FIELD OF THE INVENTION:

This invention relates to a method for processing of feeder cells suitable for adult stem cell proliferation.

This invention further relates to a cost-effective yet efficient processing of feeder cells in a way that their life span and growth supporting capability are modulated through induction of controlled growth arrest in them following treatment with mitomycin C at permutations of (1) concentration per se in the treating solution and (2) logical dose amount available to the exposed cell population. This invention also relates to using an optimized substratum consisting of feeder cells processed as above for culturing adult and embryonic stem cells for probable exploitation towards production of cost-effective organs for transplantation to treat certain ailments.

BACKGROUND OF THE INVENTION:

Culture of several adult and embryonic stem cells depends on a feeder cell layer consisting usually of growth arrested fibroblast cells like the mouse embryonic skin fibroblast cells. For example, autologous keratinocytes obtained from small uninvolved skin biopsy specimen from burned patients are cultured in vitro in presence of such feeder cells that are growth arrested by exposure to gamma-irradiation and the ensuing epidermal sheets constructed in vitro are used for autologous grafting in severely burned patients (Rheinwald and Green, 1975, Atiyeh Costagliola 2007). The growth arrest is reported to push the feeder cells permanently into a non-proliferative state and keep them metabolically active for a finite time period to impart growth stimulatory influence on target cells (Roy et al 2001; Nieto et al 2007).

Alternately, exposure to other cell proliferation-blocking agents like Mitomycin C is used to growth arrest feeders as a cost effective strategy (Navasaria et al 1994). In the prior art, use of several concentrations of Mitomycin C have been reported pointing out to inconsistent efficacy of this method while comparing with the gamma-irradiation which is considered as a standard method (Schrader, 1999; Ponchio et al 2000; Roy, 2001; Ramirez et al., 2001; Nieto et al 2007). Nevertheless, the concentration of Mitomycin C for effective growth arrest is critical; too low a concentration would result in reversal of growth arrest with resumption of proliferation, which in turn would contaminate the target cells. On the other hand, too high a concentration would result in early disintegration of feeder cells rendering them less efficient as a feeder layer in addition to deposition of its residues in target cells. The studies reported so far in prior art described feeder cell growth arrest by merely considering a range of concentrations, while grossly ignoring the precise density of exposure cell population and concluded with conflicting extents of target cell stimulation. As a consequence of such inconsistencies a uniformly effective growth arrested feeder layer has not yet been identified for a feeder based co-culture system leading to differences among the various feeder layer lines (Amit et al 2004). Moreover, occasional failure of growth arrest leading to recurrence of mitotic activity after mitomycin C treatment even at high concentrations is also reported and has no other solution than to discard such cultures (Connor 2000).

The deficiencies of both growth arresting methods compel the development of newer approaches to prepare effective feeder cells for the culture of stem cells (Browning et al 2010). Hence, there is an unmet

need to develop a culture system in which the mitomycin C induced growth arrest is not only permanent but also exerts optimal and reproducible growth supporting activity. It is now proposed that the inadequacies in mitomycin C induced growth arrest could perhaps be overcome at first, by an experimental strategy of varying cell number of feeder cells before exposure, since the action potential of a cell proliferation blocking agent has been previously shown to arithmetically depend on exposure cell density (Yerneni and Jayaraman 2003); and secondly, by deriving a range of arithmetically derived values consisting of permutations of mitomycin C concentrations and the actual dose amounts present in the final treating volume, which altogether is logically available to the respective exposed cell population. Eventually, a feeder cell growth arrest protocol would then consist of exposure to such derived volume-concentration permutations and generate a series of feeder cell batches from which an optimally performing batch will be identified in terms of maximal stimulation of adult stem cell proliferation. This strategy is proposed to cut down the cost of culture as compared to gamma irradiation technique without compromising with culture time and target cell characteristics.

OBJECTS OF THE INVENTION:

It is therefore an object of this invention to propose a method for processing of feeder cells for adult stem cell proliferation; which is simple and cost-effective.

Another object of this invention is to propose a method for processing of feeder cells for adult stem cell proliferation, which does not employ harsh chemicals, reagents or agents.

Another object of this invention is to propose a method for processing of feeder cells for adult stem cell proliferation, which stimulates maximal growth of epidermal keratinocyte stem cells in a co-culture system and is comparable with gamma-irradiated *(γ-Irr)* feeder cells.

These and other objects and advantages of the invention will be apparent to a reader from the ensuing description, when read in conjunction with the accompanied drawings.

DESCRIPTION OF THE ACCOMPANYING TABLES:

Table 1: Outcome of exposure of Swiss 3T3 cells to a 2-hour pulse of various mitomycin C doses showing their relationship with the respective concentration, exposure cell density and correlation coefficient of cell number with time.

Sl. No.	Concen-tration (µg/ml)	Exposure Density*	Dose** (pg/cell)	Linearity of cell number with time	Significance (n=5)	Growth curve Status
1	3	High	19.5	0.932	P<0.01	Stimulatory
2	4	High	26.0	-0.355	P>0.05	Stationary
3	5	High	32.5	-0.805	P>0.05	
4	3	Medium	39.1	0.364	P>0.05	
5	4	Medium	52.1	-0.978	P<0.01	Inhibitory
6	10	High	65.0	-0.882	P<0.05	
7	5	Medium	65.1	-0.891	P<0.05	
8	10	Medium	130.2	-0.920	P<0.01	
9	3	Low	164.9	-0.900	P<0.05	
10	4	Low	219.9	-0.915	P<0.05	
11	5	Low	274.9	-0.921	P<0.01	Toxic
12	10	Low	549.7	-0.900	P<0.05	

* Exposure cell densities employed are Low (0.7276 ± 0.0844 x 10^4 per cm^2), Medium (3.0711 ± 0.3021 x 10^4 per cm^2) and High (6.1556 ± 0.2309 x 10^4 per cm^2).

** Doses were Arithmetically derived by dividing the actual amount of mitomycin C present in 10 ml of medium by the cell number present at the time of exposure using the formula given in Figure 1 and arranged in increasing order from lower to higher.

Table 2: Quantitative details of mitomycin C employed for treating Swiss 3T3 feeder cells with a two-hour pulse.

Concentration µg/ml	Dose µg/cell	T75 flask*		T25 flask*	
		#Total amount (µg)	##Derived Volumes	#Total amount (µg)	##Derived Volumes
3	10	17.603	5.868	4.900	1.633
4	15	26.404	6.601	7.350	1.838
4	75	132.019	33.005	36.750	9.188
4	150	264.038	66.009	73.500	18.375
4	450	792.113	198.028	220.500	55.125
5	15	26.404	5.281	7.350	1.470
5	75	132.019	26.404	36.750	7.350
5	150	264.038	52.808	73.500	14.700
5	450	792.113	158.423	220.500	44.100
10	30	52.808	5.281	14.700	1.470

*The average Exposure cell number in T75 and T25 flasks were 1,760,247 ± 42,266 (n=22) and 490,000± 7,775 (n=6), respectively.
Total amount = Exposure cell Number (millions) x Dose per cell (pg/cell)
Volume = (Exposure cell Number x Dose per cell)/concentration

Table 3: Growth area measurement by quantitative image analysis of keratinocyte colonies grown by co-culturing 170 keratinocytes with various Swiss 3T3 feeders and colonies stained red with Rhodamine B

Feeder Group*	Total no.. of pixels of the well (A)	Keratinocyte colony pixel (B)	Per cent of Growth area (C) = (B/A) x 100	Growth area# (cm^2) (9.6/100)x C	Average area ± SD (cm^2)
4-15 (1)	1,120,088	80,886	7.22	0.693	0.613 ± 0.05
4-15 (2)	1,112,904	68,147	6.12	0.588	
4-15 (3)	1,118,724	68,426	6.12	0.587	
4-15 (4)	1,101,019	66,959	6.08	0.584	
4-150 (1)	1,041,929	135,424	13.00	1.248	1.109 ± 0.1
4-150 (2)	1,115,139	119,223	10.69	1.026	
4-150 (3)	1,130,401	129,441	11.45	1.099	
4-150 (4)	1,115,421	123,674	11.09	1.064	
γ- IRR (1)	1,118,215	58,986	5.28	0.506	0.542 ± 0.1
γ- IRR (2)	1,122,296	78,298	6.98	0.670	
γ- IRR (3)	1,117,999	65,409	5.85	0.562	
γ- IRR (4)	1,107,929	49,492	4.47	0.429	

Actual total area of the well equals to 9.6 cm^2

* Quadruplicate cultures numbered 1, 2, 3 & 4 were initiated with 1st passage keratinocytes and 144,000 feeder cells per well in 6-well plates. 4-15 and 4-150 represent feeders exposed to permutation of mitomycin C concentration of 4 µg per ml with doses of either 15 or 150 µg per million cells, while *γ- IRR* indicates *γ*-irradiated feeders.

Table 4: Growth area measurement by quantitative image analysis of keratinocyte colonies grown by co-culturing 340 keratinocytes with various Swiss 3T3 feeders and colonies stained red with Rhodamine B

Feeder Group*	Total no. of pixels of the well (A)	keratinocyte colony pixel (B)	Per cent of Growth area (C) = (B/A) x 100	Growth area# (cm^2) (9.6/100)x C	Average area (cm^2) (1+2+3+4)/4
4-15 (1)	2,067,053	286,545	13.86	1.33	1.28
4-15 (2)	2,017,667	299,975	14.87	1.43	
4-15 (3)	2,066,498	298,565	14.35	1.38	
4-15 (4)	206,6,312	209,980	10.16	0.98	
4-150 (1)	2,074,353	572,274	27.59	2.65	2.69
4-150 (2)	2,317,927	654,053	28.22	2.71	
4-150 (3)	2,089,369	646,129	30.92	2.97	
4-150 (4)	2,116,448	539,740	25.50	2.45	
γ- IRR (1)	2,088,638	523,367	25.06	2.41	1.96
γ- IRR (2)	2,066,913	370,157	17.91	1.72	
γ- IRR (3)	2,069,446	340,166	16.44	1.58	
γ- IRR (4)	2,088,217	462,831	22.16	2.13	

Actual total area of the well equals to 9.6 cm^2

* Quadruplicate cultures numbered 1, 2, 3 & 4 were initiated with 1st passage keratinocytes and 144,000 feeder cells per well in 6-well plates. 4-15 and 4-150 represent feeders exposed to permutation of mitomycin C concentration of 4 µg per ml with doses of either 15 or 150 µg per million cells, while *γ- IRR* indicates *γ*-irradiated feeders.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS:

Figure 1 depicts schematic representation of experimental derivation of doses from a given concentration of Mitomycin C by cell density titrations in Swiss 3T3 cells.

Figure 2 shows schematic representation of producing differential growth arrest by titrations of Swiss 3T3 cells with permutations of concentrations and those doses that were experimentally derived by a process shown in Figure 1.

Figure 3 shows schematic representation of demonstrating the growth stimulating potential of feeder substratums prepared by a process as in Figure 2, on epidermal keratinocyte cells.

Figure 4 shows influence of various concentrations of mitomycin C on cell extinction/proliferation of Swiss 3T3 fibroblast cells at various exposure cell densities. The cells at Low ($0.7276 \pm 0.0844 \times 10^4$ per cm^2), Medium ($3.0711 \pm 0.3021 \times 10^4$ per cm^2) and High ($6.1556 \pm 0.2309 \times 10^4$ per cm^2) cell densities were exposed to mitomycin C at concentrations of 1 μg/ml (A), 3 μg/ml (B), 4 μg/ml (C), 4 μg/ml (D) & 10 μg/ml (E) and periodical viable cell counts were performed on post exposure days of 3,6,9,12 and 20. Cell numbers are presented as the mean with standard deviation from values of triplicate cultures. Viable cell counts from the three ECNs at each time point were analysed by one way ANOVA and the significance ($P<0.02$ in A, $P<0.03$ in B & E and $P<0.001$ in C & D) is indicated by asterisk and insignificance ($P>0.05$) by NS.

