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Can insights into urodele limb regeneration be achieved with cell cultures and retroviruses?

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ABSTRACT Recent advances in the field of amphibian limb regeneration have provided insights into its cellular and molecular events. This review summarizes the development of cell lines from limb tissues and their application to the study of transdifferentiation and limb regeneration. In addition, the availability of suitable retroviral vectors for salamanders is discussed for it has opened new avenues for experimentation at the molecular level.

KEY WORDS: *amphibia, limb regeneration, cell lines, retroviral vectors*

Introduction

Historically, the field of limb regeneration has suffered because of the lack of suitable cellular and molecular tools. Newt blastema cells could not be cultured and propagated *in vitro* as stable cell lines. As a consequence, the process of blastema cell differentiation and the experimental manipulation of this process could not be readily studied. The reader should contrast this situation with the culturing of eye tissues, which has enormously facilitated the study of transdifferentiation during lens regeneration. In addition, due to our inability to culture cells *in vitro*, other experiments, such as gene transfer and genetic manipulation, have not been possible. Salamanders are also not very favorable animals for genetic manipulation. They are difficult to maintain and breed in the laboratory, inbred strains are unavailable and they have long generation times. Thus it is not possible to facilitate experimentation at the animal level and promote an understanding of the role of important genes in development as is known from other fields, such as mouse developmental genetics. In addition, lack of viral vectors capable of efficiently transferring genes of interest into the salamander genome has impeded genetic modifications in the newt. During the past few years research has however begun to yield the necessary tools, such as cell lines from the newt limb, and efficient gene transfection techniques, which will advance the field of limb regeneration.

In vitro systems

While sporadic papers on the culture of blastema cells have been published in the past, the first method to reliably grow limb cells was described by Ferretti and Brockes (1988). These investigators reported that when blastema explants from a regenerating limb or limb muscle explants are placed in culture, cells migrate out of the explants and start growing. In fact, the growth properties of cells derived from muscle explants were more impressive. Recent research identified several cell types in blastema explants. Giant multinucleated cells (osteoclasts) and signet cells (mononuclear leukocytes) are of hemopoietic origin and do not survive long in culture (Washabaugh and Tsonis, 1994). These cells appear to play no role in the differentiation process of the blastema. Another cell type is the bipolar cell, which is rare (1% of the cell population) and of unclear origin. The predominant cell type is the fibroblast-like pleiomorphic cell. This is also the only cell type that grows out of a muscle explant. It is believed that this cell type represents dedifferentiated blastema cells (Ferretti and Brockes, 1988; Washabaugh and Tsonis, 1994). These pleiomorphic cells form multinucleated myotubes during prolonged culture.

The availability of these primary cell cultures is enabling researchers to transfect cells with genes or tag them with dyes and follow their fate upon transplantation back onto an amputated limb. Such elegant experiments have demonstrated that myotubes formed by pleiomorphic cells in culture do indeed participate in the regeneration process and differentiate into muscle as well as cartilage (Lo *et al.*, 1993). These experiments revealed the potential of differentiated cells to undergo dedifferentiation and metaplasia during regeneration. Several questions can now be asked: can the growth and the biological properties of these pleiomorphic cells be altered by genetic manipulation? Can immortalized cells form stable cell lines in which different inherited properties of the cell types are manifest? Can these transformed cells differentiate upon transplantation *in vivo?* In other words, can

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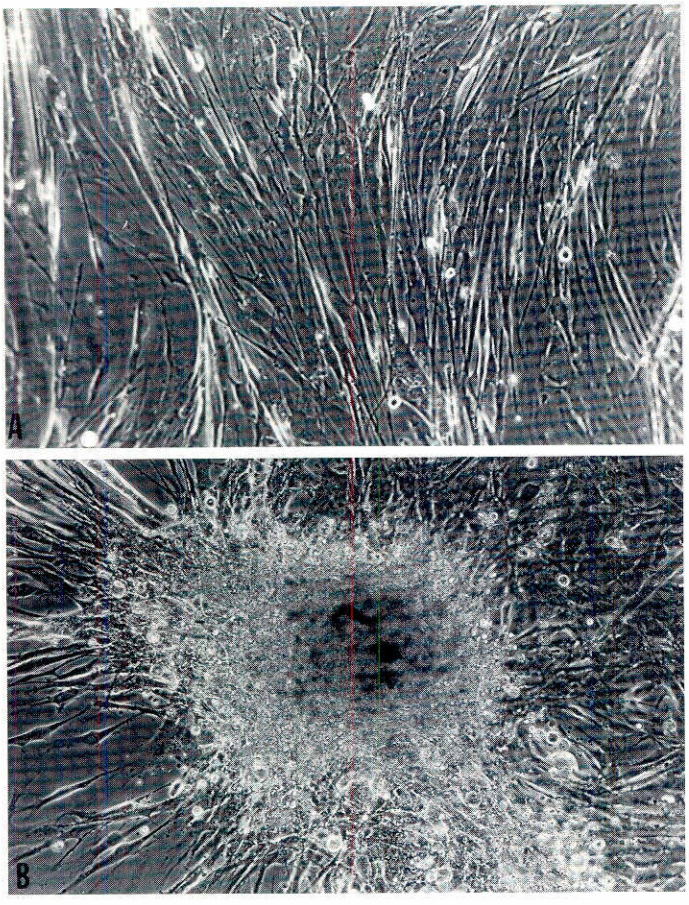

