ISOLATION AND CULTURE OF KERATINOCYTES ON MODIFIED CULTURE SURFACE FOR SKIN TISSUE ENGINEERING

A DISSERTATION SUBMITTED

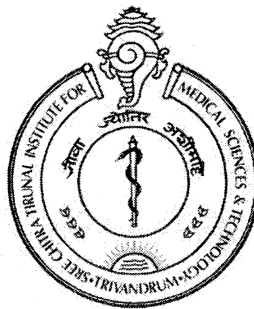
BY

KARTHIK.N.RAMESH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF PHILOSOPHY



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DECLARATION

I, **Karthik.N.Ramesh**, hereby declare that I had personally carried out the work depicted in the dissertation entitled **"Isolation and Culture of Keratinocytes on Modified culture Surface for Skin Tissue Engineering"** under the direct supervision of **Dr.AnilKumar.P.R**, Scientist C, Tissue Culture Laboratory, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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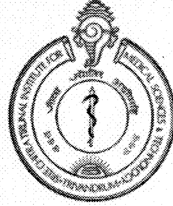

CERTIFICATE

This is to certify that the dissertation entitled "**Isolation and Culture of Keratinocytes on Modified culture Surface for Skin Tissue Engineering**" submitted by **Karthik.N.Ramesh** in partial fulfilment for the Degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by him under my supervision and guidance at **Tissue culture laboratory**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram 695011.

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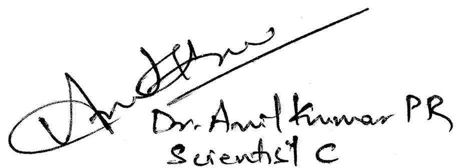

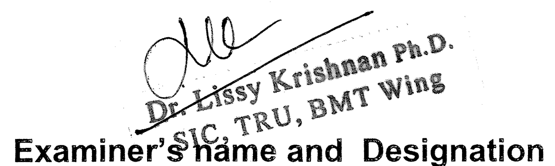

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LIST OF ABBREVIATIONS

BSA	-Bovine serum albumin
CK	-Cytokeratin
CO_2	-Carbon dioxide
DKSFM	-Defined Keratinocyte Serum free Medium
DMEM	-Dulbecco's Modified Eagle's Medium
ECM	-Extra Cellular Matrix
EDTA	-Ethylene Diamine Tetra acetic acid
ETO	- Ethylene Oxide
FBS	-Fetal Bovine Serum
FITC	-Fluorescein isothiocynate
Gal	-Galactosidase
LCST	-Low critical solution temperature
MTT	-(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NGMA	-N-Iso propylacrylamide-co-glycidyl methacrylate
OD	- Optical Density
PBS	- Phosphate buffered saline
PVDF	- Polyvinylidene Fluoride

PFA	-Paraformaldehyde
RCF	- Relative centrifugal force
TCPS	-Tissue culture Polystyrene
TRITC	-Tetra Methyl rhodamine isothiocynate

NOTATIONS

µl	- Micro Litre
cm^2	- Square Centimeter
h	- Hour
M	-Molar
min	-Minute
mg	- milli gram
ml	-milli litre
°	- Degree
° C	- Degree Celsius

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SYNOPSIS

Skin is a part of the integumentary system that acts as an interface between the body and the external environment. It plays a major role in maintaining body homeostasis by regulating temperature, retaining body fluids, and eliminating wastes. Skin has certain resident stem cells that can compensate for the cell loss in case of minor injuries. Extensive skin injuries such as burns and chronic ulcer can lead to scarring and slow wound healing. In the current scenario, the gold standard treatment for any major skin loss is an autologous skin graft. Disadvantages of this method are that it requires a second operation site and in case extensive burn injuries adequate donor site will not be available. Skin tissue engineering deals with development of skin tissue constructs, with *invitro* expanded skin cells thus waiving off the necessity for donor tissue. With most of the protocols for keratinocyte culture aimed at neonatal tissue, an optimized protocol for the isolation and culture of keratinoytes adult skin tissue is necessary. The present study aims at the optimization of isolation of keratinocytes from adult mouse skin tissue and culture of these cells on a modified culture surface.

The thesis consists of four chapters namely Introduction, Materials & Methods, Results & Discussion and Summary & Conclusion. SChapter I introduces the topic addressed in the thesis and reviews the literature related to the study. This also gives background for the present study and objectives. Skin is the outermost covering of the body and its integrity plays a key role in maintaining homeostasis in the body. When skin is subjected to extensive injuries, it can result in scarring and slow wound healing. Skin tissue engineering has evolved through ages and numerous skin tissue engineered products have been put into clinical use. Apart from the clinical use, these

invitro skin tissue constructs can be used for many *invitro* studies as an alternative to animal testing. Though, keratinocyte cell culture has well established protocols, isolation and maintanence of adult keratinocytes in culture is still a challenge. Various biomaterials have been used as a substrate to create tissue engineered products which are implanted directly on to the wounded site creating biocompatibility issues. A novel technology to develop a biomaterial free skin tissue engineered product with full efficiency is still a holy grail in tissue engineering. To achieve this, the study was aimed at

1. Optimizaion of isolation and culture of keratinocytes from adult murine skin tissue
2. Characterization of cultured primary keratinocytes using immunostaining
3. Generation of NGMA coated culture surface and characterization
4. Culture of keratinocytes on NGMA coated culture surface

Chapter II describes the experimental approach adopted which includes isolation and culture of keratinocytes from adult murine skin tissue. A commercially available defined keratinocyte medium was used for the cell culture. Optimization of the isolation and culture was done by trying two different strains of varied age group as per availability and by trying two different enzyme digestion methods, the single enzyme method and the double enzyme method. The cultured cells were characterized by immunostaining against markers such as CK-1, CK-14 and Involucrin. Immunohistochemistry of skin cryosections were also carried out to check the specificity and required dilution of the antibodies. An NGMA coated surface was created using a custom made spin coater. The coated plates were characterized using water contact angle measurement at above LCST and below LCST. The plates were checked for cytotoxicity by culturing L929 cell and L929 cell sheet retrieval was carried out to study the thermoresponsive efficacy. To study keratinocyte growth on NGMA HaCat cell line was cultured on coated plate and the cell proliferation was accessed by MTT assay. TCPS was served as control for the experiment. Primary keratinocytes were seeded

on NGMA coated plate and the growth pattern was analyzed. Image analysis was carried out using Q-Win software to compare the cell patch growth on TCPS and modified NGMA surface and to compare cell growth of cells isolated by different methods. Statistical analysis of all data was carried out using student's *t* test.

Chapter III includes results of various experiments and discussion of the data. The keratinocytes isolated by the optimized protocol were seeded and the culture was established. The cells formed of colonies and exhibited characteristic cobble stone morphology. The data showed that the cell yield from adult murine skin is independent of the age and strain of tissue source. Isolation using the double enzyme method yielded more cells with which culture could be established. Hence it was preferred over the single enzyme method. Immunostaining of the cultured cells showed positive stain for involucrin and negative for CK-1 and CK-14. The skin cryosections were positive for all 3 markers. NGMA coated surface was created and the water contact angle showed significant difference in between the measure above LCST and below LCST. The NGMA coated plates were non cytotoxic and ensured complete retrieval of cell sheet. MTT assay of HaCat cells on NGMA coated plate revealed that there was significant decrease in the growth of cells, when compared to the TCPS. Primary keratinocyte culture was established in NGMA coated plates and the cells attained their characteristic morphology. However the growth was very limited when compared to the TCPS.

Chapter IV Summarizes the whole work and provides drawn out conclusions. Adult murine keratinocytes were successfully isolated and culture was established. The isolated keratinocyte were successfully cultured on NGMA coated plate. However the keratinocyte culture did not survive first passage and the cell growth on NGMA was very low compared to the normal TCPS. Hence further optimization has to be done to maintain primary keratinocyte cells for several passages and also to increase the growth of keratinocyes on NGMA coated plate.

1. CHAPTER– I INTRODUCTION

1.1 Background

Skin is the largest organ of the body that acts as the barrier between the body and the external environment thus protecting the body from external pathogens and excessive loss of water. Skin is mainly composed of 3 layers; Epidermis, Dermis and the Hypodermis. Epidermis is the outermost part of the body which contains keratinocytes which are the principle cells in that region. Skin normally contains resident stem cells which help in the self renewal of skin, when subjected to injuries. Extensive injuries such as burns and chronic ulcers, were self renewal is not possible, can result in scar formation and slow wound healing. Currently, for any large skin loss, autologous skin transplantation is considered as the gold standard, but it again requires a second operation site which can result in another wound. In case of extensive burns, this may not be possible due to unavailability of large skin tissue for grafting [Mason.C, 2005]. Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function [Langer and Vacanti, 1993]. Methods to transplant sheets of pure epidermis was first proposed by Billingham and Reynolds in 1953 [Billingham and Reynolds, 1953]. Rheinwald and Green proposed an effective method to sequentially cultivate epidermal keratinocytes [Rheinwald and Green, 1975]. Since then, skin tissue engineering has evolved through decades and now numerous skin tissue engineered products are in market. Various methods have been used for the cell sheet retrieval from the culture, such as dispase digestion [Green et al, 1979]. One of the practical problems involved in transplantation of epidermal cell sheet is the fragility of the tissue

construct. Epicel, Myskin etc are some of the available commercial products which comprises of a substrate on which keratinocytes are grown and this can be directly transferred to the wound site without disturbing the cell sheet. Thermoresponsive polymers are those polymers that exhibit a reversible temperature-dependent phase transition in aqueous solutions at its lower critical solution temperature (LCST). Poly (N-isopropylacrylamide) (PNIPAAM) is a thermoresponsive polymer that has been used for epidermal cell sheet retrieval (Yamato *et al*, 2001). The thermoresponsive polymers surface helps in effective retrieval of cells from the culture surface without disturbing the cell-cell interaction. Transfer of these retrieved cell sheets from thermosresponsive polymer coated surface using a polyvinylidene fluoride membrane (PVDF) has been reported previously [Kushida et al, 2001]. N-isopropylacrylamide-co-glycidylmethacrylate (NGMA) is another thermoresponsive polymer, which can be used for the generation corneal epithelial constructs [N.Joseph *et al*, 2010).

Isolating keratinocytes from normal skin and expanding them in *in vitro* conditions can be used as a therapy to cure extensive skin injuries. These expanded cells can be transplanted to the donor site and induce the regeneration of skin. The advantage of this kind of cell therapy is that, autologous cells can be used for transplantation. The study focuses on optimizing the isolation and culture of keratinocytes from adult mouse tissue and their characterization. These isolated keratinocyte were cultured on a modified culture surface, coated with (NGMA).

1.2 Review of Literature

1.2.1 Skin

Skin is the largest organ of the integumentary system, which acts as an interface between the body and the external environment thus protecting the body against pathogens [Proksch et al, 2008] and excessive loss of water[Madison KC, 2003]. It contains the peripheral endings of many sensory

nerves for sensory reception, plays a key role in the regulation of body temperature and has also limited excretory and absorbing powers [William and Warwick, 1980]. The oxygen supply in the upper layer of the skin (0.25–0.40 μm depth) is provided directly from the external atmosphere where oxygen transport from the blood has little influence [Stucker et al, 2002]. Skin plays a major role in mounting a defense mechanism in response to injury and microbial insults by synthesizing antimicrobial peptides and various other host defense molecules that together demonstrate broad-spectrum activity against bacteria, fungi, and viruses [Braff et al, 2005]. The skin is primarily composed of 3 layers the Epidermis, the dermis and the hypodermis.

1.2.2 Epidermis

Epidermis is the outermost layer of the skin. It is consisted of non-vascular stratified squamous epithelium. The epidermis and its appendages such as hair, nail, sweat and sebaceous glands maintain homeostasis by constant recycling of the basal cell layer [Metcalfe at al, 2007]. It is separated from underlying dermis by the basement membrane. The epidermis mainly consist of Keratinocytes along with a few number of melanocyte and langerhans cells. Keratinocytes are principle cells of the epidermis which are of ectodermal orgin and have specialized function of producing keratin, a complex filamentous protein that forms the surface coat (straturn corneum) of the epidermis. The presence of various keratins is being used as markers to find out the degree of differentiation of keratiniocytes. Keratins are critical for normal functioning of the epidermis and keratin mutations causes of skin disease. Mutations in the genes for keratins 5 and 14 are associated with epidermolysis bullosa simplex and keratin 1 and 10 mutations are associated with epidermolytic hyperkeratosis [William et al, 2006]. Involucrin is another soluble protein precursor of the stratified squamous epithelium, which acts as an amine receptor in the transglutaminase catalyzed reaction [Rice RH et al, 1979].

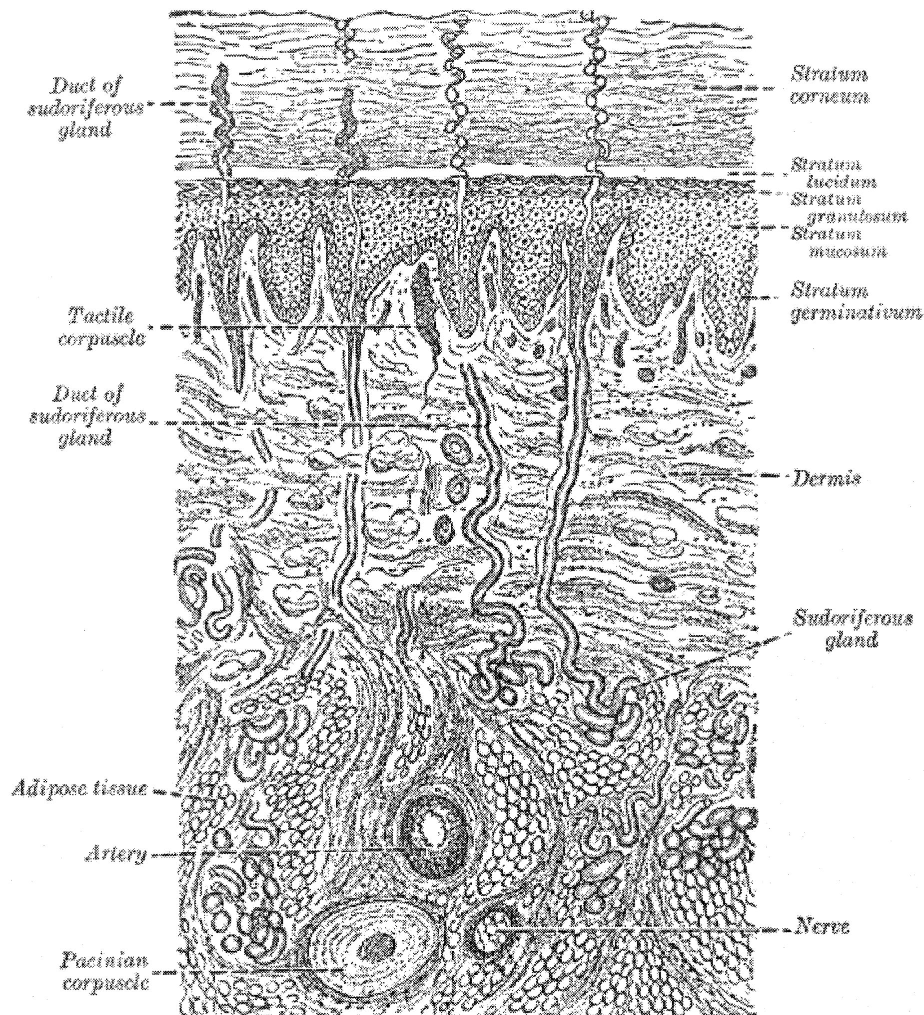


Figure 1 Layers of the skin , Grey's anatomy, 1918

The epidermis is basically divided into 5 different layers. Stratum corneum, Stratum lucidum, Stratum granulosum, stratum spinulosum and stratum germinativum [Figure 1].

