staining for the corneal epithelial marker Ck3/12, and flow cytometric data showed 76.2% of positive cells. Flow cytometric profile of differentiated rabbit oral mucosal cells 76.2% positivity for cytokeratin CK3/12 a characteristic epithelial cell marker.

## 9.2 Objectives iv-v

### 9.2.1 *Screening of the different somatic stem cells for the potential to differentiate into corneal cell lineage and Characterization of the differentiated cells*

**Hair Follicle Stem cells:** Rabbit hair follicle stem cells were attempted for differentiation to corneal epithelial lineage by culturing these cells in limbal explant condition medium for 14 and 21 days. For hair follicle stem cell differentiation the conditioned medium as diluted with SHEM medium in 1:1 ratio. Flow cytometric profile of 14 days differentiated rabbit hair follicle stem cell showing and 20.5% positivity for ABCG2 a stem cell differentiation marker and 5.7% positivity for CK3/12 a characteristic marker of the differentiated corneal epithelium. The gene expression analysis following 14 and 21 days induction did not show much variation in expression of CK3, CK12, and P63. An increase in Pax6 and ABCG2 on comparing 21 days expression with 14 days was noted although not much variation on comparing respective test and control was there. GAPDH was used as the housekeeping gene for normalization.

**Adipose-derived mesenchymal stem cells (ASCs):** ASCs were evaluated for differentiation to corneal epithelial lineage after induction with condition medium for 14 days. ASCs were differentiated from corneal epithelial lineage by incubating with a limbal conditioned

medium for 14 days. The cells attained a more broadened morphology in comparison to the non-induced ASC. The ASCs were stained positive for CK 3/12, a terminal differentiation marker after incubation with limbal conditioned media. Differentiated cells were also positive for cell junction marker Connexin 43, which is again an indicator of epithelial differentiation. The molecular phenotype determination of corneal epithelium-specific genes showed 2 to 3 fold increase in CK 3 and CK 12 genes. p63 and PAX6 also showed around 1.5 fold upregulation while ABCG2 showed around 2 fold upregulation. Flow cytometry analysis of differentiated cell population showed 29% positivity for CK 3/12 marker. Apart from the terminal differentiation marker CK 3/12, 61.6% of cells were stained positive for GAP junction protein connexin 43 (Cx 43) and less than 10% of cells were stained positive for CD 90.

**Peripheral blood mononuclear cells (PBMNCs):** PBMNCs were differentiated from corneal epithelial lineage using the limbal explant conditioned medium for 14 days at standard culture conditions. Morphology of the differentiated cells was slightly broader than the non-differentiated spindle cells. ICC analysis of differentiated cells showed high expression of Connexin 43 (Cxn 43) and CK3/12 than undifferentiated cells, an indication towards corneal epithelial differentiation. PBMNCs also showed upregulation of corneal epithelial-specific genes. There was a 1.5 fold up-regulation of in case of CK 12 while P63 showed around 2 fold up-regulation. PAX 6 a transcription factor was also up-regulated showing evidence of differentiation to corneal epithelial lineage. CK 19 an epithelial marker was also found up-regulated

**Bone marrow Mesenchymal Stem Cells (BMSCs):** Cells were differentiated in vitro for 14 days in conditioned medium for corneal epithelial differentiation. The cells showed broadening in morphology compare to un-induced cells after 14 days of induction. In FACS analysis induced cells showed 34% positivity for corneal epithelial marker CK3/12. The cells also showed positive expression for ABCG2. Gene expression analysis of CK 3 and CK 12 also showed up-regulation in the differentiated cell population in comparison with the control.

### 9.3 Objective vi:

**Synthesis of a new batch of NGMA and its characterization:** NGMA was synthesized as per our earlier published protocol from Joseph et al., 2010. Briefly, pNIPAAm Poly (N-isopropyl acrylamide) and GMA (glycidyl methacrylate) formed the copolymer in presence of nitrogen with AIBN as an initiator. NGMA was then dissolved in isopropanol and coated

in cell culture dishes for making the culture surface thermoresponsive. The dishes then dried and ETO sterilized. The polymer was characterized by Fourier transform infrared spectroscopy (FT-IR) and the lower critical solution temperature by differential scanning calorimetry (DSC). The FT-IR spectrum depicted peaks of both and GMA ensuring copolymer formation. The coated dishes also showed the same peaks ensuring even coating on the cell culture surface. The DSC thermogram depicts the LCST around 32°C. NGMA was evaluated for temperature responsiveness using L929 cells. When the temperature was brought down to less than 20°C, the monolayer of L929 detached and got adhered to the PVDF membrane placed on top of the monolayer. Cells attached to this membrane is then transferred to a new dish ensuring cell sheet transfer.

