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Invited Review

Role of diffusible and transcription factors in inner ear development: implications in regeneration

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Summary. Organogenesis involves a dynamic balance of the mechanisms regulating cell division, differentiation and death. The development of the chicken embryo inner ear offers a well-characterised model at the morphological level to study which signals are implicated in the modulation of cellular activation and commitment. The early developmental decisions that control the origin of the inner ear elements are just beginning to be identified by complementary in vivo and in vitro studies. Insulin-like growth factor-I (IGF-I) and nerve growth factor (NGF) are among the best characterised diffusible factors acting during inner ear development. Although the cellular actions of these factors are beginning to be understood, the signalling pathways triggered by them still remain largely unknown. In this context, viral vehicles can be used to deliver genes and then analyse their functional roles during inner ear development. A model is proposed where the actions of IGF-I and NGF contribute to the combinatorial expression of Jun and Fos family members in particular domains of the otic vesicle. Some of these mechanisms may be also implicated in otic regeneration.

Key words: Apoptosis, Insulin-like growth factor-I, Nerve growth factor, RCAS retroviral vectors, Jun signalling pathways

1. The early development of the vertebrate inner ear

The adult inner ear in vertebrates is a complex, highly-differentiated structure responsible for audition, the perception of movement, and the sense of equilibrium. It contains more than a dozen different cell types, which include neurons, sensory hair cells, secretory cells, and supporting cells. These cell types are organised in functional regions (www.iurc.montp.inserm.fr/cric/audition). Different regions of the ear show an elaborated spatial organisation responsible for the different functions of the organ: the vestibular portion of the ear is responsible for the senses of motion and position while the auditory or cochlear region is responsible for the sense of hearing. In both portions, hair cells convert mechanical stimulation into electrical signals, which are then processed by the nervous system. Hair cells are innervated by sensory neurons that project towards specific nuclei in the central nervous system. The development of the chicken embryo inner ear offers a well-characterised model at the morphological level to study which signals are implicated in the modulation of cellular proliferation, differentiation and programmed cell death (Fekete, 1996).

The vertebrate inner ear originates from the head ectoderm where the otic placode is formed. After it invaginates to form the otic pit that latter closes forming the otic vesicle or otocyst (Fig. 1). This is a transient structure that undergoes a distinct period of intense cell proliferation (stages 18-22 in the chicken, Hamburguer and Hamilton, 1951) that precedes the differentiation of the various cell types and compartments that will conform the adult inner ear (Bissonnette and Fekete, 1996). Neuroblasts for the cochleo-vestibular ganglion (CVG) migrate out from the medial wall of the otic vesicle. The CVG contains the afferent neurons that connect the sensory epithelium of the inner ear to the central nervous system (Hemond and Morest, 1991). Later in development, the CVG is separated into two ganglia that innervate separately the cochlear and vestibular parts of the inner ear connecting them to the central nervous system.

There are several experimental approaches to study inner ear development in the chicken embryo. In vivo studies facilitate the analysis of the expression of target genes in whole mount preparations and also the study of cell proliferation and cell death patterns through development (reviewed by Torres and Giraldez, 1998). Embryonic day 2.5 (E2.5) otic vesicles can be dissected and the explants cultured to analyse the role of extracellular factors and intracellular signals in a defined medium (León et al., 1998; Torres and Giraldez, 1998). Finally, viral vehicles can be used to deliver genes and

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then analyse the functional consequences of their over-expression (Hughes et al., 1987; Federspiel and Hughes, 1997), these genes being either wild type or dominant negative mutants. Therefore, ectopic expression of certain genes by means of retroviral vectors can provide a unique information on their functional roles during inner ear development (Federspiel and Hughes, 1997; Fekete et al., 1997). RCAS retroviral vectors facilitate the misexpresion of genes in ovo (Cepko, 1992; Fekete et al., 1997) and in cultured otic vesicles (Sanz et al., 1999) in order to study the consequences of manipulating the levels of selected extracellular factors or transducing proteins.

The basic molecules responsible for the normal ontogenesis of the inner ear have started to be unravelled. Diffusible factors form part of the environmental input that contributes to modulate the early development of the inner ear. IGF-I, retinoic acid and NGF actions have been reported to be fundamental for the correct development of the otic vesicle and CVG (León et al., 1998; Torres and Giraldez, 1998). Extracellular factors initiate intracellular signalling processes that will induce long term cellular phenotypic responses. As will be discussed in detail below, IGF-I stimulates the generation of lipidic second messengers (León et al., 1998), activates the Raf/mitogen-activated protein kinases (MAPKs) cascade (Sanz et al., 1999) and increases c-fos, c-jun and PCNA levels leading to cell growth and survival (León et al., 1998). On the contrary, NGF activates Jun N-terminal kinase (JNK) and stimulates the hydrolysis of sphingomyelin to generate ceramide, in a process that regulates apoptotic cell death in organotypic cultures of otic vesicles (Frago et al., 1998). Retinoic acid downregulates c-fos and proliferating cell nuclear antigen (PCNA) levels and induces hair cell differentiation (León et al., 1995a; Lee and Cotanche, 1996; Sanz et al., 1999). Raf overexpression by means of retroviral RCAS vectors potentiates IGF-I actions while it blocks both NGF-induced apoptosis and retinoic acid effects in the otic vesicle (Sanz et al., 1999).

