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Retinal Growth Hormone:

An Autocrine/paracrine in the Developing Chick Retina

by

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ABSTRACT

The developing chick retina is an extrapituitary site of growth hormone (GH) synthesis and action. GH, GH receptor (GHR) and their mRNAs are present in the neural retina when the neural cells are undergoing proliferation and differentiation during early embryogenesis. It is thus likely that GH acts as an autocrine or paracrine in this location. The present study shows that intra-vitreal injection of a chick GH (cGH) small interfering RNA (siRNA) into the eyes of early embryos [embryonic day (ED) 4] suppresses GH expression in the neural retina and increases the incidence of spontaneous retinal cell death. Our current work also demonstrates a reduction of local IGF-1 expression after retinal GH gene knockdown, suggesting that GH action in retinal cells is regulated through IGF-1 signalling. These results demonstrate that retinal GH is an autocrine/paracrine hormone that acts as a neuroprotective factor in the retina of chick embryos.

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AIF	Apoptosis inhibiting factor
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CNTF	ciliary-derived neurotrophic factor
Ct	Threshold cycle
DAB	Diaminobenzidine
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modification of Eagle’s Medium
DR	Diabetic retinopathy
dw/dw mouse	GH-deficient mouse
ED	Embryonic day
ELISA	Enzyme-linked immunosorbant assay
ERG	Electroretinogram
ERK	Extracellular signal-regulated kinase
EtBr	Ethidium bromide
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GCL	Ganglion cell layer
GH	Growth hormone
GHBP	GH binding protein

GHR	Growth hormone receptor
GHRG-1	GH-responsive gene-1
GHRH	Growth hormone-releasing hormone
HEK	Human embryonic kidney
IGF	Insulin-like growth factor
IGF-1R	IGF-1 receptor
INL	Inner nuclear layer
IOP	Intra-ocular pressure
IPL	Inner plexiform layer
JAK 2	Janus kinase 2
LB	Luria-Bertani
MAPK	Mitogen-activated protein kinase
MO	Morpholino oligonucleotides
mRNA	Messenger ribonucleic acid
NGF	Nerve growth factor
NT	Neurotrophin
OF	Optic fissure
OFL	Optic fibre layer
ON	Optic nerve
ONL	Outer nuclear layer
OPL	Outer plexiform layer
OT	Optic tectum
PCD	Programmed cell death

PBS	Phosphate-buffered saline
PDR	Proliferative diabetic retinopathy
PFA	Paraformaldehyde
PI3-K	Phospatidylinositol 3-kinase
PK	Proteinase K
PMSF	Phenylmethylsulphonyl fluoride
QNR/D	Quail (*Coturnix Japonica*) embryonic neural retina cell line
qPCR	Quantitative real-time polymerase chain reaction
RGC	Retinal ganglion cell
RHYNS	Retinitis pigmentosa, hypopituitarism, nephronophthisis, and mild skeletal dysplasia syndrome
RI	Regularity index
RPE	Retinal pigmented epithelium
rRNA	Ribosomal RNA
RSV	Rous sarcoma virus
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
scGH	Small chicken growth hormone
SGFS	Stratum griseum et fibrosum superficiale
siRNA	Small interfering RNA
SO	Stratum opticum
SRIF	Somatotropin release-inhibiting factor
TGF-β1	Transforming growth factor-beta1
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

Chapter One

Literature Review

1.1 Pituitary Growth Hormone

1.1.1 Overview

Pituitary growth hormone (GH) is an endocrine regulator of growth and metabolism. It is secreted by the somatotrophs of the anterior pituitary and delivered to distant target tissues, where it exerts its functions as an endocrine hormone. Its secretion from the pituitary displays a pulsatile pattern in every species studied so far. In particular, it shows a sexual dimorphism in human, rodents, and birds (Gahete et al., 2009). For instance, in adult male chickens, GH secretion occurs in discrete peaks, whereas it is less pulsatile in female chickens (Johnson, 1988). The periodic secretion of GH is mostly regulated by two hypothalamic peptides, growth hormone-releasing hormone (GHRH) for stimulation and somatotropin release-inhibiting factor (SRIF; or somatostatin) for inhibition (Gahete et al., 2009). GH secretion is also under the regulation of nutrition intake, as well as of other hormones, e.g. insulin-like growth factor (IGF)-1 and ghrelin (Gahete et al., 2009).

1.1.2 Gene and Protein Structure

The most abundant form of GH in the pituitary gland and the blood stream is a 22 kDa single chain protein consisting of 191 amino acids. The composition and structure of GH is highly conserved among species; for instance, GH of birds (e.g. chicken, turkey, and duck) has more than 90% similarity with each other, and has at least 60% identity with the mammalian GHs (Tanaka et al., 1992). In higher vertebrates, the GH gene consists of five exons and four introns. Full-length GH

mRNA with 5 exons can be translated to the 22 kDa monomer, but a heterogeneity of GH transcripts has also been found as a result of alternative splicing (Baumann, 1999; Takeuchi et al., 2001). The tertiary structure of monomeric 22 kDa pituitary GH is composed of four α-helices arranged in a left-handed orientation (Abdel-Mequid et al., 1987). It is an asymmetrical molecule with large and small intramolecular loops made up by two disulfide bridges at cysteine residues (Nicoll et al., 1986). These loops are thought to be critical for the binding of GH and GH receptor (GHR), which in turn initiates downstream signal transduction.

In chickens, numerous GH variants have also been found in the pituitary and plasma, in addition to the 22 kDa monomer (Aramburo et al., 2000), due to differences of gene transcription and post-translational modification of the protein. These differences include different protein net charge (Aramburo et al., 1989), protein glycosylation (Berghman et al., 1987; Montiel et al., 1992), phosphorylation (Aramburo et al., 1992), and proteolytic cleavage (Aramburo et al., 2001). It is known that these variants show different bioactivities (Aramburo et al., 1990) and their relative abundance changes during ontogeny (Aramburo et al., 2000).

1.1.3 GH receptor (GHR) and GH binding protein (GHBP)

The GHR belongs to the cytokine/hematopoietin receptor superfamily. Members of this family also include receptors for prolactin, erythropoietin, leptin, and multiple interleukins; receptors for interferon α, β and γ are also distantly

related (Kopchick and Andry, 2000). GHR is a transmembrane receptor with an extracellular ligand-binding domain and a cytoplasmic domain. Recent studies have indicated that the GHR exists as a preformed dimer prior to GH binding (Gent et al., 2002; Brown et al., 2005); to initiate signal transduction pathways, GH sequentially binds to the extracellular domain of dimerized GHR molecules and causes a conformational change, which then activates the receptor-associated Janus kinase (JAK) 2 tyrosine kinase on its intracellular domain (Herrington and Carter-Su, 2001; Lanning and Carter-Su, 2006).

Along with the membrane-bound GHR, a soluble truncated form of GHR also exists in the circulation system of many species. This high affinity GH binding protein (GHBP) represents the extracellular domain of the GHR, which is derived by alternative GHR mRNA splicing (in rodents) (Baumbach et al., 1989; Talamantes, 1994) or by proteolysis of the membrane bound GHR (in human, rabbits, and chicken) (Dastot et al., 1998; Vleurick et al., 1999). In the serum, GHBP binds to about half of the circulating GH (Baumann et al., 1988), and has complex actions on GH function. It is believed that GHBP acts as a reservoir for circulating GH, as it decreases the rate of GH degradation and thereby extends the half-life of GH in the serum (Baumann et al., 1988). However, since GHBP has a high affinity for GH, it may also inhibit GH signalling, acting as a competitive GHR antagonist (Mannor et al., 1991).

1.1.4 Endocrine roles of pituitary GH

It is well established that, in mammals, the main function of pituitary GH is to promote postnatal somatic growth (Ohlsson et al., 1998; Butler and Le Roith, 2001). For instance, a deficiency of pituitary GH or tissue insensitivity to GH action results in dwarfism in juveniles, whereas hypersecretion of GH before puberty causes gigantism (Okada and Kopchick, 2001). Hypersecretion of GH in adults results in acromegaly, characterized by continuous growth of acral regions (hands and feet) and by metabolic disturbances (Melmed et al., 1983).

The role for GH in growth is, however, controversial in birds. GH replacement partially restores the suppressed growth rate caused by hypophysectomy in young chickens (King and Scanes, 1986; Scanes et al., 1986), but it has no effect on the growth rate of hypophysectomized female turkey poults (Proudman et al., 1994). In addition, pulsatile infusion of turkey GH is not able to increase growth in young growing turkeys (Bacon et al., 1995). It also has been shown that pulsatile treatment with chicken GH decreases growth rate and feed intake in 8-week-old chickens (Vasilatos-Younken et al., 2000), while in other studies GH treatment is not found to affect chicken growth (Cravener et al., 1989; Rosebrough et al., 1991). This relative GH resistance in poultry may reflect their genetic selection for fast growth rates and an inability to further respond to exogenous GH, since severe postnatal growth retardation is seen in GHR-deficient (due to GHR dysfunctions) (Hull et al., 1993) and GH-deficient (caused by hypophysectomy) chickens (Harvey et al. 2001b).

In addition to causing growth, GH is considered to be an anabolic hormone because of its stimulation of protein metabolism and muscle growth in mammals. GH administration to mammalian species consistently improves their weight gain and feed intake, with a marked increase in muscle growth and reduced adiposity. These actions reflect the ability of GH to enhance amino acid uptake into skeletal muscle and to increase muscle protein synthesis. Other evidence suggests that GH also reduces protein proteolysis and oxidation, and modulates liver urea nitrogen synthesis (Moller et al., 2007; Moller and Jorgensen, 2009).

Exogenous GH administration to broiler chickens and turkeys has again shown conflicting results with respect to anabolism. Most studies report that GH treatment has no effect on lean body mass gain in young, rapidly growing chickens (Buonomo and Baile, 1988; Cogburn et al., 1989; Moellers and Cogburn, 1995), although a short-term increase in growth rate is seen in chickens subcutaneously injected with recombinant bovine GH (Buonomo and Baile, 1988). In contrast, long-term (21 consecutive days) pulsatile treatment with exogenous GH increases body weight gain in older chickens (Vasilatos-Younken et al., 1988; Scanes et al., 1990). The lack of an effective action of GH on the growth of juvenile chickens is thought to be due to their high circulating GH levels, which is known to suppress tissue GHR expression (Buyse and Decuypere, 1999). GH function in promoting avian muscle growth has also been suggested by *in vitro* studies showing that the GH treatment of chicken skeletal muscle satellite cells enhances their proliferation and inhibits cell differentiation (Halevy et al., 1996).

The fact that GH has a lypolytic effect in mammals is generally accepted. GH predominately stimulates lipolysis and lipid oxidation, resulting in the elevation of free fatty acids and glycerol flux from adipose tissues; it also impairs lipid synthesis by reducing lipogenic enzyme activity (Moller and Jorgensen, 2009; Vijayakumar et al., 2010). By contrast, GH plays a more controversial role in the lipid metabolism of birds. Studies have shown that pulsatile GH administration to 8-week-old broiler chickens for 3 weeks reduces their abdominal fat pad size and total carcass lipid content (Vasilatos-Younken et al., 1988). A reduction of abdominal fat content, a lower plasma triglyceride level, and a decrease of *in vitro* lipogenesis have also been seen in chickens treated for 7 days with pulsatile, but not continuous, GH administration (Rosebrough et al., 1991). A similar effect of GH has been seen *in vitro* as well (Harvey et al., 1977). Campbell et al. report that GH exerts a lipolytic effect to stimulate glycerol release from explants of chicken adipose tissues, and this effect can be blocked by a GHR antagonist (Scanes, 2009). The accumulation of fat tissues in GH-resistant dwarf chickens also supports the lipolytic effect of GH (Hull et al., 1993). On the other hand, Cogburn et al. observe an increase of body fat content after 2-weeks of daily GH injections in young chickens (Cogburn et al., 1989), and both continuous and pulsatile infusions of GH promoted body fat deposition in broiler chickens (Moellers and Cogburn, 1995). Furthermore, there is evidence that hypophysectomized female turkeys have lower plasma triglyceride levels, compared with their intact counterparts, and that GH can temporarily inhibit glucagon-induced lipolysis (Scanes, 2009). The role of GH in avian lipid metabolism thus seems to be more

complex and may differ among genotypes, since a lipolytic response is observed in GH-treated adipose explants from fat, but not lean, broiler chickens (Buyse and Decuypere, 1999).

In mammals, GH-induced increases in fat mobilization and utilization counteracts the ability of insulin to stimulate glucose uptake into peripheral tissues. For instance, it has been shown that GH infusion to postabsorptive individuals causes a decrease in glucose uptake and glucose oxidation of the forearm muscles (Moller et al., 1990a). Similarly, discrete GH pulses cause an acute inhibition of muscle glucose uptake and a suppression of glucose oxidation (Moller et al., 1990b; Moller et al., 1992; Gravholt et al., 1999). Studies have also indicated that the raised concentrations of blood free fatty acids (due to lipolysis) is associated with insulin resistance and leads to hyperglycemia, as well as an enhancement of hepatic gluconeogenesis (Kovacs and Stumvoll, 2005). However, it is still unclear if pituitary GH has a similar diabetogenic role in birds (Scanes, 1992; Buyse and Decuypere, 1999). The finding that hypophysectomised chickens have a decreased level of circulating glucose during fasting is one of the very few studies that suggest a similar role for GH in carbohydrate metabolism in poultry (Koike et al., 1964).

1.2 Extrapituitary GH

1.2.1 Overview

GH is traditionally considered to be an endocrine hormone only secreted by the pituitary. However, its expression in various extrapituitary sites has been

demonstrated recently. For instance, GH or GH-like proteins have been found in the nervous system, in the immune system, in the reproductive system, in the gastrointestinal tissues, and in other tissues including the lungs, the kidneys, the pancreas, the muscles, the bones, and the skin (reviewed by Harvey, 2010). Because GH mRNA is also present in the same tissues, it is believed that at least some, if not all, GH immunoreactivities are due to the translation of locally expressed GH mRNA. In addition, proteins and transcripts of the GHR are also widely distributed and colocalized with GH in most of these tissues (Harvey, 2010), suggesting that they are not only the locations of GH production, but also the target sites of GH action.

1.2.2 Distribution

The presence of GH in the nervous system of birds and rodents is well established. In the chicken, GH immunoreactivity is found in the hippocampus, the hypothalamic regions, the medial and lateral septal areas, and in the median eminence; a similar distribution of GH in the brain is also observed in chicken embryos. Furthermore, GH immunoreactivity is detected in the developing spinal cord and the optic vesicles of these embryos (Harvey, 2010). In addition to the central nervous system, GH is present in the peripheral nervous system of chick embryos, particularly within the trigeminal and vagal nerves, the extensor nerve of the limb bud, and the ethmoid nerve in the head (Harvey, 2010). At the subcellular level, GH immunoreactivity is accumulated in the cytoplasm and the nucleus or perinuclear area of the neural cells (Harvey and Hull, 2003). Since the

presence of GH protein in the embryonic nervous system is prior to the differentiation of pituitary somatotrophs (ED14-15) and to the appearance of pituitary GH in circulation (Sanders and Harvey, 2004), it likely reflects independent synthesis of GH in neural and/or glial cells. The presence of GH mRNA in the chicken brain identical to pituitary GH mRNA in size and sequence supports the possibility that neural GH is produced locally (Render et al., 1995).

GH is also widespread in chicken immune tissues including thymus, spleen, and the bursa of Fabricius, but with a much lower concentration than those in the pituitary (Luna et al., 2005). The content and composition of GH in the chicken lymphoid tissues alter with ages and tissues analyzed. Unlike the 22 kDa pituitary form of GH, the immunoreactivity found in chick lymphoid tissues is primarily associated with a 17 kDa moiety, although other variants of 14, 26, 29, 32, 37, 40, and 52 kDa are also present (Harvey, 2010). In the bursa of Fabricius, GH mRNA is mainly expressed in the cortex composed of lymphocyte progenitor cells, whereas its protein is detected in the medulla where lymphocytes mature; GH and its transcripts are also detected in the lymphocytes and supporting tissues in the bursa of Fabricius (Luna et al., 2008). In addition to in the immune tissues of birds, GH expression in peripheral blood leukocytes, the spleen, thymus, lymph nodes, and tonsils in the human and rat have been reported (Clark, 1997; Harvey and Hull, 1997). Furthermore, evidence also shows that GH and its variants can be produced *in vitro* by lymphocytes and leukocytes (Hattori et al., 1990; Kao et al., 1992; Lytras et al., 1993; Weigent et al., 1988).

Although GH was not traditionally viewed as a major player in the regulation of reproduction, numerous studies have indicated that it is produced in many reproductive tissues where it has autocrine/paracrine function in the mediation of reproduction (Hull and Harvey, 2000a, b). GH immunoreactivity is present in the ovary, the uterine tissues, the mammary tissues, the placenta, the testicular tissues as well as the prostate of the mammalian reproductive system (Hull and Harvey, 2000b). Several studies have also described the distribution of GH immunoreactivity and mRNA in chickens. GH immunoreactivity is present, before or after the onset of egg laying, in the stroma of the ovary and in small and large follicles, where it shows a higher intense in granulose cells than in the thecal cells (Hrabia et al., 2008). A coincident distribution of GH mRNA also has been demonstrated by reverse transcription polymerase chain reaction (RT-PCR; Hrabia et al., 2008). The testis is another extrapituitary site of GH expression, in which GH and its mRNA show a stage-specific expression during the process of spermatogenesis (Harvey et al., 2004; Luna et al., 2004).

Additionally, the production of extrapituitary GH in chickens is further supported by studies showing the presence of GH immunoreactivity and transcripts in many peripheral tissues such as the heart, lungs, stomach, liver, and kidneys of the chicken during early embryogenesis (Harvey et al., 2000; Beyea et al., 2005b).

1.2.3 Roles of extrapituitary GH

Although the appearance of GH and its transcripts in numerous extrapituitary tissues have extensively been found in many species, the potential roles of locally produced GH are mostly unclear. The widespread distribution of GHR and GHBP in neural tissues strongly suggests that the nervous system is a site of GH action (Harvey et al., 2003). GHR immunoreactivity has been found in the brain of rats, rabbits, and human (Lincoln et al., 1994; Mustafa et al., 1994) and in the spinal cord of rats (Thornwall-Le Greves et al., 2001). The presence of the GHR also has been shown in the developing brain and the spinal cord of chicken embryos (Harvey et al., 2001). Further, the GHR immunoreactivity in these neural tissues is colocalized with that of GH, suggesting autocrine/paracrine roles for GH in the regions (Harvey et al., 2001a; Harvey and Hull, 2003).

Autocrine/paracrine roles for GH in neural development have also been derived from rodent studies. For example, transgenic mice overexpressing the GH gene show an increase in brain and spinal weights and in the size of lumbar spinal neurons (Chen et al., 1997). GH-deficient (*dw/dw*) mice, in contrast, have a microcephalic cerebrum with retarded neural growth and poor synaptogenesis (reviewed by Harvey and Hull, 2003). Moreover, because hypomyelination due to arrested glial proliferation occurs in these *dw/dw* mice, GH may be involved in the proliferation and maturation of both neural and glial cells. This possibility is also supported by the accelerated glial cell division and myelinogenesis in *dw/dw* mice after exogenous GH treatment (reviewed by Harvey and Hull, 2003). Some studies have further suggested that both the formation of gap junctions which

provides a medium for cell-cell communication within the nervous system, and the vascularization in the brain that is essential for brain growth and development, are under the regulation of GH expression (reviewed by Harvey and Hull, 2003).

GH is also thought to have neuroprotective roles in neurogenesis. It has been found that exogenous GH is able to inhibit apoptosis in neurons injured by pilocarpine-induced epilepsy or by local hypoxic-ischemic injury (Harvey and Hull, 2003). An increased GH immunoreactivity has been reported in the cortical pyramidal neurons of the injured hemispheres and is more marked in regions that constitutively express GHR (e.g. thalamus, hippocampus and cortical pyramidal neurons), thus suggesting a role of GH in neuroprotection (Harvey et al., 2003). Similar ability of GH in preventing cell death has also been seen in avian retinal neural cells (Sanders et al., 2005).

The findings showing GHR is present in the thymus, spleen, tonsils, lymph nodes, the head kidney of fish, and the bursa of Fabricius of birds support functional significance of immune cell-produced GH (reviewed by Harvey and Hull, 1997). In mammals, membrane-binding sites of GH are present in the thymocytes, lymphocytes, and circulating monocytes (Varma et al., 1993; Badolato et al., 1994; Rapaport et al., 1995); in birds, the immunoreactivities of GHR and GHBP in the spleen, thymus, and bursa of Fabricius are mostly associated with macrophages and some mononuclear nonlymphoid cells (Hull et al., 1996). The broad distribution of GHR immunoreactivity thus suggests the involvement of GH in the regulation of physiology of immune tissues.

The effects of immune cell-derived GH have been extensively studied. It has been demonstrated that lymphocyte-produced GH is able to promote cell proliferation in an autocrine manner, as treatment of cultured lymphocytes with an antisense GH oligodeoxynucleotide decreased [3H]thymidine incorporation into cellular DNA (Weigent et al., 1991). This finding is supported by Sabharwal's study using an affinity-purified-GH polyclonal antibody to neutralize endogenously generated GH to cause a marked inhibition of phytohemagglutinin-stimulated human thymocyte proliferation (Sabharwal and Varma, 1996). In addition, other studies have shown that incubation of leukocyte cultures with GH antibodies decreases the number of cells positive for IGF-1 immunoreactivity, suggesting that leukocyte-derived GH may stimulate the synthesis of IGF-I by leukocytes and in turn promote leukocyte proliferation (Baxter et al., 1991).

Autocrine/paracrine GH action in immune cells has also been demonstrated by elevation of endogenous GH expression. Overexpression of GH in a lymphoid cell line prevents cell apoptosis induced by methyl methanesulfonate through inhibition of expression and activation of various pro-apoptotic factors (Arnold and Weigent, 2004). Related studies suggest that this antiapoptotic effect of GH may be a result of its action in promoting nitric oxide (NO) and preventing superoxide (O_2-) production in response to toxic environmental stimuli (Arnold and Weigent, 2003). In addition, the expression of IGF-1, its receptor (IGF-1R), and IGF-2R in this GH-overexpressing lymphoma cell line is significantly increased, indicating that this protection of cells from cell death may be mediated by IGF-1 and 2 (Weigent and Arnold, 2005; Farmer and

Weigent, 2007). Nevertheless, an increase of the expression of transforming growth factor-beta1 (TGF-β1), an anti-proliferative factor, has also been observed in the GH-overexpressing cell line (Farmer and Weigent, 2006). Since TGF-β1 is associated with cell death in numerous cell types (Haufel et al., 1999), the role of endogenous GH in proliferation and death of immune cells thus could be multiple and complex.

In addition to immune and neural cells, autocrine/paracrine actions of GH have also been reported for other tissues. For instance, a role for endogenous GH in early lung development has been proposed. GH expression in the alveoli of neonatal rat lungs has been induced by intratracheal microinjection of a mouse GH-overexppressing adenovirus, which leads to changes in the lung concentrations of numerous proteins (Beyea et al., 2005a). Another probable site of endogenous GH action is in developing skeletal muscle cells. It has been found that cells of a GHR-overexpressing myoblast cell line grow faster and proliferate in absence of serum (Segard et al., 2003). These effects are inhibited by application of anti-GH antibodies (Segard et al., 2003), indicating that they are a result of local GH action. In addition, GHR overexpression inhibits the formation of myotubes and the expression of markers for myoblast differentiation (Segard et al., 2003). It is therefore thought that GH acts as an autocrine to enhance proliferation and to inhibit differentiation of these myoblasts.

1.3 Retinal GH in chicken embryos

1.3.1 Retinal GH, its distribution and ontogeny in the chicken embryo

The presence of GH immunoreactivity and mRNA in the retina and the visual system has been demonstrated at least in human, rodents, teleosts, amphibian, and birds (reviewed by Harvey et al., 2007). In the chicken, the expression of retinal GH has been studied mostly during embryogenesis. It has been shown that GH immunoreactivity is present in the optic cup of chicken by embryonic day (ED) 2 of the 21 day incubation period, and is abundantly expressed throughout the neural retina and in the retinal pigmented epithelium (RPE), choroid, sclera, cornea, lens, and vitreous by ED 7 (Harvey et al., 2003, 2007; Harvey, 2010). The immunoreactivity is particularly intense in the retinal ganglion cells (RGCs) and can be traced along with their axons, which form fascicles in the optic fibre layer (OFL), to the optic nerve head, the optic nerve (ON), the optic chiasm, the optic tract, and then into the retinorecipient layer of the optic tectum (OT) in the midbrain, where the RGC axons synapse (Baudet et al., 2007b; Harvey et al., 2007; Harvey, 2010). Since *in situ* hybridization has shown full-length GH mRNA expression in the cell soma of RGCs at ED 7 (Baudet et al., 2003), the GH immunoreactivity located in the OPL is likely to be derived, at least in part, from the local production of GH in RGCs. In fact, studies have indicated that the presence of GH in the neural retina of chicken embryos precedes to the ontogenetic differentiation of somatotrophs (ED 14) as well as the appearance of circulating GH in the blood (ED 17) (Harvey et al., 2001a), and thus is believed not to be derived from the pituitary gland.

The nucleotide sequence of retinal GH mRNA in the chicken embryo is identical to that of in the pituitary, whereas the GH immunoreactivity found in the retinal tissues is predominantly associated with a 15 kDa moiety resulted from proteolysis of the 22 kDa monomers of the pituitary GH (Baudet et al., 2003). After its release from the retina, the 15 kDa GH moiety is bound to a 45 kDa proteoglycan, opticin, in the vitreous humor, in which opticin may act as a modulator of GH in regulation of retinal development or ocular function (Sanders et al., 2003). In addition, a small 16 kDa chicken GH moiety (scGH) which is derived from a truncated GH mRNA has also been discovered in the chicken embryonic retina (Takeuchi et al., 2001). By using a specific antibody, the immunoreactivity for scGH is present in most ocular tissues but is not present in the optic fibre layer, the optic nerve head, nor the vitreous. The 16 kDa moiety lacks critical residues required for GHR binding and is normally dimerized to form a 31 kDa protein (Baudet et al., 2007a; Harvey, 2010).

1.3.2 Retinal GH actions

The expression of GHR in ocular tissues of the early chicken embryo (ED 7) and its colocalization with GH immunoreactivity suggest the involvement of retinal GH in the development of the retina, especially the RGCs, during early embryogenesis (reviewed by Harvey, 2010). This possibility is strengthen by the finding that GH immunoreactivity in the RGC layer and the OFL is only present during ED 4 and ED 12 while the RGCs are projecting their axons and starting to form synapses in the OT, and is absent after ED 14 (Baudet et al., 2009). Retinal

GH is also neuroprotective for the developing retinal neural cells, as exogenous GH administration significantly reduces the occurrence of cell death in explanted embryonic chicken retinas, whereas the immunoneutralization of endogenous GH by microinjected GH antibodies substantially increases the incidence of apoptotic cells when undergoing *in vivo* developmental waves of apoptosis (Sanders et al., 2005). Moreover, the immunoneutralization of endogenous GH in immunopanning-enriched chick embryo RGC cultures indicates that RGCs may be a specific target for this neuroprotective effect of GH, as a higher ratio of apoptotic cells is observed after GH immunoneutralization (Sanders et al., 2005). This anti-apoptotic action of endogenous GH is mediated via a reduction of caspase cleavage and of caspase-independent calpain/apoptosis inhibiting factor (AIF) activation, and likely involve the activation of the phospatidylinositol (PI) 3-kinase/Akt as well as the MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) pathways (Sanders et al., 2009a).

