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# FACTORS AFFECTING MAINTENANCE OF KERATOCYTE PHENOTYPE FOR TISSUE ENGINEERING

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## ABSTRACT

In the cornea, the corneal stroma is the middle layer which represents 90% of its total thickness and has an important role in its refractive behavior. The keratocytes are specific stromal cells especially responsible for the transparency maintenance of the cornea. Faced with an injury, they may lose their differentiated phenotype, transforming into dedifferentiated repair phenotypes (fibroblasts and myofibroblasts) which can produce a fibrotic scarring, causing loss of transparency of the cornea. Tissue engineering aims at developing substitutes to restore the function of a specific tissue that is damaged and the restoration of the cornea. The use of corneal substitutes requires the determination of optimal culture conditions for maintaining the differentiated phenotype of keratocytes. Recent studies reviewed in this paper showed that various factors affecting the *in vitro* maintenance of differentiated phenotype of keratocytes, including: use of serum, calcium concentration, several growth factors (i.e., EGF, FGF-2, insulin,



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etc.), cell density and the substrate where they are grown (in culture plates, amniotic membrane, or spheroid's cultures). We proposed calcium-dependent TGF-β signaling pathways that mediate the transformation of keratocytes to its dedifferentiated repair phenotypes, regulating the effect of other agents that influence this change. The understanding of this mechanism can contribute to improve the tissue engineering development in this area.

**KEY WORDS**: keratocytes, tissue engineering, TGF-beta, calcium, amniotic membrane.

## INTRODUCTION

The cornea is a highly organized tissue with very particular characteristics which allow the well-functioning of the eye. Its regular and uniform structure, made of collagen fibers of thin diameter, as well as the absence of blood vessels, provides transparence to this structure. Additionally to its optical function, it is also able to provide protection to the intraocular contents due to its resistance to abrasion and its important regeneration ability (1). In human, five layers constitute this complex tissue, of which corneal stroma, represents 90% of the total thickness. The corneal stroma is built by an extracellular matrix which is in itself, formed by a dense net of collagen fibrils and proteoglycans, produced by keratocytes, the main mesenchymal cell from the stroma (2). In the last 20 years, the study of the differentiation processes of keratocytes has aroused great interest, due to its primary role in the transparence of the cornea. In case of injury, keratocytes may lose their original phenotype and dedifferentiate to divergent phenotypes: fibroblastic and myofibroblastic. This phenotype change acts against corneal transparence and consequently against its functioning. Additionally, construction of tissue engineered corneal equivalent could be an alternative to cornea transplant, avoiding the lack of enough donors. In order to develop artificial corneas using tissue engineering, it is necessary to identify the factors that influence both, the maintenance of differentiated phenotype of keratocytes and the proliferation of these cells, *in vitro.* The purpose of this paper is to make an extensive review of the various factors related with the maintenance of keratocytes phenotype when they are grown *in vitro*.

## PHENOTYPIC CHARACTERISTICS OF KERATOCYTES *IN VITRO*

### Morphology, motility and protein expression



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The phenotype of differentiated keratocytes obtained in culture is characterized by a dendritic morphology, similar to that of keratocytes *in situ*, which show reduced proliferative potential (3) and can develop intercellularnetworks (4). Additionally, these cells havepoor motility which is associated with the expression of type I collagen specific integrins, the lack of smooth muscle actin (αSMA) expression, and a poor organization of actin filaments around cell membrane (5).Cornea maintains high levels of keratan sulfate proteoglycans (KSPG), which are produced by keratocytes in their native state. The most abundant KSPGs in the cornea are lumican (represents 50% of corneal KSPG and is widely distributed) and keratocan, which is synthesized only in the corneal stroma (6-8). This specificity of keratocan allows it to be the most reliable marker, known up to this time, for the identification of keratocyte's differentiated phenotype. On the contrary, the proteoglycans which contain dermatan sulfate, are present in low quantities in keratocytes with native phenotype; when the ratio KSPG/DSPG decreases, it means that the differentiated phenotype is been affected[9]. Other proteins pointed as possible markers for this cell type are the aldehyde dehydrogenase (ALDH) and $CD34^+$ (8, 10).