Figure 5 is a scatter plot showing the four categories of trends of cell extinction/proliferation of Swiss 3T3 fibroblast cells over a 20 days period following a two-hour pulsed exposure to various mitomycin C doses/cell. Stimulatory growth trend was characterized by the 20^{th} day cell number significantly higher than seeded cells ($P<0.01$; $R^2=0.87$; n= 5); Stationary trend was distinguished by the inconsistent fall in cell number showing insignificant linear correlation with culture time ($R^2=0.03$, n=15); Inhibitory trend was evident by consistent fall in cell

number showing significant negative linear correlation with culture time ($P<0.01$; $R^2=0.4$; $n=30$), while the trend was assigned as toxic when the fall in cell number was rapid ($p<0.01$; $R^2=0.57$; $n=10$) with the 3rd day cell count less than 10% of seeded cells.

Figure 6 shows viable Swiss 3T3 cells recovered from T25 flasks after 2-hour pulsed exposure to mitomycin C solution having concentrations of 4µg/ml (A) and 5µg/ml (B), each of which was formulated to give dose permutations of 15, 75, 150 or 450 pg/cell. Each permutation was compared with concentrations of 3 and 10µg/ml, constituted at doses of 10 and 30 pg/cell, respectively, and included as the least and the most toxic permutations. Statistical comparisons performed by Student's t test were indicated as significant at $p<0.05$ (*) or insignificant (NS). Dose dependent fall in viability among all the permutations of 4 µg/ml (R^2 =0.927; $P<0.01$) or 5 µg/ml (R^2 =0.898; $P<0.02$) was tested by regression. Pre-exposure cell number (ECN) was determined by cell counts from three random flasks before treatment. Control represents viability in flasks sham exposed to only mitomycin C vehicle solution. Each column depicts average with standard deviation from triplicates.

Figure 7 shows differential periodic cell extinctions of Swiss 3T3 cells re-plated into triplicate wells of 24-well plates at a seeding density of 7,000 cells per cm^2 following a 2-hour pulsed exposure to mitomycin C in T25 flasks at concentrations of 4µg/ml (A & B) and 5µg/ml (C & D), each of which was formulated to give dose permutations of 15, 75, 150 or 450 pg/cell. Viable cell counts from all the dose permutations at each time point (A & C) were analysed by one way ANOVA and the P value was indicated if it was less than 0.05. The dose dependent variation in cell extinctions for each concentration (B & D) were represented by linear trend lines and R^2 values were calculated by regression.

Figure 8 shows comparative cellularity of Swiss 3T3 cells on days 6 (upper row) and 12 (lower row) after they were re-plated into 24-well plates following a 2-hour pulsed exposure to mitomycin C in T25 flasks at a concentration of 4µg/ml, formulated in a way to give dose permutations of 15 (A & B), 150 (C & D) or 450 (E & F) pg/cell. Vacuolated cells (Arrows) after 6 days were rare in feeders of 4-15 (A), few in 4-150 (C) and numerous in 4-450 (E). Overall cellularity on culture surface after 12 days post-treatment was fair in 4-15 (B), moderate in 4-150 (D) and poor in 4-450 (F). Marker length is 10 µM.

Figure 9 is a clustered column diagram showing differential periodic cell extinctions of Swiss 3T3 cells re-plated at a density of 7,000 cells per cm^2 into triplicate wells of 24-well plates following a 2-hour pulsed exposure to mitomycin C in T25 flasks at concentrations, 4µg/ml (A) and 5µg/ml (B), each of which was formulated to give dose permutations of 15, 75, 150 or 450 pg/cell. Each permutation was compared with concentrations of 3 and 10µg/ml, used at doses of 10 and 30 pg/cell, respectively, and included as the least and the most toxic permutations. Each cluster represents viable cell number from a single dose after 3, 6, 9 and 12 post-treatment days. The clusters of 3-10 and 10-30 were compared with each of the other dose permutations by paired t test and indicated as significant at $p<0.05$ (*) or insignificant (NS).

Figure 10 shows Colony Forming Efficiency of 3rd passage keratinocytes co-cultured with various Swiss 3T3 feeders, growth arrested by a 2-hour pulsed exposure to 4µg (A & C) and 5 µg (B & D) mitomycin C per millilitre, each of which was constituted to give dose permutations of 15, 150 or 450 pg/cell. Feeders exposed to 3 and 10µg/ml at doses of 10 and 30 pg/cell, respectively, were included as least and most toxic permutations, in addition to feeders growth arrested by gamma

irradiation serving as standard technique. Cultures were initiated in triplicate wells of 6-well plates with keratinocytes and feeders seeded at the rates of 250 and 144,000 per well, respectively. Colonies were counted after staining the wells with Rhodamine B (C & D). All groups were statistically compared with 4-150 group by Student's t test and indicated as significant at $p<0.05$ (*) or insignificant (NS).

Figure 11 illustrates complete color separation in a Rhodamine B stained culture plate containing keratinocyte colonies over a substratum of feeder cells, using Adobe Photoshop that facilitated quantitative image analysis. The sub title of 'Original' on the extreme left represents the untreated image of a Rhodamine B stained culture plate depicting keratinocyte growth area in red and feeder substratum in bluish-gray blue. The sub-titles of 'Keratinocytes' and 'Feeder' represent the replicated samples of the original image from which the area in red representing the keratinocyte growth area and the remaining feeder substratum in bluish-grey, respectively, are isolated to facilitate quantification of both the areas in terms of pixels. The sub-title of 'Merge' on the right shows the reconstructed combined image produced by the superimposition of 'Keratinocytes' and 'Feeder' images, to validate color isolation.

Figure 12 shows colony forming efficiency (A) and growth area analysis (B & C) of keratinocytes co-cultured with Swiss 3T3 feeders which were growth arrested by a 2-hour pulsed exposure to 4μg mitomycin C per millilitre, constituted to give dose permutations of 15 or 150 ρg/cell. Feeder cells, growth arrested by gamma irradiation (γ-Irr) serving as standard technique were included for comparison. Cultures were initiated in quadruplicate wells of 6-well plates and each well was seeded with 170 viable keratinocytes from frozen stock of 1st passage cultures

and 144,000 feeder cells. Colonies were counted after staining the wells with Rhodamine B (D). Average colony size (C) was derived by dividing the total growth area (B) by total number of colonies (A). Significant comparison ($p<0.01$) with 4-150 feeder group is indicated by black asterisk, while comparison between feeders of 4-15 and gamma irradiation is indicated by grey asterisk.

Figure 13 represents growth area analysis of keratinocytes co-cultured with Swiss 3T3 feeders which were growth arrested by a 2-hour pulsed exposure to 4μg mitomycin C per millilitre, constituted to give dose permutations of 15 or 150 pg/cell. Feeder cells, growth arrested by gamma irradiation (γ-Irr) served as standard control. Cultures were initiated in quadruplicate wells of 6-well plates, and each well was seeded with 340 viable 1st passage keratinocytes and 144,000 feeder cells. Keratinocyte colonies were stained red with Rhodamine B and subjected to image analysis to calculate per cent of keratinocyte growth area from the ratio of red pixels and total number of pixels of the well. The area of keratinocyte growth was calculated by considering actual total area of the well that equals to 9.6 cm^2. 4-15 and gamma irradiation were statistically compared with 4-150 group by Student's t test and indicated as significant at $p<0.01$.

Figure 14 includes Dot plots depicting distribution of cellularity (Top) BrdU positive cells (Bottom) across all keratinocyte colonies produced out of 210 plated 1st passage keratinocytes per feeder group. Feeder cells included 4-150, 4-15 which were growth arrested by a 2-hour pulsed exposure to 4μg mitomycin C per millilitre, that was constituted to give dose permutations of 15 or 150 pg/cell and also the gamma irradiated feeders (γ-Irr). Significance ($P<0.03$) is indicated by asterisk.

Figure 15. Pie diagram showing overall BrdU positive & negative keratinocytes with their corresponding percentages in cultures set up over feeder cells that were processed by different approaches. Feeder cells included 4-150, 4-15 which were growth arrested by a 2-hour pulsed exposure to 4μg mitomycin C per millilitre, that was constituted to give dose permutations of 15 or 150 ρg/cell and also the gamma irradiated feeders (γ -Irr). Significant ($p<0.01$) comparisons with 4-150 are indicated by white asterisk.

Figure 16 shows labelling of Bromodeoxy Uridine (BrdU) in human epidermal keratinocytes grown in the presence of mitomycin C feeders of 4-15 (A & B) and 4-150 groups (C & D). The BrdU positive nuclei are visualized as blue-green (A & C) which are merged with corresponding phase contrast images (B & D). Magnification bar = 10 μm.

Figure 17 is a clustered column diagram showing differential periodic cell extinctions of Swiss 3T3 cells re-plated at a density of 15,000 cells per cm^2 into triplicate wells of 24-well plates following a 2-hour pulsed exposure to mitomycin C in T25 flasks at concentrations, 4μg/ml (A & C) and 5μg/ml (B & D), each of which was formulated to give dose permutations of 15, 150 or 450 ρg/cell. The gamma irradiated feeders were represented by γ-Irr. Those graphs showing extinction of 7,000 plated feeders per cm^2 (B &D) are taken from Figure 9. Each cluster represents viable cell number from a single dose after 3, 6, 9 and 12 post-treatment days. The inter group comparisons between any two permutations were performed by paired t test and indicated as significant at $p<0.05$ (*) or insignificant (NS).

Figure 18 is a clustered column diagram showing the periodical growth patterns of human epidermal keratinocytes grown in presence of MMC feeders of 15, 150 and 450 ρg per cell under concentrations of 4 (Top)

and 5 (Bottom) μg per ml and compared with those of 3-10 and 10-30 feeders. The statistical comparisons between two independent feeder cell groups were performed using cell counts from all time points by two-way ANOVA with $P<0.05$ as insignificant (NS).

Figure 19 is a clustered column diagram showing the periodical growth patterns of human epidermal keratinocytes grown in presence of MMC feeders of 15, 150 and 450 ρg per cell under concentrations of 4 (Top) and 5 (Bottom) μg per ml and compared with those of γ-Irr feeders. The statistical comparisons between two independent feeder cell groups were performed using cell counts from all time points by two-way ANOVA with $P<0.05$ as insignificant (NS).

Figure 20 is a clustered column diagram showing the periodical growth output of human epidermal keratinocytes grown in presence of controls consisting of MMC feeders of 3-10 and 10-30 and γ-Irr feeders. The statistical comparisons between two independent feeder cell groups were performed using cell counts from all time points by two-way ANOVA with $P<0.05$ as insignificant (NS).

Figure 21 shows duplicate sheets of cultured epithelia produced by confluent cultures of human keratinocytes grown over feeders of 4-15 (Top row), 4-150 (Middle row) and γ-Irr (Bottom row). The epithelia were isolated 12 days after initiating with 800 Keratinocytes/cm^2 by incubating in dispase. Magnification: Original.

Figure 22 shows Hemotoxylin and Eosin stained paraffin sections of cultured epithelia produced by confluent cultures of human keratinocytes grown over feeders of 4-15 (Top row), 4-150 (Middle row) and γ-Irr (Bottom row).