In addition to cell survival, retinal GH is likely involved in early neurogenesis. Baudet et al. found that a suppression of endogenous GH in immunopanned chick ED 7 RGCs impedes axon outgrowth and elongation, whereas exogenous GH treatment significantly increases axon elongation (Baudet et al., 2009). These results therefore suggest that retinal GH acts as an autocrine/paracrine factor to promote RGC axon growth in the developing chick neural retina.

Scattered evidence corroborating roles for GH in retinal function has also been provided by some animal models. For instance, in the teleost retina, intraperitoneal administration of GH stimulates cell proliferation (Otteson et al.,

2002). In rodents, autocrine/paracrine GH action in the mouse retina has been suggested by the alterations in the expression level of four proteins involved in retinal vascularization, neural proliferation, and neurite outgrowth in GHR gene disrupted mice (GHR -/-), as compared with their wild type (GHR +/+) littermates (Baudet et al., 2008). GH also has been reported to have a role in angiogenesis in ocular tissues, as an antisense oligonucleotide targeting GHR inhibits neovascularisation in a mouse model of retinopathy (Wilkinson-Berka et al., 2007). Furthermore, neurophysiological roles of GH in the visual system have also been revealed by studies using electroretinogram (ERG) recordings (Harvey et al., 2007; Harvey, 2010).

1.3.3 Retinal GH and IGF-1

Insulin-like growth factor-1 is a critical mediator of many of GH functions. IGF-1 is traditionally considered to be stimulated by the action of pituitary GH on the liver, and then released and delivered to target tissues to modulate their growth and metabolism in an endocrine manner. Circulating IGF-1 also provides feedback to the somatotrophs to suppress further release of GH from pituitary (Buyse and Decuypere, 1999; Butler and Le Roith, 2001). However, this hypothesis has been challenged, as subsequent studies have shown that IGF-1 is also synthesized in most, if not all, tissues, and its expression is usually regulated by various local and endocrine factors (Le Roith et al., 2001). In fact, there is evidence that the expression and functions of IGF-1 in various target tissues may

or may not be GH-related, and they may or may not share the same signal transduction pathways as GH (Butler and Le Roith, 2001; Sanders et al., 2009a).

Studies in teleosts, birds, and rodents have indicated that, like GH, IGF-1 itself is involved in the regulation of embryonic retinal development. It has been shown that both IGF-1 and IGF-1 binding sites are present in the developing chick retina; moreover, transcripts of retinal IGF-1 as well as IGF-1 receptors are concentrated in the RGC layer during early stages of development in chick embryos (Bassnett and Beebe, 1990; Waldbillig et al., 1991; de la Rosa et al., 1994). These findings indicate that IGF-1 may act as an autocrine/paracrine hormone in the chick embryo retina, especially in developing RGCs. Indeed, inhibition of IGF-1 receptor signalling during zebrafish embryogenesis significantly arrests retinal development, increases cell death rate, and thus causes considerable defects in the retina (Schlueter et al., 2007). IGF-1 is thus thought to participate in the regulation of cell proliferation, differentiation, and cell survival in the embryonic retina of zebrafish. These findings are supported by another study, which showed that IGF-1 upregulation in goldfish RGCs after optic nerve injury is followed by cell survival and neurite regeneration (Koriyama et al., 2007). It has also been reported that IGF-1 has the ability to promote RGC axon growth in rats (Goldberg et al., 2002), and the ability to increase the expression of a neuroglial cell adhesion molecule G4, which is known to promote neurite outgrowth from chick embryo retinal explants (Baudet et al., 2007b).

Previous studies have indicated that, in the retina of early chicken embryos, the distribution of IGF-1 and its receptor is consistent with that of GH and GHR

(de la Rosa et al., 1994; Baudet et al., 2003), suggesting a dependent relationship between retinal GH and IGF-1. The hypothesis of this relationship is reinforced by the findings that treatment of cultured chick retina explants with exogenous GH increases local IGF-1 mRNA content (Baudet et al., 2003), and by the observation that intraperitoneal injections of GH stimulate IGF-1 mRNA expression in the retina of goldfish (Otteson et al., 2002). In addition, the rate of apoptosis of immunopanned RGCs stimulated by simultaneous immunoneutralization of endogenous GH and IGF-1 is not different from that induced by immunoneutralization of GH alone (Sanders et al., 2009a), suggesting that the anti-apoptotic effect of endogenous GH in cultured chicken embryonic RGCs is likely mediated through IGF-1 signalling. Nevertheless, direct evidence showing a functional relationship between endogenous GH and IGF-1 in the chicken embryonic retina has yet to be proven.

1.4 GH and ocular function

The possibility that GH is involved in ocular function has been suggested by previous studies. The immunoreactivities of GH and GHR have been detected in the retina and GH is present in the vitreous fluid of elderly patients with ocular disorders (e.g. diabetic retinopathy, retinal detachment, vitreous hemorrhage, and epiretinal membrane), suggesting that it may play a role in retinal function (Harvey et al., 2009). Since GH immunoreactivity is mostly present in healthy, but not in apoptotic RGCs, it is likely that the appearance of GH correlates with retinal cell survival (Sanders et al., 2009b). The existence of GH

immunoreactivity in human ocular tissues likely reflects local translation of GH mRNA, or the leakage of blood-retinal barriers, since they are thought to prevent the passage of circulating GH into the vitreous (James and Cotlier, 1983). In fact, pituitary GH has long been implicated in the pathology of retinal neovascularisation and the etiology of diabetic retinopathy (DR), and pituitary GH deficiency is also associated with many ocular dysfunctions (Harvey et al. 2007).

1.4.1 Ocular function and pituitary GH excess

Most of the ocular dysfunctions resulting from pituitary GH excess are associated with retinopathy, e.g. the retinopathy caused by complications of diabetes mellitus. Diabetic retinopathy can be characterized into two stages; non-proliferative diabetic retinopathy followed by proliferative diabetic retinopathy (PDR). Poor control of diabetes mellitus causes elevated levels of glucose in the blood. This hyperglycemia then induces the loss and dysfunction of endothelial cells and pericytes of the retinal capillaries as well as the thickening of basement membrane both that damage the blood vessels. This pathology leads to vascular leakage, and then results in local ischemia, due to lesions caused by adherent leucocytes. The lack of oxygen in the area of ischemia further causes the formation of fragile, new blood vessels in the retina and into the vitreous cavity. Vision is thus damaged by vitreous hemorrhage, vascular leakage, and retinal detachment (Wilkinson-Berka et al., 2006).

The association between GH and diabetic retinopathy was initially noticed in studies showing the therapeutic effect of pituitary ablation on severe PDR

(Beck et al., 2001). It also has been reported that the incidence of retinopathy is approximately three times higher in type I diabetic patients who are GH sufficient, than those who are GH deficient (Wilkinson-Berka et al., 2006). This correlation is also supported by studies that show the induction of retinopathy in GH-deficient patients taking exogenous GH therapy, followed by a full remission after the cessation of the therapy (Wilkinson-Berka et al., 2006). A similar effect was seen in a non-diabetic subject during separate trials of GH therapy for treating severe hyperopia (Paterson et al., 2007). GH is also thought to be involved in angiogenesis during the development of DR, as it directly stimulates the proliferation of endothelial cells from the human retinal microvasculature *in vitro* (Rymaszewski et al., 1991). Indeed, drugs that suppress pituitary GH secretion (e.g. somatostatin or analogues) are able to limit PDR progression and thus can be considered as a therapeutic approach (Harvey et al., 2007). In addition, it has been reported that acromegalic patients have a significantly greater central corneal thickness than normal controls, suggesting a positive correlation between GH excess and corneal structure (Ciresi et al., 2010).

1.4.2 Ocular function and pituitary GH deficiency

Pituitary GH deficiency impedes normal ocular function, since children with congenital pituitary GH deficiency have a greater incidence of ocular dysfunctions than normal children (Harvey et al., 2007). Hellstorm et al. have shown that GH-deficient children have significantly less retinal blood vessel branching points than healthy controls, implicating GH roles in retinal

vascularisation (Hellstrom et al., 1999). Optic disc hypoplasia, as well as septo-optic dysplasia, are also associated with pituitary GH deficiency, suggesting a relationship between GH absence and the underdevelopment of the optic nerves (Harvey et al., 2007). Abnormal emmetropization is also common in GH-deficient children. These children have a shorter axial length of their eyes, probably due to the retardation of postnatal ocular growth caused by dysfunctions in the GH/IGF-1 axis (Parentin et al., 2004). Indeed, it has been shown that well-timed GH replacement therapy results in normal emmetropization in children with GH defects (Parentin and Perissutti, 2005). It is also known that Jacobsen syndrome, characterized by gene deletions of chromosome 11q that results in pituitary GH deficiency, has been linked to many severe ocular abnormalities. Cockayne syndrome is another GH deficiency-related disorder in children, in which pigmentary degeneration of the retina has been observed (Harvey et al., 2007). People with GH resistance-induced Laron dwarfism also have visuomotor defects (Harvey et al., 2007) and refractive errors of the eye (Bourla et al., 2006). In addition, patients with RHYNS (retinitis pigmentosa, hypopituitarism, nephronophthisis, and mild skeletal dysplasia) syndrome, characterized by early retinal degeneration and reductions of photoreceptor responses, are GH insufficient (Harvey et al., 2007).

1.5 Retinal development in the chicken

1.5.1 Anatomy

The neural retina of birds consists of rod and cone receptors, vertically oriented bipolar cells, horizontal and amacrine cells, Müller glia cells, as well as ganglion neurons. These cells can be distinguished from each other based on their size, shape, molecular composition, function, and location in the strata of the retina. Most of these cell types can be further divided into several subtypes. For instance, at least seven morphological types of RGCs have been distinguished in the chicken retina (Thanos et al., 1992). The basic structure of the avian retina is very similar to that of mammals, and is characterized by five distinct layers (Fig. 1.1). The five layers are, from the ventricular to vitreal surface, the outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and the ganglion cell layer (GCL). The ONL contains cell bodies of the photoreceptors, and the INL is composed of the soma of the bipolar, horizontal and amacrine cells; the GCL is where the ganglion cells and displaced amacrine cells are located. These three nuclear cell layers are separated by two neurophil layers where synaptic contact occurs; the OPL is where the photoreceptors and bipolar/horizontal cells synapse, and the IPL is where the bipolar/amacrine cells connect to the ganglion cells. In addition, the ganglion cells project their axons along the optic fibre layer (OFL).

1.5.2 Retinal development in the chicken

The retina is derived from the optic vesicle in the posterior part of the forebrain (diencephalon). The optic vesicle invaginates to form a bi-layered optic cup, with a thickened inner layer which later forms neural retina, and an outer layer that gives rise to the RPE. The process of the optic cup folding begins when the optic vesicle contacts the surrounding ectoderm. At this early stage of eye development, all optic vesicle cells are multi-potential and capable of being induced into epithelial cells or different types of neural cells. A retinal neuroepithelium stem cell is able to keep proliferating until it undergoes a terminal mitosis and commits to its own cell type; in chickens, this process of "cell birth" begins on ED 3 and continues to at least ED 8. It has been known that most proliferating neuroepithelial cells have the potential to give rise to two or more different cell types (reviewed by Mey and Thanos, 2000).

At ED 5, the cross-section of chick neural retina can be divided into two strata, the ventricular matrix zone adjacent to the future RPE, and the inner neuroblastic layer. The ventricular zone is mainly where the proliferating cells are located. The committed neuroblasts migrate to the vitreal side after their final mitosis and differentiate to neural cells, which in turn form a new layer, the mantle layer (reviewed by Mey and Thanos, 2000). It is believed that cell proliferation at this stage is under the control of the Delta-Notch signalling system (Ahmad et al., 1997; Henrique et al., 1997). Delta and Notch are structurally related transmembrane proteins synthesized only by stem cells and new-born neurons. They act as ligand and receptor respectively to prevent stem cells from

leaving the mitotic cell cycle, and thus cells expressing Delta and Notch stay in the uncommitted state. The developing retina initially consists of a group of uncommitted, proliferating neuroepithelial cells; as development progresses, some of these cells undergo their final mitosis while other cells still keep proliferating. The post-mitotic progenitor cells begin to move toward the vitreal surface of the retina. Meanwhile they encounter different environmental signals distributed in a position-dependent manner. Cell fate is thus determined by these inductive environmental factors according to the locations of the cells in the retina. It has been shown that environmental factors including polypeptide growth factors, extracellular matrix molecules, secreted transcription factors of the hedgehog family, and the retinoids are able to influence the developmental pathway of the progenitor cells (Adler, 2000). It is also noteworthy that this model has been challenged by studies showing that different progenitor cells have bias toward the production of certain cell types and thus suggesting the existence of intrinsic mechanisms for cell fate determination (Alexiades and Cepko, 1997; Belliveau and Cepko, 1999).

In chicks, retinal maturation follows a centrifugal gradient from central to peripheral and from temporal to nasal retina. At ED 8, most of the cells in the central area are post-mitotic, whereas cells remain mitotically active for a few additional days at the periphery. Retinal neuroblasts leave the mitotic cycle in a specific order with respect to their prospective cell type. The first cell type to differentiate in the retina is the RGCs, followed by photoreceptors, then by amacrine and Müller cells, and succeeded by horizontal cells. Bipolar cells are the

last cell type that leaves the cell cycle. In contrast to the neurons, which experience an irreversible pathway once they pass the final commitment, Müller cells (glial cells) retain the ability to divide throughout whole life of the bird. As progenitor cells that move to a comparable location in the cross-section of the retina tend to turn into a certain morphological type of neurons, the chick retina thus transforms from a pseudo-stratified neuroepithelium to a multi-laminated tissue. The process of retinal stratification begins at about ED 6 and the pattern is essentially established before ED 16. The first synapses in the IPL are observed at ED 13, whereas they are not present in the OPL until ED 17. The entire process of chicken retinal development is complete at the time of hatching (21 days), but mature function as evidenced by electrophysiological recording can be obtained at ED 19 (reviewed by Mey and Thanos, 2000).

1.5.3 Development of the RGCs

Of all the retinal neurons, RGCs provide the only retina output to the brain and thus have received greatest interest. Most RGC precursor cells withdraw from their cell cycle from ED 3; at the same time they detach from the ventricular surface to migrate vertically toward the limiting membrane of the inner, vitreal side where they form the GCL. Before their translocation, precursor cells differentiate an endfoot to each side of the retina and connect with both sides while migrating. Studies have also indicated that during the process of migration, these cells may be directed by interactions with surrounding Müller glia cells. RGCs start to extend their axons directly from their vitreal endfoot right after the

detachment from the ventricular surface; in contrast, their dendritic trees develop much later than axons (between ED 7 and ED 13). A clear stratification in the IPL is not present until ED 16 (reviewed by Thanos and Mey, 2001).

Owing to the asymmetric pattern of chick retinal development, the generation of RGC axons also follows a clear central-peripheral, temporal-nasal gradient. The first axons appear in the central retina between ED 2 and ED 3. RGC axons are projected toward the vitreal side and join the nearest fascicles which then constitute the OFL. Almost all optic fibres are then directed to the optic fissure, through which they leave the eyeball and extend along the optic stalk. The first RGC axons reach the optic chiasm around ED 4 and thereafter enter the contralateral optic tract. RGC axons arrive at the anterior/ventral pole of the optic tectum on ED 6. Between ED 7 and ED 12, the elongating axons leave the anterior/ventral pole and advance to the posterior/dorsal pole of the tectum, thereby forming the superficial stratum opticum (SO) layer. RGC axons ultimately arborize and synapse with tectal neurons within the stratum griseum et fibrosum superficiale (SGFS) of the tectum. This invasion takes place gradually between ED 12 and ED 14 (reviewed by Thanos and Mey, 2001).

1.5.4 Cell death in retinal development

During the embryonic development of multicellular organisms, apoptosis, also called programmed cell death (PCD), is a general, widespread phenomenon that serves as a mechanism to regulate the size of cell populations. Apoptosis can be characterized by certain morphological changes of the cells, which include cell

blebbing, nuclear shrinkage, chromosomal DNA fragmentation and chromatin condensation; in addition, it normally involves the activation of specific intracellular signalling pathways, such as members of caspase and B-cell lymphoma 2 (Bcl-2) families. By adopting these morphological/biochemical signs as criteria, several immunohistochemical methods have been developed to identify apoptotic cells *in vitro* and *in vivo*. For instance, the nuclear staining of apoptotic cells with DAPI (4', 6-diamidino-2-phenylindole) or Feulgen leads to a stronger, compacted stain in the nucleus, with an increased number of chromosome fragments. Apoptotic cells can also be identified by using antibodies against different protein markers which participate in the apoptosis cascade. Another common method generally used is the TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay, which relies on the presence of the 3'-OH ends of DNA fragments that can be labelled by terminal deoxynucleotidyl transferase (reviewed by Vecino et al., 2004).

During the development of the chick retina, cells undergo at least two waves of programmed cell death. The first wave takes place between ED 4 and ED 7, while the retinal cells are experiencing proliferation, differentiation, and migration; the second one begins from ED 10 and peaks at ED 12, and mainly occurs in differentiated RGCs (Vecino et al., 2004; Sanders et al., 2005). As the second phase shares an overlap with the period that axons of the RGCs arrive at the optic tectum and interact with tectal cells, it is believed that the survival of the RGCs is dependent upon correct targeting to tectal sites and adequate supplement of trophic factors from the tectum (Farah, 2006).

A number of trophic factors that support retinal cell survival and thus regulate the phases of retinal cell death have been identified. It has been shown that neurotrophins and their receptors are indispensible for maintaining the viability of retinal neural cells. Members of this neurotrophin family include BDNF (brain derived neurotrophic factor), NGF (nerve growth factor), and NT (neurotrophin) -3 and 4. In particular, in the chick embryo the application of BDNF *in ovo* prevented retinal cell death during the early development (ED 5 to ED 7) of the retina (reviewed by Vecino et al., 2004). Insulin and insulin-like growth factors are similarly able to protect retinal cells from apoptosis during development (Vecino et al., 2004), and recent studies have found that GH also has the potential to promote RGC survival in chicks (Sanders et al., 2009a). In addition to these survival factors, elevation of electrical activity of retinal neurons can rapidly recruit neurotrophin receptors to the cell membrane and thus has been proposed to influence the survival of these neurons (Meyer-Franke et al., 1998).

It is now well accepted that the process of PCD during embryonic development is essential and critical for correct morphogenesis of tissues and organs, as well as healthy neonates. In the developing vertebrate nervous system, for example, approximately 50% or more neural cells die right after their birth (Vecino et al., 2004). Furthermore, dysregulation of apoptosis is thought to be causal in the generation of some disease states, e.g. birth defects resulting from diabetic pregnancy are associated with apoptosis of a great group of progenitor cells during the formation of the affected organs (Chappell et al., 2009). However, the mechanism that PCD regulates the formation of tissues and many of the

related functional implications are still unclear and thus are of interest to developmental biologists.

1.6 The chicken embryo: a model for developmental biology

1.6.1 The advantages of the chicken embryo as a model for developmental studies

The chicken embryo has been extensively used to study embryology and developmental biology. Indeed, its use as a model to gain insights into development can be traced back to the time of Ancient Egyptians and of Aristotle, and many major contributions in developmental biology have been achieved by relying on chicken embryos (reviewed by Stern, 2005). The chicken has some attributes that makes it an optimal model. Firstly, as compared to other species, chicken embryos are widely available and relatively low in cost. Fertilized chicken embryos are easily maintained in humidified chambers with a constant temperature, and the whole gestation period of chicken only takes 21 days, which is much less than that of many other species. In addition, the chicken embryo is comparatively large and floats on the egg yolk early in development, making it easy to manipulate *in ovo*.

The chick is a warm-blooded higher vertebrate that undergoes true growth during its development, in which the embryo and organs tremendously enlarge in size, and thus the progress of its embryonic development closely resembles that in humans. In addition, as the only endocrine connection with the mother is via hormone deposits left in the yolk, chicken embryonic development is relatively

independent of alterations in maternal physiology (De Groef et al., 2008). Moreover, compared with other animal species, the chicken is more susceptible to the induction of myopia, which makes it a good model for visual studies (Edwards, 1996).

With the advent of modern genetic and molecular studies, the chick lost its pioneer status for embryological and developmental research, due to the lack of genomic information. However, in 2004, the first draft sequence of the complete chicken genome was completed by a consortium led from Washington University (St. Louis, MO). It is particularly notable that the chicken genome, which has a haploid content of 1.2 X 10^9 base pairs of DNA divided into 40 chromosomes, presents about the same number of genes as humans (between 20,000 and 23,000 genes), and the genetic loci on chromosomes are highly conserved between chicks and mammals (Stern, 2005). Findings of comparative genomic analysis have indicated that, unlike teleost fishes and amphibians, the genome of the chicken has not undergone a recent duplication during its evolution, and in most cases there is a correspondent gene in the chicken to a homologous mammalian counterpart (Stern, 2005).

Knowing the genes that compose a genome allows genetic manipulations in embryos to specify the functions of a particular gene in the embryonic development. These manipulations usually involve the misexpression of a gene in a temporally and spatially controlled way. *In ovo* electroporation, a technique that is established and refined almost within the last decade, has become a generally used method that makes it possible to introduce expression constructs efficiently

into regions of any size, at almost any position, and at any stage of development (Muramatsu et al., 1997; Nakamura et al., 2004). Furthermore, a number of approaches have been developed that allow achievements of gain- and loss-of-function studies of the gene of interest. Morpholino oligonucleotides (MO) (Kos et al., 2003) and small interfering RNA (siRNA) (Nakamura et al., 2004) are good examples that have been successfully transfected into a restricted region of the chicken embryo at a specific developmental stage to locally suppress the expression of a target gene. Enhancement of gene expression in chicken embryos also has been achieved by introducing a vector expressing the desired gene into the target cells of the embryos (Swartz et al., 2001; Nakamura et al., 2004).

Transgenic animals are a powerful tool for investigating gene functions by introducing modified genetic material into a living organism and their germ cells so that the organism will express a new property and transmit it to its offspring. Unlike the successful attempts for transgenic mice, transgenic chicken technology has once faced many obstacles and thus lagged behind for years. However, significant improvements in recent years have successfully generated chicken germline transgenics that are essential for creating transgenic chickens (Mozdziak and Petite 2004; Stern 2005). It is therefore generally believed that transgenic chickens that express or lack specific genes will be available in the foreseeable future, and transgenic models will provide more insight into embryonic development.

1.7 Experimental rationale

Previous studies have demonstrated the presence of GH and GHR and their transcripts in the embryonic chick neural retina. As the immunoreactivity of GH is colocalized with that of GHR by ED 7, it is likely that retinal GH plays an autocrine/paracrine role during early embryonic development of the chick retina (Harvey et al., 2007). Indeed, studies published by Sanders et al. have suggested that GH is neuroprotective against developmental and naturally occurring cell death (Sanders et al., 2005, 2006). In those studies, GH antisera were used to immunoneutralize endogenous GH in the neural retina of chick embryos. In the present study, however, a different approach to down-regulate GH expression, siRNA knockdown, was applied. It is now well known that siRNA suppresses the expression of a specific gene through RNA interference pathway (Pushparaj et al., 2008). The possibility that GH acts as a neuroprotective factor in the developing chick retina was thus further examined by knocking down the retinal GH gene *in ovo*.

In addition, IGF-1 is generally thought to be an important mediator of a variety of GH actions which probably include GH-induced retinal cell survival (Sanders et al., 2009a). Nevertheless, direct evidence demonstrating the relationship between endogenous GH and IGF-1 in the chick retina has never been reported. It is thus of interest to know if endogenous GH knockdown causes alterations of local IGF-1 expression.

1.8 Rationale for the analysis of quail GH mRNA

Since GH and its transcripts are abundantly present in the developing RGCs and the retinofugal tract of chick embryos, and their expression mirrors the distribution of GHR within the same areas (Harvey et al., 2007), it is likely that GH has roles in modulating the development of RGCs. To specify GH actions in the RGCs rather than in other cell types of the retina, manipulations of GH expression are applied to purified RGC cultures in previous studies (Sanders et al., 2005; Baudet et al., 2009). Chick RGCs are enriched by immunopanning, using an antibody against an antigen, Thy-1, expressed on the cell membrane of mature RGCs (Barres et al., 1988; Butowt et al., 2000; Sanders et al., 2005). By applying this method, more than 90% of total cells in the primary culture are recognized as typical RGCs (Butowt et al., 2000; Sanders et al., 2005). Despite a high efficiency of enrichment, such an approach is time-consuming and the key element for capturing RGCs, Thy-1 antibody, is not commercially available for chicks. A substitute for primary RGC cultures to manipulate GH expression *in vitro* is to use a permanent RGC cell line. Owing to its property of constant division, a RGC cell line provides a renewable resource for *in vitro* experimentation. A commercially available quail (*Coturnix coturnix japonica*) embryonic neural RGC cell line (QNR/D) (Pessac et al., 1983) is hence adopted for the present studies, to demonstrate autocrine/paracrine GH functions in developing RGCs.

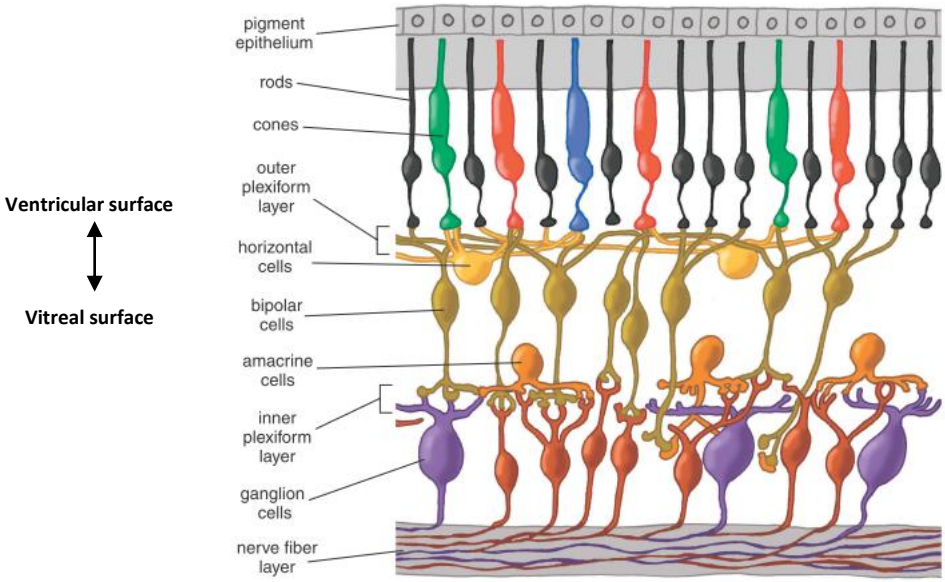
Functional actions of endogenous GH in RGCs can be determined by examining cell morphology and molecular alterations after suppression and/or augmentation of GH expression. In the previous studies of our laboratory, GH

knockdown is successfully achieved, both in immunopanned chick RGC cultures and in chick GH-expressing human embryonic kidney (HEK) 293 cells, by applying a siRNA (NR-cGH-1) designed for chicken pituitary GH mRNA (Baudet et al., 2009). It is thus essential to know if NR-cGH-1 has the potential to achieve GH gene silencing in QNR/D cells. To evaluate the potential of NR-cGH-1, the nucleotide sequence of quail GH mRNA has to be determined, to ensure that the identical sequence targeted by the chicken GH siRNA is present in quail GH mRNA. As quail GH mRNA is not listed in Genbank, its sequence was determined prior to carrying out experiments using QNR/D cells and NR-cGH-1 siRNA.

1.9 Hypothesis and aims

The hypothesis of this study is that retinal GH is an autocrine/paracrine hormone that acts as a neuroprotective factor in the early development of chick retina. The aims of this project are therefore to demonstrate if endogenous GH knockdown by RNA interference results in neural cell death in the developing chick retina, and if this neuroprotective effect of GH is through the mediation of IGF-1 signalling.