**Repair phenotypes of corneal stroma cells**

When damage occurs, keratocytes may lose their differentiated phenotype, changing to their repairphenotypes, fibroblastic or myofibroblastic (Figure 1), both of them participate in thenormal wound healing process (5). This transition of keratocytes to repair phenotypes or "activated" phenotypes, takes place after another subpopulation undergo apoptosis. At about 6 hours after the injury, activated keratocytes lose their quiescence, and migrate to the damaged area; increase their size and number of organella, and begin to change their morphology (11). The dedifferentiated repair fibroblasts phenotypes have their origin in the phenotypic transformation of keratocytes found in the surroundings of the damaged area. Simultaneously, appear myofibroblasts, another cell type which has been related with the wound contraction (characterized by higher level of the expression of αSMA), while fibroblasts can remodel the matrix by the production of proteins as the metalloproteinases, which are responsible of the matrix degradation (Figure 1) (5). Despite there is a well described pattern of morphology and protein expression for fibroblasts and myofibroblasts, there are evidences pointing that none of them are terminally differentiated phenotypes, since they can revert one into the other in response to growth factors or to culture cell density (12). A fibrotic scar of cornealwound represents



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an important physiopathological problem which may become into retinal detachment, glaucoma, secondary cataracts and retinal opacification as a result of the transformation of keratocytes into its dedifferentiated repair phenotypes (3).

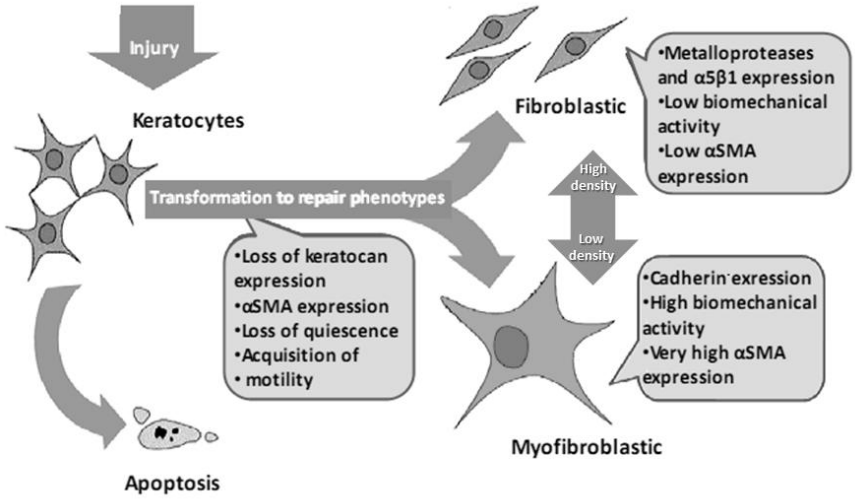


**Figure 1. Schematic representation of the main phenotypic characteristics of keratocytes and their dedifferentiated repair phenotypes, fibroblast and myofibroblast, which can switch one into the other in response to changes in the culture density.**

## Factors affecting the maintenance of keratocyte's differentiated phenotype

Once Conrad *et al.*(13) demonstrated thatcorneal stroma cells cultivated *in vitro* have a reduction of the keratan sulfate synthesis, many studies have aimed to define the ideal culture's conditions for maintaining a phenotype similar to that seen on keratocytes *in vivo (*14). These conditions have been associated with factors of different nature as described below.

## Effect of the use of fetal bovine serum in the culture medium

Fetal bovine serum (FBS) is a complex mixture of salts, proteins, lipids, growth factors and hormones. Keratocytes, isolated from rabbit's corneal stroma, and cultured with FBS, show fibroblastic and myofibroblastic phenotypes (4, 8) ; likewise it has been found that the use of serum inhibits the expression of KSPG and some marker molecules of keratocytes phenotype such as keratocan, ALDH and $CD34^{+}$ (10,



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15-17). Recently this transforming effect has been observed over limbal and scleral stromal cell cultures, finding a higher proportion of myofibroblasts in scleral cultures (18). Kirschner*et al.(*19) found that in a serum free medium (SFM), keratocytes maintain their dendritic morphology and their quiescent condition while, Beales*et al. (*4) using the same conditions found that the keratocytes express five times more KSPG than those grown in medium with FBS. Additionally, He and Bazan (20) reported that keratocytes grown in SFM are able to maintain their phenotype for over a month, but show a reduced proliferation rate.

