Steel Growth Factor in Neural Crest Cell Development.

by

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CERTIFICATE OF APPROVAL

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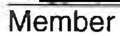

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Abstract

Neural crest is a transient structure in early development and cells within it give rise to diverse cell types. The avian neural crest cell is an ideal system to study the mechanism of cell fate determination. It is suggested that the environment neural crest cells face during migration and at final targets play instructive role in their fates. Steel growth factor is one of the environmental cues identified recently. The precise biological functions of SLF in NCC development and the mechanism of SLF action are explored in the present study. I analyzed the expression of SLF and its receptor in different tissues at different developmental stages utilizing antibodies generated from rabbits and chickens. This analysis showed that the timing and spatial expression of SLF and its receptor c-kit are consistent with their roles in NCC development. I demonstrated that NCC express both c-kit and SLF, and that SLF may work in an autocrine fashion. I demonstrated that SLF is inductive to NCC development, promoting their differentiation into melanocytes. The SLF effect on NCC differentiation is blocked when a neutralizing antibody against SLF is added. The fact that NCC express SLF, a key regulator of their development, indicates that NC cells play an active role in determining their cell fates and suggests a different developmental role for the embryonic microenvironment than what has been previously proposed. SLF and c-kit were found to be expressed in many other tissues in addition to the migration pathways and targets of NCC and their derivatives, suggesting that SLF is involved in the development of many different cell types.

I performed western-blot analysis and observed that soluble SLF is the major form in the very early embryo. At later stages, soluble SLF is still the dominant

form in brain and muscle, while membrane SLF becomes the dominant form in skin. Western-blot data directed me to study the different roles of different forms SLF at different developmental stages. To study the biological functions of SLF *in vitro*, I established a novel serum and chicken embryo extract-free medium for culturing NCC and melanocytes. This culture system allows us to study the roles of SLF in NCC development in a sensitive and less complex background. To study the function of different forms of SLF, I generated CHO cell lines which stably express a mutant form of SLF which can not be cleaved and therefore remains associated with the cell surface. I also applied an inhibitor for SLF cleavage and an inhibitor for c-kit kinase activity to dissect the differential roles of soluble/released and membrane associated forms of SLF. I demonstrated that soluble SLF promotes NCC differentiation into melanocytes in a dose-dependent manner, while the membrane bound SLF expressed by CHO cells has no effect. I showed that SLF is required for the survival of mature melanocytes, but not their precursors. I provided evidence that melanocytes use membrane SLF in an autocrine fashion for their survival. ICC on melanocytes under non-permeablizing conditions showed that melanocytes express SLF on their surfaces. ICC on E14 sections of quail demonstrated that melanocytes express membrane SLF *in vivo* as well. Melanocyte survival assay demonstrated that SLF is required for the survival of melanocytes, and that membrane SLF is sufficient for the basal level of survival of cultured melanocytes. The data presented here suggest that melanocytes may survive through autocrine action of membrane SLF via direct cell-cell contacts.

CHAPTER I--Introduction.

Avian neural crest cell as a model to study cell differentiation and migration.

Great progress has been made over the years in many areas of developmental biology. With the cloning and characterization of novel growth factors, transcription factors, and the application of new technologies to over-express or knock out specific genes, or introduce foreign genes into cells and embryos (Erickson et al., 1993; Fekete et al., 1992, 1993; Cepko, 1992; Stocker et al., 1993), we have a better understanding on many issues in development than even ten years ago. However, some of the fundamental problems are still unresolved today. For example, how does a single cell give rise to the diverse cell types of the adult body? How do cells migrate to the right targets? How do cells know when to start and stop proliferation? What tells a cell to differentiate instead of proliferate? With more and more cells produced throughout developmental, how do they know how to organize themselves?

Avian neural crest cells (NCC) have been used to address some of these questions because the following advantages (Bhattacharyya et al., 1991; Ciment et al., 1982; Dulac et al., 1992; Girdlestone et al., 1985; Le Douarin, 1973; Le Douarin 1982): 1) The developmental biology of quail and chicken has been well characterized; 2) Cell surface markers are available to detect the phenotype

of the NCC and their derivatives; 3) NCC can be acquired in a relatively large amount for biological and immunological studies *in vitro* ; 4) It is much easier to perform *in vivo* operations on quail and chicken embryos and allow them to continue development than to operate on mice.