1.2.2.1 Stratum Corneum

Stratum corneum is the outermost layer of the epidermis which acts as the principle barrier against the percutaneous penetration of chemicals and microbes, involved in the regulation of transepidermal water loss and also

capable of withstanding mechanical forces [Madison KC, 2003]. It forms a continuous sheets of protein enriched cells (corneocytes) connected by corneodesmosomes. The final stages of normal keratinocyte differentiation are characterized by the alignment of keratins into highly ordered and condensed arrays through interactions with filaggrin, a matrix protein that aggregates the keratin filaments into tight bundles. This promotes the collapse of the cell into a flattened shape, which is characteristic of corneocytes in the cornified layer thus giving its mechanical property [Palmer et al, 2006].

1.2.2.2 Stratum Lucidum

This layer is present below the stratum corneum and superficially to the granular layer , consisting of 3-5 layers of dead flattened keratinocytes[Mc Grath et al, 2004].This layer is prominent or indeed visible only in heavily keratinized areas such as glabrous skin. Glabrous skin refers to the non-hair bearing skin of the palm or the sole [Place et al, 1997]. The keratinocytes at this region contains eleidin which is a clear intracellular protein. This eleidin is later converted to keratin in the stratum corneum [William and Warwick, 1980].

1.2.2.3 Stratum Granulosum

This layer is found in between the startum lucidum and stratum spinosum. They contain certain granular cells which contain keratohyalin and lamellar granules [William et al, 2006]. These granular cells at its base consist of flattened cells with pyknotic nuclei, surrounded by numerous basophilic granules known as keratohyalin which is believed to play a role in keratinization [William and Warwick, 1980].

1.2.2.4 Stratum Spinosum

The stratum spinosum or the prickle cell layer is marked by convoluted, interdigitating profiles of the cells, the keratinocytes which are linked by

numerous desmosomes attached to internal tonofibrils of the cytoplasm. Keratinization begins in the stratum spinosum [William and Warwick, 1980].

1.2.2.5 Stratum Germinativum

This is the deepest layer of the epidermis, capable of mitotic division, mainly in a vertical direction to generate stacks of epidermal cells which are continuous to the epithelial surface. This basal layer contains some resident stem cells that helps in the renewal of keratinocytes in the epidermis. Apart from that melanocytes and langerhan's cells are also present in this layer [William and Warwick, 1980].

1.2.3 Epidermal stem cells

In an adult life, the major role of cell division is to replace cells, that has been lost due to injury thereby maintaining cell number at an approximately constant level [Hall and Watt, 1989]. This cell replacement occurs through, a distinct subpopulation of cells known as the stem cells. A stem cell is any cell that can retains the capacity of self renewal throughout and can produce daughter cells that undergo terminal differentiation [Lajtha, 1979]. Mammalian epidermis has two well characterized differentiation pathways, whose end points are the cells of the hair shaft and the outermost, cornified layers of the interfollicular epidermis. When the epidermis is injured, the hair follicle can migrate over to the basement membrane and repopulate the damaged interfollicular epidermis [Al-Bawari and Potten 1976]. Whereas, interfollicular keratinocytes grafted on to an empty hair follicle, which is in contact with the dermal papilla cells were able to form cell shaft [Reynolds & Jahoda 1992]. The dividing cells, that have the ability to regenerate the epidermis are known as stem cells and the other cells are known as transit amplifying cells which have limited self renewal capacity and are in high probability of withdrawing from the cell cycle and undergoing terminal differentiation [Potten, 1981]. A

working model for self-renewal in human interfollicular epidermis is shown in Figure 2.

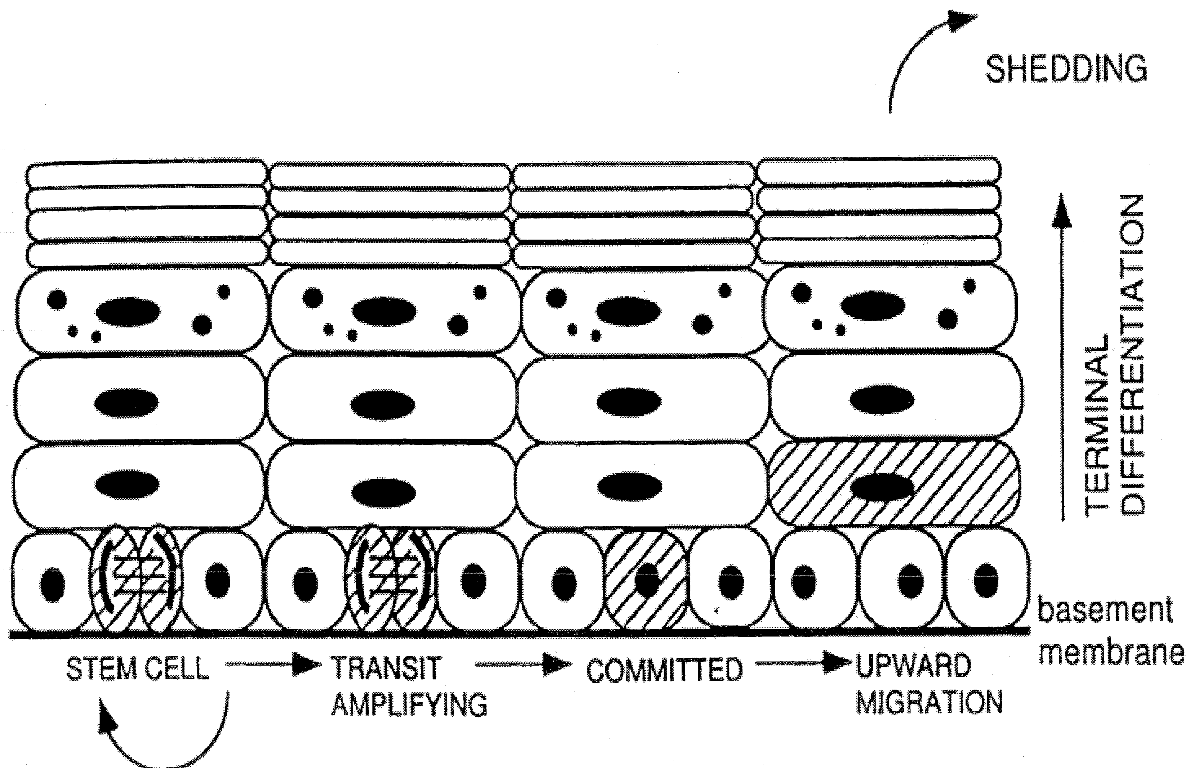


Figure 2 Diagram illustrating the organization of keratininocytes in the interfollicular epidermis [Jones and Watt, 1993]

1.2.4 TISSUE ENGINEERING

The term "Tissue Engineering" was proposed by 1987 Washington National Science Foundation bioengineering panel [Nerem RM, 1992]. Tissue engineering, as stated by Langer and Vacanti [Langer and Vacanti, 1993], is "an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" Certain applications of tissue engineering require cells or tissues without any scaffold support. In such cases *in-vitro* tissue constructs are preferred with native ECM. The disruption of Cell- Cell interaction and Cell-ECM interaction during retrieval should be avoided. This can be achieved through another approach known as cell sheet engineering using thermo responsive polymer grafted surface

[Kwon et al, 2000].In skin tissue engineering, the ultimate goal is the development of skin both *invitro* and *invivo,* meanwhile preserving all its constituents' spatial and functional three-dimensional structure as well as cosmetic properties.

1.2.5 Need for Skin Tissue Engineering

Often in case of partial thickness burns where epidermal layer is completely lost, but part of dermis remains normal regeneration is possible by replacing the lost keratinocytes, However in full thickness burns, both the epidermis and the dermis are completely lost resulting in scar formation or fibrosis. Any full thickness burn injuries more than 4cm in diameter will not heal without a surgical intervention [Herndon and Parks, 1986]. Ideally if the burn is not too extensive, autologous skin graft is used. The donor site from which the skin graft is taken will then undergo normal regeneration to restore the lost tissue. In case of extensive injuries leaving no scope for both normal regeneration and split-thickness graft, tissue engineering methods can play a pivotal role. Apart from burn injuries, tissue engineered skin can be used for chronic ulcers those requiring reconstructive surgery [MacNeil, 2007].

1.2.6 Evolution of skin tissue engineering

Methods for preparation of sheets of pure epidermis and of epidermal cell suspensions which can be transplanted successfully were first proposed by Billingham and Reynolds [Billingham and Reynolds, 1953]. These grafts were used to cover large full-thickness skin defects. Rheinwald and Green proposed a more effective method for the serial subcultivation of epidermal keratinocyte by expanding keratinocytes on lethally irradiated mouse fibroblasts in the presence of mitogens such as epidermal growth factor and hydrocortisone along with bovine fetal calf serum [Rheinwald and Green, 1975, 1977]. By this method the keratinocytes could undergo population doublings. However, this proliferating capacity decreases with age of the donor [Gilchrest, 1983]. Keratinocyte sheet grafting became a reality when the

the enzyme Dispase, a bacterial neutral protease was used to digest adhesive molecules holding the keratinocytes to the plastic substrate without disturbing the cell to cell interaction[Green et al, 1979]. The first experimental success in autologous epidermal sheet grafting came in 1981 when a group of scientists used a small piece of epidermis from an autologous source and expanded it *in vitro* and grafted it on to patients with full thickness burns. The grafts survived for an observation period of 8 months [Nicholase *et al,* 1981]. One of the major disadvantages of autologous epidermal skin grafts is the time required (2-3 weeks) to produce the grafts, which can prove fatal to the patients with severe burn injury. The use of keratinocyte allograft was proposed as a suitable solution to this problem [Hefton et al, 1983]. This was based on the study that showed, cultured keratinocyte graft in humans, do not express major histocompatability class II LA-DR antigens [Morhen et al, 1982] and also the langerhan's cells which are one of the source of class II antigens are absent in cultured keratinocyte grafts [Thivolet at al, 1986]. The practical problem involved in delivering keratinocyte as sheets is its delicacy. Another difficulty is the presence of terminally differentiated keratinocytes which are in a stage at which they cannot actively take part in restoring the damaged epithelium. One of the breakthroughs in skin tissue engineering was the Epicel, where cells are placed cell surface down onto the wound bed, and keratinocytes still capable of leaving this integrated sheet of cells enter the wound bed and form a new epithelium. The backing dressings with any residual differentiated cells are removed some 7–10 days late [Wright et al, 1998].

Considering the difficulty in handling cells alternative approaches for detaching and delivering cells have since been developed. One of them is the poly(N-isopropylacrylamide) (PNIPAM) [Yamato et al, 2001] which is thermoresponsive polymer were Cells grown into sheets on PNIPAM-coated tissue culture dishes, then the temperature is decreased to collapse the polymer chains and release the cell sheet. Keratinocytes has also been expanded in culture, made into suspensions, and sprayed onto wound beds with fibrin [Horch et al, 2001].

1.2.7 Progress and Challenges

The tissue-engineered products which are presently available can be grouped into three. There are some products which replaces only epidermal layer, some provides a dermal substitute, and a few that provide both [Sheila MacNeil, 2008]. In certain clinical conditions such as in the cases of non-healing ulcers and superficial burns, transferring laboratory-expanded cells can benefit patients. Treatment of complicated cases like the major full-thickness burns requires the replacement of both dermis and epidermis. The four major insights in this field are: improving safety, split-thickness grafts substitute, improving angiogenesis in replacement tissue once it has been grafted to the wound bed, and improving ease of use.

1.2.7.1 Safety

The prevailing culture methodology uses murine fibroblasts [Rheinwald and Green, 1975 and bovine serum [Rheinwald and Green, 1977]. Murine cells may contain viruses which are able to transform human cells, so to avoid risk murine feeder fibroblasts are treated with lethal gamma radiation and serum is tracked down from the countries where herds are free from bovine spongiform encephalitis (BSE).

Other specific safety issues have to be focused are related with the skin reconstruction using natural biomaterials. The incorporation of porcine tissues in humans i.e. Xenotransplantation carries the risk of stimulating antibodies to the Gal (galactosidase) epitope. Similar epitopes are present on porcine tissue that can result in acute rejection by activating complement system. [McPherson et al, 2000]. To avoid this problem, proper care must be taken to remove this before use.

The donor cells used for transplantation should be screened for any viral infection and the commercial products like dermagraft: biosynthetic material conditioned with donor fibroblast [Marston et, 2003], Transcyte: includes a silicone membrane that can act as a temporary epidermal barrier along with a dermal substitute [Kumar et al, 2004], Apli-graft: a combination of both

allogenic keratinocytes and fibroblast along with bovine collagen [Bello and Falabella, 2002] etc should use cells from screened cell banks. Donor cells can produce growth factors, thus can act as biological factories to stimulate wound healing, and can also be used as temporary dressings. In some cases like severe burns donor fibroblasts can be used both for chronic wound stimulation and in dermal replacement. Donor skin has been in use since the early 1980s [Hermans MH, 1989] and no problems have been reported in association with residual donor fibroblasts. Pig skin can be used as a temporary substitute for example, Permacol [Jarman et al, 2004]. The development of a synthetic dermal substitute would significantly reduce many of these risks.

1.2.7.2 Split-thickness graft substitutes

Split thickness grafts are those which include the full epidermal skin layer and part of the dermal skin layer. These Split-thickness grafts are considered as the gold-standard treatment for many clinical conditions, but limited by inadequate quantity, which can be achieved by tissue-engineered grafts. Permaderm, formerly known as Cincinnati skin substitute [Boyce et al, 2006], a Commercial product, can meet this need, but there are still substantial challenges such as the stability of the attachment of the keratinocytes to the underlying dermis in the case of deep burns. If proper dermal equivalent is not achieved by wound bed then it is very difficult for the cells to attach to the wound site. A superior approach for the development of a reconstructed skin in the laboratory in which the proper attachment between keratinocytes and dermis can be secured [Ralston et al, 1999]. The maximum thickness of the material used for the skin-replacement can easily become vascularized is 0.4 mm and if grafts used are thicker than 0.4 mm, new blood vessels cannot go through quickly enough to nourish the epidermal layer and can result in loss of grafts [Sahota et al, 2003].

1.2.7.3 Cell delivery approaches

A number of different methods are available to improve the ease of use of skin products. Keratinocytes made into suspensions and sprayed on to the wound sites with [Horch et al, 2001] or without fibrin, CellSpray [Navarro et al, 2000]. A comparison study on cell spraying with and without fibrin glue showed no statistically significant difference between the two groups and also there was no observable difference in the quality of the epithelium on histological and immunohistological analysis of either group [Currie et al, 2003]. Cells are also delivered on various carrier dressings ranging from bovine collagen [Horch et al, 2000] to a chemically defined polymer carrier dressing such as Myskin [Haddow et al, 2003]. Cells can be cultured from hair follicles and delivered as small sheets, for example EpiDex [Tausche et al, 2003].

The use of any particular form of cell delivery will finally be determined by factors such as ease of use, cost, transportation and variables other than the biological health of the cells. Accordingly, the preference is for subconfluent cells to be delivered on a chemically defined carrier dressing for both chronic wounds and burn injuries, from which the cells transfer to the wound bed. [Moustafa et al, 2004] Using the technique plasma polymerization, the inert carrier dressing can be coated with an extremely thin polymer containing acid functional groups [France et al, 1998]. Repeated applications can endorse wound healing in chronic wounds. This technique requires regular repeated applications of cells (either autologous or allogeneic) to attain proper healing in wounds that may have been for a long period of time [De Luca et al, 1989].