2. Multiple roles of IGF-I in inner ear development: antagonism with NGF actions

Insulin and IGF-I play an important role in the development, growth, and survival of normal cells. Insulin and IGF-I interact in avian cells with at least two different receptors with tyrosine kinase activity, the type 1 IGF-I and insulin receptors. In addition, a hybrid receptor composed of subunits from the insulin- and type 1 IGF-receptors has been identified. IGF-I activity is further modulated by at least ten different IGF-binding proteins (Parrizas and LeRoith, 1997; Spagnoli and Rosenfeld, 1997). Insulin-like molecules, their receptors, and binding proteins conform the IGF axis

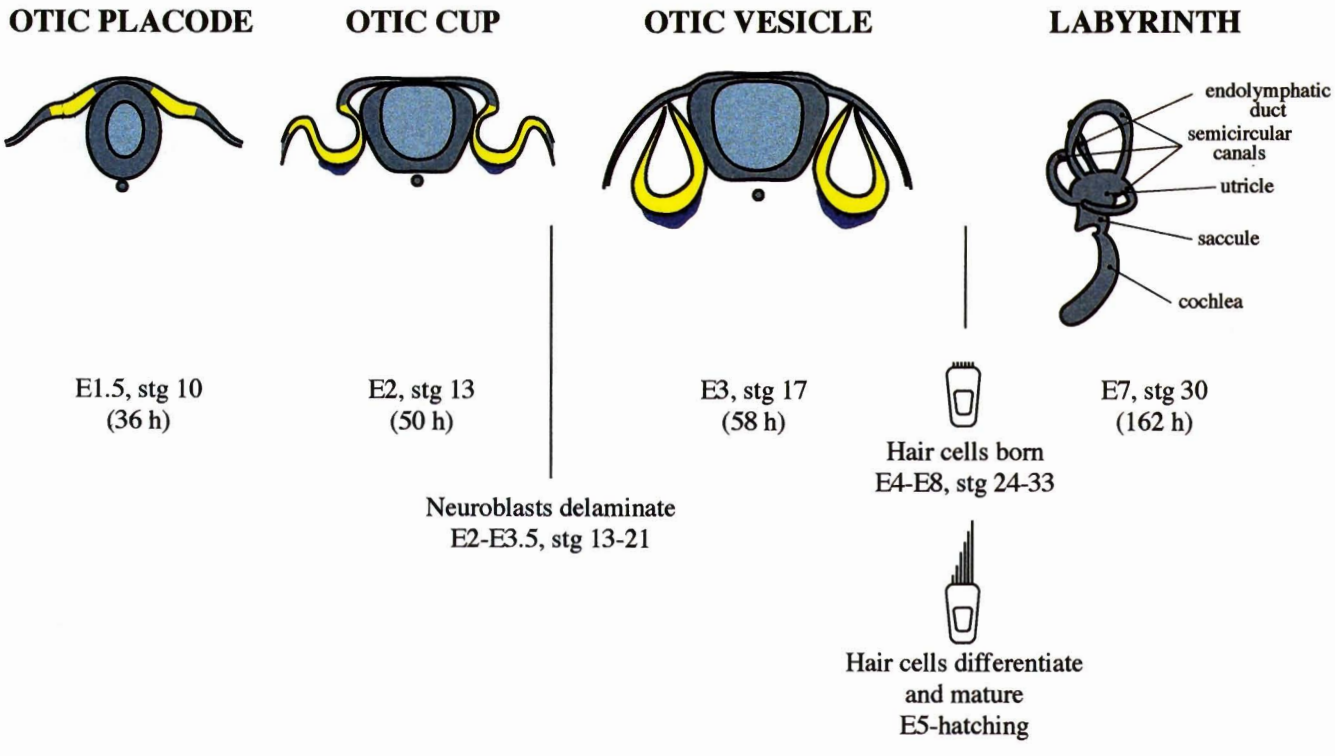


Fig. 1. Schematic drawing of the early stages of chicken inner ear development. Stages have been set following the Hamburger and Hamilton criteria.

(http://www.IGF-society.org/). Hence, there is a great potential for complex interactions between insulin family factors to regulate normal cell growth and development.