(A)



(B)

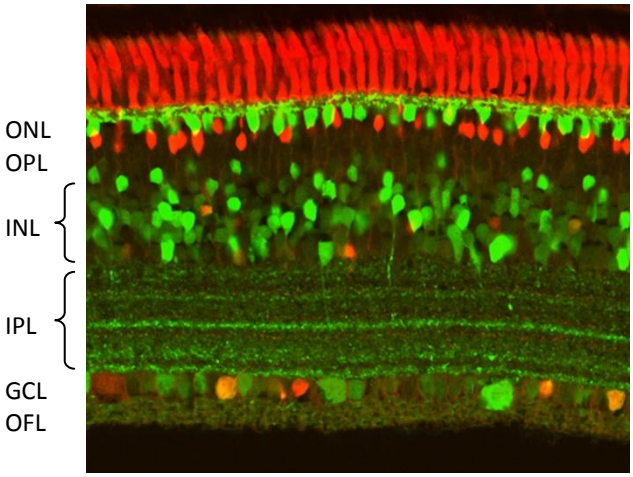


Figure 1.1. Schematic graph of discrete cell and neurophil layers in the retina (A) and a vertical section of the adult chick retina (B). ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; OFL: optic fibre layer. Figures are adapted from Kolb, 2003 (A) and the website of Retinal Microscopy at the University of Alicante, Spain (B) http://www.retinalmicroscopy.com/index.html.

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Chapter Two

Growth Hormone Expression and Neuroprotective Activity in a Quail Neural Retina (QNR/D) Cell Line

Publication and Author Contributions

The results in this chapter have been published in Sanders E. J., W-Y. Lin, E. Parker E, and S. Harvey (2010) Growth hormone expression and neuroprotective activity in a quail neural retina cell line. *General and Comparative Endocrinology*, 165, 111-119.

Quail pituitary RNA extraction, reverse transcription of extracted mRNA into cDNA, as well as TA cloning of quail pituitary GH cDNA were carried out by Wan-Ying Lin. Chicken pituitary GH cDNA was similarly cloned for comparison. These data are provided in Figures 2.1 and 2.2. The complete mRNA and amino acid sequences of quail pituitary GH have been registered in GenBank (Accession numbers FJ458436 and ACJ73931 for the GH mRNA and amino acid sequences, respectively). Wan-Ying Lin also cultured the quail cell line (QNR/D) and transfected them with the GH and control siRNA's. The manuscript was primarily written by Dr. Esmond J. Sanders and Eve Parker performed the Western blotting, ELISA and immunocytochemistry.

2.1 Introduction

Although growth hormone (GH) is widely considered to be an endocrine factor derived from the pituitary gland, it is becoming increasingly clear that it is also produced in other tissues in which it may act locally in an autocrine or paracrine fashion (Harvey and Hull, 1997). The retina is one of these extra-pituitary sites where GH is synthesized (Baudet et al., 2003; Sanders et al., 2003; Diaz-Casares et al., 2005), and subsequently secreted into, and sequestered in, the vitreous, bound to proteoglycans such as opticin (Sanders et al., 2003; Forrester, 2004; Modanlou et al., 2006; Magali et al., 2007). In this location GH appears to play a paracrine role in cell survival during retinal differentiation in the chick embryo (Sanders and Harvey, 2004; Sanders et al., 2005), perhaps controlling the developmentally-regulated waves of cell death that occur in this tissue in avians and mammals (Frade et al., 1997; Bahr, 2000; Sanders et al., 2005). We have found that GH is also present in the adult rat (Harvey et al., 2006) and human (Harvey et al., 2009; Sanders et al., 2009) retina, suggesting that it may have neuroprotective or other roles post-natally in mammals, with potential significance for retinal neurodegenerative disease.

Our investigations into the mechanisms of GH neuroprotection in cultured chick embryo retinal ganglion cells (RGCs; Sanders et al., 2006; 2008), have shown that GH uses established intracellular cell survival signaling pathways. We have proposed (Sanders and Harvey, 2008) that GH can be considered as a developmental growth and differentiation factor analogous to conventional growth factors, such as insulin, insulin-like growth factor-1 (IGF-1), fibroblast

growth factor (FGF), brain-derived neurotrophic factor (BDNF), transforming growth factor-β (TGF-β), and glial-derived neurotrophic factor (Sanders and Harvey, 2004). The influence of GH on RGC apoptosis has been demonstrated in chick embryo cells by *in ovo* intravitreal injection of GH into the eye cup at embryonic day (ED) 2, as well as in explanted retinas and in cultured RGCs enriched by immunopanning (Sanders et al., 2005).

Our previous results have thus indicated that the action of GH in the neuroprotection of embryonic RGCs involves pathways in common with other neurotrophins, supporting our view that GH can be considered to be a growth and differentiation factor in the development of the embryonic retina.

These earlier investigations were carried out on cultures enriched by immunopanning for chick embryo RGCs, in which endogenous GH levels were either supplemented by recombinant chicken GH, or depleted by immunoneutralization with GH antisera. In the present work, we have extended these investigations in two ways. Firstly, we have used a quail embryonic neural retina cell line, QNR/D, which is rich in RGC- and amacrine-derived cells (Crisanti-Combes et al., 1982; Pessac et al., 1983a, b), and show that GH is present in cells identified as RGC-derived. Cells from this particular cell line, when implanted into avian eyes, are able to incorporate into appropriate cell layers of the host, indicating that the individual cells in these cultures are recognized by the host retina (Trisler et al., 1996). Secondly, we have used small interfering RNA (siRNA) technology to deplete endogenous GH in these cultures and show that, like the immunoneutralized primary cultures of chick embryo

RGCs, siRNA-induced GH knock-down results in increased levels of apoptosis in the cultures. We have therefore validated our earlier results showing that GH is able to exert neuroprotective effects in the neural retina, and further strengthen the contention that GH is a *bona fide* cell survival agent.

2.2 Materials and Methods

2.2.1 Characterization of quail (Coturnix coturnix japonica) growth hormone mRNA

Quail pituitary GH mRNA was cloned to determine if PCR siRNAs designed for chicken pituitary GH mRNA (Baudet et al. 2009) could be used for GH mRNA expressed in cultured quail RGCs.

RT-PCR: Total RNA was extracted from adult quail anterior pituitary glands (Health Sciences Laboratory Animal Services, University of Alberta, Edmonton) using Trizol reagent (Invitrogen Canada Inc., Burlington, Ont.), and contaminating genomic DNA was removed by DNase I treatment (Invitrogen Canada Inc.). Chicken pituitary mRNA (from the heads of slaughterhouse broiler fowl, Baudet et al., 2003) was also extracted and reverse-transcribed along with quail pituitary mRNA as a positive control. First strand cDNA was synthesized by reverse transcription of 0.5 µg DNA-free total RNA using the Thermoscript RT-PCR system (Invitrogen Canada Inc.) according to the manufacturer's instructions. One µl Oligo$(dT)_{20}$ primer was used to reverse transcribe total mRNA. Two µl of first strand cDNA was amplified in the presence of 10 µM sense and antisense oligonucleotide primers (Baudet et al. 2003). cGH5 (5'

CGTTCAAGCAACACCTGAGCAACTCTCCCG 3', forward) and cGH3 (5' GCCTCAGATGGTGCAGTTGCTCTCTCCGAA 3', reverse) were used to amplify a 690 bp total-length quail GH cDNA in the presence of 10x High Fidelity PCR buffer, 50mM $MgSO_4$, 10 mM dNTP mix, and 0.2 µl Platinum Taq High Fidelity DNA polymerase. The PCR mixtures were first denatured at 94℃ for 5 min, and then subjected to 35 cycles of 94℃, 30 sec denaturation, 55℃, 30 sec primer annealing, and 72℃, 30 sec elongation, with a final extension at 72℃ for 10 min using a thermal cycler (Techgene, Fisher Scientific, Canada). The amplified cDNA was then identified by running a 1.5% (v/v) ethidium bromide (EtBr)-stained agarose gel and the size of the amplicon was compared with a 1kb plus DNA ladder (Invitrogen, Canada Inc.).

TA Cloning: The 690 bp PCR product was inserted into a PCR II-TOPO vector (Invitrogen, Carlsbad, CA), which was immediately transfected into chemically competant *E.coli* (Invitrogen Canada Inc.) following the manufacturer's instructions. Cells were allowed to grow overnight at 37℃ on Luria-Bertani (LB) plates containing 50 µg/ ml ampicillin and kanamycin. Forty mg/ml X-gal diluted in dimethylformamide was coated on the LB plates for blue-white selection prior to cell spreading. Five to ten white colonies were selected and cultured overnight at 37℃ in LB medium containing 50 µg/ ml ampicillin and kanamycin. The plasmids with the 690 bp GH cDNA were isolated using a QIAprep Miniprep Kit (Qiagen, Ontario, Canada). Three cDNAs were identified by BamHI endonuclease (Fermentas, Burlington, Ontario, Canada) digestion and visualized on a 1% (v/v) EtBr-stained agarose gel under UV light. The sequences

of each of the inserts were identical and were determined by The Applied Genomics Center of the University of Alberta, Edmonton, Canada.

2.2.2 siRNA synthesis and characterization

NR-cGH-1 siRNA,(r(UUUAGUUUCUCAAACACUC)dTdG), coupled to AlexaFluor 488, was custom synthesized by Qiagen Inc. (Mississauga, Ontario) and characterized as described previously (Baudet et al., 2009). This siRNA targets chicken GH exon 4, an exon which is present in all GH isohormones present in the chick embryo at embryonic day 7 (Takeuchi et al., 2001; Baudet et al., 2003), and in adult quail (see above). The ability of labeled siRNA to achieve a similar knockdown as non-labeled siRNA has been assessed (Baudet et al., 2009). A search using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) indicated that this siRNA, will only react with GH mRNA from chicken, turkey, duck and quail. Non-silencing siRNA, (r(ACGUGACACGUUCGGAGAA)dTdT) (Qiagen Inc.), coupled to AlexaFluor 488, was used as a negative control.

2.2.3 Quail neural retina cell line and cell culture

A quail neural retina cell line (QNR/D; American Type Culture Collection number: CRL-2532) was obtained from Cedarlane Laboratories (Burlington, Ontario).

Cells were thawed in a 37˚C water bath for 2 mins and divided into 200µl aliquots containing approximately 3 x 10^6 viable cells per ml. One aliquot was

placed in a 35mm diameter tissue culture dish and to this was added 1.8ml of DMEM medium with 10% fetal bovine serum (FBS), 2µl gentamycin and 10µl/ml of *Trans*IT-TKO transfection reagent (Mirus Bio Corp., Madison, WI, USA). Then, siRNA, either a non-silencing control, or experimental, sample, was added to the culture at a concentration of 5µl/ml (100nM). Cultures were then incubated at 37˚C for 24 hrs.

The transfection efficiency was measured by counting cells in randomly selected fields of transfected cultures in which all nuclei were labeled for 2 mins with 0.5 µl/ml 4', 6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, Oregon, USA). The transfection efficiency was found to be approximately 87% (n = 167 cells counted).

2.2.4 Western immunoblotting for growth hormone expression in quail pituitary glands

Lysates of adult quail pituitary glands were prepared in protease-inhibitor buffer, containing 15µg/ml aprotinin, 1µg/ml leupeptin, 5µg/ml pepstatin, and 1.74mg/ml phenylmethylsulphonyl fluoride (PMSF), and 20µg of this lysate protein in 2x sample buffer containing bromophenol blue and β-mercaptoethanol, were loaded in each lane of an 8% polyacrylamide gel, and run for 48 min at 140V. The Bio-Rad Protein Assay (Bio-Rad Inc., Hercules, USA) was used to ensure equal protein loading of each lane. Western blotting was carried out as described previously (Sanders et al., 2005). Negative controls were performed

using the primary antibody after pre-absorption with chicken GH as described previously (Sanders et al., 2005); in these lanes immunoreactivity was abolished.

Antibodies used in immunoblotting were as follows: a rabbit polyclonal antibody against chicken GH (bleed 1/10, diluted 1:100; Harvey and Scanes, 1997); and a sheep polyclonal antibody against chicken GH (diluted 1:100; Cheung and Hall, 1990). These are well-characterized antibodies that are pre-absorbable with chicken GH (Sanders et al., 2005).

2.2.5 Western immunoblotting for growth hormone expression in QNR/D cultures

For Western immunoblotting of siRNA-treated cultures, cells were manually removed from the culture dish using a plastic cell scraper, placed with the medium in an Eppendorf tube, and centrifuged for 8 mins at 14,000g. The supernatant was removed and 15µl protease inhibitor buffer (see above) was added to the pellet which was then sonicated for 2 secs. and centrifuged for 5 mins. The supernatant was then loaded onto the gel together with sample buffer with bromophenol blue and mercaptoethanol as described above. A naturally occurring 15kDa chicken GH fragment, provided by Dr. C. Aramburo, Universidad Nacional Autónomia de Mexico (Aramburo et al., 2000), was loaded as a positive control.

2.2.6 Enzyme-linked immunosorbant assay (ELISA) for growth hormone expression in QNR/D cultures

For the ELISA technique, a chicken growth hormone ELISA kit was used (USCN Life Science & Technology Co., Wuhan, China). After incubation the cells were manually removed from the culture dish using a plastic cell scraper, placed with the medium in an Eppendorf tube, and centrifuged for 8 mins at 14,000g. The medium was removed, the cells were resuspended in 70µl of sample diluent supplied with the ELISA kit, and sonicated for 2 secs at room temperature. The samples were centrifuged again for 5 mins., the supernatant was added to the chicken anti-GH ELISA plate, and incubated for 2 hrs. at 37˚C. All samples were treated in the same way to ensure that the final number and type of cells was the same in each experiment. Detection reagents and stop solution were added, according to the kit instructions, and the optical density of each sample was read using a microplate reader. The optical density of each sample was converted to GH concentration using a standard curve constructed using graded concentrations, between 0.2 and 10.0 ng/ml, of chicken GH supplied by the manufacturer of the ELISA kit. Statistical significance was tested using a paired t-test.

2.2.7 Immunocytochemistry

Sections of retina from embryonic day (ED) 7 quail embryos *(Coturnix coturnix japonica)*, were prepared and immunostained for GH and islet-1, after fixation in Bouin's fluid, using methods previously described (Baudet et al., 2003;

Sanders et al., 2003). In the controls, GH antiserum was replaced by rabbit serum and anti-islet-1 was replaced by mouse IgG. Embryonic day 7 is the stage from which the QNR/D cultures are derived.

For immunocytochemistry on cultured cells treated with siRNA, one 200µl aliquot of cells was diluted with 1ml DMEM medium, with 10% FBS and gentamycin. This was plated into six 35 mm. culture dishes, to each of which was added 1.8 mls., DMEM medium with 10% FBS and gentamycin. The cultures were incubated at 37˚C for 24 hrs., after which the medium was removed and replaced with DMEM medium with no serum, and 10µl/ml of *Trans*IT-TKO transfection reagent and the appropriate siRNA sample as above. After a further 24 hrs., the cultures were washed in warm phosphate-buffered saline (PBS), and then fixed with 4% paraformaldehyde for 30 mins. at room temperature.

Petri dishes bearing the cultures were washed with warm Medium 199 and fixed with 4% buffered paraformaldehyde. Immunocytochemistry was carried out as described previously (Sanders et al., 2005). Negative controls were carried out by replacing the primary antibodies with PBS, and in all cases this resulted in no immunreactivity of the cultures. The labeled cells were examined using a Zeiss LSM510 confocal microscope equipped with appropriate lasers.

The antibodies used for immunocytochemistry were as follows: a rabbit polyclonal antibody against chicken GH (bleed 1/10, diluted 1:100; Harvey and Scanes, 1997); and 39.4D5 anti-islet 1 mouse monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa), to identify avian retinal ganglion cells (Pimentel et al., 2000; Sanders et al., 2003), which has

previously been shown to be immunoreactive in embryonic quail tissue (Varley et al., 1995), including embryonic ganglion cells (Halfter, 1995).

In addition to DAPI staining, cell death was assessed using the method of terminal deoxynucleotide transferase (TdT) mediated dUTP-biotin nick end labeling (TUNEL), as described previously (Sanders et al., 2005). TUNEL labelling was combined with immunocytochemistry, by carrying out the TUNEL procedure first, followed by the immunocytochemistry procedure as above, and DAPI labeling.

Quantification of the immunocytochemical results for the presence of GH was carried out on confocal images using 'Image J' software (National Institutes of Health, USA; http://rsb.info.nih.gov/ij/). The average pixel intensity was measured over the area of each cell from a minimum of 5 cultures, and the results analyzed using an unpaired 't' test. The area of each cell was judged by the area of green label representing the presence of siRNA in the cytoplasm and nucleus. Image acquisition was standardized between all samples used for quantification, and no changes were subsequently made to them before analysis.

2.2.8 Quantification of cell death in QNR/D cultures

Apoptosis in QNR/D cultures was assessed by staining nuclei with DAPI to show shrunken and fragmented apoptotic nuclei and counting the number of apoptotic nuclei in random microscope fields. Three pairs of cultures were run, on three separate occasions, and the assessment was made blind from confocal microscope images on a minimum of three fields from each culture. The 'n' value

for each condition indicates the total number of fields counted. Results are expressed as the relative number of apoptotic cells in each condition (number of apoptotic nuclei divided by the total number of nuclei in the field of view) and the results were analyzed by an unpaired t-test.

2.3 Results

2.3.1 Comparison of the structures of quail and chick GH

Unlike chicken GH, quail GH has not previously been characterized. It was therefore necessary to examine quail pituitary GH by mRNA sequence analysis and by immunoblotting prior to carrying out experiments using QNR/D cultures and chicken GH siRNA.

A 690-bp full length GH cDNA was generated with reverse-transcribed mRNA from the quail pituitary, using primers cGH5 and cGH3. The size of quail pituitary GH mRNA was identical to the one generated from the chick pituitary (Fig. 2.1), and it's nucleotide sequence (Fig. 2.2) was 95% homologous with consensus chicken pituitary GH cDNA, based on GenBank Accession No. 211808 (Zhvirblis *et al.*, 1987); GenBank Accession No. 63406 (Lamb *et al.*, 1988); GenBank Accession No. 62909 (Baum *et al.*, 1990); GenBank Accession No. 222822 (Tanaka *et al.*, 1992); and GenBank Accession No. 9858171 (Ip *et al.* 2001). The nucleotide sequence has 30 base substitutions that would result in a five-amino acid change shown in red (isoleucine for leucine at position 12, alanine for threonine at position 13, glutamine for tryptophan at position 21, glutamic acid for glycine at position 22, and leucine for valine at position 152) in

the sequence of the predicted GH protein. Quail pituitary GH thus has 97% homology to the chicken pituitary GH. Of note, the exon 4 nucleotide sequence of quail cDNA is identical to that in the chicken, permitting the hybridization of the chicken GH siRNA NR-cGH-1. The cGH siRNA target sequence is shown in blue.

2.3.2 GH expression in the quail pituitary gland and in QNR/D cultures

Immunoblotting lysates from quail pituitary glands (Fig. 2.3, lane 1) indicated that quail pituitary GH runs at 22kDa, as does chicken pituitary GH (Baudet et al., 2003). Lysates from QNR/D cultures (Fig. 2.3, lane 3) were immunoreactive with the same antibodies at 15kDa, similar to the chicken GH variant (Fig. 2.3, lane 2), which is also found in chicken RGCs as we have shown previously (Aramburo et al., 2000; Baudet et al., 2003). Each of these samples was immunoreactive against both the rabbit polyclonal antibody against chicken GH (antibody 1/10), and the sheep polyclonal antibody against chicken GH.

2.3.3 GH is expressed by quail RGCs and by RGC-derived cells in QNR/D cultures

Immunolabelled sections of ED 7 quail retina showed GH immunoreactivity in cells in the ganglion cell layer (green in Fig. 2.4A). Sections double labeled (Fig. 2.4B) for GH (green) and the RGC marker islet-1 (red), showed co-localization of these two labels in the same cells (yellow), as it is in the chicken embryo (Baudet et al., 2003). Controls, in which the primary

antibodies were replaced with rabbit serum and mouse IgG (Fig. 2.4C), were negative.

The QNR/D cell line is derived from ED 7 quail neural retinas, and can be expected to contain several different types of retinal cells (Pessac et al., 1983a, b; Trisler et al., 1996). In our hands, as judged by immunoreactivity against the anti-islet 1 antibody, we estimated that approximately 80% of the cells present in the cultures were RGC-derived cells (n = 50 cells counted). In order to determine specifically whether GH is expressed by these cells in the cultures, cells were double labeled with GH and the antibody against islet 1, which is specific for RGCs. We found that all cells identified as RGC-derived expressed GH by immunocytochemistry. Figure 2.5 shows co-localization of GH with the RGC marker in cultured QNR/D cells. As we have shown in chick embryo RGCs (Baudet et al., 2003), both islet 1 and GH are expressed both in the cytoplasm and nucleus.

2.3.4 The effect of NR-cGH-1 siRNA transfection on the expression of GH in cultured QNR/D cells

Immunocytochemical examination of cultured and transfected QNR/D cells (green labeling in Fig. 2.6) indicated a transfection efficiency of approximately 87%. In contrast to cells transfected with control non-silencing siRNA, cells transfected with NR-cGH-1 siRNA showed a substantial decline in cytoplasmic GH immunoreactivity (red). This difference became clear in comparing the merged images (Fig. 2.6), where co-localization appears yellow in

the presence of control non-silencing siRNA, but not in the presence of NR-cGH-1 siRNA. The inset (bottom right) shows a non-transfected cell from a culture treated with cGH siRNA; in this cell GH (red) is expressed at levels comparable with control siRNA-treated cells.

Quantification of the pixel intensity of the red channel (GH) in Fig. 2.6 (right hand graph) showed a mean intensity of 147.4 ± 3.6 (SEM), n = 65) for cultures transfected with non-silencing siRNA, and 86.90 ± 3.1 (SEM), n = 81) for cultures transfected with NR-cGH-1 siRNA. This is a statistically significant difference, $p < 0.05$, and represents an approximately 40% knock-down in the GH level. By contrast, quantification of the pixel intensity in the green channel, (Fig. 2.6, left hand graph), which was indicative of transfection, showed no significance difference between the cultures transfected with non-silencing control siRNA (154.6 ± 3.8 (SEM), n = 20) and those transfected with NR-cGH-1 siRNA (165.5 ± 7.4 (SEM), n = 20). The mean levels in non-transfected cells from the cGH siRNA-transfected cultures are shown in the right-hand bars of each of these graphs (green channel: 18.0 ± 2.2 (SEM), n = 12; red channel: 174.9 ± 11.6 (SEM), n = 12).

Use of the ELISA technique to quantify this decline (Fig. 2.6, bottom panel) also showed that it was statistically significant ($p < 0.05$): the mean GH concentration in the siRNA non-silencing control treated cultures was 2.56 ng/ml ± 0.48 (SEM); n = 4, and the mean GH concentration in the NR-cGH-1 siRNA treated cultures was 1.13 ng/ml ± 0.38 (SEM); n = 4.

2.3.5 The effect of NR-cGH-1 siRNA transfection on apoptosis in cultured QNR/D cells

Immunocytochemical examination of cultured QNR/D cells transfected with NR-cGH-1 siRNA (Fig. 2.7) indicated that suppression of GH expression correlated with the increased occurrence of DAPI-stained nuclei with the apoptotic characteristics of shrinkage and fragmentation (nuclei labeled 1* in Figs. 2.7A, B, C, and D), in contrast with neighbouring cells in which GH expression was not suppressed (Fig. 2.7, cells A2, B2, B3, C2, D2, D3, D4). Quantification of the pixel intensity of the red channel (GH) in these images (Fig. 2.7E) showed a mean intensity of 159.2 ± 5.4 (SEM), n = 22) for cells showing non-fragmented nuclei, and 114.6 ± 8.0 (SEM), n = 15) for cells showing fragmented nuclei. This is a statistically significant difference; $p < 0.05$. The mean GH level in non-apoptotic cells transfected with control siRNA is shown in the right hand bar (198.0 ± 9.3 (SEM), n = 10).

The TUNEL technique was used to validate the use of nuclear fragmentation and shrinkage as a marker of apoptosis (Fig. 2.8A, B, C). Nuclei showing fragmentation (Fig. 2.8, cell A1), shrinkage (cells A2 and C1), and chromatin margination (cell B1) were TUNEL positive, in contrast with nuclei showing normal morphological features (cells A3, B2 and C2).

Quantification of the effect of NR-cGH-1 siRNA transfection on the numbers of apoptotic cells in QNR/D cultures was carried out by counting the number of nuclei with apoptotic morphology in random microscope fields (Fig. 2.8D). Four separate pairs of cultures were run on four separate occasions. In

cultures transfected with non-silencing control siRNA, the relative number of apoptotic nuclei was 5.16 ± 1.16 (SEM), n = 4 cultures, 30 cells; in cultures transfected with NR-cGH-1 siRNA, the relative number of apoptotic nuclei was 21.53 ± 1.85 (SEM), n = 36. This difference was statistically significant ($p = 0.001$).

2.4 Discussion

We have shown here that gene knock-down of GH in cells of a cultured embryonic neural retina cell line, using NR-cGH-1 siRNA, correlates with the increased appearance in the cultures of cells with apoptotic nuclear morphology. This result is consistent with our previous results in which we investigated the results of protein knock-down, by mean of immunoneutralization, on cultures of cells isolated from the chick embryo retina which had been enriched for RGCs (Sanders et al., 2006; 2008). We thus validate, using different technology and a different culture system, our contention that GH, produced locally by cells of the neural retina (Baudet et al., 2003) acts in an autocrine and/or paracrine manner to regulate cell survival in the retina (Sanders and Harvey, 2004; Sanders et al., 2005). Our previous work emphasized the developmental role of GH as a temporo-spatial mediator of cell survival using cells from embryonic day (ED) 8 chicks (Sanders and Harvey, 2004; 2008), and this conclusion is also supported by the present results, since the QNR/D cell line is derived from ED 7 Japanese quails (Pessac et al., 1983a, b). We have proposed (Sanders and Harvey, 2004) that GH, produced locally in the retina, plays a developmental neuroprotective

role similar to that of BDNF (Frade et al., 1997) in the control of the developmentally-regulated waves of cell death that occur in the embryonic retina. Our current results support this contention.

Our characterization, for the first time, of the quail pituitary GH gene indicates a 95% sequence identity with the chicken GH gene, and the sequence of exon 4, from which the siRNA probe was produced (Baudet et al., 2009) was identical between the two species. Consistent with this gene structural similarity, we found that the embryonic quail retinal GH protein is immunoreactive at the same molecular weight (15kDa; Aramburo et al., 2000) as the embryonic chicken retinal GH from RGCs. The 15kDa band may be a proteolytic fragment produced in the retinal cells themselves, as we have previously demonstrated (Harvey et al., 2007). This similarity between chick and quail GH permitted the use here of NR-cGH-1 siRNA, which had originally been targeted to chicken GH (Baudet et al., 2009). It is of interest that we find a 15kDa moiety in both of the avian species that we have examined, but not in rat (Harvey et al., 2006) or human (Harvey et al., 2009), where a 22kDa molecule is detected in the retina, similar to that found in the pituitary gland.

The cGH siRNA probe has been used previously on enriched primary cultures of chick embryo RGCs, to show that GH depletion inhibits neuron outgrowth from these cells (Baudet et al., 2009). In those experiments, cell death following treatment with NR-cGH-1 siRNA was prevented by the addition to the cultures of the broad spectrum caspase inhibitor Q-VD-OPh (Sanders et al., 2006), thus permitting examination of neurite outgrowth. It is thus apparent that local

GH has effects, presumably autocrine and/or paracrine, both on RGC survival and axon elongation in the developing retina, supporting our view that GH is a *bona fide* developmental growth and differentiation factor (Sanders and Harvey, 2004).