1.2.8 Non-Clinical uses of tissue-engineered skin

There are many non-clinical research applications for reconstructed skin tissue,especially 3D models. Engineered skin is presently used for delivering value in aspects of skin biology research as diverse as reducing animal experimentation, investigation of cell-cell and cell–extra cellular matrix interactions, wound healing, angiogenesis, regulation of pigmentation, skin contraction and investigation of skin diseases such as melanoma invasion,

psoriasis and skin blistering disorders. The design criteria for 3D scaffolds are less exact in *in vitro* than in vivo. The scaffold should allow skin cells to form an epithelium, to communicate with each other and to mimic as far as that of the normal skin cells when exposed to the biological agents. The reconstructed skin should deliver the requisite biology, but, for example, for *in vitro* toxicity studies, scaffolds made of non-biodegradable material maybe adequate [Sun et al, 2005]. If these scaffolds lack any functioning vasculature or immune system, then it cannot be used as a complete substitute for in vivo animal or human studies, but they have a major advantage in the ability to add or delete different cell types to assess their relevance to the aspect of skin biology under analysis. This cannot be done

in vivo. These models are contributing to many areas of research.

1.2.8.1 Alternatives to animal testing

Finding alternatives to animal testing for the many thousands of chemical additives used in human skin products are very much essential in the case of tissue engineered skin. The companies such as L'Oreal [Welss et al, 2004] and Skin Ethic [Netzlaff et al, 2005] has put a great investment in the development of skin models for testing pharmaceutical cosmetic and chemical compounds. This has led to the development of epithelial cell models to study agents that might cause toxicity or irritancy. In 2008, two skin irritation test methods SKINETHIC and EpiDerm were validated on the basis of the Performance Standards for applying human skin models to *in vitro* skin irritation testing [Kandarova et al, 2006]. Recent research shows that cytotoxicity testing using 3D co-cultures might be more likely to reflect true physiological responses to xenobiotic materials than existing models that rely on 2D mono-cultures [Sun et al, 2006]. The role of fibroblast in keratinocyte expansion and organization with ,which can be demonstrated even in a 3D electrospun polystyrene matrix that has none of the specialized collagen structure of the natural dermis [Sun et al, 2005].

Studies using various skin phototypes to validate a model of epidermal reconstruction with melanocytes have been carried out to understand the biology both normal and vitiligo melanocytes in reconstructed skin [Bessou et al, 1996]. The experiments led to the findings that melanocytes in 3D culture spontaneously produce pigment reflecting their donor phototype origin. This ability was not influenced by the keratinocyte environment. Based on this,the L'Oreal group have developed a model with melanocytes for investigating pigmentary responses to agents. Unexpectedly, fibroblasts were found to have a major role in determining whether spontaneous pigmentation occurs in reconstructed skin [Hedley et al, 2002]. Recent work in immune-tolerant mice grafted with reconstructed human skin has proved that human fibroblasts also function to lessen melanocyte pigmentation in vivo [Cario et al, 2006]. Thus, melanocyte behaviour is very different in the presence and absence of fibroblasts. Hypopigmentation is often a problem, when reconstructed skin is used for burns patients. The melanocytes initially present in the tissue-engineered skin may be rapidly lost when collagen is used as a dermal scaffold [Swope et al, 2006] Melanocytes can be duplicated experimentally using reconstructed human skin prepared with or with-out a basement membrane. Melanocytes fail to make a proper attachment in the absence of a basement membrane [Hedley et al, 2002].

This *in vitro* keratinocyte culture system has helped various research groups to investigate interaction between tumour cells and healthy skin cells, to examine the deliberate upregulation or downregulation of adhesion molecules on these melanomas, and study matrix metalloproteinases and inflammation. Invasion has been shown to be dependent on the interaction of the tumour cells with the adjacent skin cells [Eves et al, 2000]. Another research group used 3D skin constructs as *in vitro* model and demonstrated the role of basic fibroblast growth factor for the progression of human melanoma [Meier et al, 2000].

The tissue engineered skin has also been used to create models of some skin diseases such as psoriasis [Barker et al, 2004] and also used as the model for the treatment of the genetic disorder epidermolysis bullosa.It is

also used to develop keratinocyte adhesion. Gene therapy and grafting of tissue-engineered skin are some of the recent promising fields [Ferrari et al, 2006].

1.2.9 Challenges for the future

Tissue engineering of skin rests on a strong basement made of almost 25 years' of published research in cell biology and wound-healing knowledge. It is high time since laboratory proven experiments should move to clinical research so that these novel technologies can be put to use for the betterment of human. At an ordinary level there remains a major need to reduce the risks for patients receiving cultured cells i.e. by using a reliable and body approved xenobiotic free culture methodology, dermal substitute materials which has low risk level ,secured & proper attachment of cells .The basement formation and vascularisation are required for the survival of engineered skin after grafting process.. *In vitro* models of tissue-engineered skin focus to prevent contraction and loss of pigmentation. With respect to scar prevention, a long-established programme of research on understanding the role of the transforming growth factor-β family in wound healing [Shah et al, 1999]. With more new drugs entering human patient based trials after their success in double blind randomized trials, scarring may no longer be an inevitable consequence of modem injury or surgery and that a completely new pharmaceutical approach to the prevention of human scarring is now possible [Ferguson and O'Kane, 2004]. The cells should be user friendly and clinically accepted .Production costs must be contained so that reimbursement gives a sufficient income. Despite initial unrealistic commercial and clinical expectations, tissue-engineered skin has delivered considerable benefits to patients with burns and deep wounds and has enormous potential that is only just the beginning ,to be realized.

1.2.10 Scope Of the study

Skin tissue engineering has evolved through ages and it is now high time that all the knowledge gained from the last 3 decades, be put together to develop novel therapies for the betterment of human beings. In case of skin tissue engineering, the idea is to isolate keratinocytes from an autologous source and expand these cells invitro to induce regeneration at the wounded sites. Isolation and culture of keratinoytes has very well established protocols, with most of them optimized for neonatal skin. However, optimizing an isolation and culture method for an adult murine skin is still a challenge, since the source of tissue is very important in determining the cell quality. With the number of commercial skin tissue engineered products available, increases day by day, more safety issues also increases. Any artificial tissue constructs consisting of cells and biomaterials or scaffolds can result in biocompatibility issues. If any biomaterial free tissue construct is created, then another major issue is the cell retrieval from the culture surface. The fragility of the tissue construct can be a real practical problem in creating a scaffold free skin tissue. Therefore it is necessary to create a modified surface that can help in the efficient cell retrieval process thus creating a biomaterial free tissue construct. Thermoresponsive polymer coated culture plates are one such modified surface that exhibit a reversible temperature-dependent phase transition in aqueous solutions at its lower critical solution temperature (LCST), These polymers have the unique property of being hydrophobic above LCST and hydrophilic below LCST. NGMA (N-isopropyl acrylamide glycidyl methacrylate) is one such thermoresponsive polymer that has been previously used for corneal tissue engineering. A combination of an optimized isolation and culture protocol for adult murine keratinocyte with a culture surface modified with NGMA could pave way for the development of novel biomaterial free skin tissue construct.

1.3 Hypothesis

Keratinocyte isolated from adult murine skin by an optimized method will be helpful in establishing keratinocyte culture on modified surface.

1.4 Objectives Of the study

1. Optimization of isolation and culture of keratinocytes from adult murine skin tissue
2. Characterization of cultured primary keratinocytes using immunostaining
3. Generation of NGMA coated culture surface and characterization
4. Culture of keratinocytes on NGMA coated culture surface

2. CHAPTER II MATERIALS AND METHODS

2.1 Isolation and culture of Adult Murine Keratinocyte

2.1.1 Collagen Coating on 35mm culture plates

2.1.1.1 Materials

35mm Tissue culture polystyrene (Nunc), Type I Collagen solution (PureCol)

2.1.1.2 Method

1ml of prepared collagen solution was poured on a 35mmTCPS and filled to cover the whole surface of the plate and kept for 1min inside the biosafety cabinet. Then the excess collagen was pipetted out and coated plates were incubated at 37°C for 4-5 H or until use.

2.1.2 Tissue excision from mice cadavers

2.1.2.1 Materials

Sterile cotton, Savalon, 0.01% Pencillin-Streptomycin, Toothed forceps, sterile gloves, Sterile Scalpels (size=21), Sterile Scalpel Blade Holder, Blunt end forceps, surgical scissors, hair clipper, Mouse Cadaver (Swiss Albino/Balb C Age: 2months – 21 months)

2.1.2.2 Method

The hair on both dorsal and ventral side of the mouse cadaver was trimmed using a hair trimmer. The remaining hair was further removed using a sterile scalpel blade.Then the cadaver was wiped with sterile cotton soaked with savalon on the dorsal and ventral side of the cadaver. An approximate size of 4X4 cm^2 skin was excised (approx) using sterile surgical scissors by holding the skin with a blunt end forceps. The excised tissue was collected in 0.01%

Pencillin-Streptomycin solution. Both ventral and dorsal skin was collected seperately and transferred to the tissue culture laboratory.

2.1.3 Processing of the excised skin tissue

2.1.3.1 Materials

Sterile Scalpels, Sterile gloves, Sterile Scalpel Blade Holder, 150mm petri plate, 60mm petriplate,10% Betadiene, 0.1M PBS, 0.01% Pencillin-Streptomycin

2.1.3.2 Method

The tissue collected was transferred to 10% Betadine solution. After 2 min another fresh rinse in 0.01% antibiotic solution for 1min was given. The skin was then placed in a 150mm petridish with dermal side facing up which was identified as yellowish fatty layer with traces of blood. By holding the tissue with forceps on one side, the adipose tissue and muscle was scrapped off with a sterile scalpel blade. The tissue was then cut into four equal parts each with an approximate size of 2X2 cm^2 using a scalpel blade and then rinsed in 0.1M PBS.

2.1.4 Isolation of keratinocytes using double enzyme method from the prepared tissue sample

2.1.4.1 Material

Sterile Forceps (Blunt and Pointed), Toothed Forceps (Sterile), Sterile Scalpel Blade, Sterile Scalpel blade holder, Sterile gloves, Biosafety Cabinet, 60mm Petridish sterile, Collagen coated 35mm culture dish, centrifuge tube 15ml, Cell strainer (100 µ)/ Nylon Mesh, Micropipette (200-1000µl & 10-100µl), Sterile Tips, haemocytometer, 0.1M PBS, Trypsin_EDTA (0.25%-0.1%) in 0.1M PBS, 0.25% Dispase, Defined Keratinocyte SFM + Growth Factors (Gibco, Invitrogen), Trypan Blue, Microcentrifuge tubes, Cover slip

2.1.4.2 Method

After processing and cleaning, the tissue was transferred to a 60mm petriplate containing dispase solution and incubated 1h at 37°C or 4°C overnight with epidermal side up. The tissue was taken placed with the epidermal side facing up on another petriplate. The epidermis was scrapped off using a cell scrapper without applying excessive pressure.The scrapped off epidermis was minced using a scalpel blade and transferred to another sterile petriplate containing Trypsin-EDTA and was pipetted in and out repeatedly for (~) 10 s and was kept for incubation at 37°C for 5min. After trypsin digestion the whole solution was pipetted in and out again. The cell suspension was passed through a nylon mesh/ cell strainer and collected in a 15ml centrifuge tube. The suspension was centrifuged at 500 rcf for 8min. Supernatant was discarded and the pellet was resuspended in1ml of defined keratinocyte SFM + GF. Cell viability was determined using trypan blue. About 3.6×10^5 cells were seeded on to a collagen coated 35mmplate and incubated at 37°c and 5% CO_2. Medium was changed every 2 days.

2.1.5 Trypsin Treatment

2.1.5.1 Materials

0.1M PBS, 0.05%-0.02% Trypsin-EDTA, Micropipette, Sterile tips

2.1.5.2 Method

Medium was removed from the culture plate containing an 8day culture of keratinocyte and was washed with 0.1M PBS 3times. 1ml of 0.05%-0.02% trypsin-EDTA was added to the culture plate and incubated for 1min. After incubation the trypsin was removed and medium was added. The culture was then incubated at 37°c and 5% CO_2.

2.1.6 Subculture

2.1.6.1 Materials

0.1M PBS, 0.05%-0.02% trypsin-EDTA, Micropipette, Sterile tips, collagen coated plate

2.1.6.2 Method

Medium was removed from the culture plate containing an 8day culture of keratinocyte and was washed with 0.1M PBS 3times. 1ml of 0.05%-0.02% trypsin-EDTA was added to the culture plate and incubated for 5min. After incubation the trypsin was removed and medium was added. The medium was pipetted in and out and the cells were taken into suspension. The cell suspension was seeded on to a fresh collagen coated plate.The culture was then incubated at 37°c and 5% CO_2.

2.2 Optimization of isolation of adult Murine Keratinocytes

2.2.1 Age and strain of the Mouse cadaver

Skin tissue Samples from mouse cadaver of various age groups (2months – 21 months old) have been used for the study to isolate keratinocytes. Both Swiss albino and BALB/c were used for the experiments as per availability.

2.2.2 Tissue excision from mice cadavers

2.2.2.1 Materials

Sterile cotton, Savalon , Pencillin-Streptomycin (0.01% in 0.1M PBS), 10µg/ml Amphotericin in PBS, Toothed forceps, sterile gloves, Sterile Scalpels (size=21), Sterile Scalpel Blade Holder,Blunt end forceps, surgical scissors, hair clipper

2.2.2.2 Method

The hair on both dorsal and ventral side of the mouse cadaver was trimmed using a hair trimmer. After clipping the hair, the tissue was excised with and without shaving the remaining hair.

Collection of excised tissue in 0.01% pencillin streptomycin and 10μg/ml Amphotericin has been tried.

2.2.3 Enzymatic digestion

Two different types of enzyme digestion was done as part of the optimization of keratinocyte isolation

2.2.3.1 Single Enzyme Method

In single enzyme method the processed skin tissue was digested in Trypsin EDTA (0.25%- 0.1%) with the epidermal side facing up. Two types of trypsinisation was carried out.

- Warm trypsinisation – 2hours, 37° C [Yano et al, 2005]
- Cold trypsinisation – Overnight, 4° C [Jensen et al, 2010]

After trypsinisaton the epidermal side was scrapped off using a scalpel blade and the resulting cell suspension was centrifuged resuspended and seeded on to collagen coated culture plate.

2.2.3.2 Double Enzyme Method

In double enzyme method the processed skin tissue was digested in 0.25% Dispase followed by Trypsin EDTA (0.25%- 0.1%). After tissue processing and cleaning, it was transferred to a 60mm petri plate containing dispase solution. Dispase digestion was carried out in two different conditions

- Dispase digestion at 37° C, 1 hour [Garcia et al, 1998]
- Dispase digestion at 4° C, Overnight [Shiv Poojan and Shushil kumar, 2010]

After dispase digestion the epidermal side was scrapped off using a cell scrapper rest of the procedure was carried out according to the previously mentioned protocol.

2.3 Characterization of Keratinocytes

2.3.1 Immunostaining of mouse Skin Cryosection

2.3.1.1 Materials

Liquid Nitrogen, Isopentane, aluminum foil, blunt end forceps, Liquid nitrogen cylinder, -80oC deep freezer, Poly-L-Lysine coated slides, leica cryostat model CM3050S, Acetone, 1% BSA, 0.1M PBS, Tissue paper, Humidified chamber, Primary and secondary antibodies (Table 1), Fluorescent mounting medium (Dako Cytomation, USA), cover slips, Fluorescent microscope (leica Generic).

