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Short Communication

Zebrafish keratin 8 is expressed at high levels in the epidermis of regenerating caudal fin

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ABSTRACT Cytokeratins are structural proteins of the intermediate filament family and are mainly expressed in epithelial cells. In several vertebrates it has been shown that keratin 8 is expressed in simple epithelial tissues, some non-epithelial tissue and in hyper-proliferative tissues during development and tumor transformation. We previously cloned and characterised the zebrafish (*Danio rerio*) homologous cytokeratin 8 cDNA *(zfk8)* which was described as an epidermal marker during zebrafish development. It has been found that the *zfk8* gene is normally expressed in simple epithelia in embryonic and mature zebrafish. Using whole-mount *in situ* hybridisation, we show in this report that expression of *zfk8* is tightly linked to the regeneration of caudal fin and exclusively observed in epidermal cells. It is strongly expressed in the epidermis overlaying the inter-rays zone of regenerating caudal fin. Our results indicate that in zebrafish, cytokeratin 8 is a suitable epidermal marker during regeneration.

KEY WORDS: *zebrafish, fin regeneration, cytokeratin 8.*

Intermediate filaments (IF) are the most stable skeletal elements of the cell; they are typically 8-11 nm in diameter and are indirectly involved in cell movements (Osborn *et al*., 1981). Even if their exact role is not yet well known, they appear to play a structural role by maintaining cellular space (Lazarides, 1980) and providing mechanical strength to cells and tissues (Kopan and Fuchs, 1989; Steinert and Roop, 1988; Fuchs and Weber, 1994).

Cytokeratins constitute the main group of intermediate filaments. They are assembled as many as 30 different proteins ranging in molecular weight from 40.000 to 70.000 Da (Moll *et al*., 1982). These filaments are generally subdivided into two distinct groups encoded by two distinct gene families, type I and type II (Eichner *et al*., 1984). Type I proteins are generally acidic and small (40.000 – 56.500 Da), whereas type II are larger (53.000 – 70.000 Da) and more basic. They can also be classified based on their pattern of expression : keratins expressed in epidermal keratinocytes are called E-keratins, whereas keratins that are typical for cells forming simple epithelia are designed as S-keratins (Schaffeld *et al*.,1998). Human keratins are expressed only by epithelial cells in normal or hyperproliferative conditions. The specificity of expression of each gene is also mainteined during tumoral transformation. This is the reason why they are considered as efficient tumoral markers today (Ramaekers *et al*., 1981). It has been shown that the expression of different cytokeratin polypeptides changes in many vertebrates during development, adulthood, regeneration and hyperproliferation, suggesting that each one plays a distinct role in cell life (Fuchs *et al*., 1987; Kallionen *et al*., 1995). We focused our attention on cytokeratin 8 because it is the early and fundamental keratin expressed – together with k18 – during development of many vertebrates (Jackson *et al*., 1980; Jackson *et al*., 1981), and the main keratin present in hyperproliferative human cells (Moll *et al*., 1982, Franke *et al*., 1981). Keratin 8 plays a fundamental role in natural morphogenetic movements such as gastrulation (Torpey *et al*., 1992; Klymkovsky *et al*., 1992). Nevertheless there is evidence that in some vertebrates such as in mouse, a genetic deficiency of k8 gene allows a perfect gastrulation, but leads to high lethality at the neonatal stage (Brock *et al*.,1996). It is also known that keratin 8-deficient mice develop a severe disease of the gastrointestinal tract mainly characterised by colorectal hyperplasia and inflammation (Loranger *et al.* 1997), suggesting a basic role of this keratin in cell life.

Human k8 is a II S-type keratin normally expressed only in the tissues derived from endodermic sheet such as simple epithelia and hepatocytes. It is also expressed at high levels in many

Abbreviations used in this paper: IF, intermediate filaments; RA, retinoic acid; zfk, zebrafish keratin.