Figure 23 shows immuno-histochemical positive control images on

localization of cytokeratin 14 (CK-14), Involucrin, CK-10 and Filaggrin in human epidermis. The nuclei are stained blue with Dapi and the green deposits represent FITC tagged secondary antibody which was bound to the corresponding primary antibody.

Figure 24 shows immuno-histochemical localization of cytokeratin 14 in cultured epithelia of 4-15 (Top), 4-150 (middle) and γ-Irr (Bottom). The nuclei are stained blue with Dapi and the green deposits represent FITC tagged secondary antibody which was bound to the anti-cytokeratin 14 primary antibody. The left panel is the comparable Phase contrast image.

Figure 25 shows immuno-histochemical localization of Involucrin in cultured epithelia of 4-15 (Top), 4-150 (middle) and γ-Irr (Bottom). The nuclei are stained blue with Dapi and the green deposits represent FITC tagged secondary antibody which was bound to the anti-involucrin primary antibody. The left panel is the comparable Phase contrast image.

Figure 26 represents immuno-histochemical staining for cytokeratin 10 in cultured epithelia of 4-15 (Top), 4-150 (middle) and γ-Irr (Bottom). The sections show only the nuclei are stained blue with Dapi while there were no deposits representing cytokeratin 10. The left panel is the comparable Phase contrast image.

Figure 27 represents immuno-histochemical staining for Filaggrin in cultured epithelia of 4-15 (Top), 4-150 (middle) and γ-Irr (Bottom). The sections show only the nuclei are stained blue with Dapi while there were no deposits representing Filaggrin. The left panel is the comparable Phase contrast image.

Figure 28 consists of column charts representing non-proliferative feeder cell contamination expressed as percent of 3T3 out of total plated cells of 5000 per dish (Top) and percent of 3T3 out of total attached cells that included both 3T3 and keratinocytes (Bottom). Statistical analysis using Students t test revealed insignificant differences among the three groups.

Figure 29 shows Hoechst stained preparation in T25 flask following 3 weeks of incubating the isolated keratinocyte suspension containing 50,000 cells. A sole broad fibroblast (arrow) is seen with large nucleus containing coarsely aggregated chromatin and bright nucleoli as opposed to numerous keratinocytes containing relatively small nuclei with smoothly diffused chromatin and dim nucleoli. Magnification marker = 10 μm.

DETAILED DESCRIPTION OF THE INVENTION:

According to this invention is provided a method for processing of feeder cells suitable for adult stem cell proliferation.

In accordance to this invention is provided a process of controlled growth arrest of feeder cells by a strategy of regulating their disintegration or life span and their ability in supporting the proliferation of human epidermal keratinocyte stem cells following a pulsed exposure to mitomycin C at permutations of concentration per se in the treating solution and the experimentally recognized dose per unit feeder cell number and the subsequent identification of an optimal feeder cell processing comparable to gamma irradiation for a speedy growth of keratinocytes resulting in the formation of a stratified epithelium that could be exploited towards grafting to treat certain ailments requiring resurfacing.

This is achieved by a strategy of:

(1) Initially proving that the periodical feeder cell extinctions depend on a range of mitomycin C doses per unit cell number which are derived arithmetically after a range of feeder cell densities are experimentally pulse exposed to various concentrations of mitomycin C.

(2) Predicting an range of probable volumes of mitomycin C solutions, based on permutation of a range of such derived doses per unit cell number and the concentrations per unit volume that are short-listed on the basis of inducing significant dose dependant differential in cell disintegration.

(3) Employing the said range of volumes across the short-listed concentrations to pulse expose a constant density of qualified feeder cells to generate the respective feeder cell batches exhibiting distinct cell disintegration trends.

(4) Identifying an optimal feeder batch that is growth arrested by a specific volume of treating solution representing one of the said concentration-dose permutations by way of supporting maximal growth of keratinocytes as compared to γ-*Irr* feeders in an *in vitro* co-culture system.

(5) Establishing the ability of such an identified feeder batch in stimulating keratinocyte growth towards formation of a stratified epithelium which is comparable to that produced by γ-*Irr* feeder cells.

In this process the identification and production of best performing feeder cells is unique as it involved growth arrest through an exclusive experimental derivation of Mitomycin C dosing that is comparable to gamma irradiation but works out to be cost-effective and many fold efficient to the known mitomycin C based techniques.

The instant invention relates to a process of preparing an optimal cellular substratum demonstrated through novel experimental derivation of mitomycin C titration protocol which is shown in sequential Figures 1 to 3 employing Swiss 3T3 cell line, in a way to optimize epidermal

keratinocyte stem cell growth under artificial growth conditions. The invention is a cost-effective process for producing a comparable growth promoting feeder layer as opposed to the standard but expensive gamma-irradiation method and can be exploited to create artificial epithelia for resurfacing diseased tissues like skin, cornea etc.

A schematic representation of experimental derivation of doses from a given concentration of mitomycin C by cell density titrations by employing a popularly known Swiss 3T3 feeder cell line is shown in Figure 1. Varied number of cells (Σ) is treated to a 2 hrs pulse of Mitomycin C at different concentrations, the outcome of such exposures, which broadly included categories namely stimulatory, stationary, inhibitory and toxic, is correlated (Table 1, Figures 4 and 5) with the doses that are calculated from the following formula:-

$$\Delta = C_{(\upsilon/\Sigma}$$

Where Δ = dose / cell (pg/cell or μg/million cells)

C = concentration of MMC (μg/ml)

υ = volume of treating solution (ml) which is kept constant

Σ = exposure cell number (millions)

These experimentally derived doses and the concentrations become the basis of predicting a range of respective volumes. Subsequently, differential growth arrest was produced by exposing a desired constant density of qualified feeder cells (Σ) for 2-hr pulse of a uniform range of volumes across two short-listed concentrations representing permutations of mitomycin C concentrations per ml and the doses per cell, to achieve varied extents of growth arrest (Figure 2).

The mitomycin C concentrations used to permanently growth arrest the feeder cells are in the range of 3 to 10 μg per ml, while the doses are in the range of 15 to 450 ρg per cell or μg per million cells (Figures 6 to 9). Subsequently, the stimulatory influence of feeders is tested as shown in Figure 3 by replating the mitomycin C treated 3T3 feeder cells along with epidermal keratinocyte stem cells in Keratinocyte medium. The stimulatory influence is ascertained by the progress of growth of keratinocytes and their ability to form maximal colony forming efficiency, to cover maximal growth area (Figures 10 to 20) during comparable culture time, and ability to form stratified epidermis (Figure 21) showing the key epidermal markers (Figure 23 to 27), while keeping a minimal feeder cell contamination (Figure 28, 29). The most preferred concentration of mitomycin C is 4 μg per ml and the most preferred dose is 150 μg per million cells in terms of accomplishing not only irreversible growth arrest and also exerting maximal growth stimulatory influence on epidermal keratinocyte stem cells.

The invention is illustrated by the following examples which are only meant to illustrate the invention and not act as limitations. All embodiments apparent to a process there in the art are deemed to fall within the scope of the present invention.

EXAMPLES

General Materials and Methods:

All the described examples involved the following common methods while the additional pertinent methods are described under each specific example:

Cell culture: The Swiss 3T3 (ATCC-CCL-92) culture supplied at 115th passage was cultured in DMEM containing 10 per cent (Volume/volume)

donor calf serum and 17.86 milli molar sodium bi carbonate under cell culture conditions of a constant temperature of 37°C and humidified 5 per cent carbon dioxide atmosphere and the cells were detached by trypsinization using 0.25 percent trypsin and 0.03 per cent Ethylene Diamine Tetra Acetic acid (EDTA) in phosphate buffered saline (PBS) for sub-culturing. The cells were serially sub-cultured for 4 passages to establish a cryo-preserved master bank which was considered to be at 4th passage. A working bank was then generated from a frozen vial of master bank by sub-culturing two times until 6th passage and cryo-banked. The cultures were tested for Mycoplasma (Kumar et al 2008), doubling time, saturation density and absence of growth in methyl cellulose. The sub-culture dilutions adopted throughout the banking procedure ranged from 1:6 to1:10 while maintaining a seeding density of about 3 x 10^3 cells per cm^2 as recommended by supplier and the cultures were never allowed to grow to more than 50 per cent confluence. The 6th passage cultures were initiated from cryo-preserved working bank cells after they were rapidly thawed (per cent viability of 81.4 ± 1.1; n=22) and incubating 225,000 cells for 4 days in T75 culture flasks; the output (17.90934 ± 5.3809 x 10^4; n=22) was further sub-cultured by incubating same number of cells for 3 days; the resultant 7th passage output cells (17.60247 ± 4.2266 x 10^4; n=22) were batch-tested for irreversible growth arrest by maintaining them until all cells disintegrated subsequent to their pulse exposure to 40 µg of mitomycin C in 10 ml of solution. The other identically generated 7th passage cells from parallel working bank vials were designated as qualified feeder cells and the same were used for growth arrest experiments of examples 2 and 3, while the 6th passage output cells were used for experiments of example 1.

Keratinocyte - feeder Co-culture: Primary keratinocytes were obtained from Genlantis (Cat No. PH10205A, www.genlantis.com) which were cryo-preserved at the end of first culture of epidermal cells isolated from healthy adult human skin biopsy after growing in a feeder-free and serum-free culture system. The cells were used for co-culture experiments with the growth arrested feeders as described adopting the basic Rheinwald-Green (1975) technique (Navasaria et al.1994). The keratinocyte growth medium (KGM) comprising: Dulbecco's modified eagle medium and Ham's F-12 at 3:1 ratio, 10 percent (Volume/Volume) Fetal Calf Serum and 10 µg ciprofloxacin, 5 µg of insulin, additional 110 µg L-Glutamine, One µg of dexamethasone, 24.32 µg of adenine, 20 µg of L-serine, 0.4 µg of hydrocortisone, 10 ηg of Choleratoxin, 10 ηg of Epidermal Growth Factor (added in the medium on day 2), 1.346 ηg of Tri-Iodo-thryronine and 5 µg of Transferrin, each of which are per every ml of growth medium.

The cultures were passaged by initially treating with 0.03 grams of EDTA per 100 ml of PBS to remove feeder cells followed by detachment of keratinocytes using 0.08 grams of trypsin together with 0.01 grams of EDTA and 0.025 grams of glucose, each of which are per every 100 ml of the respective final solutions and viable cells were counted by trypan blue exclusion criterion.

EXAMPLE 1:

MODULATION OF MITOMYCIN C INDUCED FEEDER CELL GROWTH ARREST BY EXPOSURE CELL NUMBER

This example describes experimental proof to show that the periodical feeder cell extinctions depended on a range of mitomycin C doses,

expressed per unit cell number, which were derived arithmetically after a range of feeder cell densities are experimentally pulse exposed to various concentrations of mitomycin C.

Growth arrest Protocol: Working bank Swiss 3T3 cells were put in culture as described in general methods and this 6th passage output cells were seeded into T25 flasks (Nunc) containing 10 ml of medium and incubated for 5 days before treatment with mitomycin C. The cultures were initiated with seed densities of 4 x 10^2, 2.5 x 10^3 and 2 x 10^4 cells per cm^2 so as to achieve the three required Exposure Cell Numbers (ECNs) at the time of treatment to mitomycin C (Figure 1) and the resultant ECNs achieved per cm^2 of growth area were 0.7276 ± 0.0844 x 10^4 (Low density; n=6), 3.0711 ± 0.3021 x 10^4 (Medium density; n=4) and 6.1556 ± 0.2309 x 10^4 (High density; n=4), respectively. After obtaining the required ECN, the cells were exposed under the described standard cell culture conditions to a 2 hour-pulse of mitomycin C (Sigma-Aldrich, Catalogue number M4287), which was dissolved in Hanks Balanced Earl's Salts (HBES) and diluted proportionately with 10 ml of culture medium to yield a range of mitomycin C concentrations of 1, 3, 4, 5, and 10 µg per ml; higher than 10 µg per ml were not included in the study because of their acute toxicity across all the ECNs.