Table 1 Antibodies used for immnunostaining of keratinocyte culture and skin cryosections

Primary antibody	Secondary Antibody
Rabbit monoclonal Cytokeratin – 1 (ABCAM)	Anti Rabbit IgG – TRITC (Sigma Aldrich)
Mouse Monoclonal to Cytokeratin 14 (ABCAM)	Anti mouse IgG - FITC (Sigma Aldrich)
Rabbit Polyclonal to Involucrin (ABCAM)	Anti Rabbit IgG – TRITC (Sigma Aldrich)

2.3.1.2 Method

The excised skin tissue from mouse cadaver was snap frozen in liquid nitrogen cooled isopentane and stored in liquid nitrogen overnight. The sample was subsequently stored in -80°C. Cryosections (7µm thick) of the sample was taken using the leica cryostat model CM3050S. Sections were taken on to poly-L-Lysine coated glass slides air dried and stored at -80°C till use. On the day of the experiment, the sections were taken out of -80°C freezer and allowed to thaw to room temperature for 30min. Then the sections

were fixed in cold acetone (-20°C). The slides were then washed thrice with PBS 5min each. After washing the slides, the slides were wiped gently around each section. A drop of BSA solution was added on to the slide so as to cover the section completely and incubated at room temperature for 10min. After incubation BSA was poured off and the slides were wiped gently around the each section and a drop of primary antibody was added so as to cover the section completely and incubated for 1h in a humidified chamber. After incubation the slides were washed thrice in PBS, 5min each. The sections were then incubated with their corresponding secondary antibody for 1h in a humidified chamber. After incubation, the slides were again washed thrice with PBS, 5min each. After washing the slides were wiped gently around the sections. The slide was then mounted with coverslips using fluorescent mounting medium and observed under fluorescent microscope.

2.3.2 Immunostaining of cultured keratinocytes

2.3.2.1 Materials

Sterile Forceps, Pasteur pipettes, Para film, Filter paper, Humidified chamber, Microslide/ Cover slip, Fluorescence Microscope (leica Generic), 0.1M PBS, Fixative – 3.7 % Paraformaldehyde (PFA) in PBS, Blocking Solution(1% Bovine serum albumin[BSA]), 0.1% Triton-X-100, Fluorescent Mounting Medium(Dako Cytomation, USA), Primary antibodies and Secondary antibodies [Table 2]

2.3.2.2 Method

The cultured cells were analyzed for the expression of characteristic proteins such as cytokeratin – 14, cytokeratin – 1 and Involucrin. The cells grown on culture plates were fixed with 3.7% paraformaldehyde, overnight at 4°C. The fixed cells were washed thrice with PBS for 5 min and permeabilized with 0.1% Triton X-100 for 5 min. Permeabilized cells were then washed thrice with PBS and incubated with 1% BSA for 10 min to block nonspecific binding. Cells were incubated with primary antibody (1:100) for 1h in a humidified chamber. The cells were then rinsed thrice with PBS and incubated with

corresponding secondary antibody (1:100) for 1h in a humidified chamber. The cells were rinsed again with PBS and counter stained with Hoechst for 1 min and washed with PBS thrice for 5 min. The cells were then put in PBS and observed under the microscope.

2.4 Preparation of modified surface

2.4.1 Materials

4% N-isopropylacrylamide-co-glycidyl methacrylate (NGMA, In-house synsthesised), custom-made spin coater, hot air oven, sterile cold water, micropipette, 35 mm tissue culture polystyrene (TCPS, NUNC)

2.4.2 Method

A 35 mm TCPS was loaded to a custom made spin coat adaptor and 40µl of the NGMA polymer was transferred to the TCPS inside the spin coat adaptor through a sample load port on the top. Spinning was started immediately and continued for exactly 10seconds.The spin coated plates were dried in hot air oven at 60° C overnight. After drying the coated plates were rinsed with cold water (3 rinses) and dried again. After drying the plates were packed and ETO sterilized.

2.5 Characterization of NGMA coated surface

2.5.1.1 Water contact angle measurement

2.5.1.2 Materials

Deionised water, NGMA coated TCPS plates, Data Physics Water contact angle OCA20

2.5.1.3 Method

The water contact angle of the NGMA coated plates were measured using the NRL Contact Angle Goniometry (Sessile drop technique) to study the changes in the surface characteristics of the modified surface. Water contact angle was measured with both hot water and cold water to study the change in the

surface water interactions above and below LCST (Lower critical solution Temperature). Initially the water contact angle below LCST was measured with water kept at room temperature (25°C). Water contact angle at temperature above LCST was measured by using deionised water pre-heated to 40°C. The temperature of the water was checked using a thermometer. The measurement was carried out using a video based contact angle measuring device and imaging software. A minimum of 4 different fields from each sample were analyzed.

2.5.1.4 Cytotoxic evaluation of the modified surface

2.5.1.5 Materials

Confluent monolayer of L929 cells, DMEM + 10%FBS, Trypsin-EDTA (0.05% - 0.02%)Inverted Phase contrast microscope, NGMA coated culture plates, TCPS (35mm), haemocytometer.

2.5.1.6 Method

Medium was removed from the culture flask containing a confluent monolayer of L929 cells. The cells were then washed with PBS 3 times to remove the remaining medium and serum components. After PBS rinse 2 ml of 0.05%-0.02% Trypsin-EDTA was added to the flask and incubated at 37°C until the cells gets a round morphology. Then the trypsin was removed and 1ml of DMEM + 10% FBS was added. The cell count was determined using a haemocytometer. 1 X 10^4 cells were added to each 35mm Petri plate (both TCPS and NGMA coated) and were incubated after adding sufficient amount of medium. The cells were cultured at 37°C, 5% CO_2. Cells were observed under the microscope every day. Cytotoxicity was accessed by comparing the morphology of the cells under an inverted phase contrast microscope

2.5.1.7 Cell sheet retrieval from NGMA coated culture plate

2.5.1.8 Materials

PVDF (Polyvinylidene Fluoride) Membrane (Sterile), Blunt end forceps, DMEM+10% FBS, NGMA coated plate(35mm), TCPS (35mm)

2.5.1.9 Method

In order to study the thermoresponsive property of the NGMA coated culture plates, cell sheet retrieval was carried out using a PVDF membrane. L929 cells were cultured on the NGMA coated plate. Once confluent, the culture was used for cell sheet retrieval. Initially the medium was removed from the culture plate and then a PVDF membrane conditioned with the medium was placed flat on the culture surface. The plates were incubated below 10° C 1min followed by 3min at room temperature (25° C). After incubation, the cells were observed under a microscope. Once rounding of cells were observed the PVDF membrane was made to float by adding excess medium and transferred to another fresh culture plate. The PVDF membrane was positioned in such a way that the side with cells is in direct contact with the culture surface. This was kept for incubation at 37^{0} C for 1hr. After that the PVDF membrane was made to float by adding excess medium and removed from the culture plate. 1ml of medium was added to the fresh plate and observed under the microscope. Following which, the fresh plate was incubated at 37^{0}C, 5% CO2.

2.6 Culture of Keratinocytes on NGMA coated surface

2.6.1 Culture HaCat keratinocyte cell line on NGMA coated surface

2.6.1.1 Materials

0.05% Trypsin_EDTA (Paa laboratories), 0.1M PBS, Confluent monolayer of HaCat cell line, DMEM + 10% FBS, Haemocytometer, TCPS, NGMA coated plates, TCPS

2.6.1.2 Method

The medium was removed from the culture flask containing a confluent monolayer of HaCat cells. The cells were then washed with PBS 3 times to remove the remaining medium and serum components. After PBS rinse 2 ml of 0.05%-0.02% Trypsin EDTA was added to the flask and incubated at 37°C until the cells gets a round morphology. Then the trypsin was removed and 1ml of DMEM + 10% FBS was added and pipetted in and out. The cell count was determined using a haemocytometer. 1 X 10^4 cells were added to each 35mm Petri plate (both TCPS and NGMA coated 3each) and was incubated after adding sufficient amount of medium. The cells were culture at 37°C, 5% CO_2.

2.6.2 Cell proliferation of HaCat cultured on NGMA surface

2.6.2.1 Materials

MTT reagent, 0.05% Trypsin-EDTA (Paa laboratories), Isopropanol (Analytical grade), 0.1M PBS, Confluent monolayer of HaCat cell line, DMEM + 10% FBS, Haemocytometer, TCPS, NGMA coated plates, 96 well plate, Multiwell plate reader (Biotek PowerWave XS, USA).

2.6.2.2 Method

The cell proliferation of HaCat cell line in TCPS and NGMA coated plate was monitored by evaluating the cellular activity by MTT assay on 1, 3 and 5 days.

MTT is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase of the cells to an insoluble, coloured (dark purple) formazan product which are then solubilised with isopropanol and measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the increase in metabolic activity can be related to the increase in the number of cells.

After removing the culture medium from 35mm culture plate, cells were incubated with 1ml of 1 mg/ml MTT reagent for a minimum of 2 h at 37°C. After incubation MTT reagent was removed, and the cells were rinsed with PBS and incubated with equal amounts of isopropanol for 5 min with continuous shaking. The cell lysate were transferred to a 96 well plate and absorbance was measured in a multiwell plate reader (Biotek PowerWave XS, USA) at 570 nm. The results were expressed as mean ± standard deviation (n=3) and statistical significance was analyzed.

2.6.3 Culture of primary keratinocyte on NGMA coated surface

2.6.3.1 Materials

Collagen solution, freshly isolated primary keratinocytes, Defined keratinocyte serum free medium

2.6.3.2 Methods

Collagen coating on NGMA plate was carried out as previously mentioned. Primary keratinocytes isolated by double enzyme method were seeded on to this collagen pre coated NGMA coated plates and cultured using defined keatinocyte serum fee medium at 37°C, 5% CO_2.

2.7 Image analysis

Image analysis of cultured primary keratinocytes was done using the Q-Win software. The analysis was done to quantitate and compare the growth characteristics of primary keratinocytes on TCPS and NGMA coated plates. The growth keratinocytes isolated by 1h dispase digestion and overnight dispase digestion method were also compared. Initially, images of a single patch of keratinocyte in each culture group have been sought out. Images of the patch on day2, 4,6 and 8 were used for the analysis. Both cell count and the area of the patches have been calculated.

2.8 Statistical Analysis

All the data's are expressed as mean ± standard deviation. Comparison between groups were done using students 't test' and the significance of the

values were calculated based on the '*p value*' obtained. As per conventional criteria *P value* < 0.05 was considered as statistically significant. Statistical analysis was done using Graph pad software.

2.9 PREPERATION OF REAGENTS

2.9.1 DEFINED KERATINOCYTE SERUM FREE MEDIUM (Complete Medium)

(Gibco, Invitrogen)

Defined Keratinocyte serum free medium (Basal Medium) - 500ml

Growth supplements - 1 ml

2.9.2 DMEM (DULBECCO'S MODIFIED EAGLE'S MEDIUM) STOCK (Himedia)

9.6 g of DMEM powder was suspended in 930 ml sterile milliQ water. pH was adjusted to 4.0 and the medium was sterilized by autoclaving at 121°C at 15psi for 15 min. It was then stored at 2-8°C and in dark till use

2.9.3 DMEM (DULBECCO'S MODIFIED EAGLE'S MEDIUM) SERUM FREE (100ml)

DMEM stock solution - 92.07 ml

200mM Glutamine - 2.0 ml

7.5% Sodium bicarbonate - 4.93 ml

Antibiotics (penicillin/streptomycin) - 1 ml

Adjusted the pH to 7.4 and sterilized by filtration

2.9.4 DMEM (DULBECCO'S MODIFIED EAGLE'S MEDIUM) WITH 10% FBS (100ml)

DMEM SERUM FREE	-	90	ml
FBS (Fetal Bovine Serum)	-	10	ml

Adjusted the pH to 7.4 and sterilized by filtration

2.9.5 1 M PBS (PHOSPHATE BUFFERED SALINE) 10 X

NaCl	-	8.0	g
KCl	-	0.2	g
Na_2HPO_4	-	1.15	g
$KH_2 PO_4$	-	0.2	g

Dissolved the contents in 100 ml Milli Q water.

Adjusted the pH to 7 and sterilized.

2.9.6 0.1 M PBS (PHOSPHATE BUFFERED SALINE) 1 X

NaCl	-	8.0	g
KCl	-	0.2	g
Na_2HPO_4	-	1.15	g
$KH_2 PO_4$	-	0.2	g

Dissolved the contents in 1000 ml Milli Q water

Adjusted the pH to 7 and sterilized.

2.9.7 0.01% PENCILLIN-STREPTOMYCIN (1X)-WORKING

1ml of 100X stock pencillin.-streptomycin stock was diluted to 100ml in 0.1 M PBS (1X) Stored at freezer.

2.9.8 TRYPSIN –EDTA (0.05%-0.02%)- WORKING

1ml of 10X stock TRYPSIN-EDTA (PAA laboratories) was diluted to 10ml with 0.1M PBS (1X). Stored at freezer

2.9.9 TRYPSIN –EDTA (0.25%-0.1%)- WORKING

5ml of 10X stock TRYPSIN-EDTA was diluted to 10ml with 0.1M PBS (1X). Stored at freezer

2.9.10 PARAFORMALDEHYDE (3.7%)

3.7gm of paraformaldehyde was added in 100ml PBS and stirred until it dissolves in a magnetic stirrer. 1ml aliquot was made in eppendorff tube and stored at -20°C. It was thawed whenever required.

Never freeze and store after thawing.

2.9.11 TRITON X 100 - 0.1%

0.01 ml of triton X - 100 was added to 9 .99 ml of 0.1M PBS (1X).

2.9.12 BSA (1%) - 10 mg /ml (Freshly Prepared)

10 mg BSA was dissolved in 1 ml of 0.1M PBS (1X).

2.9.13 M NaOH

400 mg NaOH was dissolved in 100 ml distilled deionized water and sterilized by membrane filtration.

2.9.14 SODIUM BICARBONATE (7.5%)

7.5g of sodium bicarbonate was dissolved in 100 ml distilled deionized water and sterilized by membrane filtration and stored at freezer.

2.9.15 COLLAGEN – WORKING SOLUTION

Pure Col ™	- 8 ml
1X PBS	- 6 ml
10 X PBS	- 1 ml
0.1 M NaOH	- 1 ml

stored at 4 °C.

2.9.16 200mM GLUTAMINE

2.93g of glutamine was dissolved in 100 ml distilled water and sterilized by membrane filtration and stored at freezer.

2.9.17 0.25% Dispase Solution (Freshly prepared)

Dispase- II	- 25mg
0.1M PBS	- 5ml
DKSFM	- 5ml

2.9.18 MTT reagent (0.1%)

MTT	- 10mg
DMEM Stock Medium	- 10ml

2.9.19 Savalon Solution (1:15)

Savalon	- 1ml
Milliq Water (Autoclaved)	- 15ml

3. CHAPTER III RESULTS AND DISCUSSIONS

3.1 Isolation and culture of Adult Murine Keratinocytes

Keratinocytes were successfully isolated from murine skin tissue taken from mouse cadavers using the double enzyme method and were maintained in culture in defined keratinocyte serum free medium. The isolated cells attached within 5min after seeding on to the collagen coated plate surface (Figure 3A). Numerous round, unattached cells were also seen at this stage which remained in the plate through first medium change (Figure 3B). Cell patches were observed on day 1 and were expanding on subsequent days (Figure 3B and 3C).