In contrast to our previous experiments (Sanders et al., 2006; 2008), where we were confident that we were dealing largely with chick embryo RGCs in primary culture, the QNR/D cell line that we used here was probably derived primarily from both ganglion cell and amacrine cell lineages of the quail embryo neural retina (Pessac et al., 1983; Trisler et al., 1996). Our results indicated that our cultures were comprised of approximately 80% RGC-derived cells, and that all of these cells expressed GH. However, we also found GH expressed in cells that did not carry the RGC marker, anti-islet 1; presumably these were amacrine cells, derived from the "displaced amacrine cells" that occur in the ganglion cell layer of avian retina (Binggeli and Paule, 1969). Although, in our previous work, we have not studied retinal cell types other than RGCs, incidental observations have indicated that while RGCs are by far the primary source of GH in the embryonic retina (Baudet et al., 2003), scattered cells in other retinal cell layers do occasionally express GH. The significance of this irregular expression on other cell layers is not known.

Taken together with our previous results on chick embryo RGCs, the current work confirms, using a quail embryonic cell line, the expression of GH by embryonic avian retinal cells, and confirms by knock-down that GH in these cells promotes cell survival.

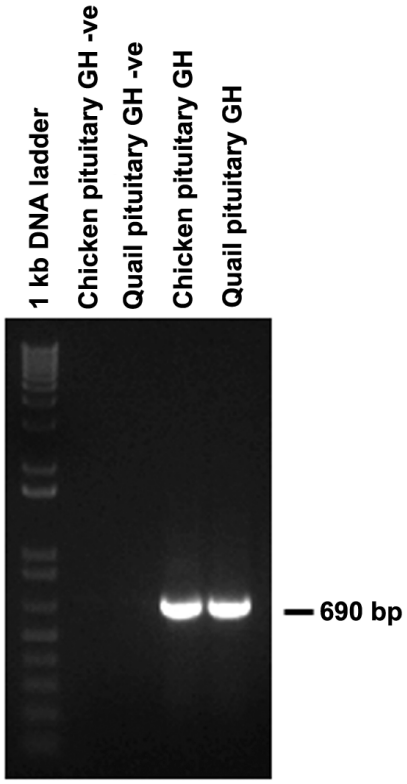
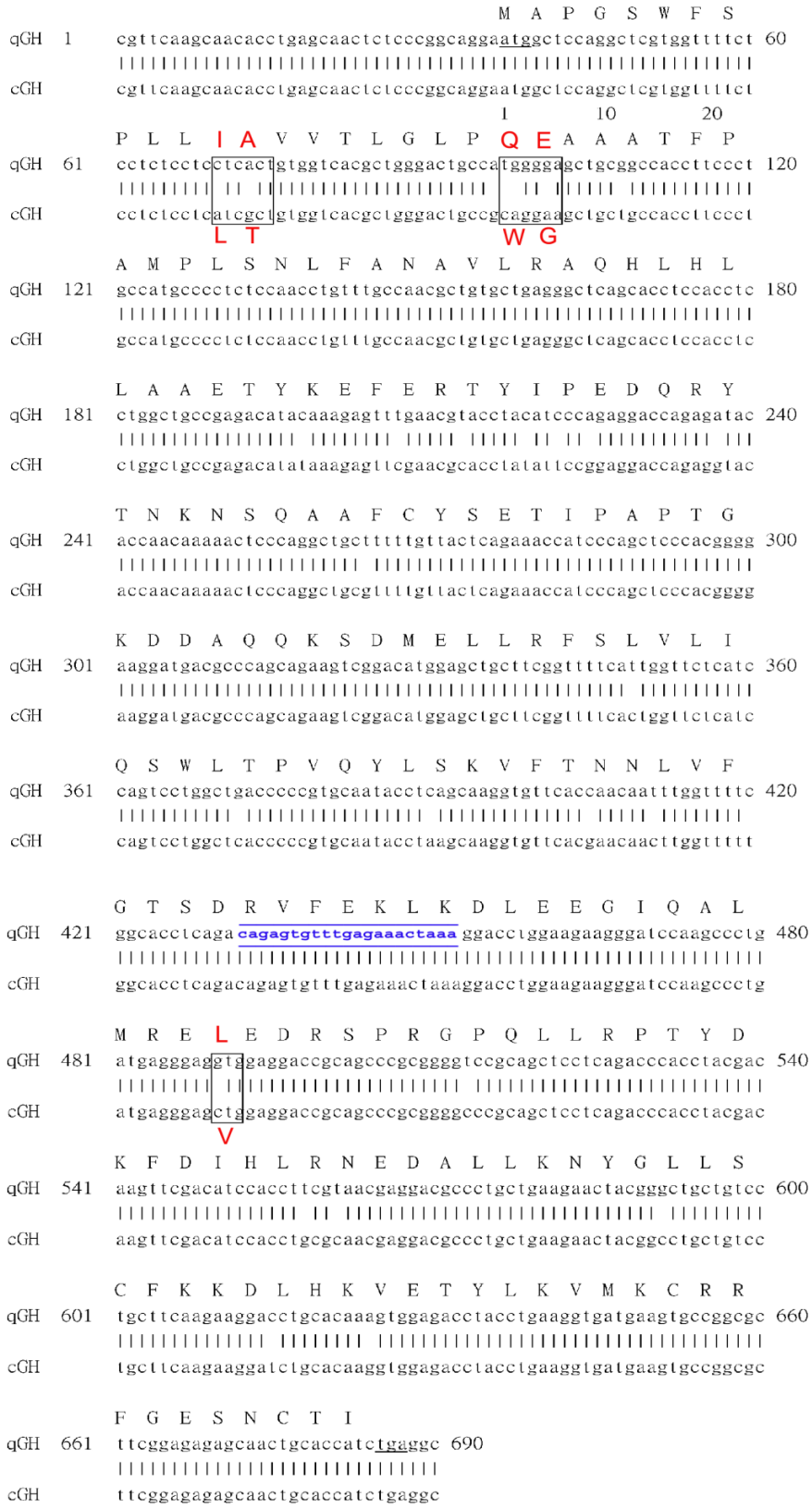


Figure 2.1. RT-PCR of reverse-transcribed GH mRNA extracted from the pituitary glands of five-week-old Japanese quail, in comparison with GH mRNA from the pituitary glands of six-week-old chickens. Reactions with mRNA in the absence of reverse transcriptase served as negative controls (-ve). Data is representative of three repeat experiments.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cGH cgttcaagcaacacctgagcaactctcccggcaggaatggctccaggctcgtggttttct
1 10 20
P L L I A V V T L G L P Q E A A A T F P
qGH 61 cctctcctcctcactgtggtcacgctgggactgccatggggagctgcggccaccttccct 120
cGH cctctcctcatcgctgtggtcacgctgggactgccgcaggaagctgctgccaccttccct
L T W G
A M P L S N L F A N A V L R A Q H L H L
qGH 121 gccatgcccctctccaacctgtttgccaacgctgtgctgagggctcagcacctccacctc 180
cGH gccatgcccctctccaacctgtttgccaacgctgtgctgagggctcagcacctccacctc
L A A E T Y K E F E R T Y I P E D Q R Y
qGH 181 ctggctgccgagacatacaaagagtttgaacgtacctacatcccagaggaccagagatac 240
cGH ctggctgccgagacatataaagagttcgaacgcacctatattccggaggaccagaggtac
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cGH accaacaaaaactcccaggctgcgttttgttactcagaaaccatcccagctcccacgggg
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cGH aaggatgacgcccagcagaagtcggacatggagctgcttcggttttcactggttctcatc
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cGH cagtcctggctcaccccccgtgcaatacctaagcaaggtgttcacgaacaacttggttttt
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cGH atgagggagctggaggaccgcagcccgcggggcccgcagctcctcagacccacctacgac
V
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cGH aagttcgacatccacctgcgcaacgaggacgccctgctgaagaactacggcctgctgtcc
C F K K D L H K V E T Y L K V M K C R R
qGH 601 tgcttcaagaaggacctgcacaaagtggagacctacctgaaggtgatgaagtgccggcgc 660
cGH tgcttcaagaaggatctgcacaaggtggagacctacctgaaggtgatgaagtgccggcgc
F G E S N C T I
qGH 661 ttcggagagagcaactgcaccatctgaggc 690
cGH ttcggagagagcaactgcaccatctgaggc

Figure 2.2. The nucleotide (cDNA) and amino acid sequence of quail pituitary GH (qGH), in comparison with chicken pituitary GH cDNA (cGH; Tanaka *et al.*, 1992). Translation initiates from the start (ATG) codon and ends at the stop (TGA) codon (underlined). Above the qGH cDNA sequence is shown the total amino acid sequence of quail pituitary GH; amino acid substitutions are indicated in red and by boxed codons. The siRNA target sequence is shown in blue. The GeneBank accession number for quail pituitary GH mRNA is FJ458436. The GeneBank accession number for quail pituitary GH is ACJ73931.

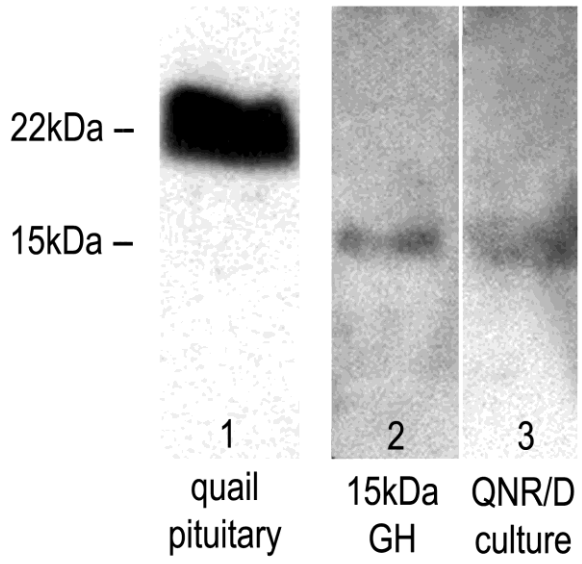


Figure 2.3. Western immunoblotting of quail pituitary gland lysate using a rabbit polyclonal antibody against chicken GH shows that quail pituitary GH is expressed as a 22kDa molecule (lane 1). Lysates from quail embryo neural retina cell line (QNR/D) cultures show that these cells express GH at 15kDa (lane 3), at the same molecular weight as the 15kDa chicken GH variant (lane 2).

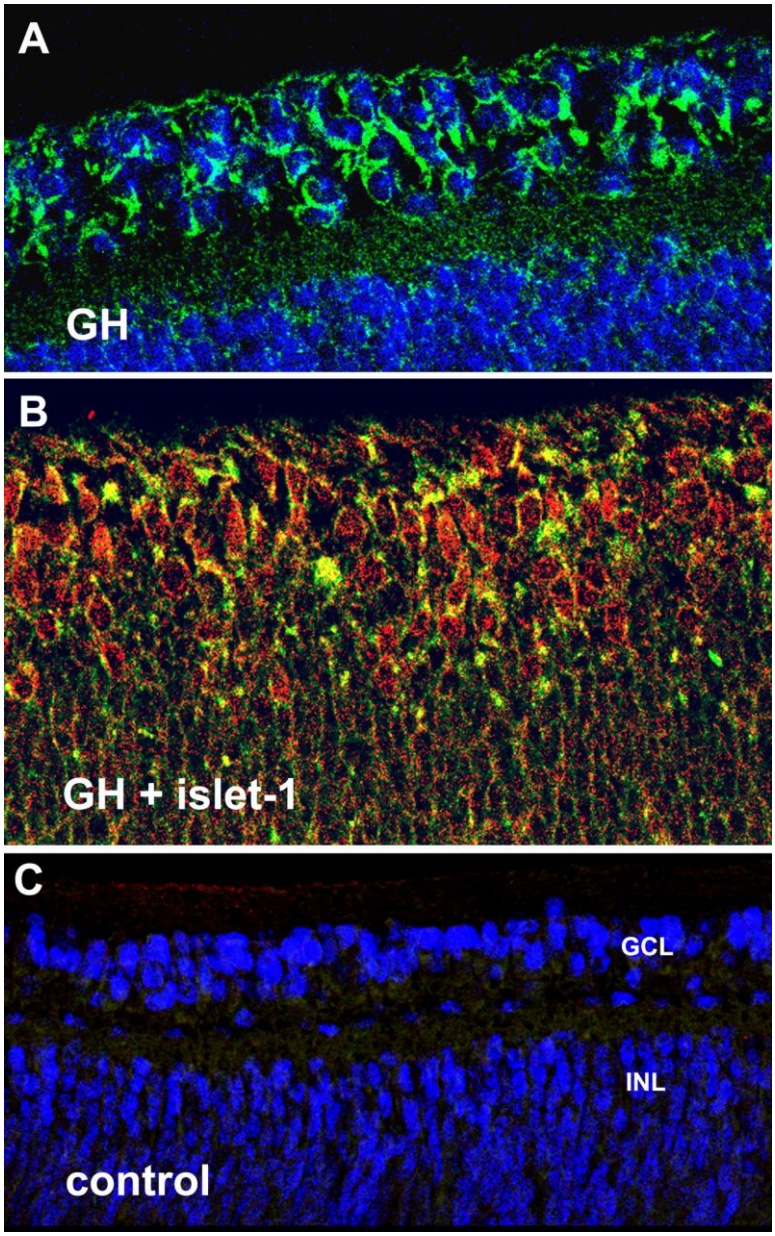


Figure 2.4. Sections of embryonic day 7 quail embryo retina immunolabelled for GH (green in A and B), and double-labeled for GH and the RGC marker, islet-1 (red in B). Co-localization of GH with islet-1 in B is indicated by the yellow colour in cells of the ganglion cell layer (GCL). Control sections, incubated with rabbit serum and mouse IgG in place of the primary antibodies were negative (C). In A and C, nuclei are labeled blue using DAPI. INL = inner nuclear layer.

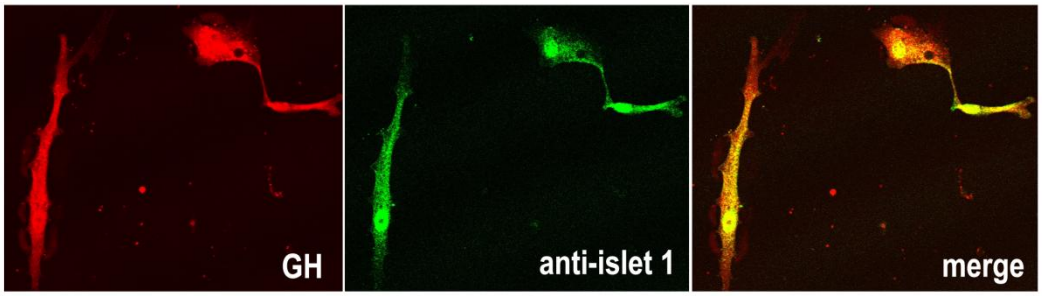
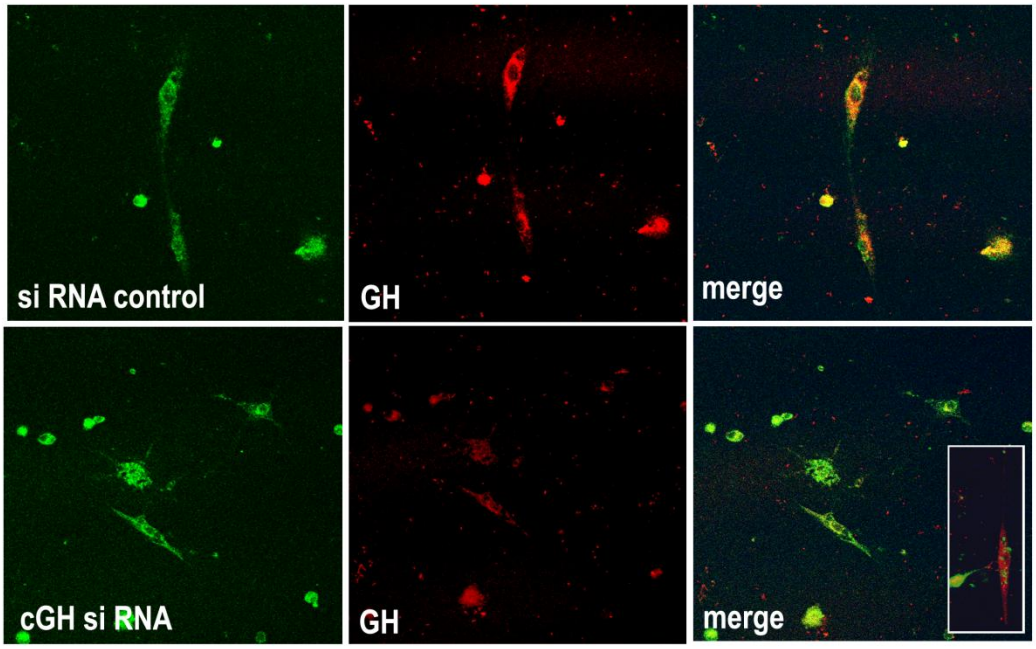
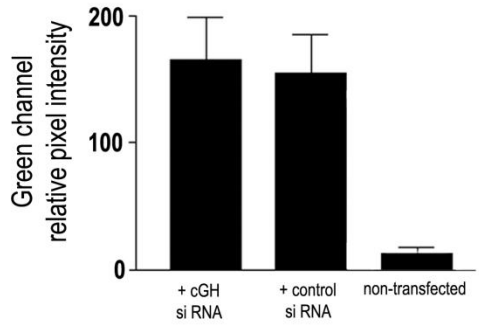
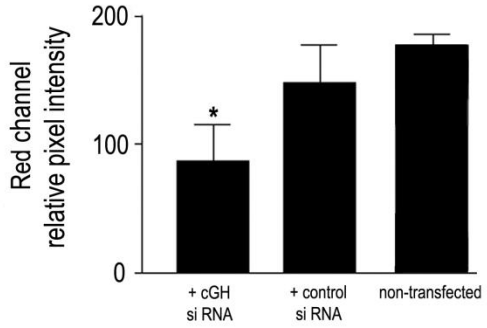
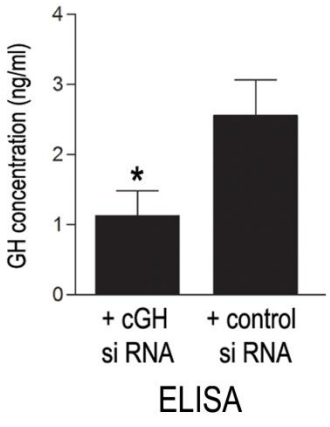


Figure 2.5. Double labeling of cells in quail embryo neural retina cell line (QNR/D) cultures with anti-islet 1 (green), a marker specific for RGCs, and GH (red) using a rabbit polyclonal antibody against chicken GH, shows that RGC-derived cells in these cultures express GH.


si RNA control
GH
merge
cGH si RNA
GH
merge


Green channel
relative pixel intensity
200
100
0
+ cGH
si RNA
+ control
si RNA
non-transfected


Red channel
relative pixel intensity
200
100
0
*
+ cGH
si RNA
+ control
si RNA
non-transfected


GH concentration (ng/ml)
4
3
2
1
0
*
+ cGH
si RNA
+ control
si RNA

ELISA

Figure 2.6. Treatment of cultured cells from the quail embryo neural retina cell line (QNR/D) with cGH siRNA reduces the expression of GH by the cells. GH expression (red) by cells in QNR/D cultures is suppressed by treatment with NR-cGH-1 siRNA (green) but not by non-silencing control siRNA (green). Co-localization of GH and siRNA is indicated by yellow in the merged images. Non-transfected cells in the cGH siRNA-treated cultures (inset, bottom right), showed GH expression levels comparable to cells in the control siRNA-treated cultures (see also graphs below). Quantification of the pixel intensity in the red channel (GH labeling, right hand graph) shows that the knock-down of expression achieved with the NR-cGH-1 siRNA was statistically significant in comparison with the effect of the non-silencing control siRNA (* = significantly different from control siRNA; $p < 0.05$). Quantification of the pixel intensity in the green channel, indicative of the presence of siRNA (left hand graph), showed no significant difference between cultures transfected with non-silencing control siRNA and NR-cGH-1 siRNA. The ELISA method (bottom panel) also showed a statistically significant ($p < 0.05$) reduction in GH expression in cGH siRNA-treated cells.

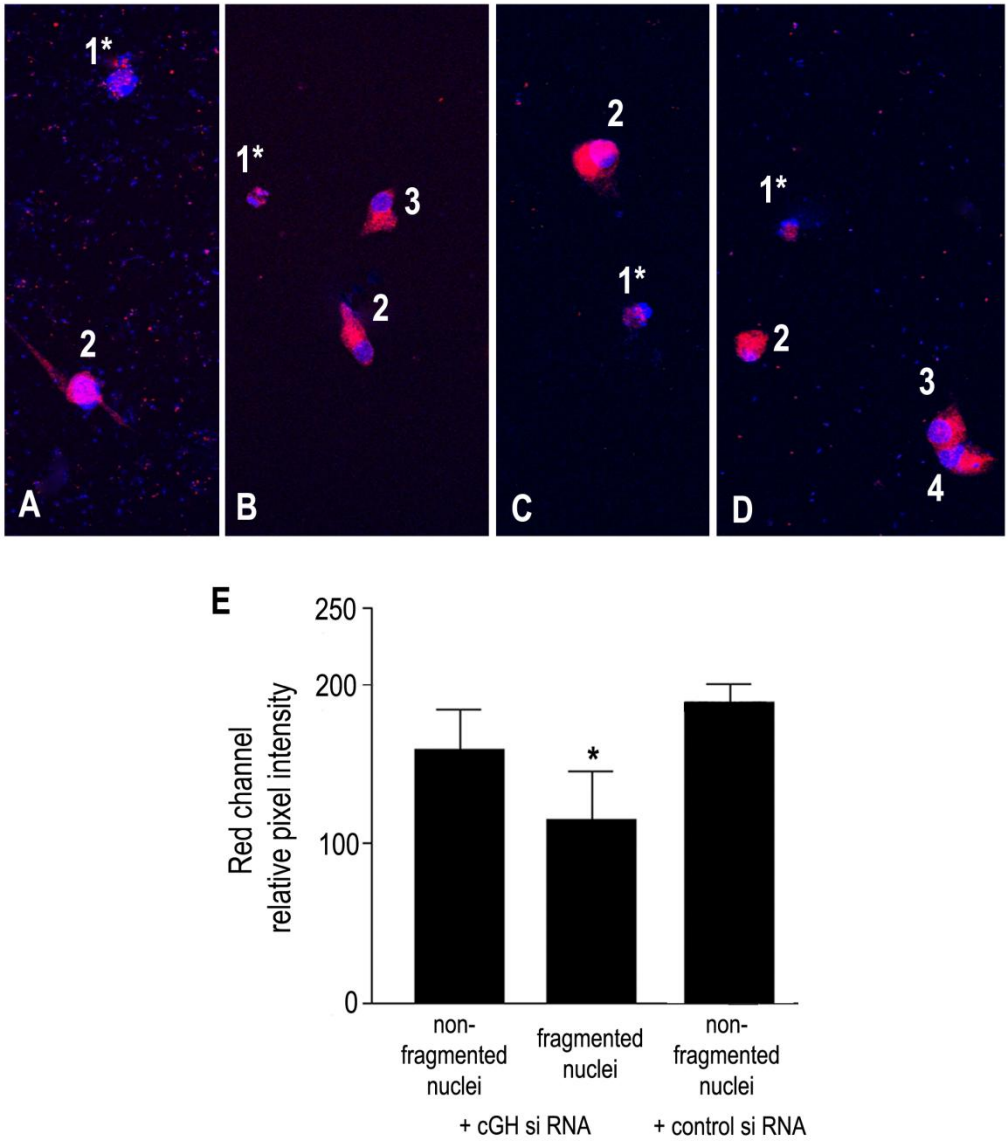


Figure 2.7. GH knockdown by cGH siRNA correlates with the appearance of apoptotic nuclei. In quail embryo neural retina cell line (QNR/D) cultures treated with NR-cGH-1 siRNA, suppression of GH expression (red) is associated with shrinkage or fragmentation of nuclei (blue) characteristic of apoptosis. Cells labeled A1*, B1*, C1* and D1* show GH suppression and nuclear shrinkage or fragmentation when compared with other cells in the field of view. Quantification of this relationship (E), shows that pixel intensity in the red channel (GH) is

statistically significantly lower in cells containing a fragmented nucleus than in cells with a non-fragmenting nucleus in cultures transfected with cGH siRNA. (* = significantly different from non-fragmented nuclei in cGH siRNA transfected cells; $p < 0.05$). Cells containing non-fragmented nuclei in cultures transfected with control siRNA showed similar levels of GH to cells in cGH-transfected cultures.

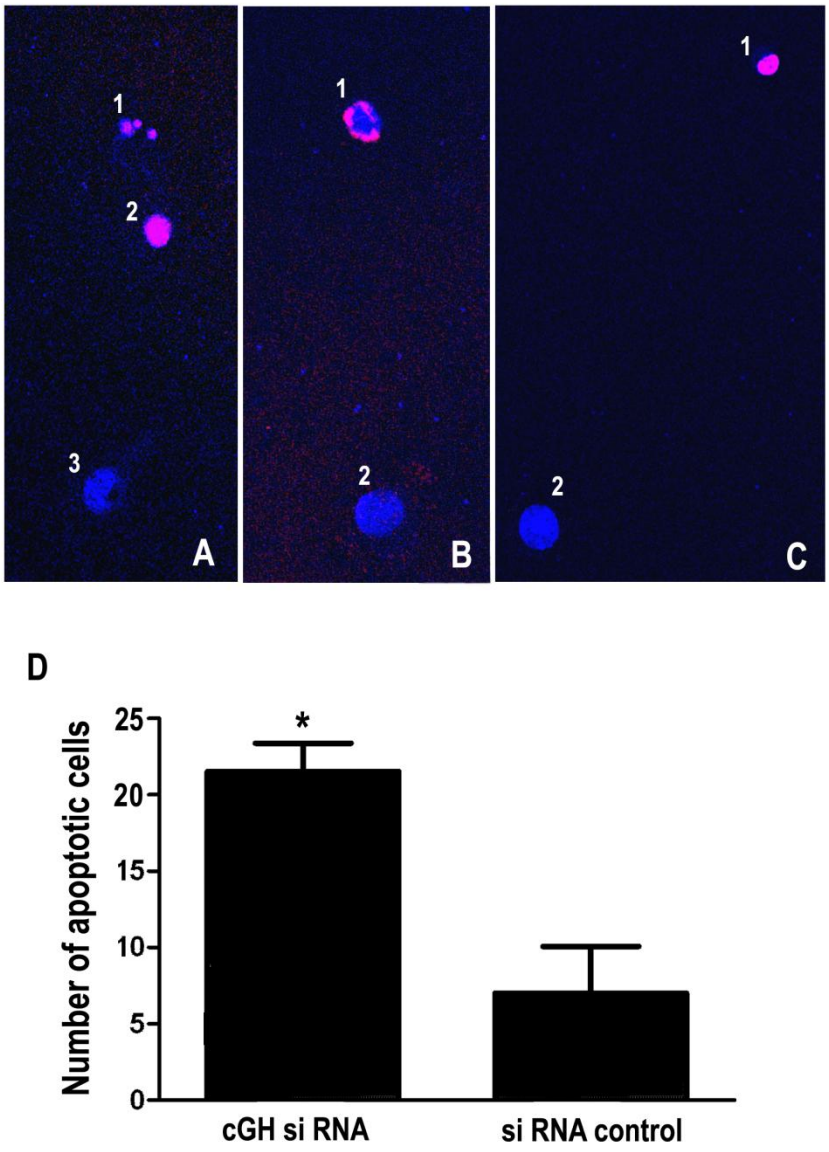


Figure 2.8. The apoptotic appearance of the nuclei of the cultured cells correlates with TUNEL-positive labeling. **A, B, C:** In quail embryo neural retina cell line (QNR/D) cultures treated with NR-cGH-1 siRNA, there is a correlation between apoptotic nuclear morphology (blue) and TUNEL labeling (red). In comparison with other nuclei in the field of view, nuclei are TUNEL-positive when they are

fragmenting (cell A1), or shrinking (cells A2, C1), or during chromatin margination (cell B1).