Cells exposed to same volume culture medium containing HBES served as control. At the end of exposure, cells were detached with solution containing 0.25 grams of trypsin and 0.03 grams of EDTA per 100 ml of PBS at pH 7.2, counted and replated into 24 well plates at a density of 14000 cells per well. The cells were detached, stained with trypan blue and viable cells were counted in Neubauer chamber at intervals of 3 days until 12 days and a final count after 20 days. Each experiment was performed in triplicate and repeated at least twice.

Statistics: The data was subjected to an analytical protocol (Yerneni and Jayaraman, 2003) to verify density dependant influence of mitomycin C on 3T3 cells. The replated control cells grew with a normal sigmoid curve as expected & reached confluence between days 6 & 9; therefore cell number comparisons between treated and control would be logically inappropriate. Therefore, independent growth curve plots were constructed for every concentration of mitomycin C tested on the three ECNs without representing control, the cell number data at each time point was analyzed by one way ANOVA and significance was considered, if $P<0.05$. Additionally, significance of variance in data resulting after the action of combinations of a given concentration with the three ECNs across Days 3 to 12 was analyzed by two-way ANOVA; day 20 data was omitted because of absence of cells in certain combinations. The dose, expressed as μg of mitomycin C per million cells, which equals to ρg per cell, was calculated by dividing the product of concentration in μg per ml and volume of treating solution in ml with exposure cell number in millions (Figure 1). The significance of linearity between viable cell number for each time point and mitomycin C dose was tested by correlation coefficient. The categories of growth arrest outcome as defined on the basis of cell extinction trend were represented in a growth curve plot and analyzed by regression.

Results and Discussion:

The lowest concentration of 1 μg per ml of mitomycin C tested on feeder cells of the three exposure cell densities (ECNs) influenced significant ($p<0.02$) ECN dependant variation in cell extinction at days 6 and 9, but thereafter there was a steady increase in cell number indicating failed growth arrest (Figures 4A).

On the other hand, concentrations of 3 and 10 μg per ml influenced significant ($P<0.03$) exposure cell density dependant variation in cell extinctions, which was particularly true during the period of 3 to 21 days with lower concentration, while it was only during the initial 9 days period with high concentration (Figure 4B & E). In fact, the significance of variance in extinctions produced by 3 μg was apparently influenced by the intensely opposite stimulatory influence exhibited by high cell density, because significant variance was not sustained when comparisons on extinction outcomes were made only in between low and medium density. On the other hand, extinction variation was consistently significant ($P<0.001$) at all time points with concentrations of 4 and 5 μg per ml (4C & D). Concurrently, each mitomycin C concentration tested across the three ECNs at all time points brought about an overall significant ($P<0.04$) variation as revealed by 2-way ANOVA, indicating that the tested concentration alone is not the determining factor but it is rather the combination of concentration & exposure cell number which are crucial to significantly influence the periodical extinction in the current experimental design.

The ECN dependant differential cell disintegration by a given concentration could be the fallout of mitomycin C acting in a range of different doses per cell that would result from the varied exposure cell number, because notionally the total amount (weight/volume) of mitomycin C, which is the product of concentration and volume of treating solution, is evenly shared by all exposed cells. This was tested, particularly at those effective concentrations of 3, 4, 5 & 10 μg per ml that brought about consistent growth inhibition on at least one of the ECNs. Hence, the extinctions were correlated with a range of such doses

that were arithmetically calculated by dividing the product of a given concentration, expressed in units of μg per ml and volume of treating solution in ml with exposure cell number in millions as depicted in Figure 1 thus the dose obtained was expressed as μg of mitomycin C per million cells, which equals to ρg per cell. After the derived doses were arranged in ascending order from lower 19.5 to higher 549.7 ρg per cell showing their relationship with the respective concentration and exposure cell density (Table 1), four distinct categories were identified amongst the total twelve tested combinations, on the basis of significant linearity of post-exposure viable cell number with time as tested by correlation coefficient. These are shown to reflect the overall growth curve status and the respective curves were plotted with regression analysis (Figure 5).

Stimulatory: The resultant growth curve after 3 μg of mitomycin C per ml were employed on high ECN which showed consistent revival with 20th day cell number higher than the seeded cell number and a significant linear positive correlation ($P<0.01$) between cell number and days of observation was assigned a final stimulatory status.

Stationary: The growth curves after using 3 μg of mitomycin C per ml to treat medium ECN and 4 and 5 μg mitomycin C per ml to treat high ECN, showing inconsistent fall in cell number with random transient revival and insignificant linear correlation between change in Cell number and progression of culture time were together assigned a final stationary status.

Inhibitory: The growth curves following the use of 4, 10, 5, 10, 3 and 4 μg of mitomycin C per ml to treat medium, high, medium, medium, low

and low ECNs, respectively, indicated consistent fall in cell number with significant ($P<0.01$ to 0.05) negative linear correlation between change in cell number and progression of culture time and were grouped together as inhibitory.

Toxic: The resultant growth curves after 5 and 10 μg of mitomycin C per ml, employed to treat the low ECN, showed rapid fall in cell number as visualized by the very first cell count at day 3 to be less than 10% of seeded cells and significant negative linear correlation ($P<0.01$ to 0.05) between change in cell number and progression of culture time and were together assigned a final toxic status.

There are certain earlier reports describing the lethal and cytokinetic effect of mitomycin C as a function of drug concentration and exposure time while studying its anticancer action (Barlogie and Drewinko 1980), while others concluded that the concentration-response curves are specific to feeder cell type (Ponchio et al 2000), but the criticality of exposure density or dose per cell was not addressed. However, the results of this example showing differential extinction, particularly in the range of effective concentrations of 3 to 10 μg per ml, is a clear indication of an empirical relationship between exposure cell density and the coefficient of post-exposure fall in viable cell number calculated against time. A dependable correlation between the exposure cell density and the action potential of melatonin which was used as a cell proliferation controlling agent was earlier described by one of the inventors to follow an arithmetic pattern (Yerneni and Jayaraman 2003). It may thus be inferred that *in vitro* effectiveness of mitomycin C and possibly several other chemical agents as well is a secondary but significant function of

dose per cell in addition to its concentration, specifically at medially effective concentrations, while lower and higher concentrations due to their weak and intense action potential, respectively, underplay the role of dose. Such a regulatory control resulting in progressive depletion of net cumulative life-span of growth supporting feeder cells remained unreported elsewhere. This relationship could in turn have a significant impact on proliferation of keratinocytes or any adult stem cells, because the ratio of metabolically active mesenchymal cells like the growth arrested fibroblasts and the target stem cells for the proliferation and/or maintenance of the later *in vitro* is very critical (Sun et al 2009; Zhou et al 2009; Jubin et al 2011). Therefore, it may be implied that oversight of this critical dose per cell consideration could have been one of the probable factors for the reported contradictory efficacy of mitomycin C approach as compared to gamma-irradiation, wherein the specific exposure density of feeder cells and volumes of treating solutions were not compared with the resultant cell extinction rates (Ponchio et al 2000; Roy et al, 2001; Schrader, 1999; Nieto et al 2007; Fleischmann et al 2009).

It may thus be proposed that the inadequacies in mitomycin C induced growth arrest at a certain given range of concentrations could perhaps be optimized by controlling the initial exposure cell number of feeder cells or the volumes of treating solutions. The significance of dose determination based on cell number could also be inferred from the recommended measures of controlling toxicity of chemotherapeutic agents in cancer patients by determining individualized dosing on the basis of recipient's lean body mass (Prado et al 2007) which is the difference between body mass and body fat, the former in turn is the product of cell number and average cell mass (Savage et al 2007). Further, it is proposed that if

exposure cell density variation strategy was to be adopted in an in vitro toxicology study design, it could possibly form a basis of calculating and predicting a compound's operational in vivo dose from the most active concentrations studied *in vitro*.

In other equivalent terms, the range of combined factors of exposure cell density of feeders and concentration of mitomycin C at its constant volume may be substituted with corresponding permutations of volume and concentration of the latter, while keeping the exposure cell density to a stably safe constant, to achieve analogous dosing and regulate live feeder cell number. There is a need to apply this alternate strategy in order to set the exposure cell number to such a constant level that the cell population does not accumulate variants through successive passaging. Because cell lines like the 3T3s are known to gradually accumulate spontaneous variants with altered characteristics when grown by sub-culturing the high density confluent populations (Rubin an Xu 1989; Matthews 1993; ATCC product information sheet for CCL-92, www.atcc.org). It was further verified in our lab that the cells maintained at confluence formed spheres when suspended in methyl cellulose, while the pre-confluent cells failed to do so and that all the cultures established from such spheres resisted low concentrations of mitomycin C, while only a few such cultures were sensitive to higher concentrations mitomycin C. Connor (2000) advised the use of mitomycin C at a high concentration of 10 μg to growth arrest mouse embryonic feeder cells, yet cautioned about feeder re-growth and offered no solution than to discard those dishes showing feeder re-growth. It is likely in the present study as well that the observed stimulatory outcome of a lower 3 μg of mitomycin C per ml employed on a high ECN of 60,000 cells per cm^2

which is a confluent population could be perhaps the manifestation of accumulated variants with resistance to mitomycin C. Therefore, a substitute strategy to achieve comparable differential cell extinction rates may be considered by employing permutations of volumes of treating solutions and preferably low concentrations of mitomycin C by setting the exposure cell number, followed by identification of the best performing combination on target cell stimulation.

It is therefore, proposed to adopt such strategy of testing a range of volumes of a given concentration of mitomycin C in the range of 3 to10 µg of mitomycin C per ml, which would be derived by dividing the product of exposure cell number in millions and dose shown as pg per cell with concentration of mitomycin C as µg per ml of treating solution (Figure 2). In this way, volume titrations of a given concentration of mitomycin C may be adopted to induce differential growth arrest through the production of varied cellular extinction rates; thereby a corresponding differential stimulation of the target adult stem cells like the epidermal keratinocytes may be obtained through altered quantitative, if not qualitative, support profile of 3T3 cells and could ultimately lead to identify the best feeder processing. The process, if proven, is likely to overcome the reported inadequacies of the cost-effective mitomycin C approach as compared to gamma-irradiation.

This assumption is tested by devising appropriate experimental designs as illustrated in subsequent examples.

EXAMPLE 2:

REGULATION OF FEEDER CELL DISINTEGRATION THROUGH PERMUTATIONS OF CONCENTRATIONS AND DOSES OF MITOMYCIN C

This example describes a strategy of controlling the net life-span of feeder cell growth arrest by regulating degree of cell disintegration through treatment of a constant exposure cell population with mitomycin C solutions constituted in a way to yield a range of volumes representing specific permutations of concentrations and the doses per unit cell number.

Materials and Methods: All experiments of this example were undertaken on 3rd day cell population of a resulting culture after one sub-culture of the identical working bank Swiss 3T3 feeder cells. This population, considered as qualified feeder cells, was batch-tested for irreversible growth arrest by maintaining them until all cells disintegrated subsequent to their pulse exposure to a concentration of mitomycin C at 40 μg in 10 ml of solution. The qualified cells grown either in T25 or T75 flasks were then titrated with a range of dose-concentration permutations of mitomycin C derived by including the average pre-exposure cell number which was determined from cell counts performed in randomly picked parallel flasks. The permutations were calculated by dividing the product of pre-exposure cell number in millions and the chosen dose expressed as ρg per cell with the concentration in μg per ml (Figure 2). Further, a broader range of doses than those derived in example 1 was chosen for titrations to increase the scope of identifying best outcome by producing more apparent differential degrees of cell extinction.