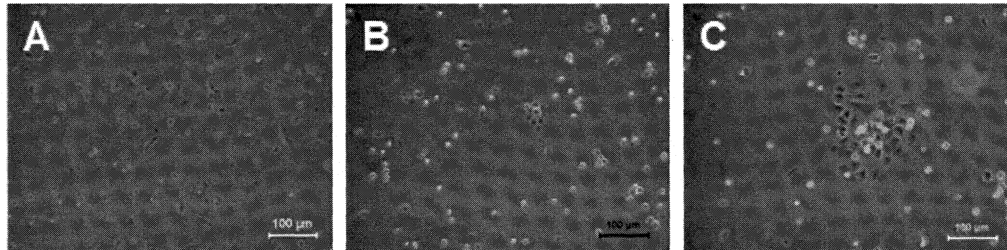


Figure 3 Primary Keratinocyte culture A, B and C shows culture of primary keratinocytes on day 0, 1 and 2.

The attached cell patches showed a steady growth till 13 days. Figure 4 A, B and C depicts the attached cell patches in culture on day 4, day 8 and day 13 respectively showing cell growth. All the unattached round cells were removed after the second medium change done on 3rd day (Figure 4A). However, few round cells remained on and around the cell patch were present on all culture days (Figure 4C). `

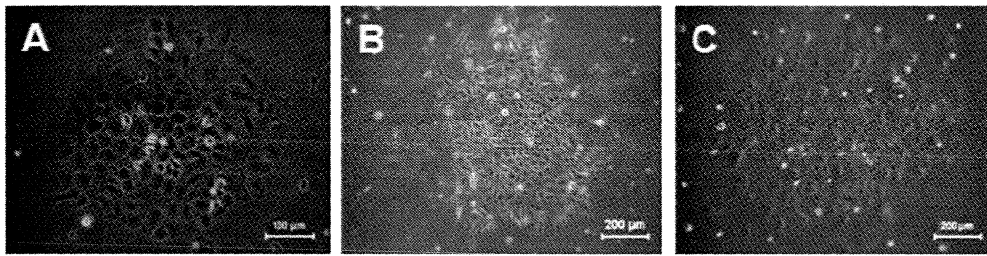


Figure 4 Primary Keratinocyte culture A, B and C shows culture of primary keratinocytes on day 4, 8 and 13.

Cells grew in the form of colonies and exhibited characteristic, cobble stone morphology. Figure 5A and 5B shows the morphology of keratinocytes on 8th day of culture in different magnifications.

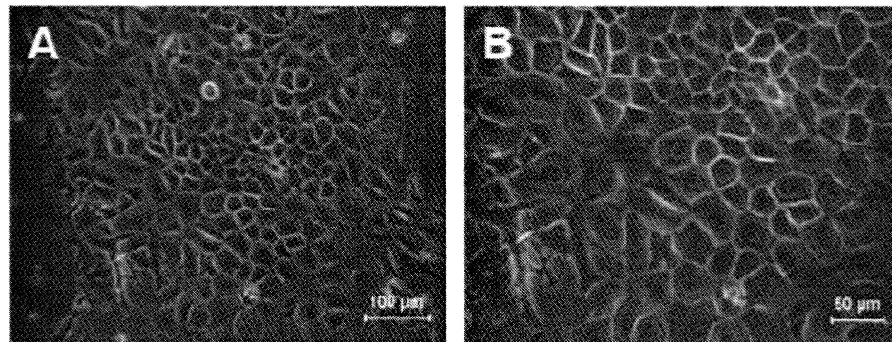


Figure 5 Primary keratinocytes on 8th day keratinocytes showed perfect showed characteristic cobble stone morphology.

3.1.1 Trypsin treatment

The 8 day keratinocyte culture was given trypsin treatment for 1min. This favoured the migration of cells from patches as shown in figure 6

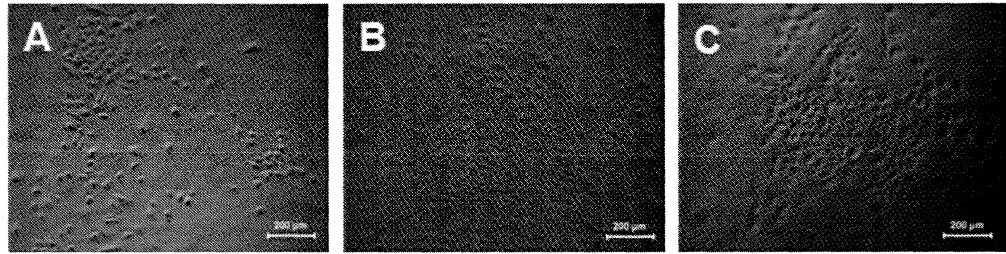


Figure 6 Keratinocyte culture after trypsin treatment A-1day, B-2day and C-4 days

3.1.2 Subculture

When the keratinocyte cells were trypsinised and taken into suspension and, the subcultred cells did not attach and grow.

It is reported that, in the absence of feeder layer, fibroblast conditioned medium and fetal calf serum, defined keratinocyte serum free medium containing supplements such as epidermal growth factor, insulin and fibroblast growth factor, could not sustain keratinocyte growth in the absence of cholera toxin in the medium [Yano et al., 2005].

In this study, primary culture of adult mouse keratinocyte has been established in defined keratinocyte serum free medium supplemented with epidermal growth factor, fibroblast growth factor, insulin and without cholera toxin. Cholera toxin is a protein which irreversibly activates adenylate cyclase by the stimulation of the nucleotide regulatory component of the adenylate cyclase system (Takeda.J et al, 1983). An earlier report shows that cholera toxin and epidermal growth factor are necessary for the sustained growth of canine keratinocytes (Wlikinson.J E et al, 1987). This can be one of the reasons behind the inability of the primary keratinocyte cells to reattach and proliferate when subcultured in medium without cholera toxin.

3.2 Optimization of isolation of adult Murine Keratinocytes

3.2.1 Age, Sex and strain of the tissue source

Tissue from two mouse strains, SWISS ALBINO and BALB/c were used for the cell isolation as per availability. Age of the cadaver from which tissue was collected for cell isolation was in the 2months, 3 months, 6months 9months and 21months. The tissues of 21months old were all from BALB/c mice. The age v/s cell yield was compared from the age group 3 (n=3),9 (n=3) and 21 (n=3) months showed almost similar cell yield (Figure 7A There was no significant difference in the cell yield when cells were isolated from tissue of different age group (p value > 0.05). Though there was no variation in cell yield of age group 3 and 9 compared to 21months, culture was established only in the group 3 and 9 months. It was also observed that, cells isolated from tissue samples of 21 months old group were more susceptible to contamination.

When the strain v/s cell yield was compared regardless of the age of the mouse (Figure 7B), no significant difference was observed (p value > 0.05) BALB/c was found to be a better source than swiss albino for the standardized protocol.

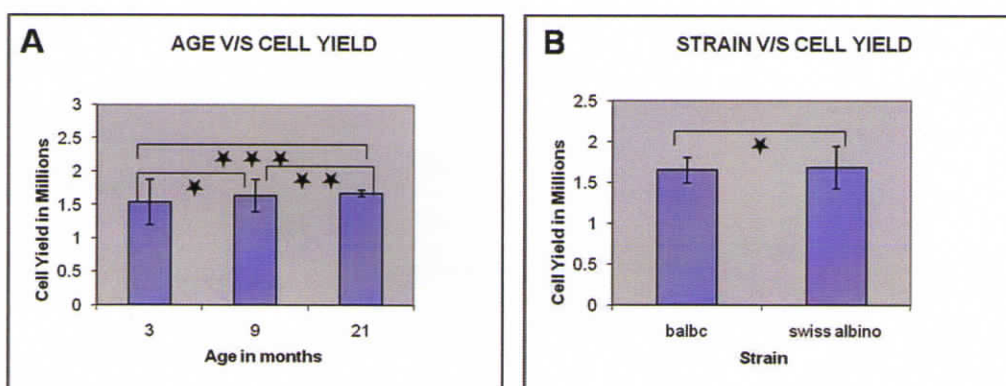


Figure 7 Comparison of cell yield with age and strain. A-Age v/s Cell yield * - p value = 0.71; ** - p value = 0.80; *** - p value = 0.54, B – Strain v/s cell yield * - p value =0.80

Hence it can be concluded that, the cell yield is independent of the age and strain of the mouse.

Hence it can be concluded that, the cell yield is independent of the age and strain of the mouse.

3.2.2 Tissue excision from Mouse cadavers

Initially the tissue was collected immediately after trimming the hair using a hair clipper. However hair clipping alone was not sufficient and resulted in interfering the cell isolation by remaining stubs of hairs in subsequent steps. Therefore hair trimming was followed by another further removal of hair using a scalpel blade. Trimming using a scalpel blade shall cause slight tissue damage, which did not significantly affect the tissue isolation.

3.2.3 Enzymatic digestion

Several methods have been used to isolate keratinocytes from skin tissue. The popular methods are by using trypsin (Hodivala -Dilke, 2002) and combination of dispase and trypsin (Shiv poojan and Shushil kumar, 2010). Both the methods have been used to isolate keratinocytes.

3.2.3.1 Single Enzyme Method

In single enzyme method the processed skin tissue was allowed to float in Trypsin solution with the epidermal side facing up. This single enzyme has been done by two different methods warm trypsinisation (2hours, 37° C) (Yano et al, 2005) and cold trypsinisation (Overnight, 4° C) (Jensen et al, 2010). In this study, both the methods were tried. However, retrieval of epidermis from the dermis was not efficient after trypsin digestion. When the epidermal side was scrapped off after trypsin digestion, it yielded some cells, but did not attach and grow. Warm trypsinisation did not yield much cells and the cells isolated did not attach and grow (Figure 8A and 8B). Cold trypsinisation also showed poor cell yield and in addition to that lot of other particles like cell debris were present along with the cells in the culture plate even after the first media change (Figure 8C and 8D).

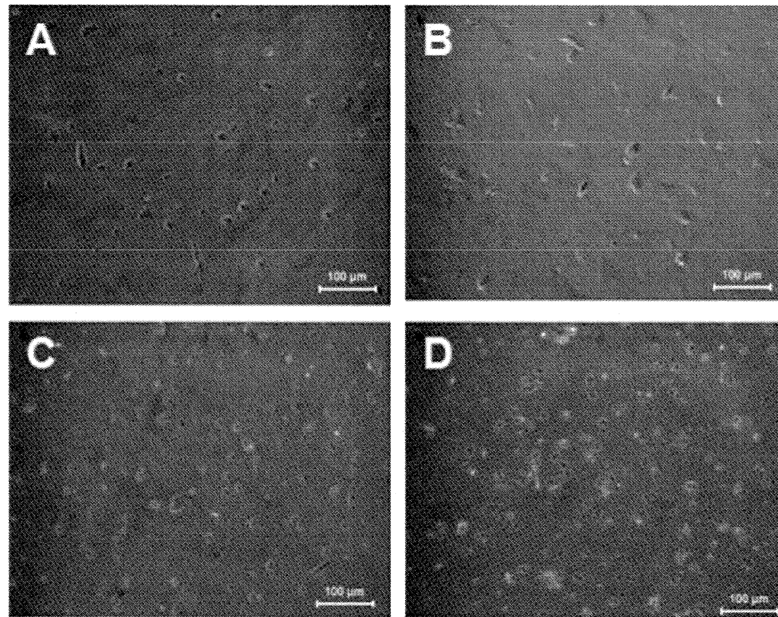


Figure 8 Keratinocyte isolation using single enzyme method A & B – Warm trypsinistion 24h and 2 Days respectively, C& D – Cold trypsinistion 24h and 2 Days respectively

3.2.3.2 Double Enzyme Method

In double enzyme method the processed skin tissue was digested in 0.25% Dispase by floating the tissue in dispase solution with epidermal side facing up and followed by scrapping off the epidermis using a cell scrapper. The scrapped of epidermis was further digested in trypsin solution. The dispase-trypsin was also carried out in two different conditions, 37° C for 1 hour [Garcia et al, 1998] and 4° C Overnight [Shiv Poojan and Shushil kumar, 2010]. In both these cases the epidermis could be effectively retrieved using a cell scrapper (Figure9).

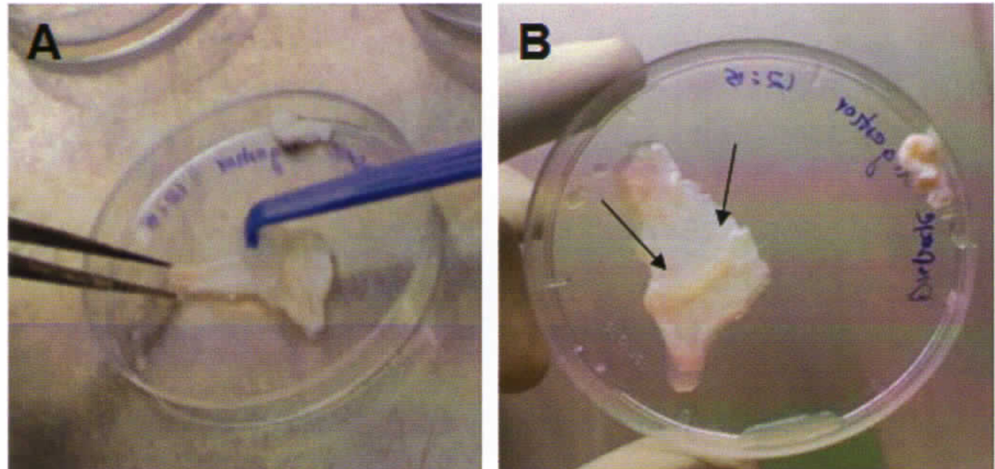


Figure 9 Skin tissue after dispase digestion A-Epidermis scrapped off after dispase digestion using a cell scrapper, B- Arrows indicating the epidermis scrapped off half way from the dermis

In both warm dispase (figure 10) and cold dispase (Figure 11) treatment, sheets of epidermis were retrieved from the skin tissue and hair follicles were seen. When the peeled off epidermis was put in trypsin, a number of cell patches and single cells which were found floating. The isolated cells were seeded on collagen coated plates and maintained. The cells grew in the form of colonies and exhibited characteristic cobblestone morphology. There was no significant difference in the morphology of cultured cells isolated by dispase 1h method (Figure 10) and the dispase overnight method (Figure 11). However both group of cells did not reach confluence and also did not survive passage 1.