The occurrence of IGF-I in the otic vesicle and CVG was explored by immunohistochemistry and by in situ hybridisation using a full-length chicken IGF-I cDNA probe (Kajimoto and Rotwein, 1989). Fig. 2A shows that IGF-I mRNA is homogeneously expressed in the CVG. However, in the otic vesicle epithelium IGF-I transcripts and immunoreactivity were concentrated in the ventral, lateral and dorsal aspects, and were apparently excluded from the medial wall facing the neural tube (Fig. 2A,B and León et al., 1995b). The CVG displayed a strong IGF-I immunoreactivity, without evidence of regional distribution. The occurrence of IGF-I receptor in the otic vesicle was studied by in situ hybridisation using a chicken IGF-I type 1 receptor probe (de la Rosa et al., 1994). Fig. 2C shows the presence of type 1 receptors homogeneously distributed in the otic epithelium. Previous data on the presence of IGF-I binding to specific receptors in the otic vesicle and CVG indicated that IGF-I binding is related to the functional expression of type 1 IGF receptors (León et al., 1995b). The localisation of IGF-I binding indicates that the ventromedial otic epithelium shows strong labelling, as does the adjacent developing CVG. Taken together, these data indicate that IGF-I factor and receptor are abundantly expressed in the chicken inner ear at E3. The pattern of expression of IGF-I binding proteins in the otocyst has not yet been reported. Our results suggest that the factor present in the dorsal areas of the otocyst may be sequestered by a binding protein that prevents effective binding to the IGF-I type 1 receptor.

Low doses of IGF-I (1 nm) stimulate proliferative growth in the otic vesicle and CVG (León et al., 1995b, 1998). Organotypic cultures of stage 18 otic vesicles treated with IGF-I increase in size and acquire the morphology that corresponds to stage 21-22 otic vesicles developed in vivo. Furthermore, the pattern of mitotic activity induced by IGF-I is similar to that of serum and concentrated in the ventral and medial regions of the otic vesicle. IGF-I treatment increases proliferative cell nuclear antigen (PCNA) levels in explanted otic vesicles (Frago et al., 1998; León et al., 1998). Chicken insulin but not bovine or human recombinant insulin is as potent as IGF-I in inducing otic vesicle growth (Varela-Nieto et al., 1991; León et al., 1998). Human IGF-II showed about half the potency of IGF-I in inducing DNA-synthesis. Data on the potency of chicken IGF-II have not been reported. These results stress the importance of using homologous species factors in *in vitro* studies on