Each permutation is depicted as a pair of whole numbers in which the left hand side of the hyphen stands for concentration and the right hand side for dose. Eventually, the permutations analogously represent a range of volumes of treating mitomycin C solutions in which the derived volume is directly and inversely proportional to doses and concentrations, respectively and both these factors along with exposure cell number determine the total amount of mitomycin C in the final treating volume. With the minimum volume of treating solution to sufficiently submerge the entire cell monolayer and the maximum capacity of a chosen culture flask being the lower and upper limits, respectively, the choice of permutations to be included in the study is restricted to only a correspondingly limited range of volumes.

In view of these considerations, several primary screening experiments were conducted by including an initial wider range of concentrations of 1, 2, 3, 4, 5 and 10 μg per ml before short-listing the effective permutations for final testing. Based on preliminary study, 1 & 2 μg per ml were excluded from dose titrations as the growth arrest produced by them was always reversible ending in re-growth of treated cells. Permutations of 3-10 and 10-30 were included as low and high concentration controls, respectively, since none of the other volumetrically permissible higher concentrations-dose permutations produced significantly deviated extinction pattern. Each of the remaining intermediate concentrations of 4 and 5 μg per ml was sub-divided into doses of 15, 75, 150, and 450 ρg per cell and the results were compared with 3-10 and 10-30. The cells were treated under the described standard cell culture conditions with a 2 hour-pulse of mitomycin C (Sigma-Aldrich, Catalogue number M4287), which was

dissolved in Hanks Balanced Earl's Salts (HBES) and diluted proportionately with culture medium to yield a desired dose permutation of mitomycin C concentrations. A median volume of culture medium containing HBES was used to expose the vehicle-control flasks which were maintained under identical culture conditions. The experiments were performed to study both short-term and long term effects of mitomycin C treatments as per the following design:

Short term influence: In order to study the 2-hour short term influence on cell viability following exposure of Swiss 3T3 cells to the short listed permutations, the 3rd day qualified cell population in T25 flasks initiated with a fixed density of 3000 cells per square centimeter was pulse exposed to Mitomycin C. This protocol yielded an average exposure cell density of 490,000 ± 7,775 (n=6) cells per flask which was used for all permutations and post-exposure viability was assessed in triplicate flasks.

Long term influence: To study the fallout of the short-listed dose-concentration permutations on cell extinctions, the 3rd day qualified cell population in T25 flasks initiated with a fixed density of 3000 cells per square centimetre was pulse exposed to Mitomycin C. This protocol yielded a highly consistent reproducible exposure cell density of 1,760,247 ± 42,266 (n=22) per flask throughout all experiments and the trypsinized cells from each permutation were replated to evaluate cell extinctions over a 12 day-period.

At the end of exposure, cells were detached with solution containing 0.1 grams of trypsin and 0.2 grams of Ethylene Diamine Tetra Acetic acid per 100 ml of phosphate buffered saline at pH 7.2 and counted. For long

term cell extinction study, the detached cells from the flasks were replated into triplicate wells of 24 well plates at a density of 14000 cells per well. Subsequently, cells were detached, stained with trypan blue and viable cells were counted in Neubauer chamber at intervals of 3 days until 12 days. Each experiment was repeated at least twice.

Statistics: The replated vehicle control cells grew with a normal sigmoid curve & reached confluence between 6 and 9 days; therefore comparison between treated and vehicle control was not undertaken as it would be logically inappropriate and a lower and a higher concentration control were included for appropriate comparisons. Each data point represents mean ± standard deviation from triplicate samples. Data were subjected to analytical procedures to verify if the cell extinctions are distinct and true reflections of dose variation. For visualizing the immediate influence of 2-hour exposure to mitomycin C on cell viability, column graphs depicting viable cell yields were constructed and each permutation was compared with 3-10 and 10-30 by Student's t test. Additionally, the 2-hour viable cell output in vehicle control was compared with pre-exposure cell number. Dose dependent fall in viability among all permutations of each concentration was tested by regression and R^2 values were calculated.

For long term cell extinctions, line diagrams were constructed by plotting viable 3T3 cell number on y-axis against post-treatment time points on x-axis to show periodical cell extinctions caused by each permutation of a given mitomycin C concentration. Viable cell numbers at each time point was subjected to one way ANOVA to verify the significance of variance among dose permutations of a given concentration. Additionally,

the overall significance of variance across all time points among all permutations under each concentration was analyzed by two-way ANOVA; Further, linear trend lines were plotted using viable cell counts on y axis against doses on x axis by least squares fit and R^2 values were calculated by regression analysis. Furthermore, column graphs were constructed in which each cluster of columns represented the specified permutation and each column represented viable cell numbers at the specified time points. Each cluster of 3-10 and 10-30 was compared with every dose permutation by Students t test after pairing the corresponding time points.

Results & Discussion:

Influence on Short-term viability: The short-listed permutations involved a derived volume range of 1.47 to 55.12 ml (Table 2) wherein the minimum volume was sufficient to uniformly submerge the cell layer in T25 flask whose maximum capacity was 65 m. Significant differences in 3T3 cell viability were observed immediately after the 2-hours pulsed exposure to mitomycin C at various permutations of concentrations and doses (Figure 6). The treatment resulted in a significant ($P<0.05$) reduction of cell viability in all groups as compared to vehicle control whose viable cell fraction was similar to that of pre-exposure cell count as determined before the start of experiment demonstrating no influence of vehicle. The intergroup comparisons indicated that the viable cell outputs in 4-15 and 5-450 were comparable to low concentration control of 3-10 and the higher 10-30, respectively, indicating that increase or decrease of action potential of mitomycin C depended on not just concentrations but also on doses per cell. Additionally, regression

analysis revealed significant dose dependent fall in viability among all the permutations of 4 μg per ml ($P<0.01$; $R^2 = 0.926$) and 5 μg per ml ($R^2 =0.898$; $P<0.02$), further indicating the importance of dose modulation in addition to concentration.

Influence on long-term extinctions: The tested permutations involved a volume range of 5.281 to 198.028 ml while the maximum capacity of T75 flask was 265 ml. The replated mitomycin C treated cells exhibited differential periodic cell extinction in a dose dependant manner within the tested concentrations of 4 and 5 μg per ml (Figure 7).

A dose per cell dependant increase in cell extinction rate was produced by the dose permutations of 4-15, 4-75, 4-150 and 4-450 (Figure 7A). The variation was significant at specific time points of days 6, 9, 12, though it was insignificant ($P<0.09$) at the earliest time point of day 3. The regression analysis on the other hand (Figure 7B) revealed that the volume titrations brought about significant dose dependant intensification of cell extinction on day 3 ($R^2=0.985$; $P<0.01$), day 6 ($R^2=0.969$; $P<0.01$), day 9 ($R^2=0.9$; $P<0.02$) and day 12 ($R^2=0.888$; $P<0.02$). Taking into account the influence of all doses within this concentration across various time points, the dose modulation was proven to induce highly significant ($P<0.001$) variation in cell extinctions as revealed by 2-way ANOVA.

More or less similarly, the permutations of 5-15, 5-75, 5-150 and 5-450 also produced a dose dependant drop in cell viability pattern (Figure 7C). Although, there was no early significant difference in post-exposure cell extinction on day 3, which was more obvious by the presentation of same

viability with doses of 15, 75 and 150 pg per cell, but the subsequent time points revealed significant variation by all doses. Equally, the regression analysis (Figure 7D) also revealed that cell viability was not significantly (R^2=0.6; P>0.05) influenced by volume titrations on day 3, while they significantly modified the extinctions at day 6 (R^2=0.987; P<0.01), day 9 (R^2=0.914; P<0.02) and day 12 (R^2=0.899; P<0.02). But across all time points on the whole, the analysis by 2-way ANOVA clearly demonstrated a highly significant (P<0.001) influence of dose permutations in inducing varied cell extinctions.

The differential cell extinctions were also microscopically noticeable among permutations of 15, 150 or 450 pg per cell (Figure 8). The vacuolated cells which are signs of cellular disintegration were rare in feeders of 4-15, less frequent in 4-150 (C), but become apparently numerous in 4-450 (E) after 6 days post-mitomycin C exposure time point. This is further correspondingly reflected by loss of overall cellularity on culture surface after 12 days which ranged from fair in 4-15 (B), moderate in 4-150 (D) to very poor in 4-450 (F). Conspicuously, the cells gradually assumed broader aspect as their number depleted.

Additional time point matched comparisons undertaken to examine differential induction of cell extinction by dose titrations using either of the intermediate concentrations of 4 and 5 µg per ml with reference to concentration controls revealed that the lower 3-10 was comparable to the dose of 15 pg per cell, while the higher 10-30 was similar to 450 (Figure 9 A & B). Furthermore, the extinction produced by 4-150 was also similar to that of 10-30, though the initial cell death was

significantly ($P<0.05$) slower on day 3. But, both 10-30 and dose of 450, either with 4 or 5 μg per ml produced an almost equally acute initial cell death in contrast to comparisons with other dose permutations demonstrating the early onset of toxicity by them.

Conventionally, *in vitro* experimental treatment protocols for effectiveness of a given drug prescribe the strength of such agents in terms of its concentration per se in treating solutions, while grossly ignoring the criticality of exposure cell density or dose per cell (Barlogie and Drewinko 1980; Connor 2000; Ponchio et al 2000). The results of example 1 and an earlier report by one of us demonstrated that it is equally important to consider the exposure cell density which altered the pharmacological anti-cancer activity of a cell proliferation blocking agent in an arithmetically derived dose dependant manner (Yerneni and Jayaraman 2003). In these examples, it was proposed that all the cells exposed to the drug in a culture flask would equally share the whole amount present in total volume of treating solution eventually leading to a range of doses per cell when exposure cell number was varied. Accordingly, the post exposure cell disintegration rate was proportional to the calculated dose increments, although, the phenomenon is limited to the moderately acting intermediate concentrations and is also comparable to their similar action after cell density titrations of example 1. The exhibition of such variation at lower and higher concentrations appears to relate with their weak and intense action potential, respectively, at the very first place.

It is worth noting that while considering the same exposure cell density as employed in this example, the normally recommended range of 10-15

ml of working volumes for T75 flasks, would correspond to dose ranges of 23 to 34 and 28 to 43 pg/cell for concentrations of 4 and 5 µg/ml, respectively, which in turn fall in between the lower tested doses of 15 and 75 pg/cell only. At the same time it is difficult to deduce probable working doses in several earlier reports, wherein the precise exposure cell density and volume of treating solution were not mentioned (Schrader, 1999; Ponchio et al 2000; Nieto et al 2007; Fleischmann et al 2009). Even after presuming confluent or sub-confluent exposure densities in such studies, the dose per cell would still be limited to only a lower range and higher doses would have been certainly unexplored. Thus, the concept of attaining higher doses of mitomycin C per cell through simpler volume titrations without raising the concentration *per se* is for the first time shown by us to produce significant alterations in post-exposure life span of feeders and is likely to influence the outcome of target cell stimulation.