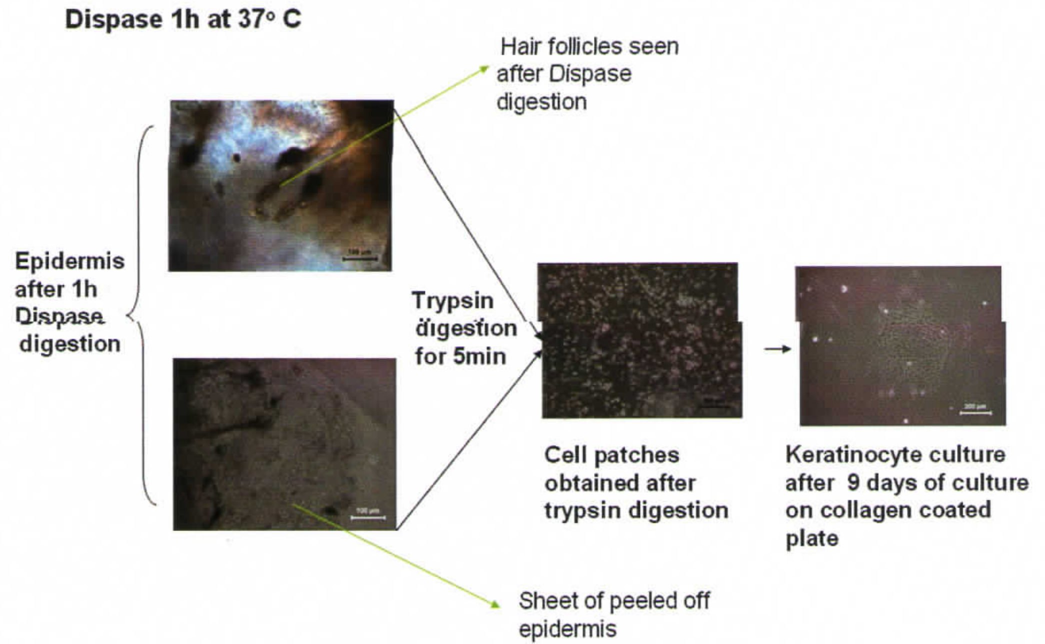


Figure 10 **Schematic showing the epidermis peeled off after dispase 1hour treatment followed by trypsin digestion and culture of the isolated cells.**

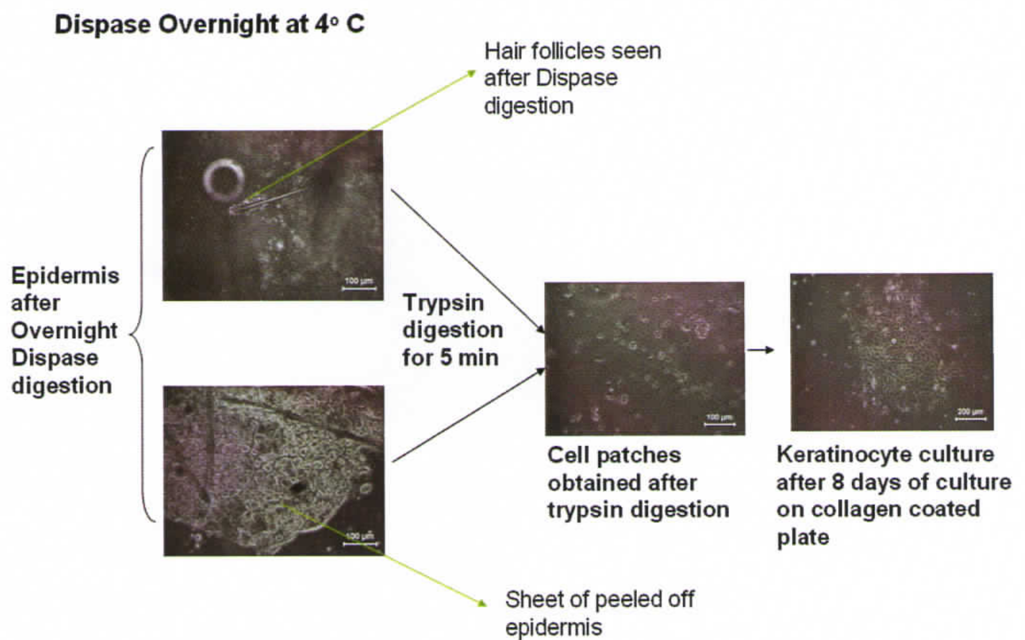


Figure 11 **Schematic showing the epidermis peeled off after dispase overnight treatment followed by trypsin digestion and culture of the isolated cells.**

The single enzyme method did not yield any cells and keratinocyte culture could not be established. The double enzyme method gave better cell yield and keratinocyte culture could be established. This result is further supported by an earlier report stating, dispase trypsin method yielded large number of viable cells and the smallest number of cells in apoptosis than other conventional methods (Gragnani et al, 2008).

3.3 Characterization of Keratinocytes

The keratinocytes were characterized by immunostaining with antibodies against CJ-1, CK-14 and involucrin.

3.3.1 Immunostaining of mouse Skin Cryosection

To confirm the specificity and dilution of antibodies against keratinocyte markers such as cytokeratin-1, cytokeratin-14 and involucrin, immunohistochemistry was performed on mouse skin cryosections using respective antibodies. It was confirmed that antibody was effectively binding and gave characteristic positive staining (Figure 12).

Cytokeratin-14 was expressed at basal layer locate apically at stratum spinosum and stratum granulosum. Cytokeratin-1 and Involucrin expression were seen specifically in the epidermis.

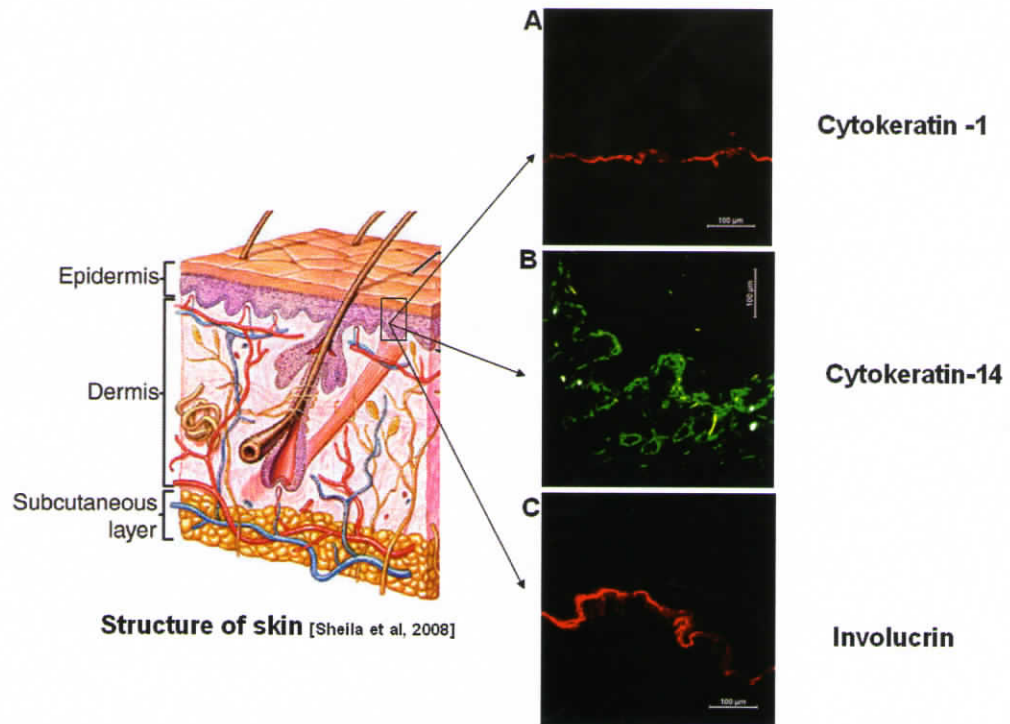


Figure 12 Immunohistochemistry of skin section showing positive stain for cytokeratin-1, cytokeratin-14 and involucrin

3.3.2 Immunostaining of cultured cells

In order to ascertain that the cultured cells were keratinocytes, immuncytochemistry of the cultured cells were carried out to screen for expression of the epidermal keratinocyte markers such as cytokeratin-1, cytokeratin-14 and Involucrin.

Cytokeratin-14 is a marker of undifferentiated epidermal cells of the basal layer and within the hair follicle (Tani et al, 1999). The cultured cells were negative for cytokeratin-14 (Figure 13).

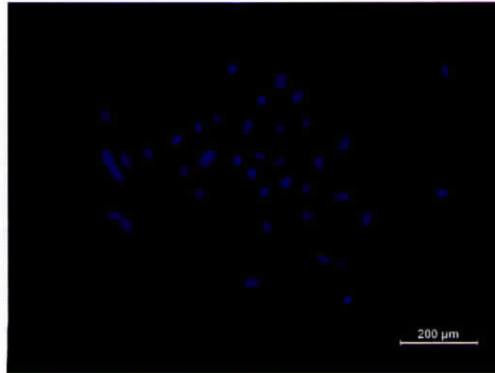


Figure 13 Immunostaining of Keratinocytes against CK-14 Fluorescent microscope images of immunostained culture keratinocytes stained for cytokeratin 14 FITC+ Hoechst showing negative expression for cytokeratin-14

Analysis of cytokeratin 1 and Involucrin on 8th day of culture showed positive expression for involucrin(Figure 12A) and negative for cytokeratin-1 (Figure 12B)

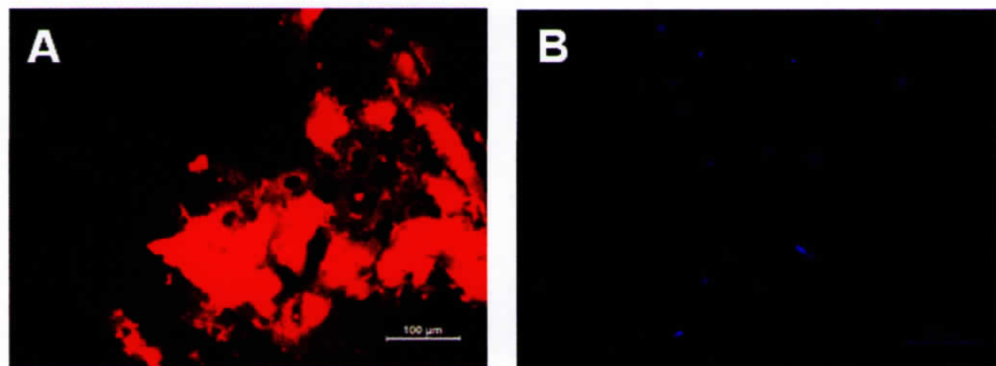


Figure 14 **Immunostaining of Keratinocytes against Involucrin and CK-1** A- Keratinocyte showing Involucrin expression (Red) B- cells were negetive for cytokeratin 1 (Red) counter stained with Hoechst (Blue)

Involucrin is a soluble protein precursor of the stratified squamous epithelium, [Rice RH et al, 1979]. It has been reported that cultured human keratinocytes synthesizes involucrin [Banks et al, 1981]. Expression of involucrin, in cultured cells confirmed that the established culture system was keratinocytes.

3.4 Preparation of modified surface

Various thermoreponsive surface have been prepared, using the thermoresponsive polymer poly(N-isopropylacrylamide) (PIPAAm) and its derivatives (Kikuchi and Okano, 2002; Gil and Hudson, 2004). These thermoresponsive polymers have been used as cell culture substrates for tissue engineering (Yamato and Okano, 2004). NGMA is one of the thermoresponsive polymers that exhibit a reversible temperature-dependent phase transition in aqueous solutions at its lower critical solution temperature (LCST) which is 31° C (N.Joseph et al, 2010). This NGMA polymer has been previously used for corneal cell sheet engineering (N Joseph et al, 2010) by coating NGMA polymer manually on the culture plate. In this study, a custom made spin coater has been used to coat NGMA polymer on 35mm culture plate. This coating method was employed in order to reduce the person to person variation in coating the polymer manually. To confirm the modification, the coated plates were further characterized before using it for cell culture.

3.5 Characterization of NGMA coated surface

3.5.1 Water Contact Angle

Water contact angle was analyzed to confirm the surface modification TCPS with NGMA (Figure 15). Uncoated plates were taken as control. The analysis was done in two different temperatures, below 25°C (Below LCST) and above 40°C (Above LCST). The values obtained are given in table 3. Data obtained is represented as mean ± standard deviation (SD).

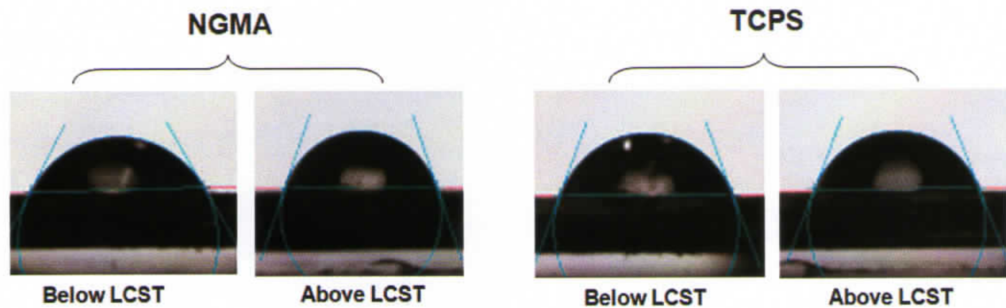


Figure 15 Water contact angle measurement on NGMA coated surface and TCPS above and below LCST

Table 2 Water contact angle of TCPS and NGMA coated plate above and below LCST

SURFACE	WATER CONTACT ANGLE	
	Below LCST (± Sd)	**Above LCST (± Sd)**
NGMA COATED	61.65° ± 1.33°	67.725° ± 2.39°
TCPS	68.2° ± 0.898°	69.5° ± 2.03°

Statistical analysis of the data showed that there was significant difference in the water contact angle of NGMA coated plates (p value = 0.0004), at two different temperatures. Whereas, TCPS did not show any significant shift in the water contact angle (p value = 0.2861) (Figure 16A).

The NGMA and TCPS were also compared for their water contact angle difference at both below and above LCST. Significant difference was observed between NGMA coated plates and TCPS below LCST (p value = 0.0002). NGMA surface performs like normal TCPS above LCST with respect to hydrophobicity. Hence, the coated and uncoated surface should not show variation. There was no significant difference in the water contact angle between the two, above LCST (p value = 0.3061) (Figure 16B).

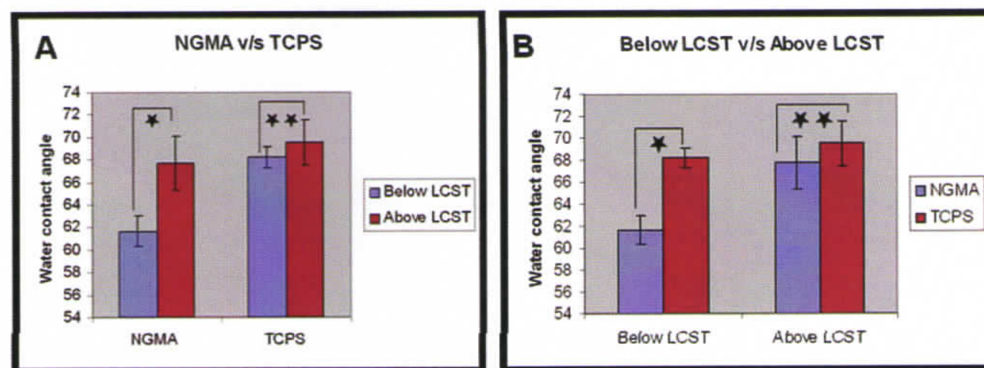


Figure 16 Water Contact Angle Measurement A- Water Contact angle measurement on NGMA plates and TCPS. * The p value was found to be 0.004. ** The P value was found to be 0.2861. B - * - p value = 0.0002; ** - p value = 0.3061

A similar kind of comparison of the water contact angle between NGMA coated plate and TCPS have been reported previously (N.Joseph et al, 2010). However, shift in the water contact angle of the two surfaces, above and below LCST has not been reported so far. The NGMA coated plate exhibits a surface property, similar to TCPS above LCST and a shift in this property, below LCST confirms the efficiency of coating. An efficient coating of NGMA on TCPS enables in using this surface for cell sheet engineering. The phase changes observed in water contact angle measurements at different temperatures shall attribute to the retrieval of cells by temperature variation.

3.5.2 Cytotoxic evaluation

The cytotoxicity of the NGMA coated surface was evaluated by culturing L929 cells on NGMA coated plates. 1 X 10^4 cells were seeded on to NGMA coated plates and TCPS was used as the. Figure 17 A, B and C shows L929 on TCPS on day 1, 2 and 3 and Figure 17 D, E and F shows L929 on NGMA coated plate on day 1, 2 and 3. No difference in the morphology of cells was observed on NGMA and TCPS. The L-929 cells retained their characteristic spindle morphology showing the non-cytotoxic nature of the NGMA coated plates.