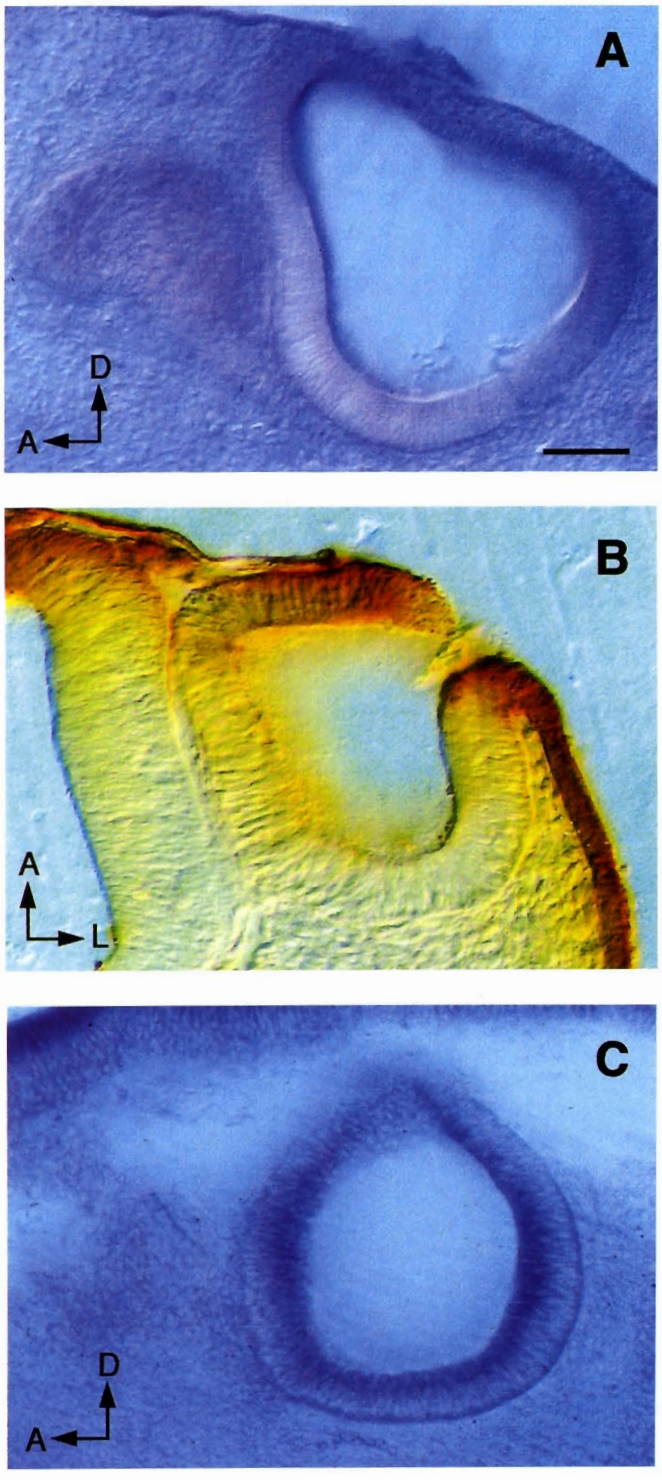


Fig. 2. Expression of IGF-I and IGF-I type 1 receptor in the developing inner ear. The occurrence of IGF-I and IGF-I type 1 receptor mRNAs in the otic vesicle and CVG was analysed by in situ hybridisation using a full length chicken IGF-I cDNA probe **(A)** or a chicken IGF-I type 1 receptor probe **(C)**. Vibratome sections were obtained after carrying out the procedure for in situ hybridisation with digoxigenin-labelled RNA antisense probes in whole mount preparations. Control specimens were hybridised with sense probes (not shown). IGF-I protein levels **(B)** were analysed by immunohistochemistry using an anti-human IGF-I antibody that cross-reacts with chicken IGF-I (León et al., 1995b). Photomicrographs from 60 μm vibratome sections of stage 19 chick embryos are shown. A: anterior; D: dorsal; L: lateral. Bars: 1, 170μm: B, C, 145μm.

the role of growth and differentiation factors during development. They also raise the question as to whether besides IGF-I there are other insulin-related peptides involved in modulating inner ear organogenesis. Therefore, otic vesicles growth can be modulated by several insulin-like molecules. On the contrary, the effects of IGF-I on cultured stage 20 CVG are not reproduced by any other factor of the insulin family. The mitogenic effect of IGF-I was dose-dependent and, like in the otic vesicle, it saturated at about 1 nM (León et al., 1995b). In addition, IGF-I, but not insulin, promotes neurite outgrowth in the CVG (León and Varela-Nieto, unpublished observation). In addition, IGF-I is a survival factor for the otic vesicle and CVG. IGF-I blocks apoptosis induced by serum deprivation and by NGF (Frago et al., 1998).

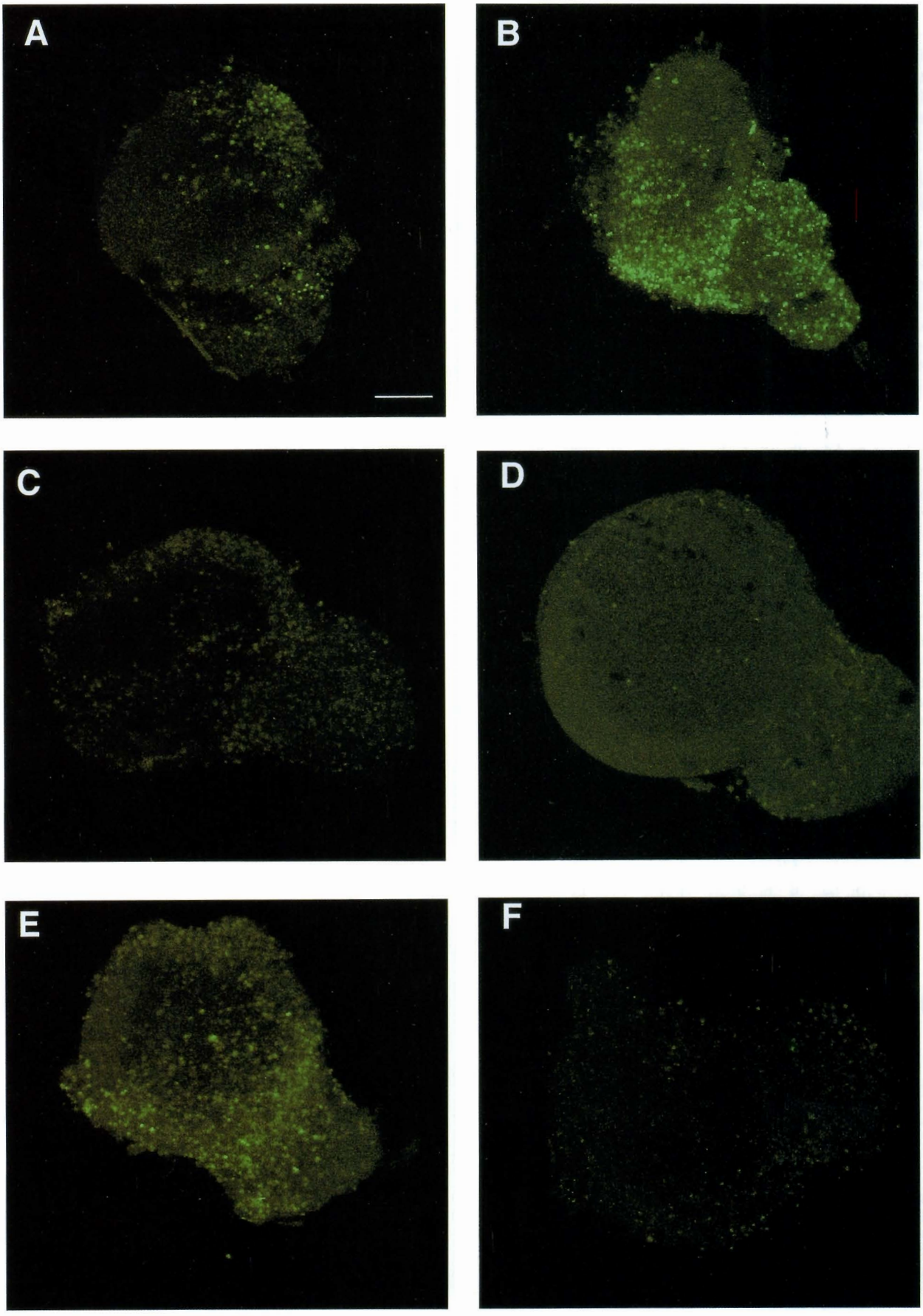
NGF induces apoptosis in specific areas of the otocyst epithelium and in the CVG (Frago et al., 1998) through its low affinity p75 receptor. NGF was initially characterised by its trophic role, including the prevention of cell death by apoptosis in specific populations of neurons in the peripheral nervous system. NGF is the first identified member of a family of factors, called neurotrophins, which promote neuronal survival in both the central and the peripheral nervous systems. This neurotrophin family also includes the brain-derived neurotrophic factor (BDNF), neurotrophin (NT) 3 and NT-4/5. All members of the neurotrophin family are known to bind two receptors, p75NFR, which binds neurotrophins with low affinity and one trk receptor. Within the family of tyrosine kinase receptor trks, three members have been described: trkA that binds NGF, trkB that binds BDNF and NT-4/5, and trkC that binds NT-3 (Chao, 1992). The role of neurotrophins in neuronal survival is mainly mediated by the activation of the trk receptors (for a review see Chao, 1992). Neurotrophins also bind the low affinity receptor p75 that is a modulator of survival and death decisions. The p75 neurotrophin receptor is structurally related to tumour necrosis factor receptor and Fas family (Beutler and van Huffel, 1994). The presence of a death domain motif has been shown in p75 NGF receptor (Liepinsh et al., 1997) which, upon neurotrophin binding, activates the sphingomyelin pathway to produce ceramide (Dobrowsky et al., 1994; Cassacia-Bonnefil et al., 1996). The presence of p75 NGF receptor has been extensively reported at different stages of inner ear development in several animal species including chicken (von Bartheld et al., 1991; Schecterson and Bothwell, 1994). The pattern of expression of p75 receptors within the otocyst epithelium is restricted and it has been associated with presumptive sensory organ areas (Wu and Oh, 1996).