Adipose-derived stem cells bone marrow MSCs, and oral mucosal progenitor cells were used for cell sheet evaluation of corneal epithelial differentiated adult stem cells.

#### 9.4 Objective vii-viii:

##### 9.4.1 *Culture of the stem cells and the semi differentiated or differentiated cells onto an in-house developed thermo responsive polymer for generation of carrier-free cell sheets and their evaluation.*

ASC sheets were constructed by culturing adipose stem cells to confluence on NGMA coated cell culture dishes at 37°C. When a confluent monolayer is formed on the NGMA coated surface, the temperature is brought down to 20°C or below. NGMA undergoes phase transition making the cell sheet to detach from the polymer surface. The cell sheet was also transferred to the new dish using PVDF as the transfer tool. Post-transfer they remained stable as sheet or clusters. Differentiated cell sheets were shown positive for CK 3/12 staining. The cell sheet transferred were viable and stable.

BMSC cultured on NGMA coated plates were induced with condition media to form a corneal epithelial cell sheet derived from BMSC culture. The induced cell sheet was retrieved from NGMA plates by lowering the temperature to 20°C. Expression of the epithelial-specific marker CK 3/12 and CX-43 was confirmed on the cell sheet.

Oral mucosal epithelial cells were cultured on NGMA modified with collagen type II. Oral mucosal epithelial cells were cultured on NGMA-col surface and retrieved as a cell sheet for ocular surface reconstruction. The cell sheet was after retrieval was found to viable by live dead staining using FDA-PI and were able to express epithelial cell-specific marker CK3/12.

In this study stem cells from different sources, bone marrow, adipose, oral mucosa, hair follicle and peripheral blood were isolated, characterized and their potential to

differentiate into the corneal lineage was evaluated. Bone Marrow-derived mesenchymal stem cells (BMSC) and Adipose-derived mesenchymal stem cells showed better results in the differentiation experiments. Oral mucosal progenitor cell also showed promising results as an alternative cell source for limbal stem cells. Hence these cell sources were chosen as the best alternatives from all the cell sources considered as alternative options to limbal stem cells.

- [1] Joseph N, Prasad T, Raj V, et al. A Cytocompatible Poly(*N*-isopropylacrylamide-*co*-glycidylmethacrylate) Coated Surface as New Substrate for Corneal Tissue Engineering. *J Bioact Compat Polym* 2010; 25: 58–74.

## **10 Detailed analysis of results indicating contributions made towards increasing the state of knowledge in the subject:**

Techniques were standardized for the isolation of autologous stem cells from different sources like Bone Marrow, Epidermis, Hair follicle, Oral mucosal epithelium, Adipose and circulating blood progenitor cells. Procedure developed for the differentiation of Bone marrow MSCs using conditioned media was found to be useful for differentiation of other stem cells to corneal epithelial lineage. Use of other autologous stem cells will be a solution for scarcity of donor tissue and being autologous, immunological issues can be avoided. Cell sheets could be developed with adipose derived MSC and Promising results were obtained when transplanted into in vivo rabbit models of corneal damage. Cell sheet transplantation did not require any other adhesive like fibrin sealant and cells maintained their naïve architecture. In the case of bilateral total limbal stem cell deficiency, ideal strategy will be the use of these alternate adult stem cells.

## **11 Conclusions summarizing the achievements and indication of scope for future work:**

Preliminary in vivo results of corneal regeneration in rabbit models of corneal damage using adipose derived MSC has produced promising results. Detailed pre clinical studies can lead to more relevant information towards clinical use. This can be further extrapolated to other cell sources.

### **11.1 S&T benefits accrued:**

#### **11.1.1 List of Research Publications**

- Bernadette K. Madathil, Sneha Sundaran P, Kumary TV, Anugya Bhatt and Anil Kumar PR, Biofunctionalised Polycaprolactone Fibrous Mat as a Transfer Tool for Cell Sheet Engineering, *Fibers and Polymers*, 2017, 18(11): 2094-2101, IF – 1.022
- Bernadette K. Madathil, TV Kumary and Anil Kumar PR, Self Organized Skin Equivalent by Epithelial and Fibroblast Cells on Polycaprolactone Electrospun Mat with Porous Fibers. *Adv Tissue Eng Regen Med Open Access* 3(1): 00056.