D: Quantification of the effect of NR-cGH-1 siRNA on apoptosis of cells of QNR/D cultures, indicates that transfection with cGH1 siRNA significantly ($p = 0.001$) increases the numbers of nuclei with apoptotic morphology in these cultures in comparison with control siRNA transfection.

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Chapter Three

Growth hormone promotes the survival of retinal cells *in vivo*

Publication and Author Contributions

The results in this chapter have been published in Sanders E. J., W-Y. Lin, E. Parker, and S. Harvey (2011) Growth hormone promotes the survival of retinal cells *in vivo*. *General and Comparative Endocrinology*, in press.

Wan-Ying Lin microinjected the GH and control siRNA's *in ovo* into the eyes of embryonic chicks and developed real-time PCR assays (see the Appendix) to quantify GH mRNA and IGF-1 mRNA expression in the embryonic chick retina and GH mRNA expression in the quail QNR/D cell cultures. These data are shown in Fig 3.3. The culture of the quail retinal cells and their transfection with the GH and control siRNA's was by Wan-Ying Lin. The manuscript was primarily written by Dr. Esmond J. Sanders and the immunohistochemical and TUNEL stainings were carried out by Eve Parker.

3.1 Introduction

Extrapituitary growth hormone (GH) is synthesized and active in a number of tissues during development and in the adult, where it appears to have autocrine or paracrine effects similar to those of conventional growth factors (Sanders and Harvey, 2004; 2008; Harvey, 2010). This extrapituitary GH is present in the chick embryo from embryonic day (ED) 2 (Harvey et al., 2000, 2001) and is active in chick development by approximately ED 6, which is significantly earlier than GH is first detected in somatotrophs, at ED12 - 16 (Józsa et al., 1979; Sanders and Harvey, 2004; 2008). One extra-pituitary tissue in which GH is particularly abundant is the retina, where it has been found in the retinal ganglion cells (RGCs) of the chick embryo (Baudet et al., 2003) and the adult rat (Harvey et al., 2006) and human (Harvey et al., 2009). In this location, and in the vitreous (Sanders et al., 2003), GH appears to function in the embryo as a paracrine anti-apoptotic factor, possibly helping to regulate the naturally occurring waves of cell death that take place in the developing retina (Vecino et al., 2004; Sanders et al., 2005), or by influencing RGC axonal outgrowth (Baudet et al., 2009). Indeed, secreted factors from transplanted embryonic RGCs appear to be able to provide paracrine survival support to adult retinal neurons (Stanke and Fischer, 2010), providing a potential therapy for human diseases of retinal degeneration related to ganglion cell death, such as glaucoma (Nickells, 2007).

In the adult human retina, it has been speculated that GH in the retina and vitreous, may be a neuroprotective factor since its concentration has been correlated with the onset of ocular disease (Harvey et al., 2009; Sanders et al.,

2009a; Ziaei et al., 2009). In this respect, GH may function in eye development and maintenance, along with other neurotrophic factors, including brain-derived neurotrophic factor (BDNF; Frade et al., 1997), ciliary-derived neurotrophic factor (CNTF; Pease et al., 2009), and insulin-like growth factor-1 (IGF-1; De la Rosa et al., 1994; Diaz-Casares et al., 2005; Sanders et al., 2009b). These anti-apoptotic effects of GH in the developing retina share common cellular mechanisms with several of these other growth factors (Sanders et al., 2006, 2008).

Previous analyses of the functional roles of GH in the chick embryo retina have focused on the consequences of GH protein knock-down by immunoneutralization, and GH augmentation using exogenous recombinant protein, both *in vivo* by means of *in ovo* microinjection, and *in vitro* using enriched cultures of chick embryo RGCs (Sanders et al., 2005, 2006, 2008). More recently, we have used a quail neural retina cell line to test the effects of GH gene knock-down using a small interfering RNA (siRNA), NR-cGH-1 siRNA (cGH siRNA), that targets chicken GH exon 4 (Baudet et al., 2009), and showed that GH gene expression is required for the anti-apoptotic effects of GH (Sanders et al., 2010).

In the present work we extend these functional studies with cGH-1 siRNA by examining the *in vivo* effects of this reagent after microinjection into the eye cup of the developing chick embryo *in ovo.* We show that intra-vitreous cGH siRNA lowers both GH mRNA and IGF-1 mRNA levels in the retina *in vivo*, and concomitantly elevates the numbers of apoptotic cells in the retina. The results

strengthen our view that a GH/IGF-1 axis in retinal cells regulates retinal cell survival *in vivo*.

3.2 Materials and methods

3.2.1 Embryo handling and microinjection

White Leghorn hens' eggs were incubated in order to bring them to ED4. Eggs were windowed and microinjected intravitreally *in ovo*, into the right optic cup (Sanders et al., 2005), using a pressure injection system (Pico-Injector PLI-100, Medical Systems Corp., Greenvale, New York, USA). The left optic cup was not injected, and served as an untreated control. The injected reagents included a chicken (c) GH siRNA that was custom synthesized by Qiagen Inc (Mississauga, Ontario, Canada), as previously described (Bauder et al., 2009; Sanders et al., 2010). This siRNA targets chicken GH exon 4, an exon that is present in all GH isoforms found in the chick embryo at ED 7 (Takeuchi et al., 2001; Baudet et al., 2003) and is present in adult quail pituitary glands (Sanders et al., 2010) and is specific for avian GH genes (Basic Local Alignment Search Tool (BLAST), National Centre for Biotechnology Information (NCBI)). The siRNA was at an original concentration of 20µM in nuclease-free water. This was diluted in serum-free DMEM culture medium (Invitrogen Canada Inc., Burlington, Ontario, Canada) containing *Trans*IT-TKO transfection reagent (Mirus Bio Corp., Madison, Wisconsin, USA; *Trans*IT-TKO : DMEM = 1:4) and 20% bromophenol blue to bring the final concentration of siRNA for injection to 10µM. Given an eye-volume of approximately 2.0 mm^3 in the ED 4 embryo (Goodall et al., 2009),

and an injection volume of approximately 100pl per pulse, we calculated a final siRNA concentration in the eye cup of approximately 30nM. Bromophenol blue (20%) was added to the siRNA in order to aid in the assessment of the accuracy of the injection. A non-silencing siRNA, (Qiagen Inc.; Sanders et al., 2010), was used as a negative control. After injection, the windows were sealed with tape and the eggs were returned to the incubator for a further 24h. After this time, the eyes and retinas of the embryos were dissected and examined for apoptotic cells or stored at -80^{o}C for mRNA analysis. Eyes intended for sectioning were fixed overnight in 4% paraformaldehyde (PFA) at 4℃ before embedding in paraffin wax and sectioning at 8µm thickness.

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3.2.2 Assessment of siRNA penetration into the retina

For assessment of the effectiveness of tissue transfection with siRNA, the siRNA was conjugated to Alexa-fluor 488 (green) and its presence in cells was visualized by diaminobenzidine (DAB) immunocytochemistry.. Eyes which had been injected and incubated with siRNA conjugated to Alexa-fluor 488, with or without a transfection reagent, were dissected, fixed overnight in PFA at 4℃, embedded and sectioned. Sections were deparaffinised with CitriSolv (Fisher Scientific, Ottawa, Ontario, Canada), rehydrated with graded ethanol solutions, and treated with hydrogen peroxide (H_2O_2) in methanol (0.3 mls H_2O_2 in 9.7mls of 50% methanol). After washing in phosphate buffered saline (PBS) for 15 mins, non-specific binding sites were blocked with 4% bovine serum albumin (BSA) for 1 hr at room temperature (RT). Sections were then incubated in an anti-Alexa-

fluor antibody (Invitrogen) diluted 1:100 in PBS, overnight at RT. After washing in PBS for 15 mins, sections were treated with a goat-anti-rabbit antibody conjugated to biotin (Invitrogen) diluted 1:50 in PBS for 1 hr at RT, followed by another wash and incubation in avidin-peroxidase reagent according to the manufacturer's instructions (ABC kit, Vector Laboratories Canada Inc, Burlington, Ontario, Canada). After another wash in PBS for 15 mins, samples were treated with a mixture of DAB and H_2O_2 according to the manufacturer's instructions (Vector Laboratories) for 2 mins, while observing the colour development under a microscope. Sections were then washed in PBS, dehydrated and mounted in Permount (Fisher Scientific). The DAB reaction product was quantified by digitally measuring its density in sections. Results were expressed as the percentage increase in density in comparison with non-injected controls. The statistical significance of the result was analyzed using Student's 't' test. In some preliminary experiments, Invivofectamine (Invitrogen) was used as an alternative to *Trans*IT-TKO for transfection.

3.2.3 Retina flat-mounts

For examination of the distribution of apoptotic cells in the chick embryo retina, eyes which had been injected with the siRNA or its control at ED4 were dissected 24h later. Retinas were prepared as whole flat-mounts by modification of the methods described by Dütting et al. (1983), Chavarría et al (2007) and Parrilla-Reverter et al. (2009). Eyes were dissected from the embryos in cold (4℃) PBS, extraneous tissue including the sclera, was removed and the tissue was

fixed for 10 mins in PFA at 4℃. After washing in cold PBS, the cornea and lens were removed. Four radial cuts were then made in the eye, avoiding the optic fissure (see below; Silver, 1977; Ozeki et al., 2000; Morcillo et al., 2006), by inserting one blade of the scissors into the pupil, thus producing four quadrants of approximately equal size in the resulting flat-mount. The vitreous was not removed. The eye was placed, with the retina side down, on the surface of a plastic dish in cold PBS, and gently flattened with camel hair brushes. The flattened eye was lifted with camel hair brushes and placed, with the retina down, on a glass slide in cold PBS. A small piece of filter paper was then placed on top of the tissue, so that the eye stuck to the paper with the retina uppermost. The tissue was fixed on the paper for 24h in 4% PFA at 4℃. The next day, the tissue was lifted off the paper with camel hair brushes and placed on a glass slide, with the retina uppermost, in two drops of cold PBS. At this point, appropriate immunocytochemical procedures were carried out on the tissue. Each repetition of the experiment consisted of three retinas processed in parallel: one from an eye injected with cGH siRNA; one from the same embryo, but not injected; and one from a different embryo injected with non-silencing siRNA. Fifteen such replicate experiments were analyzed.

In order to determine whether or not there were shorter-term effects, flat-mounts were also prepared 6h after intravitreal injection at ED 4. The technique was the same as that described above for ED 5 specimens, but identification of the optic fissure during preparation was more difficult owing to the smaller size of the samples and the poorly developed pigmented layer at this earlier embryonic stage.

3.2.4 Terminal deoxynucleotide transferase (TdT) mediated dUTP-biotin nick end labeling (TUNEL)

This method has been described in detail previously (Sanders et al., 2005). Briefly, the flat-mounted tissue was incubated with proteinase K (PK; Sigma Chemical Co., Mississauga, Ontario, Canada) at a concentration of 13µg/ml for 10 mins at 37℃, washed in PBS, and permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate for 2 mins at 4℃. After further washing, the TUNEL reaction mixture was applied for 30 mins at 37℃, according to the instructions of the manufacturer of the kit (In Situ Cell Death Detection Kit, TMR red; Roche Diagnostics, Indianapolis, IN, USA). A red fluorescent label was used for the TUNEL reaction. After washing three times in PBS, the tissue was mounted with Vectashield (Vector Laboratories) under a coverslip.

Tissue sections from embryos 6 h and 24h after injection, prepared as described above, were also labeled with the TUNEL procedure. The method used was the same as for flat-mounts, except that the PK concentration was raised to 20µg/ml and applied for 20 mins at 37℃, and the TUNEL mixture was applied for 45 mins at 37℃.

Specimens were examined using a Leica fluorescence microscope equipped with Leica Application Suite software. Photomontages were generated using the tiling function in this software.

3.2.5 Quantification and distribution of cell death in retina flat-mount preparations

Each flat-mount photomontage showed four approximately equal-sized quadrants, one of which contained the optic fissure (Fig. 1). The quadrant containing the optic fissure was designated quadrant 1, and the other three were numbered consecutively in clockwise order. Examination of the distribution of the TUNEL-positive nuclei in each flat-mount preparation indicated that these labeled nuclei were frequently clustered, usually close to the optic fissure head (Fig. 1, asterisks) and not uniformly distributed across the retina. Determination of the predominant location of the clusters, especially with respect to the position of the optic fissure, was facilitated by printing a photomontage of the flat-mount, cutting the image out, and re-constructing the eye by folding the paper and rejoining the edges (Dütting et al., 1983). In this way, it could be determined if clusters of nuclei in quadrants 2, 3, and 4 were close to the optic fissure and part of clusters in quadrant 1 that had been transected by the radial cuts in the eye.

Analysis of the degree of clustering of the labeled apoptotic nuclei was carried out by Voronoi analysis (Gaillard et al., 2009), using software available on Image J, a public domain, Java-based image processing program, developed at the National Institutes of Health. Using this software, a quantitative measure of clustering may be obtained, the Regularity Index (RI), in which random distributions approach the integer 1, while clustered distributions increasingly deviate from 1, towards zero.

Quantification of the numbers of apoptotic nuclei was carried out by counting the numbers of TUNEL-positive labeled nuclei in each quadrant. Statistical differences were determined by unpaired 't'- tests. Quantification and statistical analysis was carried out on the specimens prepared 6h and 24h after treatment.

3.2.6 Quantification of endogenous GH and IGF-1 gene expression

In order to determine the extent of gene knock-down caused by cGH-1 siRNA, the expression of GH in siRNA-treated retinas was measured by quantitative real-time PCR. Total RNA was extracted from dissected retinas using Qiagen RNeasy Mini Kit (Qiagen) following the manufacturer's instruction, and genomic DNA contamination was eliminated by DNase I treatment (Invitrogen). Total RNA concentration was quantified by measuring the optical density ratio A_{260}/A_{280} nm (>1.9) with a spectrophotometer. DNA-free total RNA was reverse transcribed using 15 U/µl ThermoScript^{TM} Reverse Transcriptase in the presence of 50 µM Oligo $(dT)_{20}$, 5x concentrated cDNA synthesis buffer, 0.1 M dithiothreitol, 10mM 2'-deoxynucleoside 5'-triphosphate mix, and 40 U/µl RNaseOUT^{TM} (Invitrogen). After reverse transcription, samples were then treated with 2 U/µl *E.coli* RNase H (Invitrogen) to remove the template RNA.

The first strand cDNA was amplified with oligonucleotide primers (Sigma-Aldrich, Ontario, Canada) for a 156-bp fragment in exon 5 of the chicken GH gene (GenBank Accession No. NM204359 ; forward primer, 5'-ACCCACCTACGACAAGTTCG-3'; reverse primer, 5;-

ATGGTGCAGTTGCTCTCTCC-3'). Quantitative real-time PCR was carried out in a Corbett Rotor-Gene 3000 (Corbett Life Science, Concord, New South Wales, Australia) using Platinum SYBR Green qPCR SuperMix-UDG Kit (Invitrogen) in the presence of 10μM of each primer and the platinum SYBR Green qPCR SuperMix-UDG. The PCR cycling parameters for GH amplification included a 2 min incubation at 50℃ to remove dU-containing products from previous reactions, an initial denaturation at 95℃ for 3 min, and 40 cycles of denaturation at 95℃ for 15 sec, with annealing at 60℃ for 30 sec, and elongation at 72℃ for 30 sec . Subsequently, a melting curve analysis was performed from 50℃ to 99℃ for each sample to ensure reaction specificity. Each sample was amplified in triplicate in comparison with no-template controls. To determine GH mRNA levels, stranded curves were constructed by Rotor-Gene Software 5.0.47 using cDNA from the chicken pituitary in ten-fold serial dilutions between 100 and 0.01pg. The data were normalized relative to chicken ribosomal 18S rRNA (GenBank Accession No. AF173612; forward primer, 5'-TTCGTATTGTGCCGCTAGAG-3'; reverse primer 5'-GCATCGTTTATGGTCGGAAC-3"). The PCR efficiencies for both GH and 18S rRNA were between 90 and 100%, and the correlation coefficient values were above 0.99.

Endogenous IGF-1 expression was determined using specific primers (forward: 5'-TATGGATCCAGCAGT-3' and reverse: 5'-CATATCAGTGTGGCGCTGAG-3') for a 158 bp fragment of the published chicken IGF-1 cDNA sequence (GenBank Accession No. NM001004384). Standard curves were constructed using ten-fold serially diluted cDNA from the

chicken liver (between 100,000 and 10 pg). The PCR parameters consisted of 50℃ for 2 min, 95℃ for 10 min, and 40 cycles of denaturation at 95℃ for 15 sec and 60 sec of annealing-elongation at 60℃. Data were again normalized relative to chicken ribosomal 18S rRNA. Statistical differences were determined by unpaired 't'- tests.

3.2.7 Quail neural retina cell line and cell culture

A quail neural retina cell line, comprised primarily of RGC-like cells (QNR/D; American Type Culture Collection number: CRL-2532), was obtained from Cedarlane Laboratories (Burlington, Ontario, Canada).

Cells were thawed in a 37˚C water bath for 2 mins and divided into 200µl aliquots containing approximately 3 x 10^6 viable cells per ml., as described previously (Sanders et al., 2010). One aliquot was placed in a 35mm diameter tissue culture dish and to this was added DMEM medium (Invitrogen) with 10% fetal bovine serum (FBS), antibiotic-antimycotic mixture (Invitrogen). Cultures were then incubated at 39˚C for 24h.

These cells were also cultured at 39˚C in 25ml flasks, passaged and subcultured every 2 days after confluence, and used between passage 20 and 40.

3.2.8 Treatment of QNR/D cultures with cGH siRNA

In order to validate the results obtained with *in vivo* treated retinas, we determined the effects of cGH-1 siRNA on GH gene knock-down in QNR/D cells,

in which we have previously determined that cGH-1 knocks down GH protein (Sanders et al., 2010).

Approximately 5 X 10^5 QNR/D cells were subcultured onto a 35 mm tissue culture dish and incubated at 39℃ until the cell density reached approximately 80% confluence. To transfect the siRNAs into cultured cells, 10 µl TransIT-TKO transfection reagent (Mirus) was mixed with 480 µl serum free DMEM and incubated at room temperature for 20 mins. Ten microliters of either cGH siRNA or non-silencing siRNA (stock concentration: 20 µM) were subsequently added, and the Trans-IT TKO/siRNA complexes were incubated at room temperature for another 20 mins. The solutions were then poured onto cells cultured in freshly changed 1.5 ml DMEM with 10% FBS to achieve a final siRNA concentration of 100 nM. Cells were incubated at 39℃ for 8 h to allow the siRNAs to successfully penetrate the cells. The medium was then replaced by fresh DMEM with 10% FBS. Cells were allowed to grow for another 16 h and were then harvested. Transfection efficiency was determined by dividing the number of siRNA transfected cells by the total number of cells in randomly selected fields. Cell nuclei were labelled with Prolong Gold Antifade Reagent with DAPI (Invitrogen) to calculate the total cell number. In order to visualize the transfected cells, siRNA conjugated with AlexaFlour 488 (Qiagen) was used. In addition, cells were cultured on 25 mm coverslips coated with 1 mg/ml laminin/poly-D-lysine (Sigma) to improve cell adhesion and survival on coverslips. After being treated with the siRNAs for 8 h, cultures were washed with 1x PBS, stained with DAPI reagent, and fixed by 4% paraformaldehyde for

20 minutes. Cells that showed scattered fluorescent in the cytoplasm were considered as siRNA transfected. The transfection efficiency was approximately 92% (n=484).

Quail GH mRNA was determined by real-time PCR, as for the determination of chicken GH mRNA, except for the use of primers directed against the quail GH gene (GenBank Accession No. FJ458436) and the use of standard curves derived from ten fold dilutions of the adult quail pituitary gland (between 1000 and 0.1 pg). Data were again expressed relative to 18S rRNA. The possibility that IGF-1 mRNA might be knocked-down in the siRNA treated quail cells was also examined, as for the determination of chicken IGF-1 mRNA, as chicken IGF-1 and quail IGF-1 are identical.

3.3 Results

3.3.1 siRNA penetration into the retina

Penetration into the retina by siRNA injected intravitreally into the eye at ED 4, was assessed using DAB immunocytochemistry on retina sections taken after fixation of the eye 24 h later, at ED 5 (Fig. 3.2). In the absence of a co-injected transfection reagent (Fig. 3.2A), penetration of Alexa-fluor 488-conjugated siRNA, as judged by the intensity of DAB reaction product, was minimal in comparison with retinas from non-injected eyes. Optimal penetration (Fig. 3.2B) was achieved using co-injection of *Trans*IT-TKO with the siRNA, and this protocol was therefore used in all subsequent experiments. Use of Invivofectamine as a transfection reagent, co-injected with siRNA conjugated to

either Alexa-fluor 488 or biotin was somewhat less effective than *Trans*IT-TKO (Fig. 3.2C).

3.3.2 Expression of GH and IGF-1 mRNA after cGH siRNA treatment

Retinas from eyes that had been injected with cGH siRNA and non-silencing siRNA were subjected to real-time PCR analysis to determine the extent to which the levels of GH mRNA and IGF-1 mRNA were affected. 6h after intravitreal injection, a significant reduction (by approximately 35%, $P< 0.001$) in the GH mRNA level was observed after GH siRNA injection, in comparison with retinas treated with non-silencing siRNA.(Fig. 3.3A). The GH mRNA knock-down was sustained for at least 24h after injection (Fig. 3.3C; $P=0.033$). This degree of knock-down was corroborated by treatment of cultures of quail retinal ganglion-like QNR/D cells with cGH siRNA and non-silencing siRNA (Fig. 3.3E). In these cultures, cGH siRNA treatment similarly resulted in a significant decrease in GH mRNA level (by approximately 41%, $P<0.001$).

In the case of IGF-1 mRNA levels, PCR on retinas tested 6h after injection with cGH siRNA showed a significant knock-down of approximately 51% ($P=0.023$), in comparison with the non-silencing control (Fig. 3.3B). This was sustained for at least 24h after treatment (Fig. 3.3D) with a reduction of approximately 46% ($P=0.01$). IGF-1 mRNA knock-down after cGH siRNA treatment could not be substantiated using the QNR/D cell line (data not shown), perhaps owing to the low abundance of the transcript.

3.3.3 Apoptosis of retinal cells after in ovo intra-vitreal siRNA injection

Figures 4A and 4C show photomontages of flat-mounts of ED 5 retinas, with the quadrants containing the optic fissure (OF) indicated. Figure 4A is from a non-injected eye, and the quadrant containing the OF (quadrant 1) is enlarged in Fig. 3.4B, which shows a relatively small number of TUNEL-positive nuclei (examples are circled). Figure 3.4C is from an eye which had been intra-vitreally injected *in ovo* with non-silencing siRNA 24h earlier, at ED 4. The quadrant containing the OF (quadrant 1) is enlarged in Fig. 3.4D, which, like the non-injected example, also shows a relatively small number of TUNEL-positive nuclei (examples are circled).

Figures 5A and B show similar photomontages of flat-mounts of ED 5 retinas, but from eyes that had been injected intravitreally *in ovo* with cGH siRNA 24h earlier, at ED 4. The quadrants containing the optic fissure are indicated (OF). Figure 3.5A shows an example in which there was clearly a higher incidence of TUNEL-positive nuclei in comparison with the examples shown in Figs. 3.4A-D. Many of the TUNEL-positive nuclei occur in clusters (examples are circled a-c and enlarged to the right) which were in close proximity to the OF. Figure 3.5B shows another example in which the clustering of TUNEL-positive nuclei was also clear (examples are circled a-c and enlarged to the right), although the overall number of TUNEL-positive nuclei in this example was lower than in Fig. 3.5A. As in Fig. 3.5A, in Fig. 3.5B the clusters were close to the quadrant containing the optic fissure (quadrant 1). In both these examples, appreciation of the proximity of the clusters to the optic fissure was facilitated by reconstructing the flat-mount

into an approximation of a sphere by cutting out and folding the image (data not shown).

Quantification of the total numbers of TUNEL-positive nuclei in retinas subjected to the three conditions (non-injected; 24h after injection with non-silencing siRNA; and 24h after injection with cGH siRNA) is shown in Fig. 3.6A. In comparison with the non-injected eyes, retinas from the cGH siRNA-injected eyes showed a significantly higher number of TUNEL-positive nuclei ($P < 0.05$; n = 15), retinas from eyes injected with non-silencing siRNA did not. Although quadrant 1 tended to show higher numbers of TUNEL-positive nuclei in comparison with quadrants 2-4 (Fig. 3.6B), this was not statistically significant. Reconstructing the flat-mount into an approximation of a sphere showed that all 4 quadrants contained cells which shared proximity to the optic fissure, and contained the clusters of TUNEL-positive nuclei shown in Fig. 3.5. Analysis of the distribution of the apoptotic cells, using the Voronoi method for determining the Regularity Index (RI; Fig. 3.6C), indicated that the apoptotic cells induced after 24h cGH1 siRNA treatment showed a significantly more clustered distribution across the retina than were cells in retinas not so treated ($P < 0.01$; n = 15). The increased apoptosis induced by GH withdrawal occurred in clusters of retinal cells, rather than with a random distribution.

When eyes were examined in flat-mount only 6h after injection (Fig. 3.7), the situation was not quite so clear as for 24h., because at this point in development there was appreciable spontaneous background cell death in the region of the optic fissure in the non-injected controls (Fig. 3.7A, asterisk). While

injection of the non-silencing control siRNA did not appreciably affect this distribution of apoptotic cells (Fig. 3.7B, asterisk), treatment with cGH siRNA appeared to raise the incidence of dying cells in five out of seven specimens examined, but the high background precluded statistical significance. As at 24h, the apoptotic cells were specifically in regions close to the optic fissure (Fig. 3.7C, asterisk, and enlargements a and b). Voronoi analysis indicated that, like the 24h data, the cGH siRNA-induced apoptotic cells showed a significantly more clustered distribution than the controls ($P< 0.05$; n = 5; data not shown).

The clustering of TUNEL-positive nuclei close to the optic fissure was examined in sections of eyes at ED 5, after injection at ED 4 with non-silencing siRNA or cGH siRNA, as well as in non-injected eyes (Figs. 3.8 and 3.9). Retinas in eyes injected with cGH siRNA clearly showed the increased incidence of TUNEL-positive nuclei in proximity to the optic fissure in 8 out of 10 samples examined (Figs. 3.8A-C). At ED 5, there were relatively few TUNEL-positive nuclei elsewhere in the neural retina. It was also clear from these sections that the TUNEL-positive nuclei in the vicinity of the optic fissure were spread throughout the layers of the neural retina. This result was in contrast to that obtained from sections of non-injected eyes (Fig. 3.9A) and eyes injected with non-silencing siRNA (Fig. 3.9B), which did not show the higher incidence of TUNEL-positive nuclei in the vicinity of the optic fissure (n = 8).