3. Cross-talk between IGF-I and NGF signalling

3.1 Lipidic messengers: ceramide and ceramide-1-P

The turnover of lipids at the plasma membrane of cells plays a critical role in signal transduction and cellular activation. The interaction of extracellular agonists with specific membrane receptors results in the activation of specific enzymes, phospholipases, which generate intracellular second messengers from lipid precursors. Sphingolipids have long been considered as structural units of the plasma membrane of cells. However, it has been recently shown that these molecules have multiple biological activities, and many have been described as potent second messengers and regulators of cell activation (Kolesnick and Kronke, 1998). Ceramide can be considered as the sphingolipid core from which sphingolipids that are more complex are derived. In addition, the sphingomyelin/ceramide cycle functions as a second messenger system. The physiological significance of ceramide accumulation has been related to the induction of cell differentiation and programmed cell death. Several extracellular agonists induce the hydrolysis of sphingomyelin to generate ceramide. Among them, NGF acts through its low affinity p75 receptor (Dobrowsky et al., 1994). Short-chain ceramide analogues (C_2-ceramide) are useful tools to study the biological effects of natural ceramides, since the natural long-chain ceramides are not permeant to cells. Ceramide can be converted to ceramide-1-P by a Ca^{2+}-dependent kinase (Dressler and Kolesnick, 1990). In turn, a phosphatase has been characterised that specifically hydrolyses ceramide-1-P in the plasma membrane (Boudker and Futerman, 1993). The conversion of ceramide-1-P into ceramide decreases DNA synthesis and promotes apoptosis. These results suggest that ceramide-1-P may play an important role in cell activation. C_2-ceramide induces internucleosomal DNA fragmentation which leads to programmed cell death or apoptosis in the otic vesicle (Fig. 3 A,B). On the contrary, ceramide-1-phosphate is a cytoprotector for otic vesicle explants that acts as a suppresser of cell death upon serum withdrawal (Fig. 3C). NGF induces sphingomyelin hydrolysis and ceramide release in the otic vesicle in association with cell death in the otic vesicle and the CVG. NGF-induced apoptosis is apparent in specific areas and it contributes to the formation of the endolymphatic duct and to the neurogenesis of the CVG (Fig. 3E). IGF-I treatment effectively blocks ceramide and NGF effects (Fig. 3D,F). Furthermore, IGF-I blocks NGF-induced ceramide generation and co-operates with ceramide-1-P in cell survival (Frago and Varela-Nieto unpublished