Primarily, the necessity to fix exposure cell density to such a constant level that the cell population does not accumulate variants through successive passaging, arises because cell lines like the Swiss 3T3 are known to gradually accumulate spontaneous variants with altered characteristics when grown by sub-culturing the high density confluent populations (Rubin and Xu 1989; Matthews 1993; ATCC product information sheet for CCL-92, www.atcc.org). It was further verified in our lab that the cells maintained at confluence formed spheres when suspended in methyl cellulose, while the pre-confluent cells failed to do so and that all the cultures established from such spheres resisted low concentrations of mitomycin C, while only a few of them were sensitive to higher concentrations of mitomycin C (Data not shown). Considering the

occurrence of such probabilities, it may be presumed that the observed stimulatory outcome of 3 μg of mitomycin C per ml employed on a confluent high ECN of 60,000 cells per cm^2 in example 1 which referred to a calculated dose of 19.5 pg per cell, is perhaps a possible manifestation of such accumulated variants expressing resistance to low concentration of mitomycin C, while showing susceptibility with higher concentrations. In fact, it is apparently this stimulatory influence that largely contributed to the overall reflection of significant variation in extinction profile in 3 μg group involving titration with all other densities and similar outcome may not be expected if presence of variants is controlled by taking a sub-confluent population for mitomycin C exposure. Concurrently, such an overall variation in extinction following volume titrations with any range of doses under 3 μg is abolished in the current example in which the high exposure density is replaced with a less than sub-confluent safe population of cells.

It is of particular interest to observe that the significant differences in cell extinction rates influenced by volume titrations with intermediate concentrations of 4 or 5 μg per ml were as consistent as with cell density titrations. It is of utmost importance to note that the total amount of mitomycin C at these middle order concentrations in their respective comparable doses turn out to be same, but are basically distributed at different dilutions and concurrently the statistical analysis between the matched volume titrations of these two concentrations (not shown in results) proved no significant deviation in cell extinctions. This again stresses the importance of considering all pertinent factors like concentration, dose per cell or exposure cell density while validating the influence of mitomycin C on feeder cell growth arrest. Considering the

broader aspect of titrations with either exposure cell densities or volumes (dose and concentration), it may be stated that the cell density variation strategy alone may not precisely project the effective combinations of concentrations, doses and exposure cell densities, but potentially provides primary estimates of probable range of such useful permutations for the subsequent convenient testing by corresponding volume titrations. It is thus shown that the volume titrations of a range of given concentrations, but limited to a median effective range, were instrumental in either diminishing or accentuating the net cell viability and could exert objective-specific array of consequences. Particularly, in the context of proliferation and maintenance of feeder cell based keratinocyte or any adult stem cell culture, the demonstrated control of net life span, which determines the net ratio of growth arrested but metabolically active fibroblasts and the target cells, could be exploited to optimize such cultures, since such a ratio is a well identified key growth regulating factor *in vitro* (Sun et al 2009; Zhou et al 2009; Jubin et al 2011).

In routine toxicological evaluation studies, concentration is the term of reference for *in vitro* studies and the *in vitro* dose response curve truly represents concentration dependant evaluation, while dose is specific for *in vivo* studies (Eisenbranda et al 2002). In the current example, both concentration and dose per given cell population were considered as discrete variables while making endpoint observation on cell death. Therefore, it is tempting to propose that if volume variation strategy was to be adopted following cell density titrations in an in vitro toxicology and/or pharmacological study design, it could perhaps form the basis of extrapolating a compound's operational *in vivo* dose from the most active

permutation of concentration and dose studied on a fixed population of cells *in vitro*. Moreover, such an approach could further help in predicting accurate effective dosing of anti-cancer agents in a pre-clinical efficacy evaluation setting, particularly while testing moderately toxic concentrations in an attempt to simulate containment of unwanted side-effects in vivo. At the same time, it is suggested that further studies are necessary to explain specific mechanisms for modified biological outcome by the permutations of dose and concentration of mitomycin C in contrast to the reported correlation of concentration with cytotoxic effects (Barlogie and Drewinko 1980).

Considering the impact of volume titrations while using a range of less toxic intermediate concentrations of mitomycin C in regulating the viability of feeder cells unreported in prior art, it may be proposed that the approach could be superior, reliable and convenient for rendering the feeder cell growth arrest than by the cell density regulation alone. Therefore, a strategy may be adopted to test the range of feeder cell batches produced by volume titrations as demonstrated in this example in an epidermal keratinocyte co-culture model to verify if they influence differential stimulation of target cells and to subsequently identify the best outcome while comparing with the standard γ-*Irr* feeders. The process, if proven, will overcome the reported inadequacies of the cost-effective mitomycin C approach as compared to gamma-irradiation technique.

EXAMPLE 3:

STIMULATION OF KERATINOCYTE GROWTH DEPENDS ON FEEDERS PROCESSED BY CONCENTRATION-DOSE PERMUTATION.

So far, it has been shown in an embryonic skin fibroblast cell line model consisting of a given population of Swiss 3T3 cells that their extinction

profile following growth arrest with a pulse exposure to mitomycin C was regulated by a strategy of simpler volumetric titrations, in which the range of volumes is broader than the recommended. It is postulated that the resultant net life-span of growth arrested feeders determines their net ratio with human epidermal keratinocytes, when both cell types are co-cultured in a medium that favors their maintenance *in vitro* and only those feeders that possess a specific extinction profile support the highest proliferation of keratinocytes. The present example describes experimental evidences demonstrating the differential ability of feeder cells growth arrested by using various concentration-dose permutations of mitomycin C to exert varied growth stimulatory effect on the human epidermal keratinocyte cells and identifying the best outcome.

Materials and Methods:

Several preliminary experiments were conducted to short-list the most effective concentration-dose permutations and identify feeder-keratinocyte cell seeding ratio for assessing growth stimulatory influence of feeders on epidermal keratinocyte cells in a co-culture system. Initial screening indicated that the feeders of 4-150 exposure maximally stimulated the 3rd to 5th passage keratinocytes, while feeders exposed to 75 ρg mitomycin C dose per cell produced a keratinocyte stimulation that was comparable to that of 15 ρg per cell. Subsequently, out of several Keratinocyte-feeder ratios tested using feeders of 4 μg mitomycin C per ml concentration group, a ratio of 1:2 with keratinocytes and feeders seeded at densities of 7,500 and 15,000 per cm^2, respectively, was found to be optimal in which keratinocytes reached maximal growth by day 9. With the raise in feeder cell seeding density from 7,000 per cm^2, whose extinctions were tested in example 2,

to optimal 15,000 per cm^2, the later were additionally evaluated alone for periodical extinctions in order to check their similarity in extinction with those of the former and gamma irradiated feeders (*γ-Irr)* were further included for comparison. Therefore, the short-listed doses included for growth assessment were 15, 150 & 450 ρg per cell, each combined with concentrations of 4 and 5 μg per ml, respectively. Feeder groups of 3-10 and 10-30 were included along with *γ-Irr* feeder cells as controls for comparison. Keratinocytes used in the experiments included either the frozen 1st passage cells as received from the supplier (Genlantis) or the 3rd passage cells produced by culturing the frozen 2nd passage cells, both of which were subcultured using 15000 feeders of 4-150 group per cm^2.

Feeder performance on epidermal keratinocytes at clonal density: Colony forming Efficiency (CFE) and digital image analysis for growth area assessment were performed plating low density keratinocytes over various feeder groups in 6-well plates with each well containing 15,000 feeder cells per cm^2. CFE was performed using 250 keratinocytes of 3rd passage and feeders treated with all the short-listed permutations. Subsequently, separate experiments were performed to estimate average colony size and total growth area with 170 and 340 viable 1st passage keratinocytes plated per well, respectively, and the feeders were the best performing 4-150 along with the sub-optimal 4-15 and γ-Irr. Plates were incubated for 9 days, with change of culture medium every alternate day, fixed in 4% para-formaldehyde prepared in phosphate buffered (pH 7.2) saline for 45 minutes, stained with 1% Rhodamine B in distilled water for 30 minutes, washed in distilled water for color differentiation of areas of keratinocytes and feeder cells and air-dried. CFE comprised of counting of discrete keratinocyte colonies of 8 or more cells, expressed as

percentage of plated cells. Estimation of average colony size was based on growth area assessment and CFE, wherein small colonies of highly irregular shape containing broad, flattened and terminally differentiated cells were considered as aborted and the rest constituted the proliferative colonies. Experiments for CFE were performed in triplicate while growth area assessment was performed in quadruplicate per feeder group.

Digital Image Analysis: The stained plates were illuminated with constant light and photographed using Nikon field camera producing digital images having a resolution of 9×10^4 pixels per $inch^2$. The images were subjected to image analysis using Adobe Photoshop version 7 as per an earlier reported technique (Kumar and Yerneni 2009). The basic technique comprised of separate selection of similar color feature on digitized-images. The selection of pixels of desired colors wherein red and pale bluish pink colors representing keratinocytes and feeders, respectively, was a procedural selection using the magic wand tool. The selection process was manually controlled by the necessary corrective measures using options in Magic Wand palette and Select options in menu bar. After ensuring accurate color separation, the images were triplicated, out of which two were used for isolating the two colors, while the third un-treated one was referred for comparison. Once the respective colors were isolated, they were superimposed to verify the reproduction of an image similar to the original. This was followed by attainment of quantification, in terms of number of pixels within the selected red area, using the Histogram command in the Image menu. The percentage of number of red pixels out of total number of pixels of the well was calculated and area of keratinocyte growth was derived from the known total area of the well. In case of wells plated with 170 keratinocytes, the calculated area in square centimeters was divided by the total number of colonies counted in the respective well to obtain average colony size.

BrdU labelling:- Mitotic Index was estimated in slide flasks (Nunc) in which cultures were initiated with 70 viable 1st passage epidermal keratinocytes per flask containing the best performing feeders of 4-150 along with 4-15 and □-Irr at a density of 15,000 per cm^2 and incubated with change of culture medium every alternate day for 10 days. Feeder cells were selectively removed with 0.02 % EDTA and the wells were incubated in non radio-active Bromodeoxy Uridine (BrdU) for 1 hour, fixed in Cornoy's fixative, treated with 4M HCl for antigen retrieval, neutralized with 0.1M sodium Borate, incubated in primary mouse monoclonal anti-BrdU antibody (Cat No.sc-32323, Santacruz) followed by visualization of fluorescence labelled nuclei after incubating in FITC labeled anti-mouse secondary goat polyclonal antibodies (Cat No. sc-2010, Santacruz Biotechnology Inc.). Every colony from triplicated slide flasks per feeder group was differentially counted for labelled and un-labelled nuclei. Briefly, each colony was photographed in both phase contrast and fluorescence modes on Nikon Diphot 300 microscope at 20X objective using Evolution QEi monochrome camera (Media Cybernetics); multiple images were taken if the colony was larger than the field and overlapping margins were demarcated by matching the images before counting of nuclei was performed using manual tag tool of Image Pro-Express express software version 6.0.

Feeder performance on mass cultures of epidermal keratinocytes: Co-cultures were initiated in 24 well plates using 3rd passage primary epidermal keratinocytes, in the presence of short-listed feeder groups with change of culture medium every alternate day until differential cell counts performed from triplicate wells per group at days 3, 6 & 9. For differential cell collection, the cultures were first treated with 0.02 %

EDTA to selectively remove the feeder cells that were collected in keratinocyte medium followed by collection of keratinocytes in separate vials after detaching them using a 3 times diluted solution of 0.25 % trypsin, 0.03 per cent EDTA and 0.025 per cent glucose. Viable cell counts were performed in Neubauer chamber after trypan blue exclusion.