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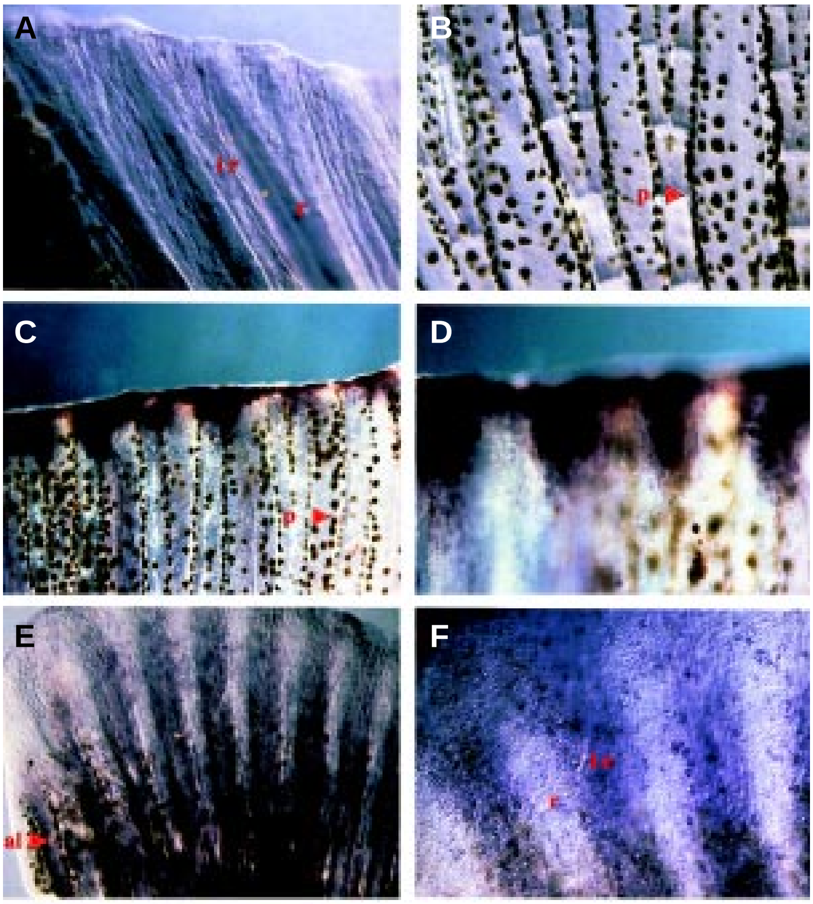

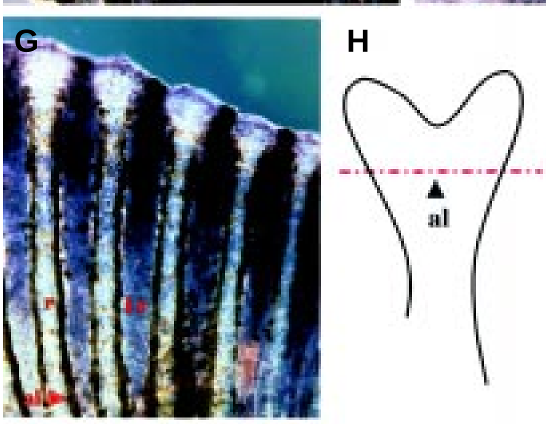

Fig. 1. Expression of *zfK8* mRNA in regenerating caudal fin. (A,B) *Control, non-regenerating caudal fin.* **(C,D)** *1 day after amputation.* **(E,F)** *3 days after amputation.* **(G)** *6 days after amputation.* **(H)** *Schematic representation of amputation level (al). Fig. 1 A,B shows the expression levels of the k8 gene in the epidermis of non-regenerating caudal fin. A red-violet labeling indicates the presence of k8 mRNA in the inter-rays zone, (ir) of the regenerating fin. Brown spots and lines represent pigments of melanin (p). K8 gene expression is not evidenced in the epidermis under the rays (r). Magnification in A,E, 15X; in C,G, 20X; in B, 25X; in D,F, 40X. Expression was detected by whole mount in situ hybridisation with a Dig-labelled antisense probe.*

hyperproliferative types of carcinomas (epithelial tumors) and in some cultured cell lines of carcinomas (Moll *et al.*, 1982). On the other hand it is absent in stratified epithelia such as the epidermis. In adult elasmobranchs (shark, *Scyliorhinus stellaris*) it is shown that the homologue of human k8 is a II S-type keratin (sstk8 IIS), and that it is expressed only in epithelial cells (Schaffeld *et al.*, 1998). On the contrary in amphibians (newt, *Nothophtalmus viridescens*), in the rainbow trout (*Oncorhyncus mykiss*) and in zebrafish (*Danio rerio)* the homologue of human k8 has been found also in mesenchymal cells in adult and during limb regeneration (Markl and Franke, 1988; Ferretti *et al.*, 1989; Ferretti *et al.*, 1993). We recall that teleost zebrafish and amphibian newt share the possibility of regenerating their appendices, even if their anatomy differs for the absence of cartilage and muscles in zebrafish fins (Ferretti and Géraudie, 1998). With antibodies against human k8 Ferretti has found an expression of Nvk8 (the homologues of human k8) in mesenchymal and not in epithelial cells between the seventh and the twenty-second day of limb and jaw regeneration (Ferretti *et al.*, 1989; Ferretti and Ghosh, 1997). With the same approach Conrad detected the expression of zfk8 protein in adult zebrafish, in simple epithelia and in some non mesenchymal tissue, (Conrad *et al.*, 1998). For the first time we investigated by *in situ* hybridization the expression of *zfk8* during caudal fin regeneration. We previously cloned and characterized an homologue k8 cDNA (Imboden *et al.*, 1997) by screening a lambda Zap cDNA library prepared from poly (A+) RNA of zebrafish embryos at gastrula stage (100% epiboly). The nucleotide sequence comparison and characteristics of the deduced polypeptide indicate that it belongs to the cytokeratin type II subfamily (Imboden *et al.*, 1997).