Fig. 1. Blastema cells in culture. (A) *Untransfected newt cells derived from a muscle explant and cultured for two weeks. Note that the cells have started to align and form multinucleated myotubes.* **(B)** *The same cells transformed with SV40 large T antigen and propagated in culture for four weeks. Note absence of myotube formation and the transformed phenotype with formation of a pile.*

we generate different lineages of blastema cell lines which could help identify factors which are crucial for the differentiation which characterizes the regeneration process?

Recent work with blastema cell cultures has begun to address some of these questions. Pleiomorphic cells have been isolated from muscle explants and transfected with a plasmid carrying cDNA for a dominant selectable marker and also the SV40 large T antigen. The T antigen represents a viral protein that is routinely used to immortalize cells from different tissues and species, including mammalian germ cells (Hofmann *et al.*, 1992, 1994). Subsequent selection of the transfected cells resulted in the survival of cells with a transformed phenotype. These cells, unlike their untransfected counterparts, did not form myotubes after prolonged incubation, but rather formed piles characteristic of transformed cells (Fig. 1). If differences exist between immortalized cell lines they might be exploited for identifying the factors responsible for these differences. The molecular nature of the transformation has also been examined. It was found that the plasmids were episomal and that no integration had occurred (Fig. 2). This may be an advantage for transplantation experiments, because the reporter gene is constitutively expressed independent of host genomic regulation, thus permitting detection of the plasmid gene products during regeneration. Reporter gene expression has been monitored by ß-galactosidase activity in normal cells and by immunostaining for the large T antigen in immortalized cells (Fig. 3). In both cases, high levels of reporter gene expression have been noted. In fact, the episomal plasmid is capable of expressing the reporter gene for long periods of time (up to four weeks in the transplantation experiments).

When these cell lines (normal untransfected or immortalized) were tagged with a fluorescent dye and reimplanted onto an amputated limb, they readily participated in the regeneration process. Marked cells were observed several weeks later as terminally differentiated cells in the musculature and cartilage of the regenerate (Tsonis *et al.*, 1995).

These experiments confirm the results of similar studies by Lo *et al.* (1993). Importantly, the transformed cells participated in regeneration of the limb as well as the untransformed cells, thus suggesting that immortalized cells can differentiate into different tissues of the regenerate *in vivo* despite their inability to do so *in vitro*. The current hypothesis is that the regeneration

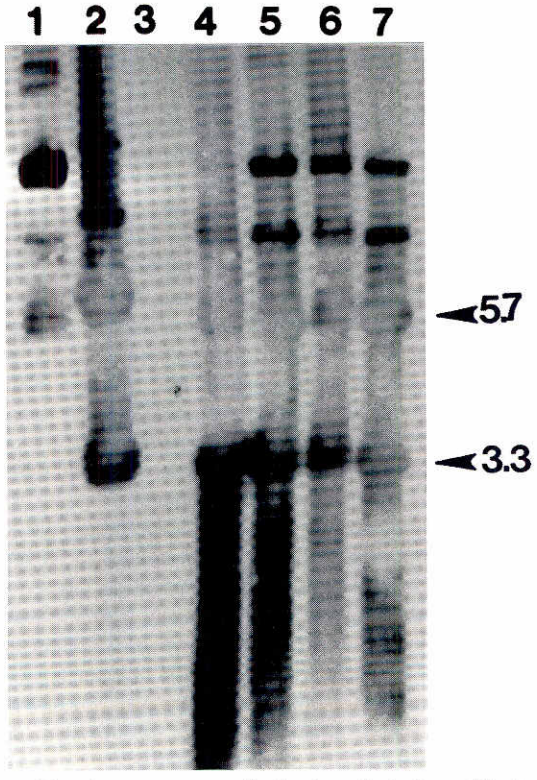

Fig. 2. Large T antigen sequences in the transfected newt limb cells. (Lane 1) *pSV3-neo plasmid, undigested.* **(Lane 2)** *pSV3neo plasmid digested with Bam HI generating a 3.3 and 5.7 kb fragment.* **(Lane 3)** *Genomic DNA from untransfected cells digested with Bam HI.* **(Lanes 4 and 6)** *Genomic DNA isolated from the two immortalized cell lines and digested with Bam HI.* **(Lanes 5 and 7)** *Hirt DNA (fraction contains only episomal plasmids) from the same cell lines digested with Bam HI. Note the presence in all DNA preparations of the 3.3 and 5.7 kb fragments as well as uncut forms of the plasmid. The similarity in the digestion profile seen in lanes 4-7 indicates that the plasmid is episomal and not integrated.*