Preparation of stratified epithelium: The artificial epithelia which are equivalent of cultured epithelial autografts were prepared using the best performing feeder cells of 4-150 and the other sub-optimally performing feeders of 4-15 along with γ-Irr were included for comparison. Triplicate cultures were initiated in 6-well plates by seeding 800 viable 1st passage keratinocyte cells per cm^2 and 15,000 feeder cells per cm^2. Culture medium was changed every alternate day until confluence and Epidermal growth factor (EGF) was added to the culture medium after 48 hours. Two wells were used for histological and immunohistochemical studies, while the third was assessed for feeder cell contamination and growth in agar-methyl cellulose. The stratified epithelium from confluent keratinocyte cultures was recovered by incubating in solution containing 2 mg of Dispase per ml of keratinocyte culture medium without serum at 37^{0}C for 50-70 minutes.

Histological evaluation: The epithelia were fixed with 4% paraformaldehyde and processed for paraffin embedding by dehydrating through graded series of alcohol followed by clearing in xylene and embedding in paraffin. 5 µm thick sections of cultured epidermis were deparaffinized, hydrated, stained with Haematoxylin and Eosin and mounted in DPX after dehydrating.

Immunohistochemistry: Parallel sections were deparaffinized in xylene and hydrated through graded series of ethanol. Antigens of filaggrin, cytokeratin-10 (CK-10) and cytokeratin-14 (CK-14) were retrieved by

immersing the slides containing sections in 10mM of sodium citrate buffer for 30 minutes at 90°C followed by cooling for 30 minutes, while involucrin was retrieved by treating for 7 min at room temperature with freshly prepared 0.1% trypsin and 0.1% $CaCl_2$ in Tris Buffered Saline (TBS). Sections were subsequently washed with TBS, blocked with 2 % of normal goat serum (SC-2043) for 1 hr at 37°C in a humidified chamber and incubated overnight at 4°C in mouse monoclonal primary antibodies (Santacruz Biotech) against filaggrin (SC-25896), Involucrin (SC-21748), CK-10 (SC-51581), CK-14 (SC-58724) diluted at a ratio of 1:50. Sections were then rinsed with TBS before incubating for 1 hr at 37°C in FITC-tagged goat anti-mouse IgG secondary antibody (SC-2010) diluted at 1:100 followed by mounting in DAPI containing medium (SC-24941). Negative controls were equivalently processed except for incubating in plain TBS in place of primary antibody. Normal human skin processed similarly served as positive control.

The third confluent keratinocyte culture was disaggregated using a 3 times diluted solution containing 0.25 % trypsin, 0.03 per cent EDTA and 0.025 per cent glucose and the cell suspension was used for assessing the feeder cell contamination and growth of keratinocytes in agar methyl cellulose.

Feeder contamination: The isolated keratinocyte suspension from the 3 feeder groups was assessed for both (a) non-proliferative and (b) proliferative feeder cell contamination by Hoechst staining.

(a) **Non-proliferative contamination**: The cell suspension generated by the three feeder groups was plated in 60 millimeter dishes without feeders at a density of 5000 cells per dish; cells were allowed to attach overnight by incubating in KGM and number of feeder cells were counted after Hoechst staining.

(b) **Proliferative contamination:** The trypsinized cells were plated in T25 flasks at a density of 50,000 cells per flask and incubated in KGM for a period of 3 weeks and processed for Hoechst staining.

Hoechst staining: Cultures at the end of respective incubation periods were fixed in a chilled solution of three parts of methanol and one part of glacial acetic acid, stained with Hoechst 33258 (Sigma, H-6024) at a concentration of 0.125 µg per ml of Hank's balanced salt solution, in the dark for 10 minutes. The dishes were washed with distilled water and observed in a fluorescence microscope (Nikon Diaphot 300) fitted with excitation filter of 330–380 nm and emission filter at 420 nm. 3T3 cells were distinguished from Keratinocytes on the basis of their different nuclear size, morphology and fluorescence pattern (Alitalo et al., 1982).

Transformation assay: 5 X 10^4 isolated cells from trypsinized confluent keratinocyte cultures grown in presence of the three feeder groups were suspended in Methyl cellulose (Methocel, Sigma-Aldrich). Methyl cellulose was prepared at a final concentration of 0.8% in 3T3-CBS medium, poured over a base of 0.6% agar in 35 millimeter dish, incubated at standard culture conditions and examined under Nikon inverted phase contrast microscope after 2 weeks to look for keratinocyte growth.

All experiments were performed using keratinocytes from two separate lots, each originating from human subjects of a 53 years old female (abdominal skin) and a 54 years old male (facial skin), respectively.

Statistics: For extinction of feeders plated at a density of 15,000 per cm^2, column graphs were constructed in which each cluster of columns

represented the specified permutation and each column represented viable cell numbers at the specified time points. Clusters of 3-10 and 10-30 or γ-Irr feeders were compared with other dose permutations by Students t test after pairing the corresponding time points.

Experimental groups for CFE, growth area assessment and non-proliferative feeder contamination involving 4-15 and γ-Irr were statistically compared with 4-150 by Student's t test and considered as significant, if $p<0.05$. Separate dot plots were constructed to represent total number of cells and percent of BrdU positive cells per each colony in every feeder group. Variance in distribution of BrdU positive cells among colonies of the three feeder groups was analyzed with Kruskal-Wallis H test and comparisons between two feeder groups were undertaken by Mann-Whitney non parametric U-test, while the overall BrdU positivity between two groups of feeders was tested by Chi square.

For feeder extinctions column graphs were constructed in which each cluster of columns represented the specified permutation and each column represented viable cell numbers at the specified time points. Each cluster of 3-10 and 10-30 was compared with every dose permutation by Students t test after pairing the corresponding time points. Similarly, mass growth assessment studies involving high density epidermal keratinocytes, periodical cell counts were plotted in clustered column graphs with each cluster representing the specified concentration-dose permutation of mitomycin C used for feeder cell growth arrest and each column represented mean cell number at the

specified time point. Every cluster representing permutations of 3-10 and 10-30 or γ-Irr feeders that served as control groups was compared with tested permutations; significance of variance in their growth promoting influence was evaluated by two-way ANOVA in which the variance in between two permutations were evaluated by inputting triplicated cell counts of two independent feeder cell groups against the three time points in 2 x 3 tables. The calculated P values, when $P<0.05$, are represented in the graph to denote significant variation in between two feeder groups. The calculated critical values for the Tukey HSD test were used for post-hoc analysis to estimate the real significant difference between comparable time points of two feeder groups.

Results & Discussion:

Colony Forming Efficiency: Experiment involving the comprehensive screening of all short listed feeder groups for inducing CFE on 250 keratinocytes revealed that 4-150 stimulated the formation of highest number of colonies of 67.7±0.6 among all the tested groups which is significantly higher as compared to 56.7±2.5, 59.7±3.2, 55±2.6, 49.7±1.5, 36±3.6 by 3-10, 4-15, 4-450, 10-30, γ-Irr, respectively, while there were 60.7±1.2 colonies in 5-150 feeder group which is significantly higher to 3-10, 5-450 (53±3.6), 10-30, and γ-IRR but insignificant compared to 5-15 which influenced formation of 59.3±1.2 colonies (Figure 10). In fact, 4-150 produced highly significant ($P<0.001$) stimulation than 5-150; hence subsequent experiments on growth area included appraisal of 4-150 in comparison with 4-15 and γ-Irr, which are considered as standard feeders.

CFE & Digital Image analysis: A complete color separation was possible in Rhodamine B stained preparations using Adobe Photoshop-based

image analysis, since this staining resulted in two distinct shades of color, viz., red, representing keratinocyte growth and bluish grey, depicting feeder area (Figure 11). The color selection was found to be foolproof as the superimposed merge image of the two isolated color images revealed a near perfect match with the untreated original image in all the cases.

At first, CFE with a clonal density of 170 viable keratinocytes of frozen 1st passage supplied sample was evaluated, so as to accomplish counting of discrete colonies and image analysis to obtain average colony size in addition to total growth area. Data of pixels representing the two areas of keratinocyte growth and the back ground area of feeders and their calculated growth area are given in Table 3. The results showed that feeders of 4-150 facilitated growth of 76.7±4.11 proliferative keratinocyte colonies, which is significantly different ($P<0.01$) as compared to 53.5±4.12 and 61.25±4.57 in 4-15 and γ-Irr, respectively; (Figure 12 A). It is interesting to observe that number of colonies in γ-Irr was significantly higher than in 4-15. On the other, the occurrence of aborted colonies was more or less similar in all.

The image analysis of total growth area revealed that 4-150 produced largest keratinocytes growth area of 1.109±0.1 cm^2 and is significantly higher than in 4-15 (0.613±0.05 cm^2) and γ-Irr (0.542±0.1 cm^2), but the difference in between these less performing groups was not significant (Figure 12 C and Table 3), reflecting differences in colony size. This became evident after dividing the growth area by total number of colonies, revealing significantly higher average colony size of 1.009±0.05 in 4-15 than 0.787±0.11 mm^2 in γ-Irr, but 4-150 remained superior among all with an average colony size of 1.335±0.18 mm^2 (Figure 12 B and Table 3).

Lastly, the experiment to assess growth stimulating potential of feeders on 340 keratinocytes plated per well which is higher than clonal density revealed a reproducibly higher keratinocyte growth area of 2.69±0.21 cm^2 with 4-150 feeders than 4-15 and γ-Irr feeders which yielded 1.28±0.29 cm^2 and 1.96±0.38 cm^2, respectively (Figure 13). But, growth area produced by γ-Irr was significantly larger than 4-15 which is in contrast to the insignificant outcome when clonal density of 170 keratinocytes were plated per well. This appears to be the consequence of cumulative growth from coalescing of high number of smaller colonies resulting from a high plating density of 340 cells per well, because, the frequency of colony formation per total plated cells is proportionately dependent on the plating density of keratinocytes, exhibiting higher the plating, higher the frequency and expectedly higher the differential because of increased resolution.

BrdU labeling studies: The overall distribution of either cellularity or BrdU labeled keratinocytes across colonies was not significantly varied among the tested feeder groups (Figure 14). Nevertheless, the percent of BrdU positive keratinocytes in 4-150 feeders at 30.1% (3243/10790) from 27 colonies was significantly ($P<0.01$) higher than 4-15 which revealed 20.5% (2000/9754) positivity from 20 colonies (Figure 15). This elevation appears to be contributed by the additional colony formation in 4-150 resulting in significant ($P<0.03$) distribution of labeled cells per colony in this group (Figures 15 and 16). On the contrary, the percent of labeled cell number in γ-Irr was found to be 28.9% (3270/11321) from 21 colonies and was not significantly different from that in 4-150, but at the same time, γ-Irr was found to be significantly superior to 4-15. The

results indicated that it was the stimulation of colony initiation rather than increase in mitosis per colony that turned out to be the advantageous consequences of fine-tuning of mitomycin C treatment by including both concentration and dose per cell. It is apparent that feeders of 4-150 were superior to 4-15 in inducing a higher BrdU label, in spite of absence of significant variation in average cellularity per colony, indicating that an overall high cell turn over would eventually set in. The importance of dose titration of feeders with mitomycin C is further highlighted through improvement of the otherwise sub-optimal performance to match with that of γ-Irr feeders in achieving faster growth of keratinocytes.

Feeder performance on mass cultures of epidermal keratinocytes

Feeder cell disintegration analysis following initial plating of 15,000 cells per cm^2 from feeder groups of the short-listed permutations revealed differential dose dependant cell extinction patterns that were comparable to that observed after plating with 7,000 feeders per cm^2 (Figure 17). The time point matched inter group comparisons in between various MMC treated feeders under each of the concentrations of 4 μg per ml (Figure 17A) and 5 μg per ml (Figure 17B) showed significant differential. Furthermore, the cell death in γ-Irr feeders was significant and the most rapid compared to any of the MMC feeders.