It has been shown that various keratins are expressed at high levels in regenerating tissues (Smoller *et al.*, 1989; Ferretti and Brockes, 1991; Tsonis *et al.*, 1992; Ferretti *et al.*, 1993), but the expression of k8 gene has never been investigated in zebrafish during fin regeneration. By experiments of whole-mount *in situ* hybridisation we revealed an important expression of *zfK8* transcript only in epidermis of regenerating caudal fin.

As shown in Fig. 1 an intense epidermal signal for this mRNA is clearly evident in the blastema of regenerating caudal fin 1, 3 and 6 days after amputation. In Fig. 1 C,D, we can observe that the signal is restricted to the wound epidermis of 1 day of regeneration. At 3 and 6 days (Fig. 1 E,F,G) k8 gene expression is linked to the proximal extremity of the blastema, and goes decreasing just to the wound. In all cases the signal is much more intense in the epidermis under the rays. On the other hand, in Fig. 1 A,B, we can perceive the absence of signal in non regenerating caudal fin, as in accordance to the results of Conrad (Conrad *et al.*, 1998).

It is known that the first phase of regeneration is the migration of keratinocytes from lateral position to cover the surface of the wound (Ferretti and Géraudie, 1998), without forming a basal membrane. In this case the wound maintains a direct contact with the underlying blastemal cells that falls in an hyperproliferative stage. In Fig. 2, cross sections reveal that the expression of *zfk8* transcript is restricted to the epidermis and completely absent in mesenchymal cells (Fig. 2 A,B,C). It is also clear that the signal is much more exalted in the inter-rays tract. We know that the inter-rays space contains mesenchymal cells and lacks bony structures. We don't know the reason of this gradient, but the accumulation of k8 at this level may strengthen a fragile regenerating zone that lacks resistant scaffolding such as bony rays. The expression of the *zfK8* mRNA in regenerating caudal fin is absent at the lateral extremity of the fin (Fig. 2C). We also made amputations in a step-way (Fig. 2D) in order to observe on the same cut the difference of expression between a regenerating bit and a zone adjacent to it (Fig. 2 E,F). In fact, it is possible to observe that only the regenerating extremity (**re**) presents an important expression of zfk8 transcript.

Gene expression analysis during embryonic development of caudal fin revealed the same pattern of expression (data not shown).

We finally assayed the effects of all-trans retinoic acid (RA) on the expression of *zfK8* gene in caudal fin during the process of regeneration. In fact it is known that in some vertebrates, such as amphibians, some keratin mRNAs are regulated by retinoic acid in different ways depending on the tissue localisation during development and regeneration (Ferretti *et al*., 1991; Ferretti, 1995). The exact role of this endogenous RA is not really known, but it seems possible that it could promote the formation of blastemal cells by de-differentiation of other cellular types (Viviano *et al*., 1995). Exogenous RA appears to affect the process of regeneration at different levels by inducing a number of striking morphological changes on the regenerate, such as the fusion of the rays (White, 1994; Geraudie *et al*., 1995). By *in situ* hybridisation we did not note an important difference of expression of the k8 transcript in regenerating caudal fin treated with RA 10^{-6} M and, that is important, no morphological changes was detected (data not shown). The *cytokeratin 8* gene is expressed at high levels in many vertebrate tissues during several related processes such as development, hyperproliferation and regeneration, suggesting that it may be necessary for the maintenance of undifferentiated and proliferative states.