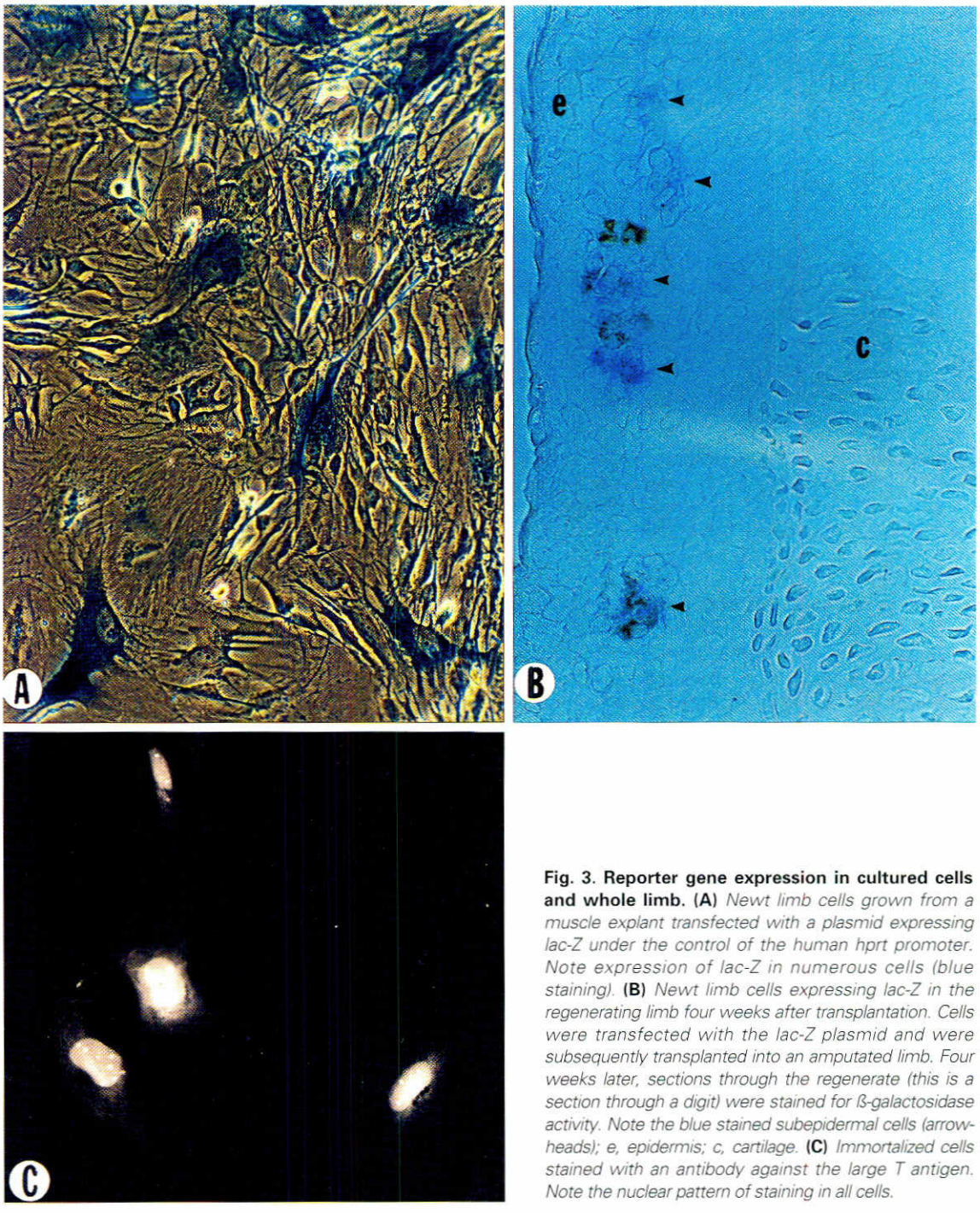

Fig. 3. Reporter gene expression in cultured cells and whole limb. (A) *Newt limb cells grown from a muscle explant transfected with a plasmid expressing lac-Z under the control of the human hprt promoter. Note expression of lac-Z in numerous cells (blue staining).* **(B)** *Newt limb cells expressing lac-Z in the regenerating limb four weeks after transplantation. Cells were transfected with the lac-Z plasmid and were subsequently transplanted into an amputated limb. Four weeks later, sections through the regenerate (this is a section through a digit) were stained for ß-galactosidase activity. Note the blue stained subepidermal cells (arrowheads); e, epidermis; c, cartilage.* **(C)** *Immortalized cells stained with an antibody against the large T antigen. Note the nuclear pattern of staining in all cells.*