## EFFECT OF SPECIFIC CYTOKINES AND GROWTH FACTORS:

**Platelet derived growth factor (PDGF):** It has been demonstrated that human keratocytes, as well as corneal epithelium and endothelium cells, synthesize and express PDGF. The expression of this factor by epithelial cells has been related with the activation of quiescent keratocytes, inducing their proliferation and migration during wound healing (3). It has been found that the presence of PDGF in the culture medium, in bovine and rabbit keratocytesstimulates the contraction of collagen gels and the dedifferentiation to repair phenotypes (3, 8, 17, 21). Jester *et al. (*3) found that this dedifferentiation process to fibroblasts is blocked by neutralizing PDGF using specific antibodies, in a medium containing TGF-β

**Transforming growth factor (TGF- β)**

TGF-β is a cytokine secreted by almost all nucleated cells, which is involved in many aspects of cellular behavior, and has a role in many processes like proliferation, differentiation, wound healing, senescence and apoptosis. It is present in wounded and pathological corneas, since it is secreted by epithelial cells upon an injury. It has been reported to stimulate the proliferation of keratocytes (15). TGF-β is known as a strong inductor of keratocytes dedifferentiation to myofibroblasts, even in a serum free medium. The specific effect has been confirmed by the inhibition of the transformation, when blocking with antibodies anti-TGF-β (22, 23). This transformation induction is associated with the fact that TGF-β promotes the expression of αSMA, a characteristic protein of myofibroblasts, one of thededifferentiated repair phenotypes, (2, 11). Likewise, this growth factor is able to activate the expression of biglycan, which seems to be an important factor in fibrotic scarring; it also reduces the KSPG levels, as well as it causes an increase of DSPG, and the consequent reduction of the ratio KSPG/DSPG, typical change observed in



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dedifferentiated repair phenotypes (15). At concentrations as low as 100 pg/mL, TGF-β causes the loss of dendritic morphology and the loss of CD34 expression (10).

Jester *et al*. (3, 17) studied the possible synergic actions among TGF-β, PDGF and the integrins, using peptides which block the integrin binding to extracellular fibronectin, and using specific antibodies to neutralize PDGF or TGF-β. They found that the blockage of any of these factors avoids the differentiation to myofibroblast, inhibiting the expression of αSMA and the formation of focal adhesions. These results suggest that TGF-β induces transformation to myofibroblasts but requires synergic interactions with integrins and several growth factors.

Likewise, the blockage of matrix assembly and stress fiber formation causes the inhibition of the TGF-β-induced αSMA expression (23). In those studies, there is a positive correlation between the activation of TGF-β signaling and the loss of differentiated phenotype, confirmed by the loss of dendritic morphology and a dramatic reduction of keratocan and CD34 expression (24, 25).

All these evidences pointed that TGF-β plays a central role not only in the transformation to myofibroblast but, in general, in the whole process of loss of differentiated phenotype and the substitution for the dedifferentiated repair phenotypes (Figure 2).

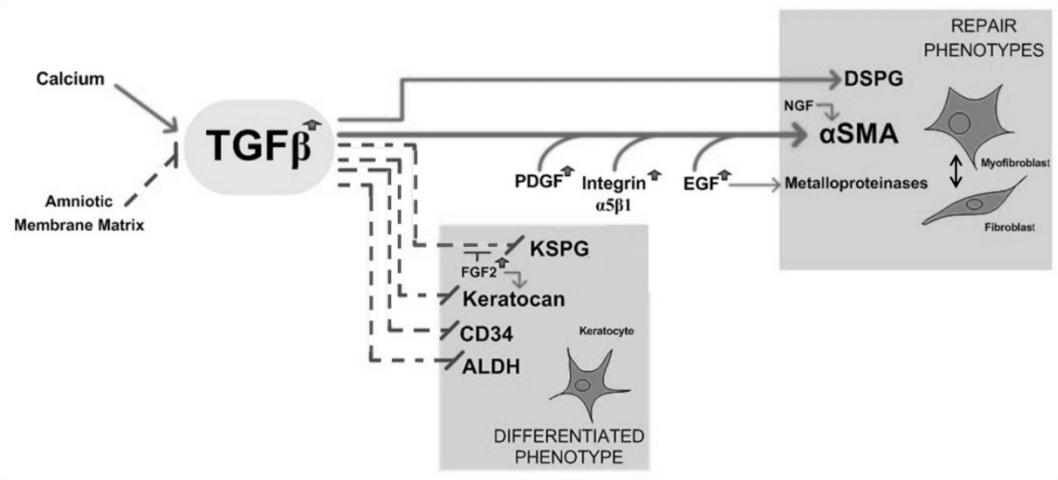


**Figure 2. Schematic representation of the interaction between TGF-β and other factors that affect the protein expression in the different corneal stromal cell phenotypes. TGF-β signaling is favored by high concentrations of calcium and is inhibited when the keratocytes are grown on amniotic membrane matrix. TGF-β down regulates the expression of the differentiated phenotype proteins (inhibition is**