As with the flat-mount analysis shown in Fig. 3.7, sections of the eye taken 6h after injection (Fig. 3.10) clearly showed the relatively high level of spontaneous background apoptosis, close to the optic fissure, in non-injection

controls (Fig. 3.10A, asterisk), which was not changed by injection of non-silencing siRNA (Fig. 3.10B, asterisk). Consistent with the flat-mount results, treatment with cGH siRNA for 6h, increased the incidence of apoptotic cells in this region in 13 out of 15 samples examined (Fig. 3.10C, asterisk).

3.4 Discussion

This work, using siRNA-mediated GH gene knock-down in an *in vivo* system, provides further support for our contention that GH is an endogenous modulator of cell death in the developing retina (Baudet et al., 2003; Sanders et al., 2009b), an effect which may persist in the adult (Harvey et al., 2009; Sanders et al., 2009a; Ziaei et al., 2009). We have previously demonstrated a 15kDa GH moiety in the chick embryo retina at ED 6 (Baudet et al., 2003; Sanders et al., 2009b) and ED 4 (not published) and proposed that this is an active paracrine anti-apoptotic molecule. In the current work we have used the PCR technique to demonstrate the effectiveness of our siRNA treatment for GH gene knock-down.

We have previously demonstrated that this siRNA molecule specifically targets the avian (chicken and quail) GH gene *in vitro* (Baudet et al., 2009; Sanders et al., 2010), and results in significant knock-down of the 15kDa GH moiety. Here, we use this technique *in vivo*, and show that the siRNA persists in the developing eye cup for up to 24h after injection. Evidence in the literature suggests that siRNAs in plasma are rapidly degraded (Layzer et al., 2004; Morrissey et al., 2005), but that their physiological effects may last for at least 4 days after treatment (Schröder et al., 2007). By contrast with the systemic route,

siRNAs after intravitreal injection, may persist for as long as 5 days (Shen et al., 2006), and their physiological effects presumably last correspondingly longer. In the current work, we were able to demonstrate significant GH mRNA knock-down 6h after *in ovo* intravitreal injection of the siRNA, persisting up to 24 h after injection. Correspondingly, the physiological effect of GH gene knock-down on apoptosis in the retina, apparent by 6hrs after injection, also persisted for at least 24h after injection indicating a lasting effect, even assuming that conditions in the vitreous favor early siRNA degradation.

Systemically, the effects of GH on growth, differentiation and cell survival are mediated by IGF-1, and changes in the circulating level of one influence the level of the other (Stewart and Rotwein, 1996; Nyberg, 2009). Like GH, IGF-1 is present and active in the developing retina (De la Rosa et al., 1994; Diaz-Casares et al., 2005; Schlueter et al., 2007), having paracrine physiological effects on cell survival which overlap with those of GH . We have shown previously (Sanders et al., 2009b) that the levels of GH and IGF-1 in the retina are linked, and that the neuroprotective effects of GH are probably at least partially mediated by IGF-1. In the current work, we show that partial silencing of the GH gene in the retina also results in a lowering of the IGF-1 mRNA levels 6h after cGH siRNA injection, which persists for up to 24h. Whereas the GH knock-down in the retina was supported by similar results using the QNR/D cell line, the IGF-1 mRNA knock-down in the retina following cGH siRNA treatment could not be duplicated in the QNR/D cultures, possibly owing to the low abundance of the IGF-1 transcript in the cultured cells.

The increased incidence of retinal cell apoptosis that we found in response to GH gene knock-down, at both 6h and 24h post-treatment, is consistent with our earlier *in vitro* results using GH protein knock-down (Sanders et al., 2005, 2008, 2010), and supports the proposal that GH is an endogenous neuroprotective agent in the retina. The apoptotic cells resulting from treatment were neither uniformly distributed across the retina, nor were they restricted to the retinal ganglion cell layer. The dying cells appeared in clusters, most obviously positioned close to optic fissure, suggesting that cells in this location may be more sensitive to apoptotic stimuli.

The optic fissure**,** or choroid fissure, is a transient cleft in the optic cup at the ventral pole of the embryonic eye, which allows the migration of mesenchymal cells into the developing eye cup (Silver, 1977; Ozeki et al., 2000; Morcillo et al., 2006). As the fissure seals during further development, its edges fuse and give rise to the optic stalk and optic nerve head. Failure of the optic fissure to close, results in the condition known as coloboma (Sehgal et al., 2008). In birds, the optic fissure is the site of the future pecten oculi, an elaborate pigmented structure of uncertain function (Seaman and Storm, 1963; Wolberg et al., 1999).

The retinal regions of the optic fissure and the optic nerve head in the developing chick embryo at ED4-6 normally show a higher level of cell death than other regions of the retina (Schook, 1980; Diaz et al., 1999; Ozeki et al., 2000; Mayordomo et al., 2003), with an abundance of clustered dying cells in these regions. This preferential, clustered, distribution is maintained even when

further apoptosis is induced by various treatments of the eye (Diaz et al., 2000; Mayordomo et al., 2003). Our current results agree with this, and confirm the patterning and apoptotic pre-disposition of cells in the region of the optic fissure when we withdraw GH. We found that the apoptotic cells induced by GH withdrawal were not only concentrated in the region of the optic fissure, but were also distributed in clusters. The clustering may indicate that there are sub-populations of retinal cells in this region that are more susceptible to death-inducing stimuli than other sub-populations in this and other regions of the retina.

The increased level of apoptosis induced by cGH siRNA was clearer at 24h post-injection than at 6h post-injection, which is close to the peak of the early wave of naturally occurring cell death in the retina near this time (Frade et al., 1997; Sanders et al., 2005). The additional incubation time resulted in a natural developmental decline in the spontaneous background cell death in the region of the optic fissure in the normal untreated retinas, making the increase in the abundance of apoptotic cells after treatment more apparent.

The question arises as to why retinal cells in the regions of the optic fissure and optic nerve head are normally selectively pre-disposed to apoptosis and also highly susceptible to apoptotic stimuli, such as GH withdrawal. Because the optic fissure is a transient embryological structure, cell death in this region is an example of "morphogenetic degeneration" (Glücksmann, 1951; Valenciano et al., 2009); one of a number of such examples of cell death during early development, which are perhaps related to early cell proliferation or the phase of the cells in the cell cycle (Valenciano et al., 2009). Cell death in this region may

also be related to the establishment of the initial patterning of optic fiber growth and the guidance of growth cones in early axon migration, as the fibers leave the retina via the optic nerve (Cuadros and Rios, 1988). Further, the first RGCs to appear in the retina are close to the optic fissure (Roger et al., 1993), and there is a subsequent retinal centre-to-periphery gradient in the initial generation of axons by RGCs (Thanos and Mey, 2001). This suggests a relationship between early axon outgrowth and neurite activity, and the susceptibility to apoptosis; a possibility which is supported by examination of the relative distributions of early RGC axons and apoptotic cells (Vecino et al., 2004).

Although we did not measure the intra-ocular pressure (IOP) in the injected eyes, it is clear that the intravitreal micro-injection process raises the IOP, at least temporarily. Raised IOP is a major cause of the apoptotic death of RGCs seen in glaucoma (Quigley, 1998; Nickells, 2007). The question then arises as to whether or not our intravitreal injections non-specifically precipitated retinal cell death. Reports of the length of time that IOP needs to be raised experimentally in order to cause apoptotic cell death vary, however the estimates range from approximately 1 week to 3 months, with a possible peak after 3 weeks (Johnson et al., 2000; Cordeiro et al., 2004; Guo et al., 2005). It seems unlikely, therefore, that raised IOP for 24h or less in our experiments would appreciably raise the level of apoptosis in RGCs, and in any case, injection of non-silencing siRNA did not raise the number of apoptotic cells when the eyes were examined 24h after injection.

Our previous work (Harvey et al., 2009; Sanders et al., 2009a; Ziaei et al., 2009) has implicated retinal GH in human visual dysfunction, including the death of RGCs. It has been speculated that the loss of RGCs specifically at the optic nerve head in glaucoma may be attributable to neurotrophin deficiency, and that this neurodegeneration may be therefore be analogous to the loss of mis-targeted neurons during embryogenesis (Quigley, 1998). The current results support our view that retinal GH is a significant paracrine survival factor for RGCs during development, and are consistent with the conjecture that this effect of retinal GH may persist in the adult.

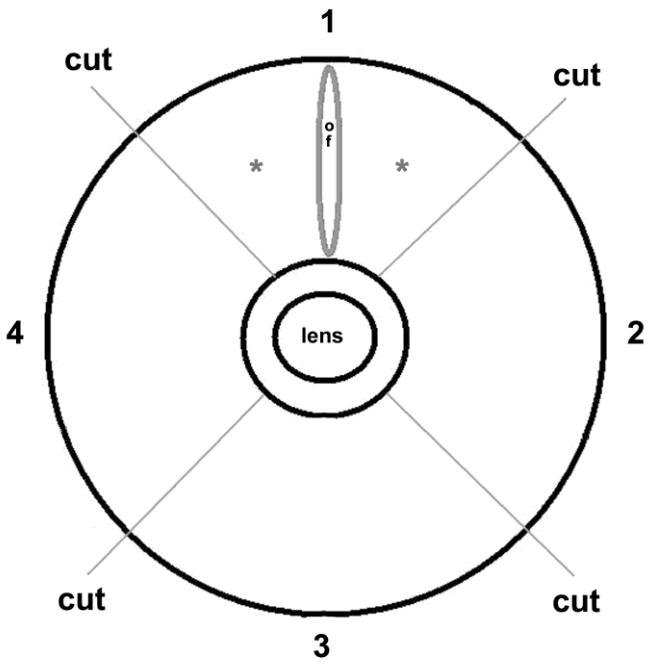

Figure 3.1. Diagram of the ED4 eye flat-mount. Four cuts were made as shown, dividing the eye into four quadrants, labeled 1 – 4. The asterisks indicate the predominant location of clusters of apoptotic cells in cGH siRNA-treated specimens. of = optic fissure.

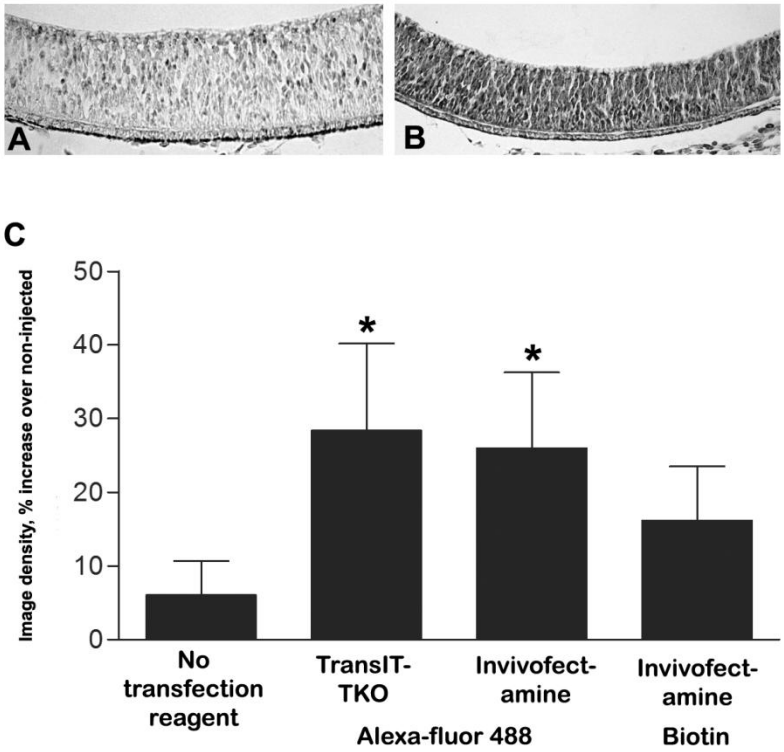

Figure 3.2. The effect of various transfection conditions on the penetration of siRNA reagents into the ED5 retina. Chick embryo eyes were microinjected *in ovo* at ED4, and penetration of the reagents was assessed using diaminobenzidine (DAB) immunocytochemistry on retina sections taken after fixation at ED5. Color development time was standardized to 2 mins in all samples. Penetration was assessed by digitally measuring the color intensity of the sections. A. An example of the color intensity resulting when no transfection reagent was co-injected with the siRNA reagent (see C, lane 1). B. An example of the color intensity resulting when *Trans*IT-TKO transfection reagent was co-injected with the siRNA reagent (see C, lane 2). C. Quantification of colour intensity in sections of retina after *in ovo* injection of siRNA with and without transfection reagents, and using different immunocytochemical techniques. Intensities were

measured in arbitrary units by the microscope software, and are expressed as a percentage of non-injected controls. Lane 1: siRNA coupled to Alexa-fluor 488 with no transfection reagent; DAB immunocytochemistry using anti-Alexa-fluor antiserum; goat-anti-rabbit antibody conjugated to biotin; avidin-peroxidase and DAB. Lane 2: as for lane 1, but co-injected with *Trans*IT-TKO transfection reagent. Lane 3: as for lane 1, but co-injected with Invivofectamine; Lane 4: as for lane 2, co-injected with Invivofectamine, but using siRNA coupled directly to biotin followed by DAB immunocytochemistry using avidin peroxidase. * = significantly different from Lane 1 ($P < 0.05$).

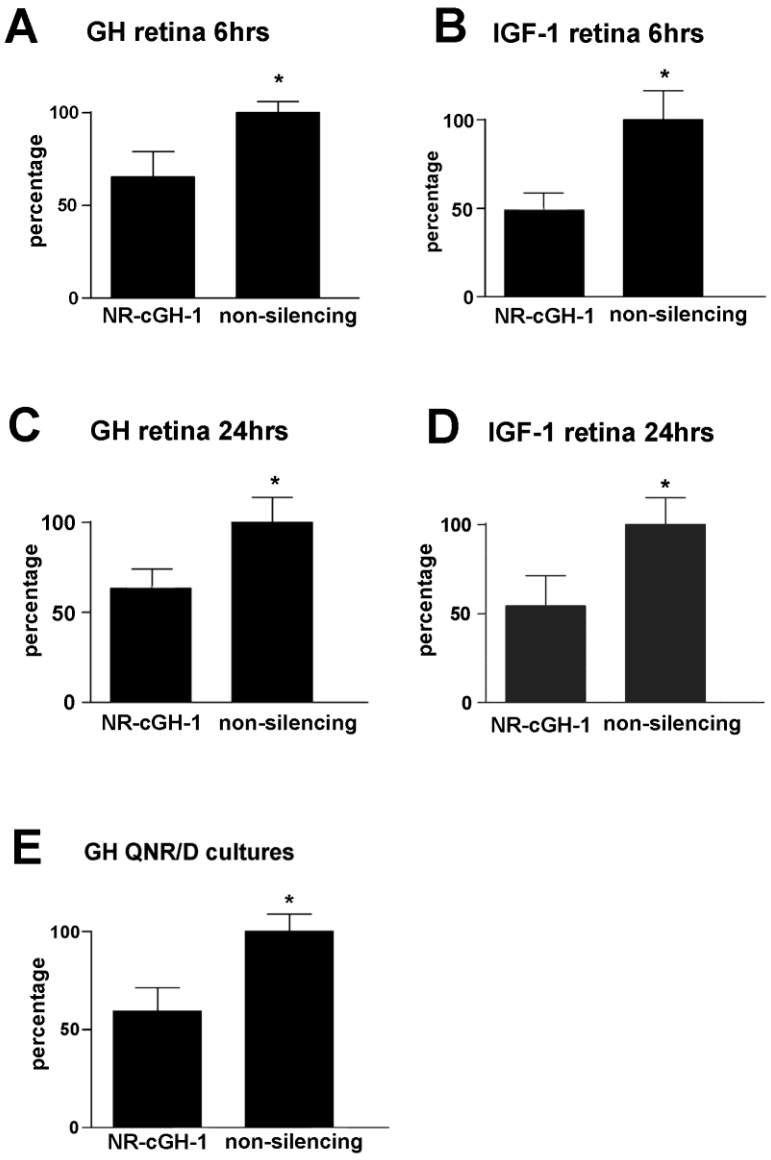

Figure 3.3. Real-time PCR assessment of siRNA-induced GH mRNA and IGF-1 mRNA knock-down in chick retinas and in cultured QNR/D retinal ganglion-like cells. Results are shown as a percentage of the non-silencing siRNA treatment, where the latter is expressed as 100%. A. GH mRNA in retina tissue 6h after treatment. B. IGF-1 mRNA in retina tissue 6h after treatment. C. GH mRNA in retina tissue 24h after treatment. D. IGF-1 mRNA in retina tissue 6h after

treatment. E. GH mRNA in cultured QNR/D retinal ganglion-like cells at 24h after treatment. * = significantly different Error bars = standard error of the mean.

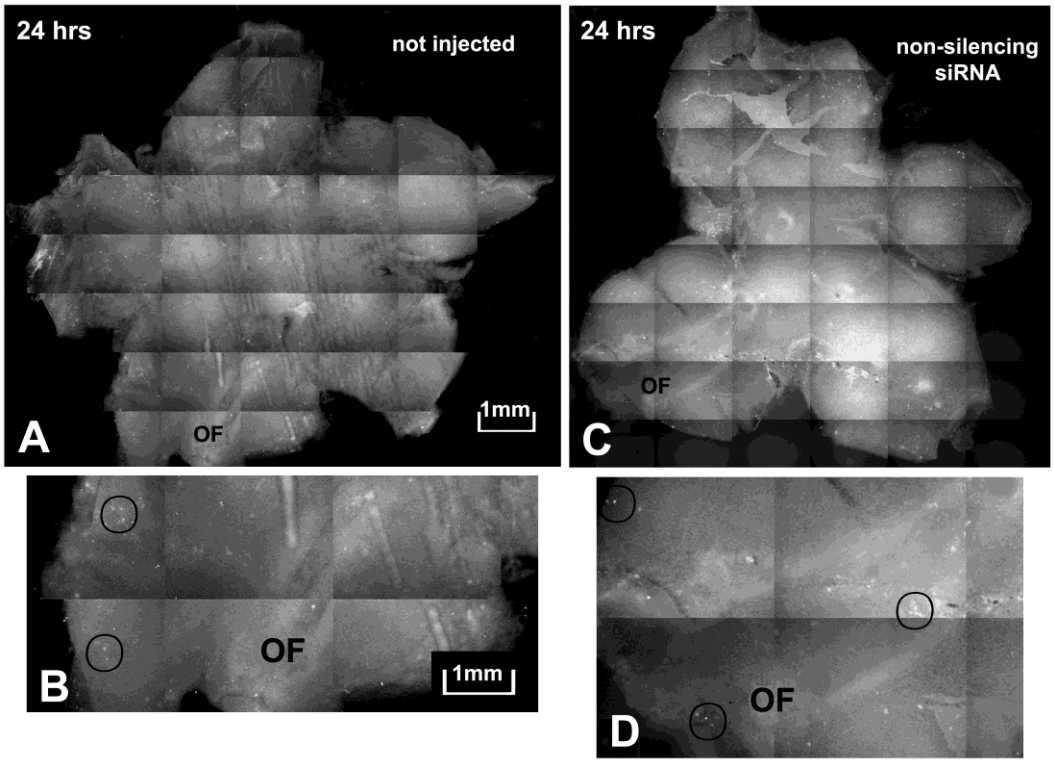

Figure 3.4. Photomontages of flat-mounts of ED5 retinas that were subjected to the TUNEL technique 24h after injection to show apoptotic nuclei. A. A retina from a non-injected eye. B. Higher magnification of the optic fissure (OF) region of A, showing relatively few TUNEL-positive nuclei (examples are circled). C. A retina from an eye injected with non-silencing siRNA. D. Higher magnification of the optic fissure (OF) region of C, showing small groups of TUNEL-positive nuclei (examples are circled).

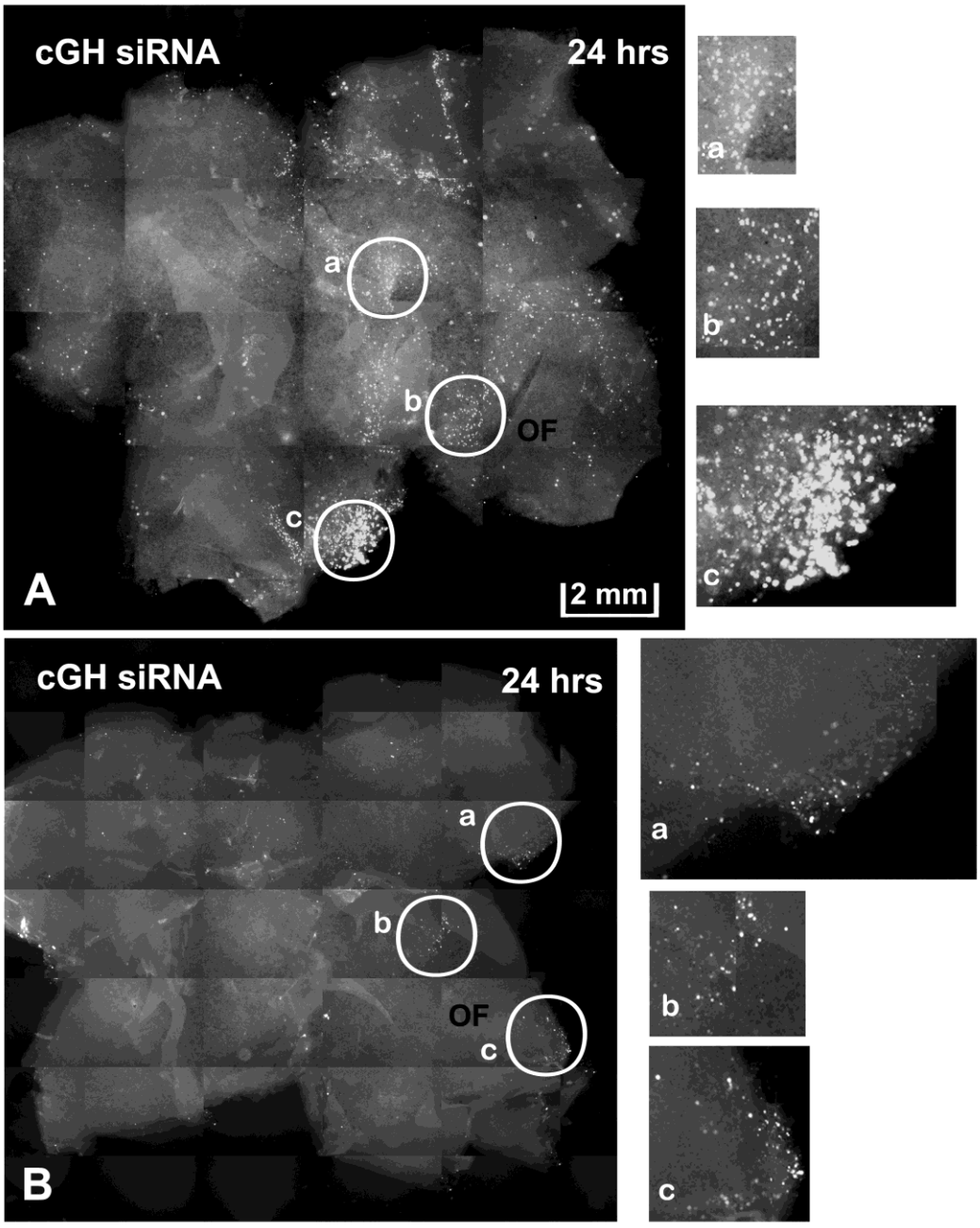

Figure 3.5. Photomontages of flat-mounts of ED5 retinas that were subjected to the TUNEL technique 24h after injection to show apoptotic nuclei. A. A retina from an eye injected with cGH siRNA. Examples of clustered TUNEL-positive nuclei are circled (a-c) and enlarged to the right. Note that the clusters, although not necessarily in the quadrant of the retina containing the optic fissure (OF;

quadrant 1), are within a radius of approximately 1mm of the optic fissure. B. Similar to A, again showing clusters of TUNEL-positive nuclei (examples are circled; a-c). As in A, these clusters are close to the optic fissure, though not necessarily in the quadrant containing the optic fissure owing to the cuts made in flattening the retina.

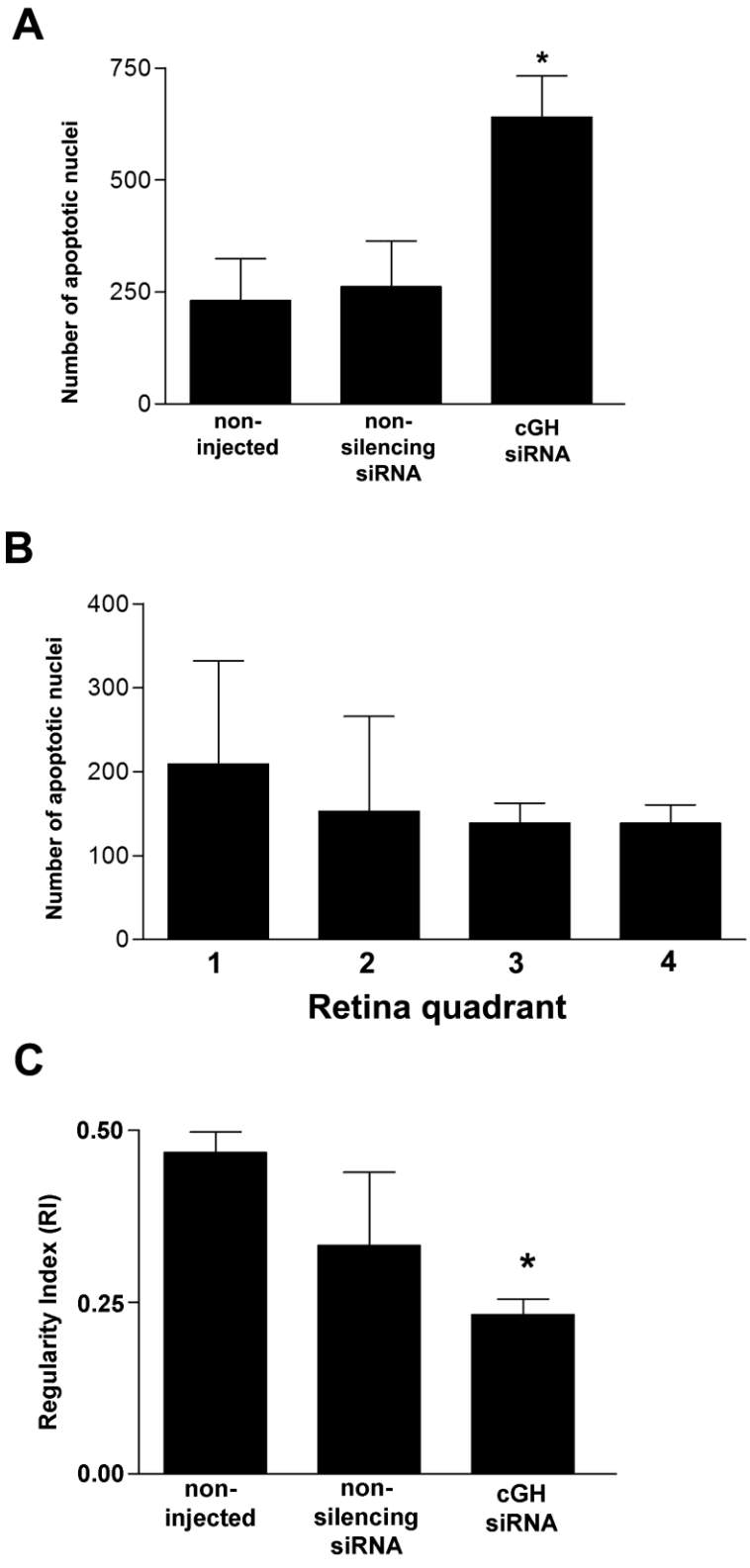
A
750
500
250
0
Number of apoptotic nuclei
*
non-injected
non-silencing siRNA
cGH siRNA
B
400
300
200
100
0
Number of apoptotic nuclei
1
2
3
4
Retina quadrant
C
0.50
0.25
0.00
Regularity Index (RI)
*
non-injected
non-silencing siRNA
cGH siRNA

Figure 3.6. Quantification of the numbers and distribution of apoptotic nuclei in flat-mount preparations of ED5 retinas using the TUNEL technique, 24h after injection. A. A comparison of the total number of apoptotic nuclei in retinas from eyes that were: not injected; injected with non-silencing siRNA; and injected with cGH siRNA. * = significantly different from non-injected eyes ($P < 0.05$; n = 15). B. A comparison of the numbers of apoptotic nuclei in the 4 quadrants of the flattened retinas from eyes injected with cGH siRNA. Quadrant 1, which contained the optic fissure, while showing the highest number of apoptotic nuclei, did not show a significantly higher number (n = 15). Reconstruction of the image of a flattened retina into a sphere, showed that all quadrants contained areas within a radius of 1mm of the optic fissure. This radius was the zone showing the highest number of TUNEL-positive nuclei. C. A comparison of the Regularity Index of apoptotic nuclei, 24h after treatment, in retinas from eyes that were: not injected; injected with non-silencing siRNA; and injected with cGH siRNA. Apoptotic nuclei in retinas treated with cGH1 siRNA were less regularly distributed than the other conditions. * = significantly different from non-injected eyes ($P< 0.01$; n = 15). Error bars = standard error of the mean.