environment forces the transformed cells to differentiate normally. Such experiments may provide new insights into the events leading to tumorigenesis. As has been established in the past, tumorigenesis cannot be achieved in the regenerating limb. It is believed that the transformed phenotype can be controlled under the influence of regeneration. Such a control might exist to ensure proper differentiation of the blastema cells which, like cancer cells, are dedifferentiated (Del Rio-Tsonis and Tsonis, 1992).

Gene transfer

As mentioned above transfection experiments with blastema cells do not result in stable integration of the foreign gene into the salamander genome. Thus far, no system currently exists for the development of transgenic newts. The effects of a foreign gene can however be studied *in vivo* after the cells have been transfected *in vitro* and transplanted as described above. As an alternative method, Pecorino *et al.* (1994) have utilized the biolistic

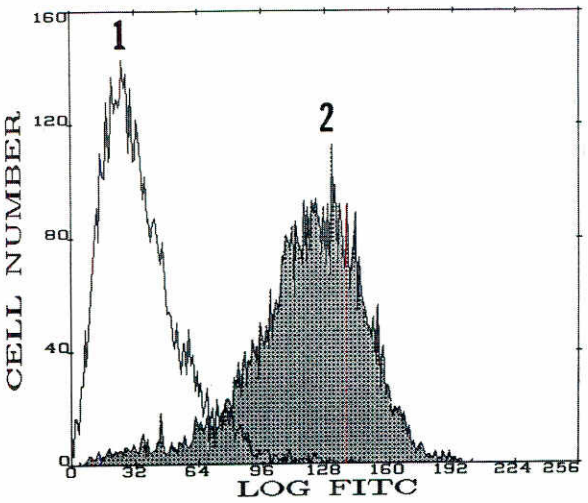

Fig. 4. Flow cytometric analysis of ß-galactosidase activity in newt limb cells. *Peak 1, mock infected cells; peak 2: cells infected with the retroviral vector LRgeoL-G. Almost 90% of cells in peak 2 lie outside the distribution of cells in peak 1, indicating reporter gene expression in these infected cells.*

particle gun to transfer plasmids into limb cells. The resulting episomal transformation has provided important information. For example, by using retinoid reporter genes, assays of the actions of retinoic acid were performed. These assay results led to the conclusion that differences exist in retinoic acid-dependent gene expression between the proximal and distal blastema. Similarly, it was found that different receptors for retinoic acid might account for different actions of this substance on cell proliferation and differentiation (Brockes, 1992; Schilthuis *et al.,* 1993). Unfortunately, such techniques cannot be used to create a truly transgenic limb. In other organisms, gene transfer mediated by murine retroviruses has been useful in creating transgenic animals. Since retroviruses enter cells through contact with specific receptors in the cell membrane, the use of retroviruses is restricted to cells that express the appropriate receptors. A new class of pantropic retroviral vectors has been developed with a broad host cell range (Burns *et al.,* 1993). In these vectors, the retroviral envelope protein has been replaced with the envelope glycoprotein of vesicular stomatitis virus (VSV-G). This protein interacts with phospholipid components of the plasma membrane, thus conferring a broad host range on the pseudotyped particles.

Future prospects

These pantropic retroviral vectors can infect, stably integrate, and express the reporter gene products in the cultured limb blastema cells (Burns *et al.,* 1994). Indeed, in one experiment activity of ß-galactosidase was detected in nearly 90% of the infected cells (Burns *et al.,* 1994) (Fig. 4). Such vectors have already been used to infect *Xenopus* and fish embryos and to produce transgenic fish (Lin *et al.,* 1994; Burns *et al.,* submitted). Clearly, the use of pantropic retroviruses holds great promise for the start of genetic experimentation with live salamanders. The role of several genes in limb regeneration and other regenerative and developmental phenomena in urodeles can now be studied *in vivo*.

While work with urodeles has posed interesting challenges for researchers, standard modern cellular and molecular techniques can now be applied to these animals. Such approaches have already shed light on mechanisms of dedifferentiation, functions of retinoic acid, and gene regulation, and will undoubtedly contribute to further understanding of regenerative phenomena and their regulation in amphibia.

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