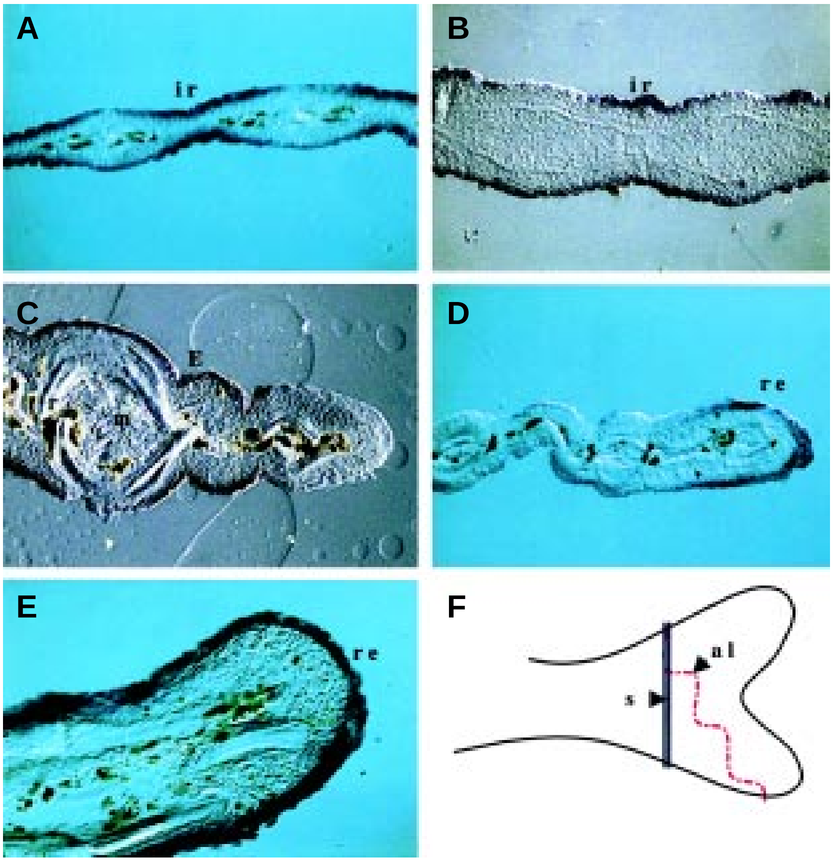

Fig. 2. Cross sections of regenerating caudal fin stained with the *zfk8* probe. (A,B) *1 day after amputation.* **(C)** *3 days after amputation (proximal section).* **(D)** *Step-cut section (s) of 1 day regenerating caudal fin.* **(E)** *Step-cut section of a 3-day regenerating caudal fin.* **(F)** *Schematic representations of step-cut (al) and of regenerating sections (s). An anatomical view is given in Fig. 2C in order to evidence the labeling only in the epidermis during regeneration. Here, the gradient of expression which is shown in A-B is not appreciable, because it is a proximal section. r, ray; ir, inter-ray; al, amputation level; m, mesenchyme; E, epidermis; re, regenerating extremity. Whole mount in situ hybridisation (magnification in A,D, 20X; in B,C, 40X; in E, 80X).*

Experimental procedures

Zebrafish (*Danio rerio*) embryos and adults utilised in the experiments were bought from SIDOLI (France). Wildtype fishes were directly imported from India. Mature fishes were kept at 28°C in photo-controlled aquariums and were anaesthetised with EMS 222 before cutting their fins. The different stages of regeneration were analysed by removing the regenerating caudal fin after 1, 3 and 6 days after the first amputation. All the fins were fixed overnight in 4% paraformaldehyde / phosphate buffered saline (PBS) at 4 °C, dehydrated in methanol (MeOH) at room temperature and stored at –20°C. Control sense RNA probes were synthesised with T7 RNA polymerase using Bam HI cleaved Bluescript SK as template, while antisense RNA probes specific for zfK8 mRNA were synthesised with T3 polymerase after plasmid linearization with Hind III purchased from Pharmacia.Digoxigenin (DIG)-labelled RNA probes used for *whole-mount hybridisation* analysis were produced with the DIG RNA labelling kit of Boehringer Mannheim.

Samples, containing 5-10 zebrafish caudal fins for each time and fixed embryos, were rehydrated in MeOH/PBST, permeabilized and hybridised as described by Xu (Xu *et al*., 1995). The experiments were carried out about six times for each point. The staining reaction was carried out in alcaline phosphatase buffer containing additional nitroblue tetrazolium and5-bromo-4-chloro-3-indolul phosphate as described in the Boehringer Mannheims instruction manual for DIG detection. The staining reaction took place within a period shorter than 2 h. After a *whole-mount hybridisation* of the embryos and adult tissues, respectively, paraffin and cryostat sections were prepared following conventional protocols.

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