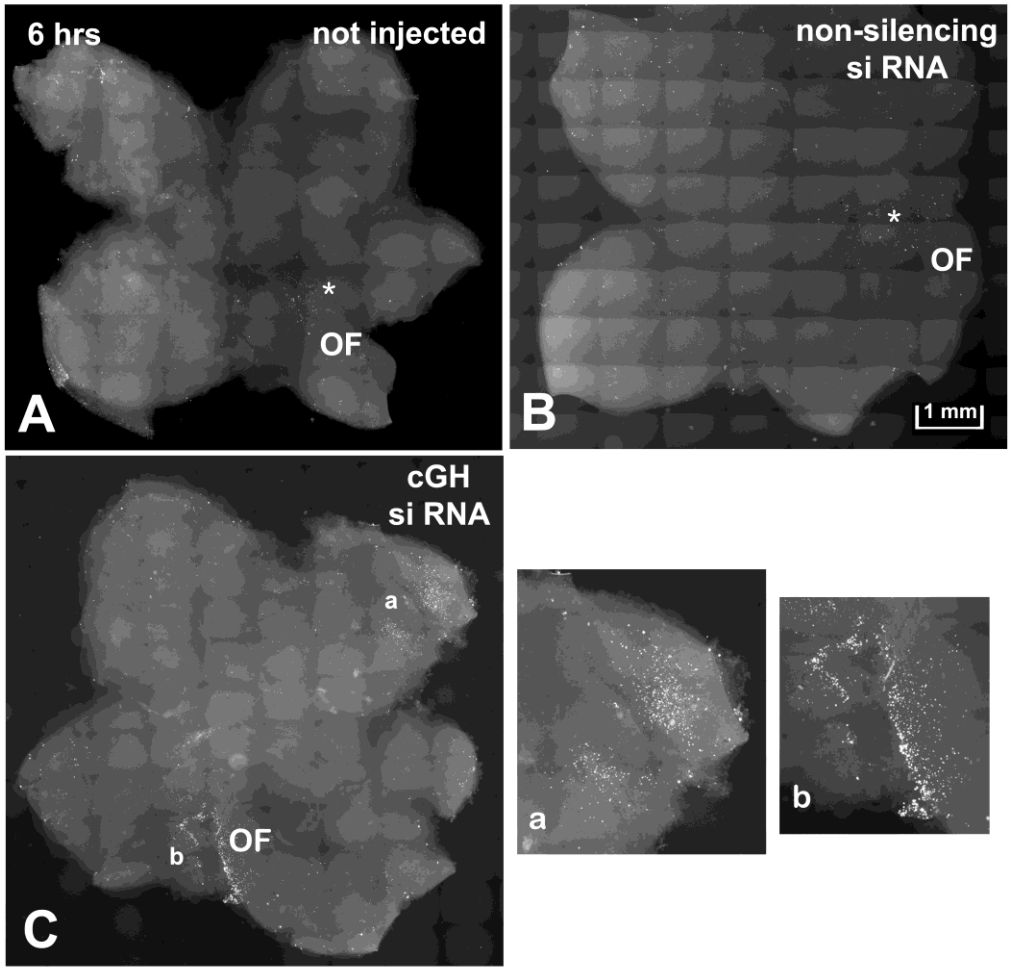

Figure 3.7. Photomontages of flat-mounts of ED4retinas that were subjected to the TUNEL technique 6h after injection to show apoptotic nuclei. A. A retina from a non-injected eye. B. A retina from an eye injected with non-silencing siRNA. Note in both A and B that there is a concentration of apoptotic cells in the region of the optic fissure (OF, asterisks). C. A retina from an eye injected with cGH siRNA. Large clusters clusters of TUNEL-positive nuclei are present close to the optic fissure (a and b) and enlarged to the right.

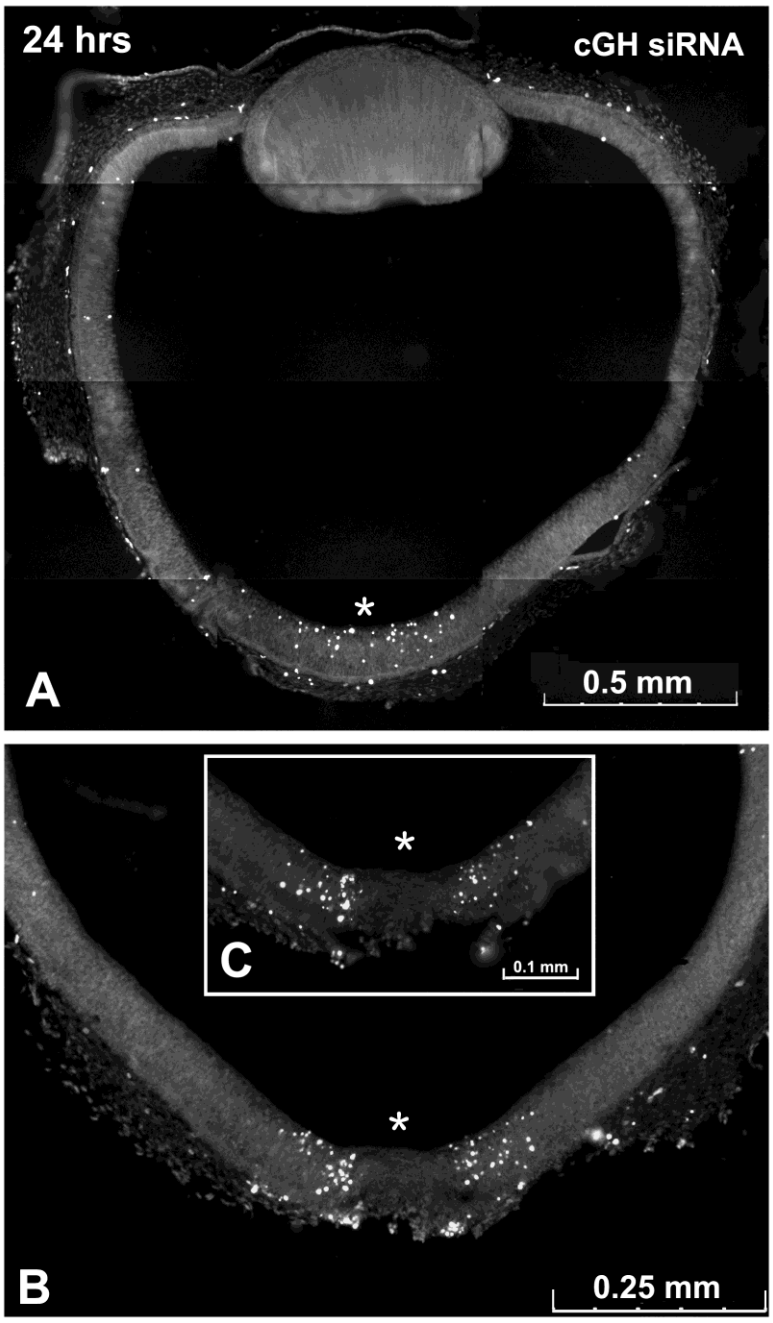

Figure 3.8. Sections of ED5 eyes 24h after intravitreal injection of cGH siRNA, treated with the TUNEL technique to label apoptotic nuclei. Three different specimens are shown (A – C). In each case, brightly fluorescent apoptotic nuclei are clustered close to the optic fissure at the back of the retina (asterisks).

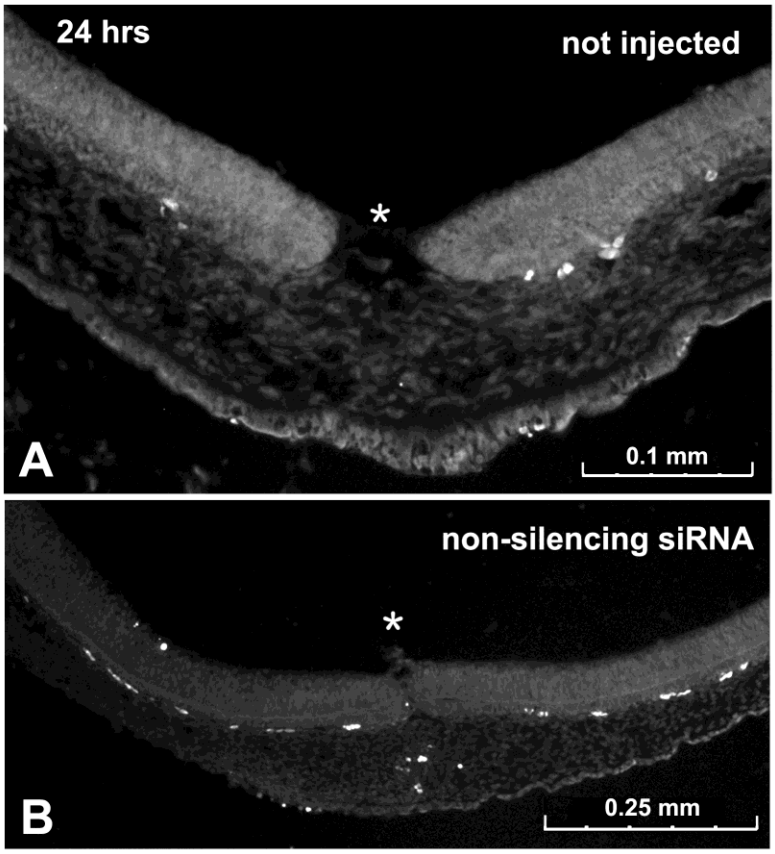

Figure 3.9. Sections of ED5 eyes 24h after injection, treated with the TUNEL technique to label apoptotic nuclei. The back of the retina is shown, similar to that shown in Figs. 6 B and C. A. A non-injected eye. B. An eye injected with non-silencing siRNA. In neither case is clustering of apoptotic nuclei close to the optic fissure seen (asterisks).

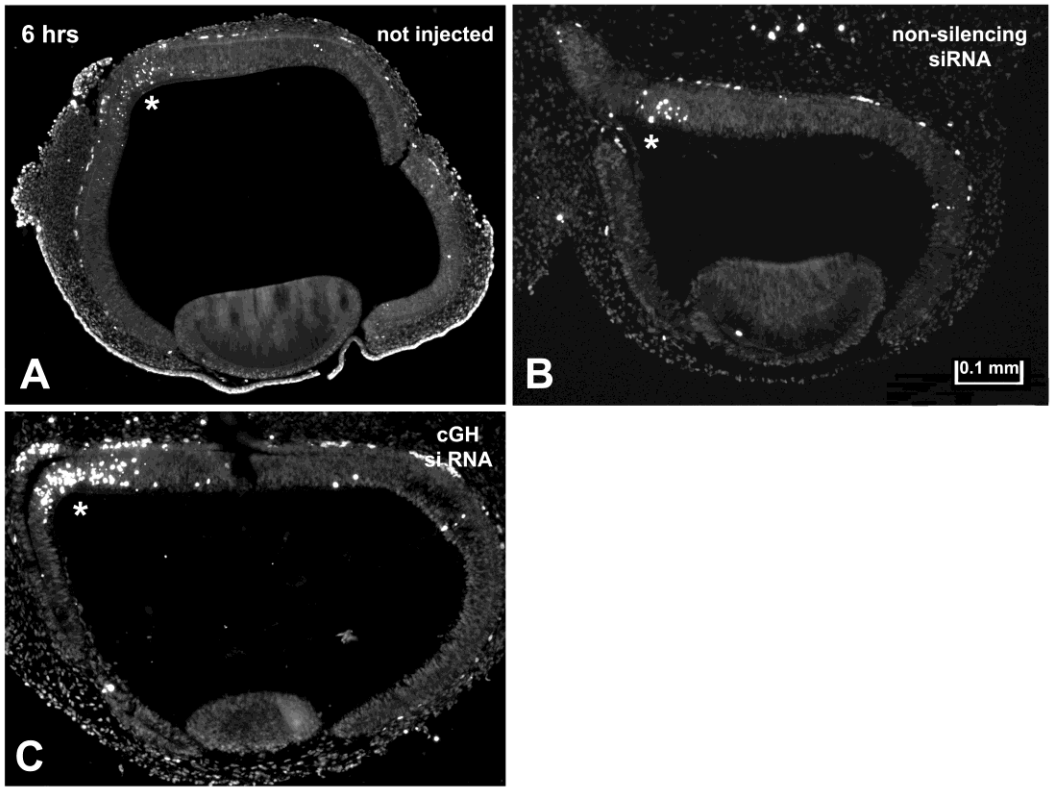

Figure 3.10. Sections of eyes 6h after intravitreal injection. A. A non-injected eye. B. An eye injected with non-silencing siRNA. Note that in both A and B there are concentrations of apoptotic cells in the region of the optic fissure (asterisks). C. An eye injected with cGH siRNA. Note the high concentration of apoptotic cells in the region of the optic fissure in comparison with A. and B (asterisk).

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Chapter Four

General Discussion

4.1 Overview

These results demonstrate that suppression of locally synthesized GH expression in the developing chick retina results in an increased incidence of retinal neural cell death, indicating a role of retinal GH in promoting neural cell survival in the early development of the chicken retina. The current study further reveals that chick retinal GH exerts this neuroprotective effect through a positive regulation of local IGF-1 gene expression. These findings are consistent with the previous studies about endogenous GH actions in the retinal development of chicks (Sanders et al., 2009a; Harvey, 2010), thereby reinforcing the hypothesis that GH is an autocrine/paracrine hormone in the retina of chick embryos.

4.2 GH is an autocrine/paracrine hormone in the developing chick retina

GH gene expression in a number of extrapituitary tissues has been demonstrated in recent years (Harvey, 2010), which challenges the traditional view that GH is only produced in the pituitary gland, with endocrine actions on target tissues (Butler and Le Roith, 2001). The new viewpoint suggests GH is an autocrine/paracrine hormone that is synthesized and acts in its target tissues, such as the nervous and immune systems (Harvey, 2010). This autocrine/paracrine GH production has functional significance, especially when it happens in early embryogenesis before pituitary development (Harvey, 2010).

GH is present in the chick retina before it is present in the pituitary gland. It is synthesized in the neural and epithelial retina of the chick, and is particularly abundant in RGCs, during early embryogenesis (Harvey, 2010). Within the retina,

GH is functionally involved in RGC survival during developmental waves of apoptosis, since the immunoneutralization of endogenous GH *in ovo* (Sanders et al., 2005) or the knockdown of GH gene expression in immunopanned chick RGCs (Baudet et al., 2009), promotes cell death. The current work supports this point of view by showing an increase incidence of apoptosis in developing neural cells after GH knockdown in the retina of ED 4 chick embryos. These results provide evidence that GH is synthesized in neural retinal cells, and has autocrine/paracrine effects on retinal function in the chick embryo.

These results specifically indicate that the actions of GH occur during a limited window of time (between ED 4 and ED 5) during the first phase of retinal cell death (Sanders et al., 2005). These dying cells may result from the so-called morphogenic cell death that occurs in the development of the vertebrate retina. According to Valenciano et al., this phase of cell death mainly affects proliferating neuroepithelial cells, and is distinguishable from the second developmental wave of cell death (neurotrophic cell death), which primarily happens in differentiated neurons (Valenciano et al., 2009). It has also been noted that morphogenic cell death is related to invagination of the optic vesicle and the closure of the optic fissure (Valenciano et al., 2009). In our study, the timing and location (not restricted to the RGC layer) of the dying retinal cells suggest that they are in the phase of morphogenic cell death. Further, as the cells dying after GH withdrawal are present in clusters, it is likely that sub-populations of retinal cells exist that are more susceptible to death-inducing stimuli. It would therefore be of interest to know if the cells within the same clusters display the same

characteristics and develop into the same neural type. Further studies combining immunohistochemistry with specific cell markers (e.g. islet-1 for RGCs) would thus be necessary to precisely define the characteristics of these dying cells.

4.3 Neuroprotective factors in retinal development

Spontaneously occurring neural cell death in a restricted time period is a crucial physiological process for embryo development (Vecino et al., 2004). It is now well known that these phases of apoptosis in the developing retina are under rigorous control by a number of neurotrophic factors including NGF, BDNF, NT-3 and NT-4 (Vecino et al., 2004). For example, it has been reported that, in the chick retina, *in ovo* administration of BDNF prevents retinal cell death during the early period (ED 5 to ED 7) of development, whereas NGF counteracts the survival effect of BDNF by killing new-born neurons acting via binding to a specific low affinity receptor p75 (Frade et al., 1999). The results of the current study show in agreement with previous *in vitro* and *in vivo* studies (Sanders et al., 2005; Sanders et al., 2009a), suggesting that GH is a member of this family of neurotrophic factors.

4.4 The actions of GH in the developing chick retina are mediated by IGF-1 signalling

It is well established that the production of circulating IGF-1 by the liver is stimulated by the action of endocrine GH, and liver-derived IGF-1 is once considered as the only source of IGF-1 stimulating body growth and development

(Daughaday and Rotwein, 1989). Further research has, however, suggested that it is the local production of IGF-1 that is involved in the growth and metabolism of tissues (Le Roith et al., 2001). In contrast to liver-derived IGF-1, the mechanism for local IGF-1 synthesis seems to be more complex and not necessarily GH-dependent. For instance, Ogunkolade et al. find that the regulation of IGF-1 expression by GH is tissue specific and only specific tissues (e.g. the liver and breast) have significantly higher levels of IGF-1 mRNA in wild-type mice than in GHR-deficient mice (Ogunkolade et al., 2005). Moreover, Lupu et al. demonstrate that GH exerts IGF-1-independent effects on body growth, after comparing the body length of mice with GHR gene knockout, IGF-1 gene knockout, and GHR-IGF-1 double knockouts (Lupu et al., 2001). Therefore, it is likely that, in addition to the physiological functions that both GH and IGF-1 cooperatively contribute to, they also possess independent roles in the development and growth of their target tissues, and their functional relationship is likely to be tissue-specific.

Evidence suggesting a functional relationship between GH and IGF-1 in the vertebrate retina is scarce. Otteson et al. show that GH injections raise retinal IGF-1 mRNA expression, which in turn promotes stem cell proliferation in the retina (Otteson et al., 2002). An increase in IGF-1 expression has also been seen in cultured chicken retinas treated with recombinant GH (Baudet et al., 2003). The results of the present study are, however, the first to show a dependent relationship of locally synthesized endogenous GH and IGF-1 expression. Our data show that endogenous GH in the developing chick retina is capable of

stimulating local IGF-1 expression in an autocrine/paracrine manner, as local GH gene knock down led to a reduction of IGF-1 mRNA levels.

4.5 Autocrine/paracrine GH and ocular function

Local GH expression in the retina is not unique to the chick embryo, since it has also been found in fish, in perinatal and adult rodents, as well as in humans (Harvey, 2010). In addition, it is thought that the blood ocular barriers in the retina exclude endocrine GH in the circulation from entering into the eye (Cunha-Vaz, 2004), and thus the GH immunoreactivity in the human retina and vitreous (Harvey et al., 2009) likely reflect local GH production. This possibility is supported by the lower GH concentrations measured in human vitreous compared with that in the bloodstream, and by the finding that there is no decline in vitreous GH concentrations in aged people, whereas GH concentrations in human plasma are distinctly age-related (Ziaei et al., 2009). The human eye is also likely a site for autocrine/paracrine GH to exert its action, since pituitary GH has long been implicated in retinal vascularization and the etiology of retinopathy (Harvey et al., 2009). However, direct connections between retinal GH and ocular function are lacking, although studies have suggested GH roles in RGC survival (Sanders et al., 2009b), neurogenesis (Baudet et al., 2008), and angiogenesis (Wilkinson-Berka et al., 2007; Baudet et al., 2008) in the human or mouse retina. Other studies have also indicated a decrease of GH concentration in the vitreous of diabetic patients who usually suffer from retinopathy (Ziaei et al., 2009).

In the present study our findings provide evidence to support the involvement of autocrine/paracrine GH in normal ocular function. In particular, our results indicate that GH withdrawal-induced cell apoptosis in the early development of chick retina occurs surrounding the optic fissure. This structure is an important channel for both RGC axons to leave the eye and for the hyaloid artery to enter the eye (Chow and Lang, 2001; Morcillo et al., 2006), and improper differentiation of this channel results in an ocular disease known as coloboma (Cunliffe et al., 1998). A deficiency of retinal GH may contribute to the etiology of this disease. This possibility is also supported by the studies suggesting a relationship between GH deficiency and CHARGE syndrome, a genetic disorder characterized by several specific symptoms including coloboma (Pinto et al., 2005).

A deficiency of retinal GH may also be involved in the etiology of glaucoma, an ocular disease that results from selective RGC loss. It has been reported that the deprivation of neurotrophic support, resulting from exposure of the eye to an elevated intraocular pressure, is the causal factor in glaucoma (Johnson et al., 2009). In view of its neuroprotective role, the loss of GH in the RGCs of elderly individuals (Sanders et al., 2009b) may thus contribute to the etiology of this disease.

4.6 Quail QNR/D cell line and its relevance as an avian RGC model

The quail QNR/D cell line is derived from neuroretinas of ED 7 quail embryos (Pessac et al., 1983). The isolated primary cells are then infected with a

transformation-defective thermosensitive mutant of Rous sarcoma virus (RSV) to create a permanently established cell line. For unknown reasons, the same attempts to derive permanent cell lines from chicken neural retinas are unsuccessful (Pessac et al., 1983). Although this quail neuroretina cell line hasn't been generally used as a model for *in vitro* study in avian retinal development, it has some advantages. Firstly, these QNR/D cells can be labelled with antibodies raised from chick retinal ganglion and amacrine cells, indicating that the clone is derived from these cell types, and more importantly, that QNR/D cells may share high similarities in molecular and immunochemical properties with chicken RGCs and amacrine cells (Pessac et al., 1983). Moreover, it has been shown that QNR/D cells produce stable action potentials when stimulated with brief depolarizing currents, which suggests that these cells are able to exert normal electrophysiological functions (Pessac et al., 1983). Studies have further shown that after being injected into the vitreous of embryonic chick eyes at the time that ganglion cells in the central retina is maturing (about ED 9), most (98%) of the QNR/D cells migrate to and remain in the appropriate strata of the retina, i.e., in the RGC and amacrine cell layers (Trisler et al., 1996). These cells are thus likely to have some, if not all, the molecular characteristics of ganglion/amacrine cells *in vivo*. Our data similarly show that a high percentage of RGC-derived cells are present in the QNR/D cells, since approximately 80% are immunostained with islet-1 antibody, a specific marker for RGCs in chickens (Sanders et al., 2005; Baudet et al., 2007b) and quail (Halfter, 1998).

The present study identified a 15 kDa GH moiety to be the major GH isoform in QNR/D cell extracts by immunoblotting with two polyclonal anti-chicken GH antibodies (Harvey and Scanes, 1977; Cheung and Hall, 1990), and this finding is consistent with the presence of 15 kDa GH moiety in the culture media of embryonic chicken retinas (Baudet et al., 2003). In addition, immunocytochemical examination has further shown that these antisera immunoreacted with GH in the nucleus and cytoplasm of QNR/D cells, as in chicken RGCs (Baudet et al., 2007a).

Taken together, the present study demonstrated that quail GH is very similar to chicken GH, both at the mRNA (95% homology) and protein (97% homology) level. The similar molecular/immunochemical properties that QNR/D cells exhibit supports their use as an alternative to immunopanned chicken embryonic RGCs for *in vitro* studies on RGC function. Also, the average incubation period for quail embryos is 23 days, which is very close to that in chicken (21 days). These QNR/D cells, derived from ED 7 quail embryos, may thus exhibit molecular and cellular characteristics similar to the corresponding cells in the chick retina at parallel embryonic stages. The quail QNR/D cell line is thus a useful model for chick RGC studies, especially as it eliminates animal use and the need to enrich isolated chick RGCs using scarce Thy-1 antibodies.

4.7 Future studies

The present study show that GH produced in the retina of chicken embryos plays a neuroprotective role to promote cell survival. The experiments

are, however, restricted to a window of time between ED 4 and ED 5. Since the early phase of retinal cell death in the chicken persists to and peaks at ED 7 (Sanders et al., 2005), we may have underestimated the number of dying cells involved. The effect of longer and later periods of GH withdrawal on retinal cell death should therefore be determined. It would also be of interest to see if local GH deficiency results in alterations in the cell composition in the neural retina, as blocking of the signalling of another neurotrophic factor, (pro)insulin, at early phase of embryonic development (between ED 2 and ED 6) halves the ultimate number of RGCs (Valenciano et al., 2009). In addition, in the absence of GHR signalling, neonate mice have been shown to have a shorter axial length of their eyeballs, a smaller neural retina with decreased width of OFL and IPL, but an unchanged thickness of the RGC layer, in comparison with the wild type mice (Baudet et al., 2008). A relative long-term effect of GH deficiency on histological changes of the chick embryo retina however has not yet been shown and is thus of interest.

In order to demonstrate long-term autocrine/paracrine effects of GH in the chick retina, an approach that can chronically knockout GH expression is required. Our current results confirm the ability of the siRNA to silencing GH gene within 24 hours post-injection. It is unclear and probably unlikely, however, that the injected siRNA stays and is still functional in the retina for much longer, as nucleases abundant in the developing retina easily degrade the RNA molecules (Behlke, 2008). Therefore, a chemically modified siRNA (Behlke, 2008) and/or a virus-delivered short hairpin RNA (shRNA) (Pushparaj et al., 2008) would

provide a better experimental approach to prevent nuclease degradation in further long-term studies. Indeed, a few studies have successfully induced silencing of target genes in the mouse retina for up to 5 days by applying chemically modified siRNA (Shen et al., 2006), as well as in the chick retina until 5 to 6 days after delivery of shRNA-expressing virus (Harpavat and Cepko, 2006).

In addition, as some downstream genes of GH signalling are GH-specific, changes in the expression of these genes would provide an additional index of GH action. In chicks, expression of a cysteine protease inhibitor GH-responsive gene-1 (GHRG-1) is considered as a specific marker of GH-activated intracellular signaling, as GHRG-1 is not present in GHR-deficient dwarf chickens, and its expression is upregulated by exogenous GH (Harvey et al., 2001, 2002). The presence of GHRG-1 mRNA in the neural retina of the chicken embryo has been demonstrated (Harvey et al., 2002; Baudet et al., 2007b), suggesting it participates in the autocrine/paracrine roles of GH in the developing retina.