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**indicated by dotted line): keratan sulfate proteoglycans (KSPGs), keratocan, CD34$^{+}$andaldehyde dehydrogenase (ALDH), while resulting in an increaseof thequantitativeexpression(induction is indicated bycontinuous line) of dermatansulfateproteoglycans(DSPG) and-smooth muscle actin (αSMA), both markers of dedifferentiated repairphenotypes. Other factors affect the expression of αSMA, either independently (neural growth factor -NGF) or synergistically with TGF-β (platelet derived growth factor -PDGF, Integrin α5β1, and epidermal growth factor - EGF). Additionally, EGF promotes the expression of metalloproteases (characteristic of a fibroblastic phenotype). Otherwise, fibroblast growth factor 2 (FGF-2) favors keratocan expression, and counteracts down-regulationof KSPG induced by TGF-β.**

## Fibroblastic growth factor 2 (FGF-2 or basic FGF)

Long *et al.(*26) made studies with bovine keratocytes and found that the addition of FGF-2, FBS, TGF-β or PDGF, stimulates cell proliferation, but only FGF-2 is able to stimulate the secretion of KSPG and even to counteract the FBS's inhibitory effect over the KSPG secretion. They also found that this factor stimulates the expression of lumican, mimecan and, especially keratocan. Furthermore, Maltseva*et al*. (12) reported that, in activated phenotypes, FGF-2 in presence of heparin and serum, promotes the change from myofibroblastic to fibroblastic phenotype, reverting the TGF-β-induced effect on fibroblast to myofibroblast differentiation. FGF-2 and TGF- β pathways seem to converge on the regulation of SMAD proteins, having opposite effects, since FGF-2 may avoid the translocation of SMAD into the nucleus, which is required for the transcriptional activation induced by TGF- β.

## EPIDERMAL GROWTH FACTOR (EGF)

It has been reported that keratocytes have only low affinity type receptors for EGF, reason why its growth-promoting effect is weak (27). However, He and Bazan (20) demonstrated that besides the effect of EGF over proliferation it also promotes the expression of αSMA and induces the expression of proinflammatory cytokines such as interleukin-1α and interferon-gamma (IFN-γ), together with metalloproteases, a distinctive characteristic of fibroblastic phenotype. They demonstrated a synergic action of EGF and TGF- β showing that a higher percentage of keratocytes dedifferentiate to fibroblasts in cultures treated with both factors than with each one separately.

## INSULIN

Insulin is a factor present in the FBS which modulates metabolic processes, but is able to act like a mitogenic agent for some cell



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types. Musselmann*et al (*8) compared the isolated effect of insulin, FGF-2 or PDGF-BB, on bovine keratocytes, evaluating their proliferation, morphology and expression of specific markers. They found that all the factors stimulate the proliferation, but insulin cultures showed higher proliferation than the others. Western-blot analysis revealed that all the factors used allow maintaining the expression of ALDH and keratocan, but only the cultures with insulin preserve the morphology of keratocytes, while the cells grown with other factors showed a fibroblast-like morphology, demonstrating that morphology is not strictly associated to marker proteins production.

## OTHER GROWTH FACTORS

Nerve growth factor (NGF), interleukin-1α (IL-1α), and insulin like growth factor I (IGF-I) are reported to possibly have an effect on keratocytes phenotype. NGF is able to modulate the fibrogenic activity in human keratocytes *in vivo.* It was found that this factor stimulates in a dose dependent manner the migration of keratocytes, and the transformation to myofibroblasts, because it promotes the expression of αSMA and the contraction of collagen gels (28). IL-1α and TNFα, which are secreted by the overlay epithelium upon injury, have been involved with the apoptotic response of keratocytes; however, *in vitro* studies have shown that IL-1α as well as IGF-I, promote cell proliferation without altering the phenotype of keratocytes (29).