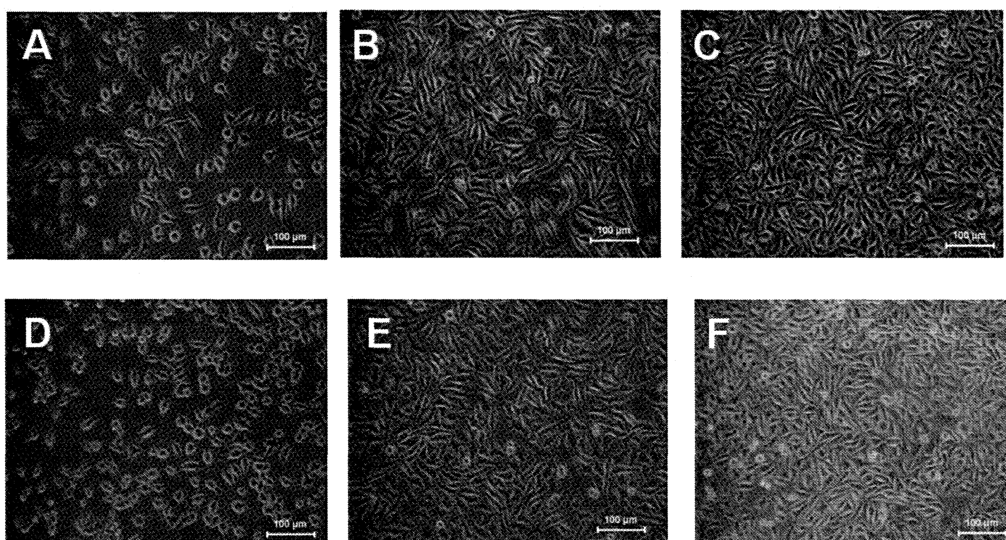


Figure 17 Cytotoxic evaluation of NGMA coated plates, L929 cells seeded on TCPS A- 1 day, B – 2 days, C – 3 Days. L929 cells seeded on NGMA coated plates D – 1 day, E – 2 Days, F – 3 Days

3.5.3 Cell sheet retrieval from NGMA coated culture plate

Cell sheet retrieval was carried out using a PVDF membrane as support in order to study the thermoresponsive efficacy of the NGMA coated culture plate. The monolayer of L929 cells on the NGMA plate was incubated below 10° C with a PVDF membrane placed over it (Figure 18 A). Cells adhered on NGMA surface showed change in morphology on temperature variation. Cells around the PVDF membrane expressed round morphology after low temperature treatment. At this stage, when the membrane was removed, the cells on NGMA which was in direct contact with the PVDF membrane showed was removed (Figure 18B) as cell sheet. This PVDF membrane with the cells were transferred to another fresh TCPS and incubated for 1h at 37° C. The cell patches on the membrane adhered to the fresh culture surface (Figure 19A). These cells when cultured in optimal conditions grew to form a monolayer in 5 days (Figure 19 B).

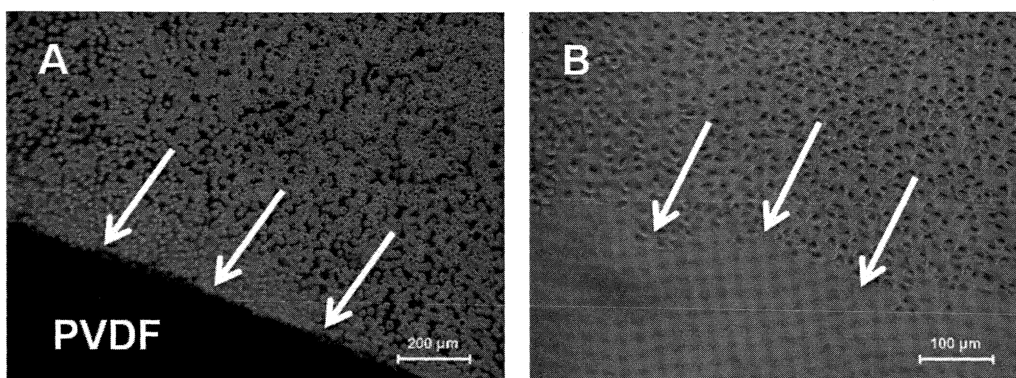


Figure 18 Cell sheet retrieval using PVDF membrane. A – PVDF membrane on the monolayer. B – NGMA coated plate after PVDF mediated cell transfer

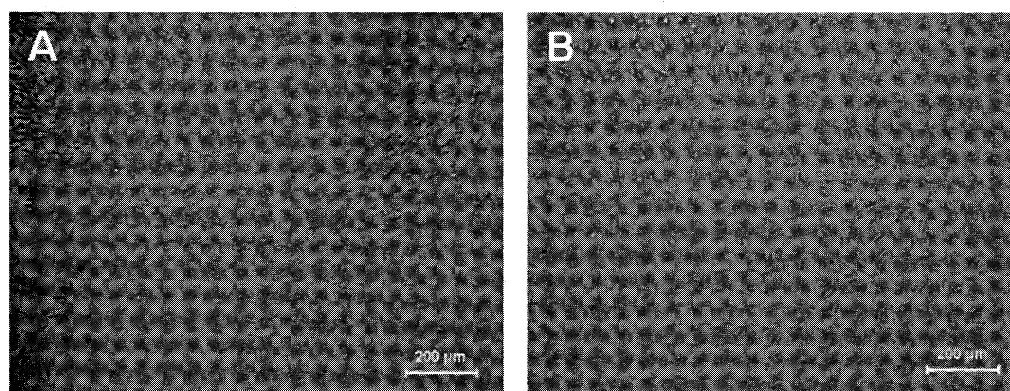


Figure 19 Transferred cell patches in culture A – 0 h and B - 5 days

3.6 Culture of Keratinocytes on NGMA surface

3.6.1 Culture HaCat keratinocyte cell line on NGMA surface

HaCat human keratinocyte cell line was cultured on NGMA coated plate to study the support the growth and proliferation of keratinocytes. TCPS was used as control for the experiment. The cells exhibited perfect cobble stone morphology after 24 hr of culture (Figure 20A) and the morphology was maintained till the end of 5 days of culture (Figure 20B). There was no significant difference in the morphology of the cells cultured on TCPS (Figure 20C and 20D) and NGMA coated plate.

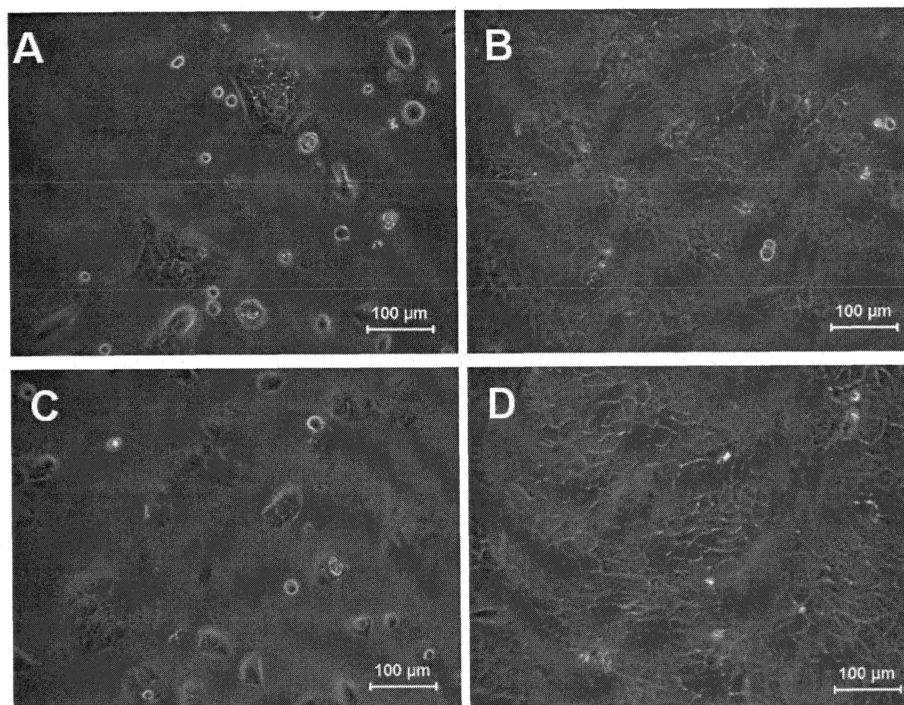


Figure 20 Culture of HaCat cell line on NGMA coated plate and TCPS
A and B showing HaCat cell line cultured on NGMA coated plate on day 1 and day 5 respectively. C and D shows HaCat cell line cultured on TCPS on day 1 and day 5 respectively

3.6.2 Cell proliferation of keratinocytes cultured on NGMA surface

In order to study the cell proliferation of keratinocyte on NGMA coated plate, HaCat cells were cultured and analyzed by MTT assay. TCPS was used as the control. MTT assay performed at different time intervals, showed metabolic activity of the cells and thereby indirectly the proliferation.

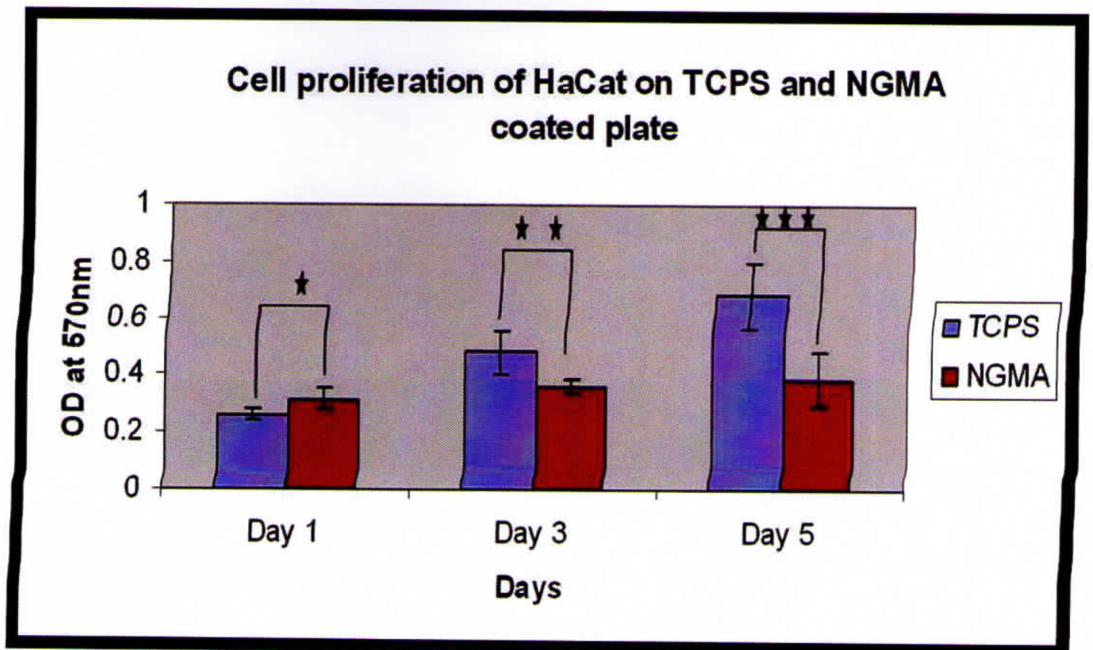


Figure 21 MTT profile of HaCat cell line seeded on TCPS and NGMA coated plates MTT readings on day 1, day 3 and day 5, n = 6. (*p value = 0.0070, ; ** p value = 0.0058, ; *** p value = 0.0006,.

There was a significant increase in the cell number on day 1 in NGMA plates when compared to the TCPS (p value = 0.007). Where as TCPS showed better cell proliferation on day 3 (p value = 0.0058) and day 5 (p value = 0.0006) when compared to the NGMA coated plates (Figure 21). N Joseph *et al.*, (2010) has reported a decrease in cell proliferation in NGMA coated plates after 72h of culture of L929 cells when compared with TCPS. It was stated that, the reduced roughness of the culture surface due to NGMA coating results in lower cellular proliferation.

When the proliferation on the NGMA coated plated were compared (Figure 22 B), there was a statistically significant difference in growth between day1 and day 3 (p value = 0.0261). Whereas, there was no significant increase in the cellular growth on day 5 when compared to day 3 (p value = 0.56). In contrast to this, the TCPS (Figure 22 A) showed a significant increase in the cell number after day1, day3 and day 5.

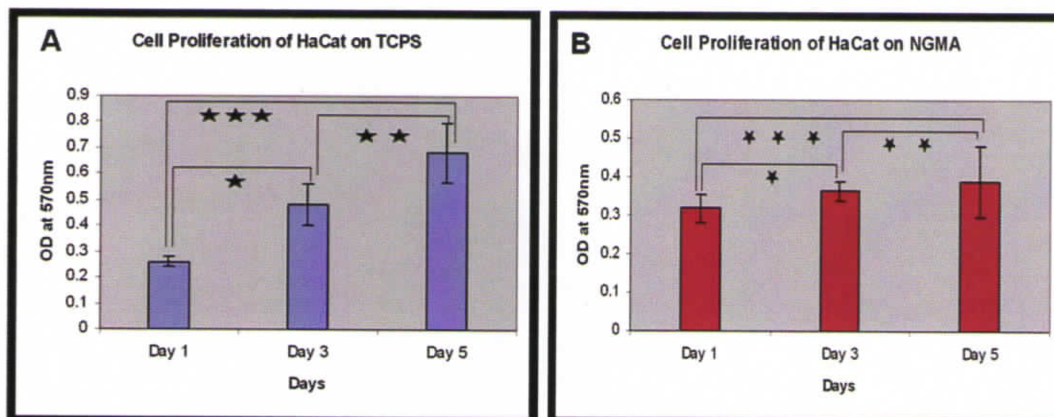


Figure 22 MTT profile of HaCat cell line grown on A- NGMA and B-TCPS

A - MTT profile of HaCat cell line grown on TCPS Day1, Day 3 and Day 5 (n=6) (* pvalue = 0.0001; ** p value =0.0054 ; *** p value = 0.0001)

B - MTT profile of HaCat cell line grown on NGMA coated plate on Day1, Day 3 and Day 5 (*p value – 0.02;**p value – 0.56; *** p value – 0.11) (n=6),

The results show that, HaCat cells when cultured on NGMA coated plated can retain their characteristic morphology, but the proliferation of the cells is lower compared to the TCPS.

3.6.3 Culture of primary keratinocyte on NGMA coated surface

Isolated Keratinocytes were seeded on NGMA coated plates which were pre coated with collagen. Cells attached to the surfaces. Sustained growth of patches till the 8th day was observed (Figure 23). The cultured cells attained their characteristic cobble stone morphology. However the cells did not become confluent and requires further optimization. The data suggests that primary keratinocytes culture could be established on NGMA coated plates. Optimizing the culture conditions to increase the growth rate is required. Cells adhered on the NGMA plate formed colony and cells proliferated over a weeks time to form large patches. This shows that NGMA support adhesion and proliferation of primary keratinocytes. Collagen coating on NGMA spin coated surface might have a critical role in cell adhesion and proliferation This

could explain why HaCat cells expressed a static growth compared to primary cells on collagen coated NGMA.