Fig. 3. Distribution of apoptotic cells in the otocyst. Apoptotic cell death is revealed by in situ DNA-end labelling technique (TUNEL protocol) in cultures of otocysts from embryonic day 2.5. Optic sections of 2.5 μm are shown for the following conditions: otocysts were isolated and grown for 8 hours in serum-free medium **(A)**, 5 μM C2-ceramide **(B)**, 25 μM Cer-1-P **(C)**, 5 μM C2-ceramide plus 10 nM IGF-I **(D)**, 4 nM NGF **(E)** or 4 nM NGF plus 10 nM IGF-I **(F)**. Bar: 70 μm.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observations). These results indicate that NGF and IGF-I signalling pathways frame a network of intracellular signals that regulate early inner ear development via induction of regionally restricted areas of cell death and cell proliferation (Fig. 4).

3.2 A strict control of c-Raf kinase levels is essential for early inner ear development

c-Raf is a cytoplasmic serine/threonine protein kinase involved in signal transduction from the plasma membrane to the nucleus (Mark and Rapp, 1984; Daum et al., 1994; Naumann et al., 1997). Increased Raf kinase activity is associated with an increase in the degree of phosphorylation of mitogen-activated protein kinase (MAPK) during the cellular response to various mitogenic agents including IGF-I in a variety of cell types (Marshall, 1994). c-Raf is a member of a small family of proteins essential for growth and development. Knockout and transgenic chimeric c-Raf-deficient mice show growth retardation (Naumann et al., 1997; Wojonowski et al., 1998). Our group has recently shown that during the early organogenesis of the inner ear there is a sustained expression of Raf kinase. This suggests that Raf is required for the intense mitotic activity reported during this period (Sanz et al., 1999). c-Raf activity is increased in response to IGF-I and the activation by IGF-I of the c-Raf kinase pathway is a requirement to turn on cell proliferation in the otic vesicle. The role of c-Raf kinase was further explored by misexpressing both c-raf and a dominant negative c-raf mutant (Raf-C4) cDNA by means of RCAS retroviral vectors. Overexpression of c-raf in E2.5 explants increases the proliferative response to low serum and IGF-I and blocks differentiation induced by retinoic acid. The increase in c-Raf levels also prevents NGF-dependent induction of programmed cell death and potentiates IGF-I actions as a survival factor. Consistent with these results, the expression of a dominant negative c-Raf mutant potentiates retinoic acid action and decreases the rate of cell proliferation (Sanz et al., 1999).

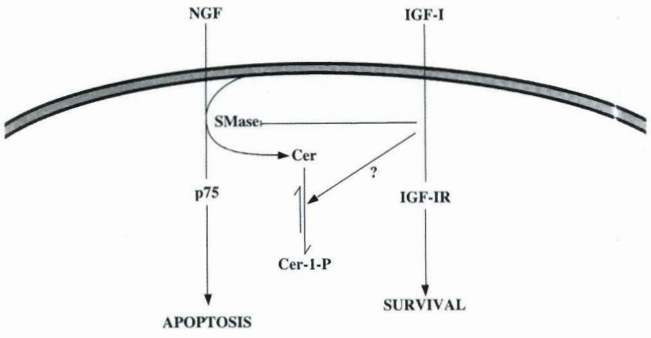

Fig. 4. Cross-talk between IGF-I and NGF signalling in the otic vesicle. Upon NGF binding to low affinity, p75 NGF receptor ceramide is generated leading to apoptosis. IGF-I acts as a cytoprotector by blocking ceramide generation and possibly inducing ceramide-1-phosphate formation from ceramide.

Therefore, the unbalance of c-Raf levels will alter early otic vesicle morphogenesis by increasing the total cell number. These data suggest that modulation of c-Raf levels contributes to increase specificity in the response to a given growth factor or to the local combination of extracellular stimuli, that encounters a developing cell. We propose that a strict control in the levels of c-Raf kinase is essential for the normal progression of inner ear development, thus allowing the system to balance the main biological processes of cell growth, morphogenesis and apoptosis (Fig. 5).

3.3 Role of transcription factor AP-1 in inner ear development

Transcription factors are central for achieving the specificity in the cellular response to extracellular signals that is required during development. Jun family of proteins are together with c-Fos a major component of the AP-1 transcription factor (Angel and Karin, 1991). Jun proteins show tissue-specific and differential expression during development and in adult organisms (Wilkinson et al., 1989). Jun family members present a high homology in the N-terminal sequence and DNA