The neural crest (NC) was first described in 1868 by Wilhelm His, a Swiss embryologist (His, 1868). He was studying the development of the spinal cord and its associated peripheral structures in embryonic chick. He observed a band of cells between the epidermal ectoderm and the neural tube, which he called intermediate cord, these cells migrated to the periphery and formed the spinal ganglia and cranial ganglia. Due to its presumed derivatives, the structure between epidermal ectoderm and neural tube became known as the ganglion crest. However, studies in the next several decades using surgical ablations and transplantation demonstrated that numerous other structures and cell types are also derived from the intermediate cord. In addition to the spinal ganglia and portions of the cranial ganglia, dental papillae, components of the cardiac outflow tract, some skeletal and connective tissues of the head, neurosecretory cells and pigment cells are also derived from this structure. The structure was therefore renamed as the neural crest to reflect its broader derivatives.

The NC is a transient structure observed during neurulation stages in all vertebrates (Le Douarin, 1982). It arises between presumptive neural and epidermal ectoderm as the neural folds close to form the neural tube. Weston (1963) and Johnston (1966) studied the fates and pathways of NCC by ^{3}H-

thymidine radio-labeling NCC from cranial and trunk regions of chicken embryos, and grafting them into unlabeled hosts. Weston found that NCC in the trunk region migrated along two major routes. Some cells followed a dorsolateral pathway, entered skin and then differentiated into melanocytes Others migrated ventrally through the somitic mesenchyme, forming dorsal root and sympathetic ganglia. Johnston observed that cranial NCC gave rise to neuronal derivatives, cartilage, connective tissue and bone of the face and head, and some of the enteric ganglia of the gut. Later, studies on the migration and differentiation of NCC were carried out in Le Douarin's lab using chimaeric embryos and chimaeric tissues (Le Douarin, 1982). They took advantage of the distinct staining patterns of quail and chicken nucleolus they observed, transplanted quail tissues into chicken embryos and followed the migration and differentiation of quail cells. More recently, Bronner-Fraser and Framer group analyzed avian and murine NCC pathways in detail first using a vital dye (Serbedzija et al., 1989; Serbedzija et al., 1992), lysinated rodamine dextran (LRD). LRD is membrane impermeant, hence can be passed from the injected cell only to its progeny by cell division. Therefore, all labelled cells in the embryos at later stages must be derived from the same injected precursors. They performed cell lineage analysis of avian NCC, especially the NCC of trunk region by intracellular microinjection of LRD into the premigratory NCC. They then further studied the timing and patterns of avian trunk NCC and that of murine cranial NCC by injecting DiI into the lumen of the neural tube (Bronner-Fraser et al., 1991; Fraser et al., 1991). DiI is very hydrophobic and can be incorporated almost irreversibly into the plasma

membrane of cells it contacts, hence it will not spread from labelled to unlabelled cells.

These studies provided a lot of information on NCC development and inspired many scientists to explore further the fundamental questions of developmental biology with this system. These results suggest that NCC disperse and migrate from the dorsal neural tube immediately following neural tube closure. Migration starts in mid- brain, then extends progressively to caudal regions of the neural tube. Trunk NCC migrate along two distinct pathways: a ventral pathway, through the rostral portion of each sclerotome and around the dorsal aorta; and dorsolateral pathway, under the epidermis, as well as adjacent intercalation through the dermamyotome. In addition, NCC populated their derivatives in a ventral to dorsal order, with the latest emigrating cells moving along the dorsolateral pathway. In mouse and avian embryos, the dorsolateral migration does not occur until approximately 24 hours after the initiation of ventral NCC migration. Research in recent years indicates that trunk NCC can travel along more than one ventral pathway. Some crest cells migrate to the spaces between somites, while others migrate between somites and the neural tube (Le Douarin, 1973, Newgreen et al., 1990). In avian and mammalian embryos, crest cells migrate under the dermamyotome, mixing with cells in the rostral portion of the somitic sclerotome (Serbedzija et al., 1989; Serbedzija et al., 1990).