- Venugopal B and Kumary TV. Adipose derived mesenchyme stem cells - A promising population of adult stem cell population for regenerative therapy. IJSER.2018 Aug; 9(8):1328-32. IF- 4.4
- Mathews S, Prasad T, Venugopal B, Palakkan AA, Anilkumar PR Kumary TV. Standardizing transdifferentiation of rabbit bone marrow mesenchymal stem cells to corneal lineage by simulating corneo- limbal cues. J Stem Cell Res Med 2017. Doi: 10.15761/JSCRM.1000119.

***Abstracts published***

- Venugopal B, Kumar AP, Sreenivasan K and Kumary TV (2016). Bio functionalized thermo responsive Matrix for Corneal cell sheet Engineering. Front. Bioeng. Biotechnol. Conference Abstract: 10th World Biomaterials Congress. doi: 10.3389/conf.FBIOE.2016.01.02793

***Conferences***

- Balu V Gopal, Shabeena E A, AnilKumar PR, Kumary TV. Corneal Surface Reconstruction Using Transdifferentiated Mesenchymal Stem Cell Sheets. Indo - Australian Conference on “Biomaterials, Tissue Engineering, Drug Delivery System and Regenerative Medicine” (BiTERM-2015). Anna University. February 5-7, 2015 (Oral).
- Balu V Gopal, Shabeena E A, AnilKumar PR, Kumary TV. Mesenchymal Stem Cell Sheets as Scaffold Free Constructs for Corneal Surface Therapy. International Society for Stem Cell Research – Annual Meeting 2015. Stockholm, Sweden. June 24-27. 2015.
- Balu V Gopal, Sumitha Mohan, AnilKumar PR, Kumary TV. Scaffold free Mesenchymal stem cells sheet derived from thermo responsive N-isopropyl –co-glycidyl methacrylate coated surfaces for Corneal surface reconstruction. Nominated for Best Paper Award. Kerala Science Congress 2017. Thiruvalla, Kerala. Jan 28-31. 2017
- Balu V Gopal, Sachin J Shenoy, Sumitha Mohan, Anil kumar PR, Kumary T V. Trans Differentiated Mesenchymal stem cell sheets as an alternate to Limbal stem cells for corneal surface reconstruction. 6th Asian Biomaterials Congress. October 25-28, 2017. Thiruvananthapuram, Kerala

**11.1.2 *Manpower trained on the project***

- i. Research Scientists or Research Associates: One (Research Associate)
- ii. No of PhDs produced: One
- iii. Other technical personnel trained: One (Project Assistant)

## 12 Patents taken if any: Nil

## 13 Financial Position:

No	Financial Position/ Budget Head	Funds Sanctioned	Expenditure	% of Total Cost
I	Equipment	3,45,000	3,45,000	100
II	Salaries/Manpower Cost	30,55,000	11,51,464	99.48
III	Supplies & Materials		1749920	
IV	Contingencies		1,37241	
V	Travel		600.00	
VI	Overhead Expenses	3,00,000	3,00,000	100
VII	Others if any	The excess amount of Rs.9378 towards equipment accessories is adjusted in the contingency head. Unutilised balance of Rs.15,775/- was returned to DST		
	<b>TOTAL</b>	37,00,000	36,84,225	99.57

### 13.1 Procurement/Usage of Equipment

### 13.2 Procurement

Sl. No	Sanctioned List	Model & Make	Cost (Rs)	Date Of Installation	Utilization rate	Remarks regarding maintenance/ breakdown
1	Easy Sep Magnet with specific beads	Stem cell technologies EASYSEP™ Magnet 18000	1,66,678		60%	NIL
2	Table Top Spin Coater	Holmark spin coater Model HO-TH 05	1,87,700		90%	NIL

### 13.3 Plans for utilizing the equipment facilities in future.

The laboratory is committed in developing newer techniques and exploring frontier areas of research in the field of Tissue engineering and regenerative medicine. Towards this goal cell sheet engineering using thermoresponsive polymers remains a stronghold of the laboratory. We continue to focus our research in developing newer bioconjugates of the thermoresponsive polymer that would in the long run modulate the growth and differentiation of the cells cultured in vitro. The use of stem cells in our line of research would greatly aid in the therapeutic potential of the cell sheets generated. Both the equipments procured in this

project ie., the table top spinner for the large scale generation of thermoresponsive substrates and the easy sep magnet for the separation of our desired cells would continue to aid and support our technical skills and substantiate our research methodologies.

Name and Signature with Date

a.

(Principal Investigator)

b. (Co-Investigator)