4.8 Conclusions

The presence of GH and GH receptor and their transcripts in a variety of tissues of many species has replaced the traditional view of GH of being an endocrine, with a broader understanding that GH also acts as an autocrine/paracrine in the regulation of physiological functions (Harvey, 2010). The current study confirmed that suppression of locally produced GH in the developing chicken retina significantly increases the incidence of neural cell death, and the action is likely mediated through the regulation of local IGF-1 gene

expression. These results are consistent with the conclusions based on the previous studies (Sanders et al., 2005, 2009a, b). Taken together, the present study strongly suggests a neuroprotective role of retinal GH for developing retinal neural cells, and further strengthens the view that GH is an autocrine/paracrine in the developing chick retina.

4.9 References

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Appendix

Quantification of GH and IGF-1 mRNA using real-time PCR

A.1 General principles of real-time PCR

Quantitative real-time polymerase chain reaction (qPCR) is a technique that enables nucleic acid quantification and analysis during PCR amplification (Deepak et al., 2007). Real-time PCR is a technique for monitoring the amount of PCR product throughout the PCR process, thus combining amplification and data collection into a single step. It is achieved using different fluorescent chemistries that correlate the amount of PCR product to the fluorescence intensity detected. Unlike traditional PCR, which takes measurements at the plateau phase (end-point) of PCR amplification, at which time the reaction begins to slow down and stop due to depletion of PCR materials, real-time PCR collects the fluorescence data during the exponential phase (Fig. A.1), where exact doubling of product is occurring (close to 100% of reaction efficiency) and thus avoids the drawbacks of end-point detection (Kainz, 2000). This technique is highly sensitive and can even detect a single copy of a specific target nucleic acid (Palmer et al., 2003). Indeed, it has been reported that real-time PCR assays are 10,000- to 100,000-fold more sensitive than RNase protection assays, and are 1000-fold more sensitive than dot plot hybridization (Wong and Medrano, 2005).

Quantification of PCR products with respect to specific fluorescence intensities can be achieved by a variety of fluorescent chemistries (Wilhelm and Pingoud, 2003). Among them, SYBR Green I binding is a convenient and economical method that is commonly used. SYBR Green I is a fluorescent dye (excitation and emission maxima at 494 and 521 nm respectively) that binds to any double-stranded DNA, and therefore its fluorescence intensity is proportional

to the amount of amplified DNA. Fluorescent molecules are monitored with a real-time PCR thermocycler that provides fluorescent excitation and detection of fluorescent emission.

Non-selective binding of SYBR Green I to any double-stranded DNA is the main defect of this method, as nonspecific PCR products and primer-dimers can also contribute to SYBR Green signals. Melting curve analysis, a method to identify PCR specificity, is therefore usually performed after PCR cycling in the same reaction tube. After PCR, the annealed products are melted at a constant rate, and the decrease in fluorescence is monitored as the strands dissociate. Products of the same size denaturate at a certain temperature based on their GC content, length, and sequence. Therefore the desired products can be distinguished from the undesired products by different dissociate temperatures (Ririe et al., 1997).

A.2 Quantification of PCR products

Target PCR products can be quantified by either absolute or relative quantification. Absolute quantification determines the absolute amount of the product and is usually expressed by copy number or concentration, whereas relative quantification shows the ratio between the amount of the target and the amount of a reference gene that is evenly expressed under different experimental conditions (usually a “housekeeping gene”). Relative quantification is thus generally used to compare differential gene expression in distinct samples. The experimental designs and methodologies for relative quantification can vary depending on the needs of the study and the precision desired. For example, the

relative standard curve method, the comparative Ct method (△△Ct), and Pfaffl method are generally used to quantify gene expression (Wong and Medrano, 2005).

A.3 Determination of relative quantities of GH and IGF-1 mRNA with the relative standard curve method

In the present study, siRNA-induced GH gene knockdown and subsequent suppression of IGF-1 expression in the retina of chicken embryos are determined by real-time PCR using the relative standard curve method. With this method, serially diluted standards of known RNA concentration are used to generate standard curves (Larionov et al., 2005; Rutledge and Cote, 2003). Reverse transcribed mRNA, extracted from chicken pituitaries, is used to generate the standard curve for measurement of mRNA GH levels, and serially diluted reverse transcribed RNA samples from the chicken liver are used to construct a standard curve for measurement of reverse transcribed IGF-1 mRNA. The standards are run with the experimental samples (chick retinas injected with cGH siRNA) and the calibrator samples (controls injected with non-silencing siRNA), and the quantities of these samples are estimated in comparison with the standard curves. The values of GH and IGF-1 quantities in each sample are then normalized to the quantity of 18S ribosomal RNA (rRNA, serves as a reference gene) in each sample measured with the same method.

Standard curves are drawn by Rotor-Gene Software 5.0.47 (Corbett Life Science, Concord, New South Wales, Australia). Fluorescence detection is carried

out in the extension step of PCR cycling. Amplification of GH gene, for instance, is initially displayed as a sigmoidal-shaped (linear scale) plot, in which the fluorescence intensity is plotted against the number of PCR cycles (Fig. A.1A). The software then converts the plot into a logarithmic graph that enables easy identification of the exponential phase of the PCR (Fig. A.1B). From the logarithmic graph the software defines a threshold within the exponential range of the amplification curve; fluorescence under the threshold is considered as baseline. Usually the fluorescence that reaches the threshold is ten times higher than the standard deviation of the baseline (Wong and Medrano, 2005). Figure A.1B shows the threshold for GH mRNA, but different thresholds are separately set for different target genes.

The cycle at which the amplification curve crosses the threshold (meaning a significant increase of fluorescent signals is detectable), is called the threshold cycle (Ct) or crossing point. The Ct value can be related directly to the starting amount of the template in samples. Plotting the Ct values of serial diluted standards for chicken GH against their input amounts creates a linear regression curve (Fig. A.2C). The relative quantity of GH mRNA in each sample is first determined by interpolating its Ct values into the regression curve (Fig. A.2C), and then expressed relative to the amount of 18S rRNA (Fig. A.3, A-C and Table A.1A). This normalized quantity of GH mRNA in the calibrators is designated as 100%, and the quantity of the experimental samples is shown as a fold-difference relative to the calibrator (Chapter 2, Fig. 3.3). Because the experimentally derived quantity is then divided by that of the calibrators, the units from the standard

curve are eliminated. A similar example of the standard curve constructed for IGF-1 mRNA, and normalization of IGF-1 levels are shown in Fig. A.4 and Table A.1B.

A.4 Melting curve analysis and PCR specificity

A melting curve analysis with SYBR Green I is performed from 50℃ to 99℃, immediately after the PCR cycle. Fluorescence data are continuously collected and converted into melting peaks by Rotor-Gene Software 5.0.47. The melting peaks are plotted as negative derivative of fluorescence (-dF/dT) against temperature (Fig. A.2D). The specific temperature at which PCR products dissociate is shown as a single sharp peak in each melting curve, indicating amplification of a specific target during PCR cycling. Panel D of Fig. A.2, A.3, and A.4 shows melting peaks for PCR products of GH, 18S rRNA, and IGF-1, respectively. The size of the amplified fragments for GH and IGF-1 are also confirmed by gel electrophoresis (Fig. A.2E and A.4E).

A.5 Advantages of the relative standard curve method for real-time PCR quantification

The slope of a standard curve can be used to determine the efficiency PCR reaction with the following formula: Efficiency = $[10^{(-1/slope)}]$-1 (Wong and Medrano, 2005). PCR efficiency is a critical factor that significantly influences quantification. For instance, if the reaction efficiency is 1 (each cycle results in twice as much template), the increase of the template after 10 cycles will be 2^{10}

(equal to 1024) times, as fold increase in target gene after n cycles equals to $(1+\text{efficiency})^n$ (Schefe et al., 2006); by contrast, if the efficiency is just 0.9, the increase will be $(1.9)^{10}$ (equal to 613) times only. Therefore, PCR amplification of even a fixed starting amount of template under different efficiencies can cause a huge difference in fold increase. Most of the commonly accepted PCR quantification methods, however, are based on the assumption that the PCR efficiency is constant over time and has the same value in all studies samples (Ramakers et al., 2003). The comparative Ct method, for example, assumes a constant efficiency equal to one for both the target and reference genes. With this method, the amount of the target gene in test samples normalized by a reference gene and related to a calibrator sample is calculated as $2^{[(-\text{Cttaget,test-Ctreference,test})-(\text{Cttarget,calibrator-Ctreference,calibrator})]}$ (Ramakers et al., 2003). Nevertheless, it has been reported (Wong and Medrano, 2005), and has been seen in our current work, that in most cases the PCR efficiencies of target and reference genes are not equal and can lead to a big error in calculation of fold-difference. This variation of PCR efficiency, in contrast, is not a concern in the relative standard curve method, as a standard curve is always run with samples and thus the PCR efficiency of a particular assay is always taken into account for mRNA quantification. The efficiencies of target and reference genes therefore do not have to be equivalent.

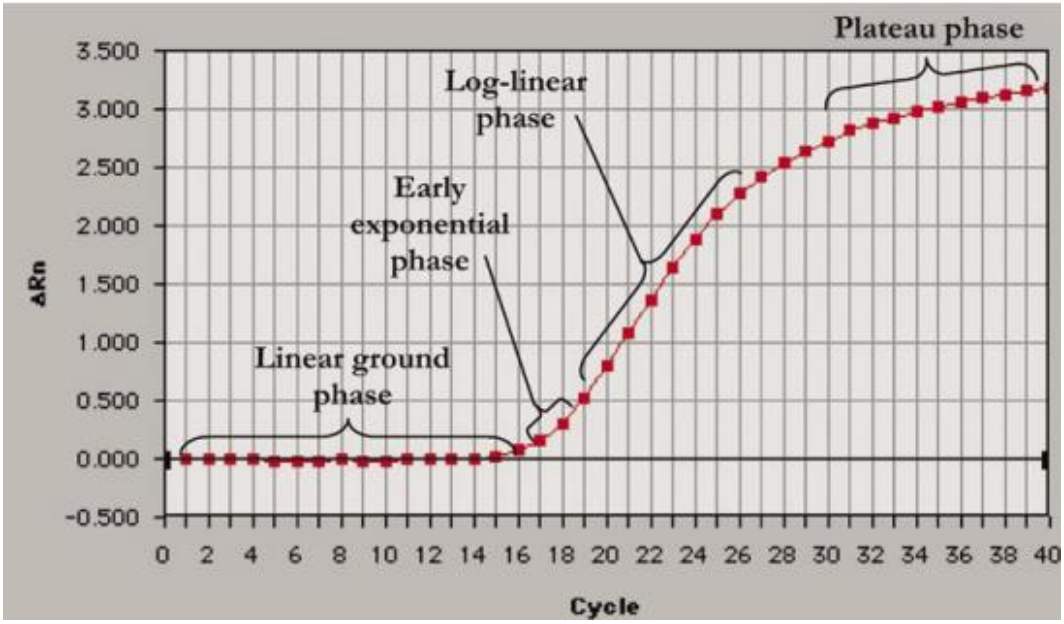
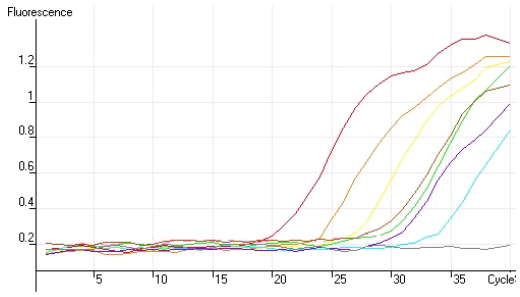
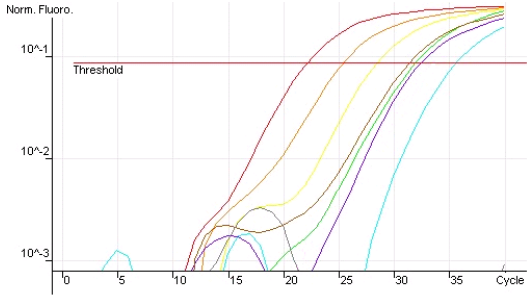


Figure A.1. Phases of the PCR amplification curve in linear view. The process of PCR amplification can be divided into mainly four phases: the linear ground phase, exponential phase, linear (log-linear) phase, and plateau phase. Rn is the intensity of fluorescent emission of the reporter dye divided by the intensity of fluorescent emission of the passive dye (a reference dye incorporated into the PCR master mix to control for differences in master mix volume). ΔRn indicates the difference in Rn values of a sample and background, and thus represents the magnitude of signal generated during PCR. The graph is adapted from Wong and Medrano, 2005.

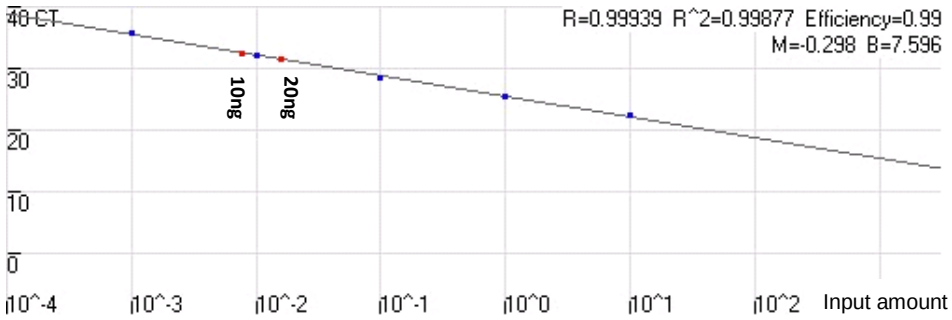
(A)



(B)



(C)



Colour	Name	Type	Input Amount (pg)	Calculated Amount (pg)	Ct
	Dilution 1	Standard	10.0000	9.0577	22.28
	Dilution 2	Standard	1.0000	0.9809	25.52
	Dilution 3	Standard	0.1000	0.1185	28.6
	Dilution 4	Standard	0.0100	0.0109	32.08
	Dilution 5	Standard	0.0010	0.0009	35.76
	NR 10ng	Sample		0.0078	32.57
	NR 20ng	Sample		0.0158	31.54
	No-template	NTC			

(D) (E)

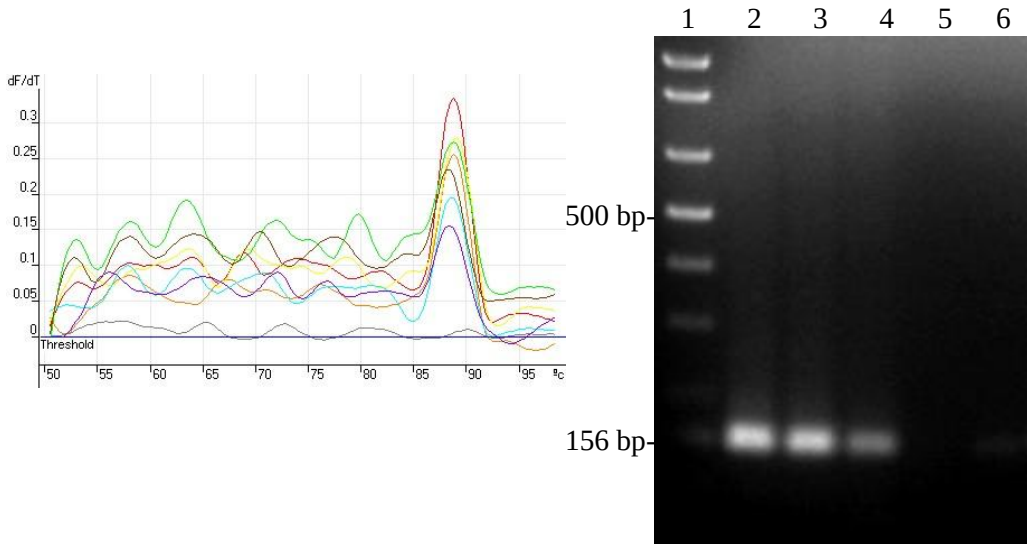
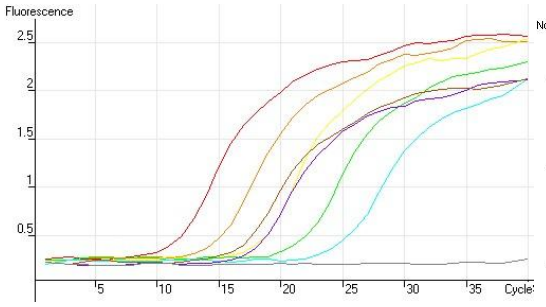


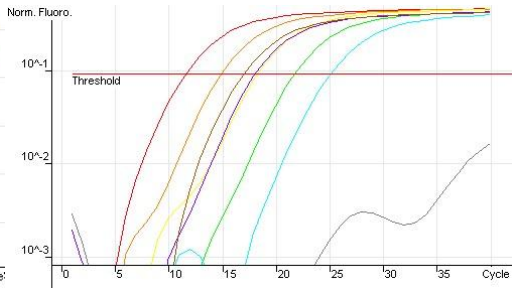
Figure A.2. Quantification of GH mRNA levels in the ED 5 chick neural retina. (A) Linear-scale graph showing the normalized fluorescence intensity data of standards generated from chicken pituitary RNA extracts (10-fold dilutions from 10 to 0.001pg are indicated in red, orange, yellow, green, and light blue, respectively) and of chick neural retina samples with different loading amounts of total RNA (NR; 10ng in purple and 20ng in brown). DNA amplification was not detected in no-template control (NTC, in grey). (B) Threshold set within the log-scale graph. The point at which the threshold crosses the amplification curve of each standard/sample is collected as its Ct value. (C) The regression curve generated as Ct values of standard dilutions against the logarithm of their input amounts. The red dots indicate where the Ct values of the neural retina samples are located. The R value indicates the correlation coefficient of the created standard curve. R value above 0.99 describes a good fit of the standard dilutions to a straight line. The efficiency of this PCR amplification is 99%. The table

shows how the unknown amount of a sample is calculated by interpolation. (D) Graph showing that the melting curve of every standard/sample has a particularly significant peak. Note that the curve for no-template control doesn't have this peak. (E) Electrophoresis in 2% agarose gel showing PCR amplification of reverse transcribed GH mRNA from the neural retina of ED 5 chicken embryos, in comparison with that from the chick pituitary. Lane 1: 1 kb plus DNA ladder; lane 2 and 3: the PCR product of GH mRNA from the chick pituitary; lane 4: the PCR product of GH mRNA from a non-injected chick retina; lane 5: PCR reaction with chick retinal mRNA reverse transcribed in the absence of reverse transcriptase; lane 6: no-template control.

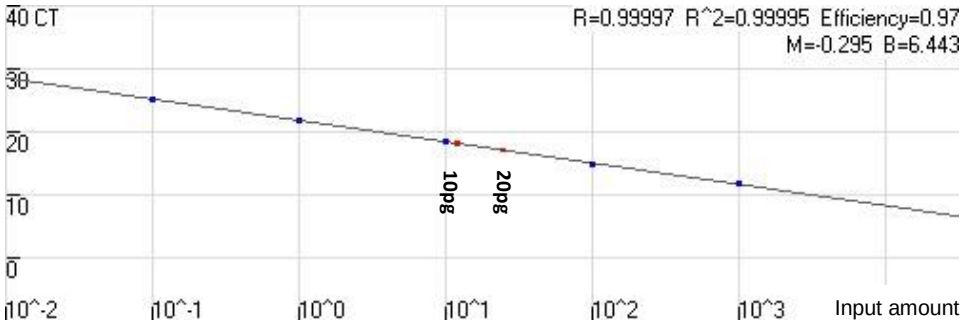
(A)



(B)



(C)



Colour	Name	Type	Input Amount (pg)	Calculated Amount (pg)	Ct
	Dilution 1	Standard	1,000.0000	971.6007	11.7
	Dilution 2	Standard	100.0000	103.7272	14.99
	Dilution 3	Standard	10.0000	10.1369	18.41
	Dilution 4	Standard	1.0000	0.9773	21.85
	Dilution 5	Standard	0.1000	0.1002	25.2
	NR 10pg	Sample		11.8530	18.18
	NR 20pg	Sample		24.3706	17.12
	No-template	NTC			

(D)

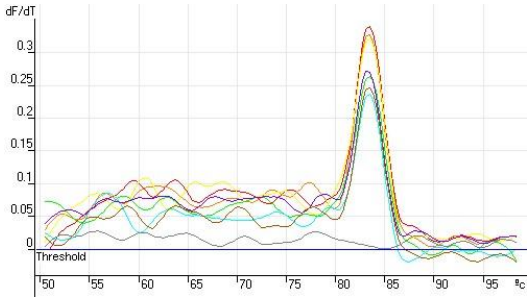
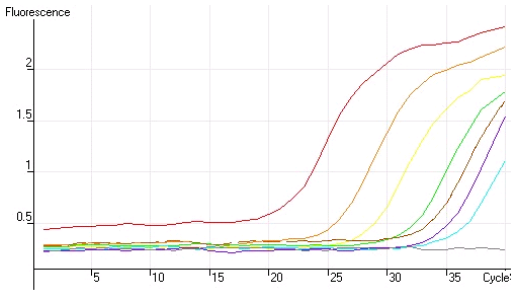
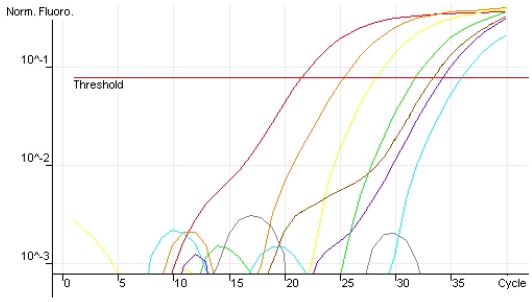


Figure A.3. Quantification of 18S rRNA levels in the ED 5 chick neural retina. (A) Linear-scale graph showing the normalized fluorescence intensity data of standards (10-fold dilutions from 1000 to 0.1pg: red, orange, yellow, green, and light blue) and neural retina samples with different loading amounts of total RNA (NR; 10pg in purple and 20pg in brown). The grey curve indicates no-template control (NTC). (B) Threshold set within the log-scale graph. (C) Generated regression curve (Ct values vs. the log of input amounts). The red dots indicate where the Ct values of retina samples are located. The efficiency of this PCR amplification is 97%. The correlation coefficient of the regression curve is very close to 1. (D) Melting curve analysis showing the specific amplified product of 18S rRNA.

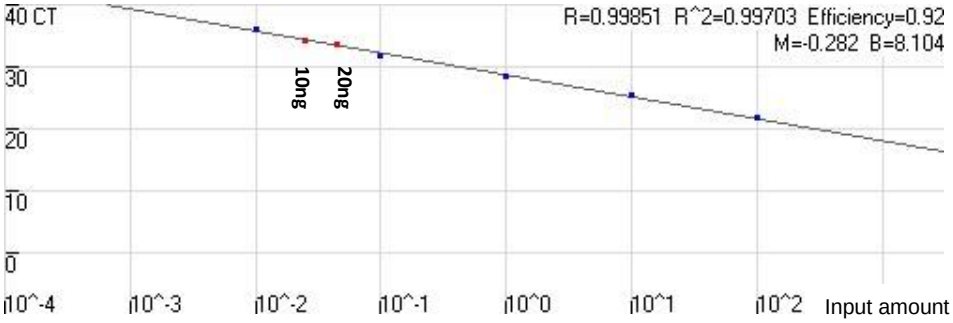
(A)



(B)

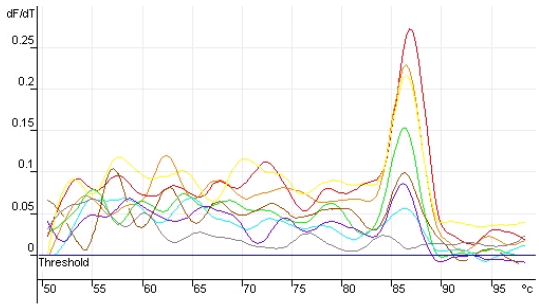


(C)



Colour	Name	Type	Input Amount (ng)	Calculated Amount (ng)	Ct
	Dilution 1	Standard	100.0000	95.7338	21.68
	Dilution 2	Standard	10.0000	8.4086	25.42
	Dilution 3	Standard	1.0000	1.2346	28.37
	Dilution 4	Standard	0.1000	0.1227	31.92
	Dilution 5	Standard	0.0100	0.0082	36.08
	NR 10ng	Sample		0.0249	34.37
	NR 20ng	Sample		0.0439	33.5
	No-template	NTC			

(D) (E)



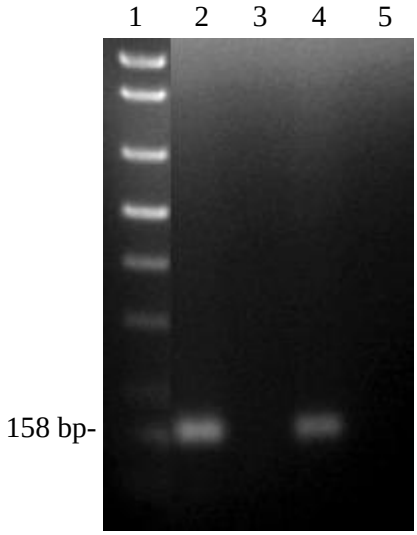


Figure A.4. Quantification of IGF-1 levels in an ED 5 chick neural retina. (A) Linear-scale graph showing the normalized fluorescence intensity data of standards (10-fold dilutions between 100,000 and 10pg: red, orange, yellow, green, and light blue) as well as chick neural retina samples (NR; loading amount 10ng in purple and 20ng in brown). The grey curve indicates no-template control (NTC). (B) Threshold set within the log-scale graph. (C) Generated regression curve (Ct values vs. the log of input amounts). The efficiency of this PCR amplification is 92%. The correlation coefficient of this regression curve is 0.9985. (D) Melting curve analysis showing the specific amplified product of IGF-1. (E) Electrophoresis in 2% agarose gel showing amplification of reverse transcribed IGF-1 mRNA from the neural retina of ED 5 chicken embryos, in comparison with that from the chick liver. Lane 1: 1 kb plus DNA ladder; lane 2: the PCR product of IGF-1 mRNA from the chick liver; lane 3: PCR reaction with retinal mRNA reverse transcribed in the absence of reverse transcriptase; lane 4: the PCR

product of IGF-1 mRNA from a non-injected ED 5 chick retina; lane 5: no-template control.

(A)

Treatment	Relative quantity		GH normalized to 18S rRNA($*10^{-3}$)	cGH-1 group relative to control
	GH	18S rRNA		
cGH-1	0.0187	45.26		
	0.0191	44.16		
	0.0204	45.18		
Mean±SEM	0.0194±0.0005	44.87±0.35	0.43±0.01	0.65±0.02
control	0.0303	46.54		
	0.0308	47.63		
	0.0331	47.42		
Mean±SEM	0.0314±0.0008	47.20±0.33	0.66±0.02	1.0±0.02

(B)

Treatment	Relative quantity		IGF-1 normalized to 18S rRNA($*10^{-3}$)	cGH-1 group relative to control
	IGF-1	18S rRNA		
cGH-1	0.0118	43.40		
	0.0126	41.94		
	0.0129	44.16		
Mean±SEM	0.0124±0.0003	43.17±0.65	0.29±0.01	0.47±0.02
control	0.0299	46.54		
	0.0293	46.97		
	0.0280	47.42		
Mean±SEM	0.0291±0.0006	46.98±0.25	0.62±0.01	1.0±0.02

Table A.1. Example of normalization of the amount of GH (A) and IGF-1 (B) mRNA to 18S rRNA in the experimental (cGH-1) and calibrator (control) groups. SEM: standard error of the mean.

A.6 References

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