Cell migration is believed to involve interactions between the cell surface and the extracellular matrix (ECM). A variety of ECM and cell surface components

have been identified (Erickson et al., 1993) in different species during NCC migration, including fibronectin, hyaluronic acid, collagen type I, collagen type III, and laminin. Fibronectin is considered as a candidate for guiding neural crest migration (Duband and Thiery 1982; Krotoski and Bronner-Fraser 1986; Krotoski et al. 1986). In the cranial regions, a good correlation between the patterns of fibronectin distribution and the routes followed by NCC has been observed. However, in the trunk, fibronectin distribution seems to be more prevalent around tissues where there is little neural crest migrating, rather than along the routes of NCC migration (Rickmann et al. 1985; Krotoski et al. 1986). Laminin has been found to have a similar pattern of immunoreactivity to that of fibronectin, and plays similar roles in NCC migration. In addition to fibronectin and laminin, glycosaminoglycans have been detected along the routes followed by neural crest cells. It is believed that hyaluronate, which is very hydrophilic, may promote migration by opening up spaces which facilitate movement of cells through tissues. Other molecules, some forms of chondroitin sulphate, for example, inhibit NC migration *in vitro*. It is believed that fibronectin, laminin, hyaluronic acid, and other molecules can serve as permissive substrates for NCC migration, whereas other less adhesive substrates may inhibit their migration.

Also of great interest are the molecules expressed on the surface of NCC themselves. The distribution of neural crest adhesion molecule (NCAM) changes during neural crest cell development. NCAM has been detected on the surface of both premigratory and postmigratory NCC in avian embryos, but has not been

observed on the surface of migrating NCC (Thiery et al., 1982). These data have been interpreted to suggest that NCAM may be important only to NCC dispersion. The mechanism of NCC migration is still poorly understood. So far, no molecules have been shown to have the ability to attract or repulse NCC and hence influence their migration to the right targets. Erickson and Goins (1995) showed that avian NCC can migrate along the dorsolateral pathway only if they have been "specified" as melanocytes in culture under certain conditions for a certain period of time. They proposed that melanocytes are directed, by "virtue of their phenotype" into the dorsolateral path. It is possible that melanocytes or melanoblasts are expressing one or a few specific surface molecules which can sense a gradient/ gradients of certain soluble long-range molecules. Tosney (1992) provided evidence that dermatome from stage 21 avian embryos could attract NCC when grafted into younger embryos. However, a dermis-associated chemotactic factor or factors have not been identified so far.

NCC are multipotent.

Understanding how different crest-derived lineages become segregated from parental cells has been a major goal of many research groups. It is possible that following dispersion from the neural tube, NCC could undergo a series of divisions producing multipotent cells whose fates are gradually determined by instructive environmental cues encountered during migration and at their final targets. An alternative possibility is that NCC are pre-determined to distinct

lineages or subsets of lineages at the onset of their migration. In this case, NCC undergo a series of divisions and become further restricted by the permissive environment along the pathways and at their targets.

Most studies have supported the notion that most NCC are multipotent at the onset of their migration. A common approach used to demonstrate multipotentiality is clonal analysis *in vitro*. A variety of such studies found that clones derived from cells migrating away from either cranial or trunk neural tube were to give rise to diverse derivatives (Baraffio et al., 1988; Sieber-Blum and Cohen, 1980; Bronner-Fraser et al., 1980; Baroffio et al. 1988; Sieber-Blum, 1989). Multipotency of early NCC has been demonstrated by tracing the cell lineage of individual cells microinjected with fluorescent dextran *in vivo* as well. Using this method, the progeny of some of the labeled cells have been found in all of the different locations to which NCC migrate, including dorsal root and sympathetic ganglia, peripheral nerves, the adrenal glands, and the skin (Bronner-Fraser and Fraser, 1989; Fraser and Bronner-Fraser, 1991). In 1991, Frank and Sanes (Frank et al., 1991) injected a retrovirus expressing the *E.coli* lacZ gene into the neural tube of avian embryos to follow the fates of NCC. They detected lacZ positive cells, presumably representing clones of an individual early NCC, that had characteristics of neurons, Schwann cells, and satellite cells.