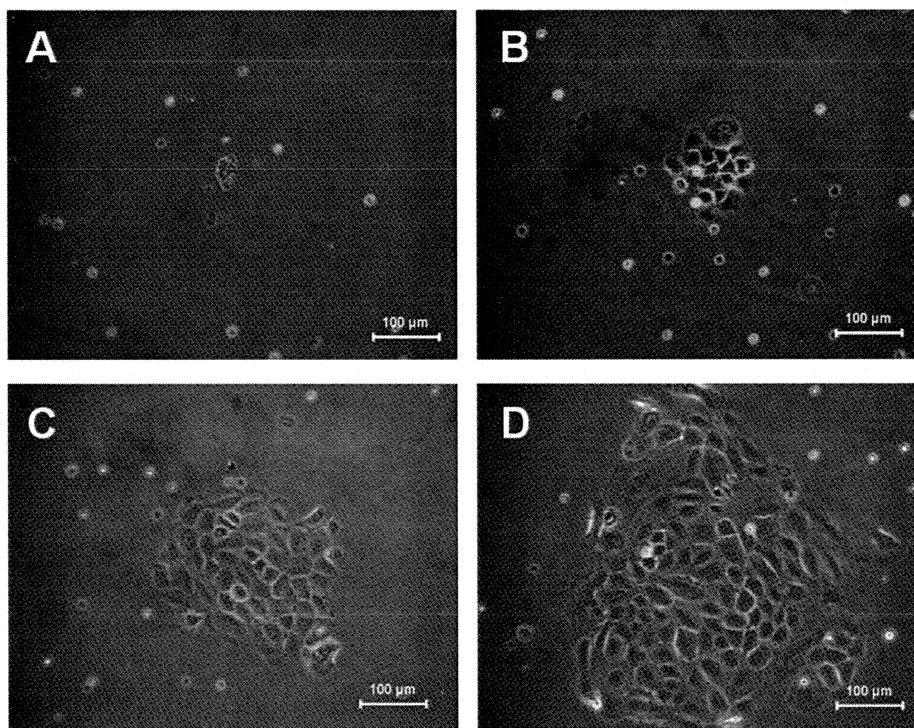


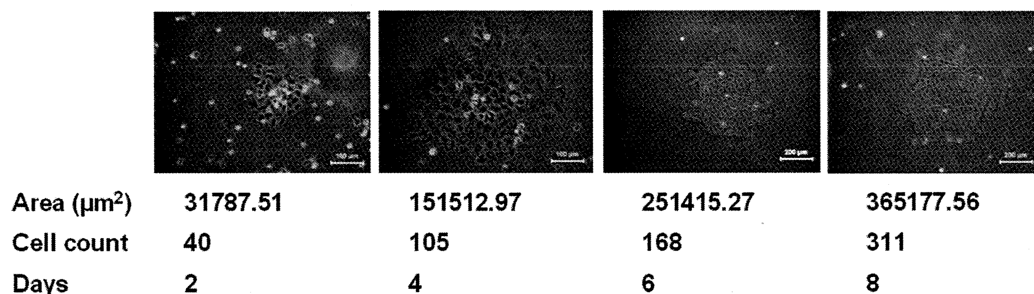
Figure 23 Primary murine keratinocytes cultured on NGMA coated plate
Primary murine keratinocytes cultured on NGMA coated plate showing characteristic cobblestone morphology. A-D denotes cultures on day 2, 4, 6 and 8 respectively.

3.7 Image analysis

Image analysis of keratinocyte cultures was performed using the Leica QWIN software. The growth characteristics were quantitated and the pattern of the keratinocyte cells culture was studied. The increase in cell count and area of patch of cells were calculated.

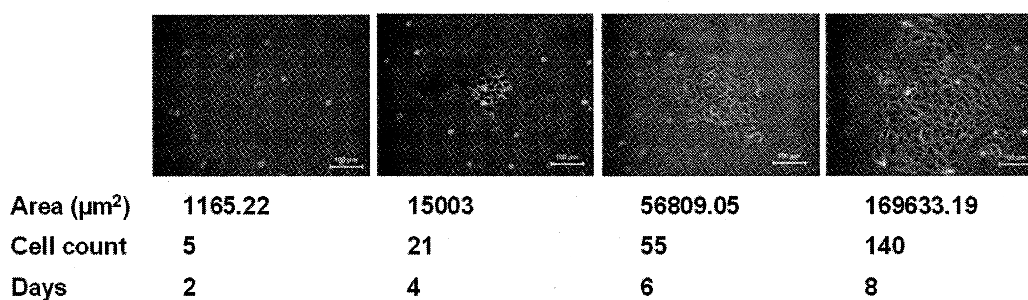
2 sets of images of keratinocytes cells isolated by Dispase 1h method were analyzed for their growth pattern. One set contained images of keratinocyte grown on TCPS (Figure 24) and the other set contained keratinocyte grown on NGMA coated plate (Figure 25). The increase in area and the increase in cell count of the cell patch over number of days were

calculated. Both the surface showed better growth of cells on all culture days. The cells used for both these groups were obtained from dispase 1h method.



Area (μm^2)	31787.51	151512.97	251415.27	365177.56
Cell count	40	105	168	311
Days	2	4	6	8

Figure 24 Shows keratinocyte cells isolated by Dispase 1h digestion method grown on TCPS on day 2, 4, 6 and 8

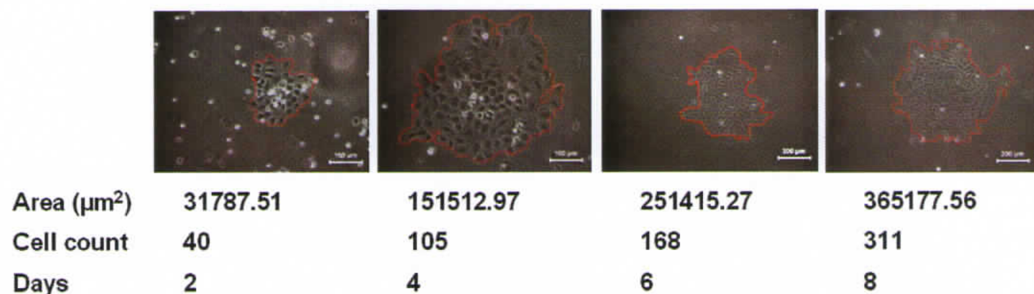


Area (μm^2)	1165.22	15003	56809.05	169633.19
Cell count	5	21	55	140
Days	2	4	6	8

Figure 25 Shows keratinocyte cells cultured on NGMA coated plates on day 2, 4, 6 and 8

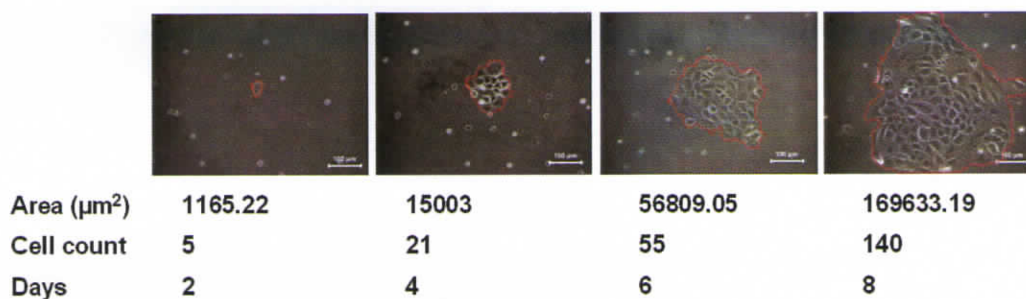
Similarily, the images of cultured keratinocyte cells isolated by overnight dispase method were also analyzed (Figure 26). The increase in the patch area and increase in the cell count over number of days were calculated.

calculated. Both the surface showed better growth of cells on all culture days. The cells used for both these groups were obtained from dispase 1h method.



Area (µm²)	31787.51	151512.97	251415.27	365177.56
Cell count	40	105	168	311
Days	2	4	6	8

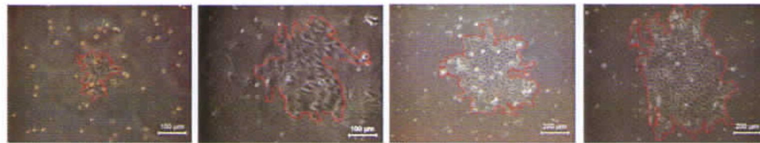
Figure 24 Shows keratinocyte cells isolated by Dispase 1h digestion method grown on TCPS on day 2, 4, 6 and 8



Area (µm²)	1165.22	15003	56809.05	169633.19
Cell count	5	21	55	140
Days	2	4	6	8

Figure 25 Shows keratinocyte cells cultured on NGMA coated plates on day 2, 4, 6 and 8

Similarily, the images of cultured keratinocyte cells isolated by overnight dispase method were also analyzed (Figure 26). The increase in the patch area and increase in the cell count over number of days were calculated.



Area (µm²)	14628.35	98366.39	290055	468622.72
Cell count	28	74	168	358
Days	2	4	6	8

Figure 26 Shows keratinocyte cells iaolated by Dispase Overnight digestion method on day 2, 4, 6 and 8

The growth pattern of keratinocytes isolated by warm and cold dispase method were compared. (Figure 27).

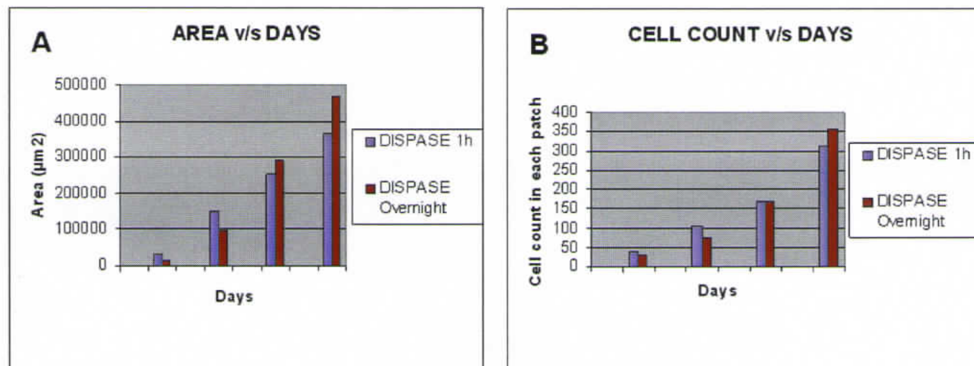


Figure 27 Graph quantitatively comparing the growth pattern of primary keratinocytes isolated by dispase 11h method and dispase overnight method A –Increase in area of the patch with the number of days in culture, B – Increase in the cell count in the patch with the number of days in culture

On comparing the two dispase treatment methods it is observed that the growth pattern of the two groups is almost similar till 8 days of culture, though the dispase overnight treatment showed a slight increase in area and cell count.

On the whole, Keratinocytes were isolated using the standardized protocol and culture could be established. The culture of primary keratinocytes on NGMA coated plates proved that keratinocyte culture could be established on NGMA coated plates. However further optimization has to

be done to maintain the cells in culture for several passages and to increase the growth of the cells on NGMA coated plate.

4. CHAPTER IV SUMMARY AND CONCLUSION

Skin is the largest organ of the body that acts as a barrier against the body and the external environment. Epidermis is the outermost part of the skin, which is composed of stratified squamous epithelium. The maintenance and self renewal of skin is achieved by resident stem cells present in skin. Conditions, such as in burns or chronic ulcers where self renewal of skin does not happen, can result in scar formation and slow wound healing. Currently, the gold standard treatment for any large skin loss is autologous skin transplant. The disadvantage of this treatment is that this can result in another wound. In case of extensive burn injuries, adequate tissue may not be available for grafting. Hence, there is an increased need for *invitro* engineered skin tissue constructs that can compensate for the cell loss at the wounded site. Many commercial skin tissue engineered products are already available in market and has been showing clinical success.

The idea of skin tissue engineering is to isolate keratinocytes from a compatible tissue source and expand these cells *invitro.* With most of the murine keratinocyte isolation protocols optimizes for neonatal skin, isolation and culture of keratinocytes from adult murine skin is still a challenge. The difficulty in getting good quality cells that can proliferate in culture and maintaining them in culture makes the necessity for an optimized protocol for isolation and culture of keratinocytes. Efficient retrieval of the cells from the culture is very important to improve the quality of the cells to be grafted. Hence a combination of optimized keratinocyte isolation and culture method and a modified culture surface that can efficiently retrieve cells from the culture surface, can be a big breakthrough in skin tissue engineering. The

present study aims to optimize keratinocyte isolation from adult murine tissue and culture it on modified with NGMA. The study was conducted in 4 phases.

The first phase was the optimization of isolation and culture for keratinocyte culture. Skin tissue from mouse cadavers of age group 2 months 3months, 9 months and 21 months were used for the isolation as per availability. Collected tissue samples included strains such as Balb/c and Swiss albino. Statistical analysis revealed that the cell yield was independent of the age or strain of the mouse. As part of the optimization of the isolation of keratinocytes, single and double enzyme digestion methods were adopted. The enzyme treatment involving two enzymes, dispase and trypsin was found to be effective in isolating keratinocytes and establishing a culture. This double enzyme method was again done in two different types, one with dispase 1h digestion at 37°C and another one dispase overnight. Culture was established using both the methods and there was no significant difference observed in the growth pattern of the cells.. The cultured Cells had grown in the form of colonies and exhibited characteristic, cobble stone morphology.

The second phase of the study was the characterization of the isolated cells. Initially to confirm the specificity and dilution of antibodies against keratinocyte markers such as cytokeratin-1, cytokeratin-14 and involucrin, immunohistochemistry was performed on mouse skin cryosections using respective antibodies. It was confirmed that antibody was effectively binding and gave positive staining. The cultured cells were then stained against 3 markers. The cells showed a negative expression for cytokeratin 14 and cytokeratin 1, but showed a positive expression for involucrin. The expression of Involucrin confirmed the established culture was of keratinocytes.

The third phase of the study involved the generation of the modified surface using an in-house synthesized polymer and its characterization. The culture surface was modified by coating NGMA, a thermoresponsive polymer using a custom made spin coat adaptor. The coated plates were characterized using water contact angle measurement. Water contact angle was measured at both temperatures, above and below LCST and it was

observed that there was a significant difference in the water contact angle of NGMA coated plate at different temperature. The plates were tested for cytotoxicity by culturing L929 cells and were found to be non-cytotoxic.The thermoresponsive efficacy of the cells was also studied by retrieving cell sheet of l929 cells from the NGMA coated plates. The study featured, perfect cell sheet retrieval from NGMA coated plates.

The fourth phase of the study involved the culture of keratinocytes on the NGMA coated plate. HaCat keratinocyte cell line was cultured on NGMA coated plate and MTT assay was done to study the growth and proliferation of keratinocyte cells on NGMA coated plates. The study showed that there was a significant decrease in the proliferation of keratinocyte when compared with the cells cultured on TCPS. However, the cells attained perfect morphology which showed the non cytotoxic nature of the modified surface. Primary keratinocytes were also cultured on NGMA coated plates. These cells attained perfect cobble stone morphology. Image analysis was done in order to compare the growth pattern of cell patches of primary keratinocytes grown on TCPS and NGMA coated plates. The growth of keratinocytes on NGMA coated plate was much lower, when compared to TCPS. Hence further optimization has to be done for the culture of primary keratinocytes on NGMA.

Conclusion

The result of the present study shows an optimized protocol for isolation of, keratinocytes from adult murine skin. Established culture showedpositive staining for involucrin which is the terminally differentiated stage of the cells. A modified culture surface was also synthesized by coating thermoresponsive NGMA polymer and characterized for its stimuli responsiveness. The modified surface was analyzed for keratinocyte growth using HaCaT cell line showed its suitablitiy for skin tissue engineering but with a lower proliferation compared to TCPS. Culture of primary keratinocytes on collagen precoated NGMA coated plate confirmed the adhesion and growth of keratinocyte on NGMA surface. Difference in culture of cell line and primary cells could be due to the role of collagen in keratinocyte proliferation.

Future Prospects

Further optimization has to be done in the cell isolation protocol to maintain the cells in culture for several passages. The growth of the cells on NGMA coated plate should also be analyzed further. This technology can be used for the efficient transfer of keratinocyte cell sheets expanded in *in vitro* conditions to the wounded sites without enzymatic intervention. These findings may support the development of novel biomaterial free tissue engineered skin constructs in the future.

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