## EFFECT OF CELL DENSITY

Cell density is an additional factor which has been related with the maintenance of keratocyte's phenotype, this effect has been associated with the possible interactions between these cells and it depends on the distance between them. A high cell density allows a better communication, either by factors secreted to the medium, or by cell-cell interactions (through cadherins and conexins mediated junctions). Stem cells show an increment in cadherin levels when they differentiate to keratocytes, indicating that the differentiation to this cell type involves the formation of intercellular networks (30). Musselmann*et al. (*8) evaluated the effect of various growth factors (FGF-2, insulin, PDGF, etc.) over keratocyte's phenotype, using cultures seeded with high (20.000 cells/$cm^2$) or low (5.000 cells/$cm^2$) cellular density. Their results show a clear influence of density over the behavior of keratocytes *in vitro*. They found that, in general, high cell density cultures secreted higher amounts of ALDH and keratocan to the culture medium than low density cultures, been it more notable in the case of keratocan levels. Further,



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keratocytes cultured at low density and in absence of growth factors, showed degradation of proteoglycans secreted to the culture medium, suggesting that the presence of growth factors avoids their proteolysis. On the contrary, the proteoglycans produced in high density cultures, are not degraded in absence of growth factors, suggesting a role of cell density in the stability and accumulation of proteoglycans, probably associated with components secreted by keratocytes, which interacts with the proteoglycans in the culture medium (8).

## CALCIUM CONCENTRATION EFFECT

It has recently been reported that low concentrations of calcium in the culture medium promotes the keratocyte's phenotype (14). Kawakita*et al*. (25) found that using a low calcium serum free medium (commercial medium denominated KSFM, Gibco®) supplemented with EGF and FGF-2, could enhance the *in vitro* maintenance of keratocyte phenotype and obtain higher proliferation rates than using DMEM (higher calcium concentration) supplemented with insulin, transferrin and selenium. Additionally the same group evaluated the role of calcium in the modulation of the differentiated phenotype, considering its effect in combination with FBS. Kawakita*et al* .(25) assayed four different conditions: KSFM, KSFM+calcium, KSFM+10%FBS, and KSFM+calcium+10%FBS. They found that, in absence of FBS, the increase in the calcium concentration did not affect neither the activation of TGF-β promoter nor protein expression (keratocan, ALDH and $CD34^{+}$), but using FBS and increasing calcium concentration at the same time, can promote the activation of TGF-β promoter and reduces the expression of keratocyte´s marker proteins. These results suggested a synergic effect between calcium and FBS, on which the increase in the calcium concentration is not enough by itself to modify keratocytes phenotype, but it is able to modulate the effect of TGF-β over the cultures, meaning that a low calcium medium, plays an important role decreasing the effect of TGF-β signaling (25). This is consistent with the finding of Ryan *et al* .(31) about the expression of the calcium-binding protein S100A4, which is activated when keratocytes dedifferentiate to fibroblasts and myofibroblasts; so they hypothesize, in base of the patterns of expression and distribution, that this calcium-binding protein has a role in the transformation between keratocytes and their dedifferentiated repair phenotypes.

### Substrate culture system effect



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Extracellular matrix is able to influence the differentiation process of connective tissue cells, affecting the cell shape and its adhesionto the substrate, which can be done through physical or chemical interactions.

**Culture using plates treated to promote cell adhesion (plastic culture):**

When keratocytes are cultured in plastic (understanding by plastic, the traditionally used culture plates) in a medium with serum, these cells rapidly lose their dendritic morphology, acquiring the fibroblastic morphology. Simultaneously, they begin to express α5β1 integrins and αSMA, especially when cultures are made at low density, with an important reduction in the ratio KSPG/DSPG (6). In contrast, if the "plastic" culture is made using a serum free medium, the dendritic morphology and the ratio KSPG/DSPG are maintained. Under such conditions, keratocytes secrete lumican, keratocan and mimecan. However, because of the low proliferation rate, this serum free culture method excludes the possibility of *ex vivo* expansion and subculture (6).

**Culture using a decellularized human amniotic membrane matrix:**

The amniotic membrane, with its thin basal membrane and its avascular stromal matrix, has been used with success to treat various conditions requiring the ocular surface reconstruction (32). España*et al* .*(*6) found that keratocytes cultured over a matrix obtained from decellularized human amniotic membrane showed dendritic morphology, even with high serum concentrations (10% FBS), developing an intercellular net in a tridimensional pattern. The culture of keratocytes in amniotic membrane counteracts the decrease of the CD34 expression, which occurs in presence of serum[10]. Moreover, Kawakita*et al* .*(*24) reported that this culture substrate allows maintaining keratocan expression and dendritic morphology for 8 passages, as well as it is able to down-regulate the promoter activity of TGF-β and its receptor.