All these data support the scenario that most premigratory NCC are multipotent, or at least, only partially restricted to particular developmental potentials. However, numerous studies have also shown that phenotypically distinct

subpopulations of NCC do exist before their migration along different routes. Several groups (Anderson, 1989; Ciment and Weston, 1983; Barald, 1982; Girdlestone and Weston, 1985) have demonstrated that some premigratory and early crest cells in culture express antigens recognized by cell-type specific antibodies. Heterospecific grafting experiments (Le Lievre et al., 1980; Ciment and Weston, 1985; Fontaine-Perus et al., 1988), and clonal analysis (Baroffio et al., 1988; Bronner-Fraser and Fraser, 1989; Dupin et al., 1990; Frank and Sanes, 1991) also supported the existence of a distinct subpopulation before the onset of migration of NCC. In these studies, various early NC derivatives were isolated and found to give rise to only a limited number of phenotypes. Clones of some NCC both *in vitro* and *in vivo* only gave rise to one or two cell types. However, it is possible that factors necessary for the development of other cell types were not included in these experiments. Collectively, the above data suggest that NCC is a heterogeneous population of cells: a small portion of crest cells may have restricted developmental potential, while most of them are partially restricted or multipotent at the onset and during the process of migration.

Environmental cues play important roles in the developmental fates of NCC.

It is believed that NCC become committed to particular phenotypes through a series of events, rather than by a single determinative interaction (Wessells, 1977). Moreover, the developmental process is influenced by both environmental cues

(Glimelius et al., 1981; Loring et al., 1981; Sears et al., 1988; Stocker et al., 1991; Vogel et al., 1988) faced by the migrating NCC and by the response of cell surface receptors expressed by NCC at particular times in particular cells or tissues. In 1980, Sieber-Blum and Cohen demonstrated that extracellular matrix could enhance the proportion of clones containing catecholaminergic neurons. Recently, Stemple and Anderson showed that clones of rat NCC grown on fibronectin plus poly-D-lysine gave rise to Schwann cells and neurons, but gave rise to mainly Schwann cells when grown on fibronectin alone. Studies by several groups in the early 90's observed the influence on the fates of NCC by some unknown molecules in the serum and extracellular matrix (Maxwell et al., 1988; Barald, 1989; Morrison-Graham et al., 1990).

The observations that some cell types in the adult organism can be interconverted in the presence of certain growth factors also support the notion that the environment is instructive to cell fates. Small intensely fluorescent cells of sympathetic ganglia, which share phenotypic characteristics of chromaffin cells and neurons, have been shown to transform into sympathetic neurons in the presence of nerve growth factor, but transform into chromaffin cells when glucocorticoids were present (Doupe et.al., 1985). On the other hand, chromaffin cells from either embryonic or adult rat adrenal glands can be converted to sympathetic neurons when treated with basic fibroblast growth factor (bFGF) or NGF (Doupe et al., 1985; Seidl et.al., 1989; Stemple et.al., 1988). There is evidence that such interconversions can occur *in vivo* as well. Injecting

glucocorticoids or an antibody against NGF increases the number of small intensely fluorescent cells, while injecting NGF results in the appearance of sympathetic neurons in the adrenal glands (Aloe et.al., 1979; Carnahan et.al., 1991).

There is evidence supporting the existence of another NC-derived bipotent intermediate which can give rise to either Schwann cells or melanocytes. Melanocytes are found in both the iris and skin of most higher vertebrates. They produce various types of melanins, which shield underlying epithelial cells in the skin from ultra-violet radiation, and absorb scattered light as it passes through the retina in the eye. Melanins are also important for scavenging harmful free radicals and are determining factors for the color of skin and coat furs. A variety of extirpation and grafting experiments indicate that, except for the optic cup-derived pigment cells of the retina, all pigment cells in vertebrates are derived from the NC (Le Douarin, 1982). It is believed that almost all Schwann cells are NC derivatives (Le Douarin et al., 1991; Jessen and Mirsky, 1992), although a modest number may arise from the ventral neural tube (Rickmann et al., 1985; Lunn et al., 1987). The Schwann cells enfold axons in peripheral nerves and produce myelin, which insulates peripheral axons and increases their conduction velocities. They also synthesize all of the basal membrane components of neurilemma, and produce neurotrophic agents during axonal outgrowth and regeneration (Heumann, 1987.) In 1986, Ciment et al. demonstrated that dorsal root ganglion (DRG) or peripheral nerve explants from 7 to 9 days Japanese quail embryos gave