The keratinocyte growth experiments performed as co-cultures with the main short-listed permutations along with controls of MMC feeders proved that 4-150 was the only feeder group that produced significantly higher keratinocyte growth than the MMC control groups. The most intriguing point is that the influence of controls of 3-10 and 10-30 were

comparable to 4-15 and 4-450, respectively and both pairs exhibited equivalent potential, where as none of the varied dose MMC permutations with 5 μg per ml produced such variation (Figure 18). In a more or less similar fashion, the feeders of 4-150 were significantly superior not only among the other MMC groups, but also to γ-Irr feeders while such a trend was not repeated with permutations of 5 μg per ml (Figure 19). The only similarity was that feeders of 5-15 produced a moderately higher stimulation than the γ-Irr feeders but this significant difference ($P<0.02$) was apparent only on the last day 9 with $P<0.01$, as indicated by post-hoc analysis in contrast to significant difference on both days 6 and 9 in comparison to 4-15 ($P<0.001$). As a whole, the co-culture using 4 μg per ml MMC feeders, were superior to those of 5 μg per ml. The subsequent comparisons made among control feeders of 3-10, 10-30 and γ-Irr revealed insignificant differences in periodical growth output (Figure 20) which points out that γ-Irr feeders were equivalent to the sub-optimally performing feeders of 3-10 and 10-30.

Stratified epithelium: Generally, Keratinocyte colonies of 2 to 5 cells appeared in the well by 2nd day in cultures. After one week, large keratinocyte colonies with specific areas of differentiation appeared. Keratinocyte colonies eventually coalesced and the confluent cultures gave rise to stratified squamous epithelium in 10-13 days which were isolated from culture surface by dispase treatment. The isolated epithelial sheets contracted to about ¾ the size of the well, appeared as thin, fragile, semi- translucent, free-floating tissues (Figure 21). Preliminary experiments with a constant feeder density of 15,000 cells per cm^2 and 1st passage epidermal keratinocytes of 400, 800 or 1700

viable cells per cm^2 which were confluent in 13, 12 & 10 days, respectively, proved that the intermediate density produced epidermis of a more or less uniform thickness and integrity. Hence, epithelia from this 800 seeding group grown in presence of feeders of 4-15, 4-150 and γ-Irr were analyzed for histological characteristics.

Histological characterization: The H & E stained paraffin sections revealed that the epidermal constructs grown over feeders of 4-150 uniformly presented the basal and the differentiated compartments with more or less even stratification, where as the epidermal thickness resulting from its growth in presence of either γ-Irr or 4-15 MMC feeders was highly varied from one cell to 8 cells (Figure 22). Particularly, the sheets from γ-Irr group were very inconsistent with intermittent regions of completely differentiated segments in between zones of good thickness showing well formed basal compartment.

All the four markers were immunohistochemically demonstrated with their specific staining pattern in normal human epidermis (Figure 23). The distribution of cytokeratin-14 was not restricted to the basal compartment as in native epidermis but present throughout the cultured epidermis in all the feeder groups (Figure 24). On the other hand, there were feeder group dependent differences in the presentation of involucrin; those grown in presence of 4-150 MMC feeders exhibited a well demarcated and uniform distribution in the less nucleated supra-basal zone, but 4-15 and γ-Irr groups displayed its poorly demarcated non-uniform distribution (Figure 25). The major deviation with normal human epidermal distribution of markers was with cytokeratin 10 (Figure 26) and filaggrin (Figure 27) which were conspicuously absent in cultured epithelia.

Feeder contamination:

(a) **Non-proliferative contamination**: The disaggregated cultured epithelia grown in presence of feeders of 4-15, 4-150 and γ-Irr revealed 0.46±0.03, 0.54±0.04 and 0.56±0.09, respectively as percentages of attached 3T3 cells out of 5 X 10^3 plated cells (Figure 28), which worked out to be 3.3±0.2, 3.5±0.3 and 3.6±0.6, respectively, as percentages of feeder contaminants out of the total cells attached and the rest were keratinocytes. The data showed no significant differences.

(b) **Proliferative contamination:** The Hoechst stained preparations in T25 flasks after 3 weeks of incubating the disaggregated cell suspension containing 5 X 10^4 cells from epithelial constructs showed replicated keratinocytes characterized by the presence of relatively small nuclei with smoothly diffused chromatin, but did not reveal any proliferative feeders which contained large nucleus with coarsely aggregated chromatin (Figure 29).

(c) **Transformation assay:** None of the 5 X 10^4 isolated cells from trypsinized confluent keratinocyte cultures grown in presence of the three feeder groups showed any growth in Methyl cellulose after 2 weeks of incubation.

Primarily, those MMC concentrations which are closer to the normally employed volumes (doses) alone appeared to modify the mass growth supporting ability of feeders in a concentration dependant manner with middle order concentrations of 4 and 5 μg per ml doing better than the rest. This is comparable to the reported optimal performance of those feeders that were exposed to intermediate durations and both higher and lower exposure durations were sub-optimal (Zhou et al 2009). Subsequently, upon further titration with arithmetically derived doses at

such concentrations, again the intermediate dose of 150 ρg per cell was the optimal, while the lower 15 and higher 450 were suboptimal and comparable to lower and higher concentrations, respectively, yielding further improved results by fine-tuning action of doses. Further, the superior growth potential of 4-150 feeders on keratinocytes plated at clonal density clearly demonstrated their outperformance achieved by dose titrations. The most intriguing point of this investigation has been the least performing γ-Irr feeders, as evident by their exhibition of mass growth support which was equivalent to the sub-optimally performing concentrations of a lower 3 μg per ml and a higher 10 μg per ml and at low clonal density plating γ-Irr feeders turned out be even poorer performers.

Most of the early literature mentioned the use of low concentrations of MMC in the range of 4-5 μg/ml (Barlogie and Drewinko 1980; Watt 1984; Blacker et al 1987) as effective, which over the time was gradually raised to concentrations as high as 10 μg per ml and it appears that this shift could have been influenced by the sporadic exhibition of re-growth of treated cells for which no positive solution was offered (Connor 2000). Although sensitivity to MMC was reportedly dependent on feeder cell type (Ponchio et al 2000), we suggest from our investigation that prior to qualifying the feeder cells for growth arresting, the cell banks should be established through selection of an appropriate sub-culture protocol which should be simultaneously tested for absence of resistance to mitomycin C.

The most important outcome of this investigation on feeder cell titrations vertically with various concentrations of MMC and also subsequent

lateral titrations with arithmetically derived doses is that the degree of feeder cell extinctions proportionately rose with not just concentration but also dose, which is largely ignored so far (Schrader, 1999; Connor 2000; Ponchio et al 2000; Roy et al, 2001; Nieto et al 2007; Fleischmann et al 2009; Zhou et al 2009 & 2014). An experimental approach to derive such effective doses has also been demonstrated (Figure 1) along with validation of such doses (Figure 2). Further on, the results of co-culture experiments are coherent with the postulation that highest proliferation of keratinocytes which was determined by a preferred MMC dose used to growth arrest the feeder cells, would depend on the net resultant ratio between regressively depleting feeders and progressively growing human epidermal keratinocytes. Such a ratio is a well identified key growth regulating factor *in vitro* (Sun et al 2009; Zhou et al 2009; Jubin et al 2011). Accordingly, if such a ratio was to prevail maintaining higher net feeder cell density, as in case of lower doses like 4-15 due to slower extinction, the keratinocytes would be left with less space on 2-D culture surface resulting in their hindered growth. Similar lowering of growth is also likely to occur, if feeder cell number would fall short of minimum necessary to support the growth of keratinocytes, due to their faster depletion as with high dosing such as 4-450.

Therefore, it is more likely that 4-150 could have worked as an ideal intermediate dosing producing maximal keratinocyte proliferation through balancing of feeder extinction rate in a way to achieve optimal net ratio. Such a fine tuning approach, while still retaining a low and safe treating concentration of 4 μg per ml possessed high resolution in terms of achieving a narrow but significantly varying range of feeder cell extinctions, which may not materialize by just concentration modulation

and is scientifically unknown in the public domain. Thus, this novel process ultimately identified a comparatively faster and economical method of culturing human epidermal keratinocytes for both clonal and mass cultivation without compromising with qualitative characters of cultured epidermis.

WE CLAIM:

1. A culture system for the growth of epidermal keratinocyte stem cells comprising,

a. processing the feeder cells economically with Mitomycin C; and

b. providing a substratum in the form of such feeder cells;

c. achieving better growth stimulation of epidermal keratinocyte stem cell growth than γ-irradiated feeder cells;

characterized in that the optimal concentration of mitomycin C is combined further with respect to the dose per cell to derive a range of concentration-dose permutations, where the concentration of mitomycin C is in the range of 3 to 10 μg per ml and the dose is in the range of 15 to 450 ρg per cell.

2. The culture system as claimed in claim 1, wherein, the dose of mitomycin C is per cell which is in addition to concentration per ml.

3. The culture system as claimed in claim 1, wherein the adult stem cells are epidermal keratinocyte stem cells and the feeder cells are any feeder cell.

4. The culture system as claimed in claim 1, wherein the concentration of mitomycin C is preferably 4 μg per ml and the dose is 150 ρg per cell.

5. The culture system as claimed in claim 1, comprising (a) exclusive culture medium for feeder cells which consisted of DMEM with 10% v/v donor calf serum and 17.86 milli molar sodium bicarbonate and (b) keratinocyte culture medium which consisted of DMEM and Ham's F-12 at 3:1 ratio, 10 percent (Volume/Volume) Fetal Calf Serum and 10 μg ciprofloxacin, 5 μg of insulin, additional 110 μg L-Glutamine, One μg of dexamethasone, 24.32 μg of adenine, 20 μg of L-serine, 0.4 μg of

hydrocortisone, 10 ηg of Choleratoxin, 1.346 ηg of Tri-Iodo-thryronine and 5 μg of Transferrin, 10 ηg of Epidermal Growth Factor (added in the medium only on day 2), each of which are per every ml of growth medium.

6. A process for the controlled growth arrest of feeder cells comprising of exposing a range of feeder cell densities to different concentrations of mitomycin C, following which the doses were calculated from the following formula:-

$$\Delta = C_{\upsilon/\Sigma}$$

Where Δ = dose / cell (pg/cell or μg/million cells)

C = concentration of MMC (μg/ml)

υ = volume of treating solution (ml) which is kept constant

Σ = exposure cell number (millions),

predicting a range of probable volumes of mitomycin C solutions from a list drawn on the basis of dose per unit cell number and concentration per unit volume by the following formula:-

$$\upsilon = \frac{\Sigma \Delta}{C}$$

where

Σ = exposure cell number (in millions) is kept constant in a way to prevent inclusion of mitomycin C resisting variants

inducing significant dose dependent differential in cell disintegration,
subjecting a constant density of feeder cells to expose to the listed range of volumes within the listed concentrations and identifying the feeder cell batches exhibiting distinct cell disintegration trends,
identifying an optimal feeder batch, growth-arrested by a specific volume of the specific mitomycin C concentration, which supports maximal

growth of keratinocytes as compared to *γ-Irr* feeders in an vitro co-culture system.

7. The process as claimed in claim 6, wherein the feeder cells are any feeder cell, preferably Swiss 3T3 feeder cells.

8. The process as claimed in claim 6, wherein the mitomycin C concentration is in the range of 3 to 10 µg per ml.

9. The process as claimed in claim 6, wherein the dose of mitomycin C is in the range of 15 to 450 pg per cell.