**Spheroids culture:**

Funderburgh*et al. (*33) reported that growing keratocytes in presence of insulin and FGF-2 can induce the formation of spheroids. Those structures are solid cellular aggregates (0.1 to 1 mm in diameter) spherically organized on which cells remain viable, and in some cases, it favors the maintenance of differentiated phenotype. The inmunofluorescence assays confirmed the expression of keratocan and CD34 in such cultures, in accordance to the results obtained by us. Figure 3 shows the spheroids in culture obtained from human corneal stroma using microbiological plates and



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serum-free medium supplemented with FGF-2 and insulin. Our results also confirmed the presence of significant amounts of keratocan, which is consistent with results obtained by others authors (14), suggesting the presence of highly differentiated keratocytes in these structures. In spheroids it has been detected the expression of the corneal extracellular matrix components, such as lumican, and type I, V and VI collagen, the abundance of extracellular matrix demonstrates the presence of a substrate for the cell-matrix interactions. The main difference between both culture methods seems to be the absence of a rigid substrate and the great cellular density present in spheroids[14]. Yoshida *et al .(*2) reported that they could make subcultures of mouse keratocytes in high amounts, without the use of serum, using the spheroids technique. These subcultures maintained the expression of all the evaluated markers (keratocan, lumican, CD34 and vimentin) without observable changes, even after 10 passages. However keratocan expression *in vitro* was lower than in the corneal stroma.

In the same way, the transforming effect of PDGF and TGF-β decreased in keratocytes spheroids cultured comparing with monolayer cultures. Funderburgh*et al .(*14) found that for keratocytes monolayer cultures the expression of genes involved in the transition to dedifferentiated repair phenotypes is 8 to 10 times higher than in the spheroids cultures. One possible reason for this behavior is that adherence to rigid substrates promotes expression of αSMA in response to TGF-β signaling, whereas in cultured keratocytes in spheroids it is not promoted (14). Another possible reason is that the spheroid's structure favors an extremely high cellular density, which promotes the communication between cells, favoring the maintenance of differentiated phenotype. Additionally, it has been found in monolayer cultures that over 90% of keratan sulfate is secreted into the culture medium, whilst in the spheroids cultures, significant amounts of KSPG remain associated to the cells in the extracellular matrix inside the spheroids (14). This indicates that in the spheroids occurs the secretion of extracellular matrix with a three-dimensional arrangement that resembles more the stromal tissue *in vivo.*



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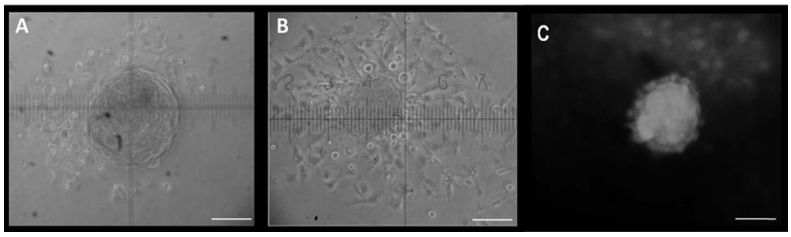


**Figure 3. Keratocytes cultures in spheroids using fetal bovine serum free medium. Cultures were carried out in DMEM supplemented by insulin and FGF-2, using microbiological plates to promote no-cell´s adhesion and formation of spheroids. a) Spheroid in culture. b) Spheroid adhering to the surface of the culture plate. There are cells migrating from the structure that showing a dendritic morphology, suggesting that cells grown in such structures preserve their differentiated phenotype. Maintenance of differentiated phenotype was confirmed by inmunofluorescence assay c) Keratocan staining using a goat anti-keratocan polyclonal antibody (Santa Cruz Biotechnology). Bar, 50 µm.**

## SUMMARY

Getting keratocytes retain their differentiated phenotype becomes a complex challenge for tissue engineering, as it depends on several variables, such as those we reviewed. We found that the recommended culture conditions are those which allow cells to maintain a three-dimensional space distribution and the establishment of cell-cell contacts, such as high cell density, culture over human amniotic membrane or in spheroids. Additionally, this review highlights the central role of TGF-β in the keratocyte transformation to its dedifferentiated repair phenotypes, proposing that it is involved in most of the pathways affecting the phenotype of keratocytes *in vitro*. We can conclude that the culture conditions which seem to be the most favorable for the maintenance of differentiated phenotype and the proliferation capability are the culture in serum free low calcium medium, spheroids or using a more complex matrix like amniotic membrane.

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