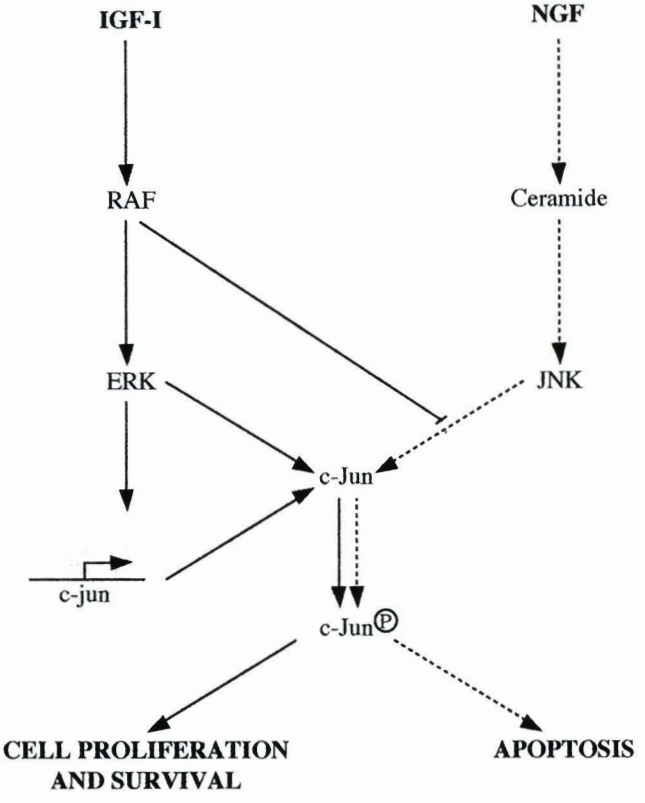

Fig. 5. NGF and IGF-I signalling pathways converge on c-jun activation in the otic vesicle. Scheme of signalling to c-Jun in the otic vesicle. IGF-I activates the Raf/ERK kinase cascade which in turn induces c-Jun expression and phosphorylation leading to cell proliferation and survival. On the contrary, if c-Jun is activated by NGF via JNK-dependent phosphorylation it mediates apoptotic cell death.

binding domain. Jun proteins can form either homodimers or heterodimers with different proteins that include the Fos-related proteins, ATF-2 or CREB. Dimerization takes place trhough leucine zipper domains and allows binding to DNA sequences, denominated TRE and CRE, that are present in the promoters and enhancer elements of target genes (for a review see Karin et al., 1997). c-jun, junB and junD differ in their binding properties to AP-1 and they display distinct and even opposite roles. Therefore, there are a large number of different combinations of dimers that allow for specific transcriptional responses by activating different sets of target genes depending on the physiological situation of the cell. c-jun knock out mice die in utero and cells lacking c-jun presented retarded cell growth in culture (Johnson et al., 1993) This and earlier reports pointed to c-jun as a masterpiece in the control of cell proliferation in response to multiple extracellular stimuli and during cellular transformation. Jun signalling has also been implicated in the regulation of cell differentiation. Finally, c-jun and junD are activated in response to apoptotic stimuli in a variety of cell types (Karin et al., 1997). Thus, the different AP-1 components can achieve diverse functions. Regarding the signalling mechanism that orchestrates Jun activation, Jun proteins can be activated by phosphorylation through two distinct pathways that have been extensively characterised in PC12 pheocromocytome cells and in mature oligodendrocytes (Karin et al., 1997). After growth factor binding to tyrosine kinase receptors the Raf/MAPKs cascade is turned on. The activation of c-jun by a subgroup of MAPKs, the extracellular signal regulated kinases (ERKs), is associated with the stimulation of cell proliferation and differentiation. On the other hand, serum-deprivation, ceramide accumulation, or NGF binding to p75 receptors activates other members of the MAPK family; the JNKs that phosphorylate c-jun in the serines 63 and 73. This phosphorylation step activates c-jun distinctly and leads to an increase in apoptosis.

The early development of the inner ear provides a useful model to study the complexity and specificity of AP-1 signalling. c-Fos is expressed in the wall of the otic vesicle and in the CVG during those stages of early inner ear development exhibiting a high proliferation rate (León et al., 1995a). The expression pattern of c-fos indicates that it is more abundant in the ventromedial epithelium of the otocyst and in the CVG. At embryonic day 2.5 the otic vesicle is formed by a morphologically homogeneous epithelium but it presents high mitotic activity in the ventromedial areas (Torres and Giraldez, 1998). The expression of c-Fos is induced by IGF-I and is required for otic vesicle proliferation (León et al., 1995b). In cultured otic vesicles, IGF-I increases the levels of Jun proteins and antisense c-jun oligonucleotides partially block the cellular responses to IGF-I (León et al., 1998). Thus, there is an association between the simultaneous expression of Fos and Jun with the mitogenic effects of IGF-I. However, quiescent otic vesicles maintained in culture exhibit a restricted expression of c-jun transcripts in the dorsal aspect that co-localises with an area where cells could be labelled by the TUNEL technique indicating apoptosis (Frago et al., 1998). These data suggest that Jun family proteins could play a dual role during early inner ear development. Indeed, whole mount in situ hybridisation analysis of jun expression indicates that c-jun and junD transcripts are expressed in the otic pit and otic vesicle as well as in the CVG with distinct expression patterns that co-localise with areas of high apoptosis. Furthermore, phosphorylation of c-Jun in the serine 63 residue occurs within the same area in the stages studied. The role of c-Jun phosphorylation during apoptosis was confirmed by studying the response to NGF in cultured otic vesicles and in otic vesicles overexpressing Raf. Upon NGF treatment of the explants, c-Jun phosphorylation was increased. When NGF responses were blocked, by co-treatment with IGF-I or by an increase in the intracellular Raf levels, c-Jun was not phosphorylated at the serine 63 (Fig. 5). These results describe an intermediate transcription step which links extracellular apoptotic signals to long term cellular responses during organogenesis (Sanz, Giraldez and Varela-Nieto, unpublished observations).

4. Molecular mechanisms in organogenesis: implications in regeneration

The damage of mechanosensory cells in hearing organs causes hearing loss with a severity proportional to the number of hair cells missing. Hair cells of the adult inner ear are lost during ageing and also following acoustic trauma or treatment with certain drugs (www.nih.gov/nidcd; www.iurc.montp.inserm.fr/cric/audition). In the mature mammalian organ of Corti, the sensory cells are not replaced and the deficits are permanent. In contrast, new hair cells are produced to replace those that have been lost in the bird cochlea and in the vestibular sensory epithelia of birds, fish and mammals (for recent reviews see Cotanche, 1997; Staecker and Van de Water, 1998; Stone et al., 1998). The regeneration of hair cells is preceded by renewed mitosis in the sensory epithelium. Although in the past ten years numerous studies have been directed to examine the events and regulatory mechanisms that control hair cell proliferation and differentiation, the factors that induce hair cell regeneration remain largely unknown.

In the otic vesicle and CVG, IGF-I modulates cell proliferation, differentiation and survival (this review and León et al., 1995b; Frago et al., 1998). The importance of IGF-I in ear development is stressed by the report of a clinical case of a 15-year-old boy with severe prenatal and postnatal growth failure and sensorineural deafness who had a homozygous partial deletion of the Igf-I gene (Woods et al., 1996, 1997). Targeted disruption of the genes for IGF-I and type 1 IGF receptors has conclusively demonstrated an

essential role for IGF-I in prenatal growth and development. Postnatal mouse growth is also dependent on normal levels of these genes (Baker et al., 1993; Liu et al., 1993; Beck et al., 1995; Cheng et al., 1998; Gao et al., 1999), but these studies did not give specific details on the development and maturation of the inner ear. IGF-I gene is transiently expressed during the maturation of the rat auditory system (Bondy, 1991) and, as discussed above, there are IGF-I binding sites and IGF-I in the avian otic vesicle and ganglion. These results, taken together with the high sensitivity to exogenous IGF-I suggest that the proliferative period of ear development will be seriously affected. There might also be other factors stimulating growth in parallel with IGF-I and partially rescuing the increase in cell number, but they may or may not mimic IGF-I completely. In this context, the analysis of the details of the mutant phenotype will be a substantial step forward in the knowledge of the functions of IGF-I in the inner ear.

On the other hand, both avian and rat adult hair cells express IGF-I and insulin receptors (Lee and Cotanche, 1996; Saffer et al., 1996). IGF-I receptors are expressed in high levels in inner ear epithelial cells after injury or deprivation of hair-cells (Jennische et al., 1987; Lee and Cotanche, 1996) and epithelial cell proliferation is inhibited by antibodies against IGF-I (Swanson et al., 1990). In addition, it has been reported that: i) IGF-I and/or insulin promote cell growth in cultures of mature avian and rat utricular epithelial cells (Oesterle et al., 1997; Zheng et al., 1997); ii) in vivo treatment of the vestibular sensory epithelium with IGF-I combined with other factors increases hair cell regeneration in the guinea pig and proliferation in the rat (Kopke et al., 1996; Kuntz and Oesterle, 1998); and iii) high doses of insulin have been shown to regulate avian CVG neuritogenesis and growth, acting as a cofactor for neurotrophins (Sokolowski, 1997).

Cell proliferation of hair cell progenitors is the early major event occurring during hair-cell regeneration after acoustic trauma or exposure to ototoxins. It has recently been reported that supporting cells and transducing hair-cells share a common progenitor (Fekete et al., 1998) These results suggest that an increased proliferation of supporting cells coupled to their transdifferentiation may lead to the formation of new hair cells. On the other hand, injury may induce inner ear epithelium to acquire immature properties typical of earlier developmental stages. Therefore, understanding the mechanisms regulating cell proliferation in the inner ear will help to understand hair cell regeneration and functional repair. In this context, IGF-I is a good candidate to regulate proliferation during the regenerative response. In addition, manipulation of the signalling pathways that control apoptosis in the otic vesicle and CGV may contribute to cellular regeneration. This is the case of the JNK inhibitor CEP-1347 that promotes survival of cochlear neurons and attenuates hair cell loss following trauma (Pirvola et al., 1999).

In summary, the insulin family of growth factors are potentially useful agents for the regeneration of inner ear cells and treatment of hearing impairments caused by ageing or ototoxic drugs, either alone or in combination with other growth factors.

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