rise to melanocytes when cultured in the presence of phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA). These melanocytes migrated into the overlying epidermis and contributed to feather pigmentation after grafting into White Leghorn chicken embryos, which normally have unpigmented feathers. One explanation for this is that TPA promotes melanocyte differentiation by reversing the commitment of these cells in the explant culture from a neuronal, Schwann cell lineage to melanocytic lineage (Ciment et al., 1986; Ciment et al. 1990). Furthermore, since this effect was observed in peripheral nerve explants, it is likely that TPA caused the transdifferentiation of Schwann cell precursors into melanocytes. Sherman and Ciment (Sherman et al., 1991; Sherman et al., 1993) found that basic fibroblast growth factor (bFGF) induced pigmentation in peripheral nerve and DRG explant cultures. They also found that TPA increased bFGF protein expression in these cells in the presence of serum. They proposed that TPA caused transdifferentiation of Schwann cells into melanocytes through up-regulation of bFGF in these cells. Interestingly, another growth factor, transforming growth factor-β1 (TGF-β1) (Gatherer et al., 1990; Jakowlew et al., 1992; Jakowlew et al., 1994; Rogers, 1992), showed antagonizing effects to TPA and bFGF induced pigmentation of DRG cultures (Jaye et al., 1992; Johnson et al., 1993; Morrison et al., 1993; Yayon et al., 1990). Recently, Shah et al. showed that TGF-β1 drove virtually all NCC to a smooth muscle fate. They suggested that the choice of each of several alternative fates of NCC can be instructively promoted by different environmental signals. They also showed that BMP2, a member of the TGF-β superfamily of growth factors, promoted rapid induction of

the autonomic lineage-specific basic helix-loop-helix protein MASH1 and autonomic neurogenesis *in vitro.*

Studies of a mouse mutant, Patch (Ph), in which pigment cells and other non-neurogenic crest derivatives develop abnormally (Morrison-Graham et al., 1992; Wehrle-Haller et al., 1996), probably due to a deletion of the gene for platelet-derived growth factor (PDGF) α, indicate that PDGF may play important roles in NCC differentiation into pigment cells. The exact role played by PDGF during melanocyte commitment, however, is unclear. PDGF has not been shown to influence melanocyte growth and differentiation *in vitro.* Some data, however, suggest that the genomic deletion in Patch might disrupt adjacent genes, such as c-kit , and the defect of the adjacent gene or genes cause the pigment phenotype.

Steel growth factor and its receptor c-kit's roles in NCC development.

Mutations at the dominant white spotting locus (W) of mice have been shown to have pleiotropic effects on both embryonic development and the regulation of hematopoiesis in adults (Besmer et al., 1993; Mayer et al., 1968; Nocka et al., 1990; Tan et al., 1990). Homozygotes of the W locus mutation show extensive white-spotting because of the disappearance of melanocytes. In 1987, Yarden et al. cloned a human proto-oncogene encoding a receptor tyrosine kinase. In 1988, Qiu et al. and Geissler et al. showed that the dominant-white spotting locus of the mouse encodes the same kinase, the c-kit proto-oncogene. C-kit is structurally

related to receptors for colony stimulating factor (CSF) and PDGF (Qiu et al., 1988; Blechman et al., 1995). These receptors are characterized by the presence of a split kinase domain separated by a short stretch of amino acids unique to each member of this class of receptors. The extracellular domain of c-kit includes a 500-amino acid ligand binding domain with five immunoglobulin (Ig)-like regions. It also contains several intramolecular disulfide bonds and potential N-glycosylation sites. A 23-amino acid hydrophobic domain anchors the receptor in the plasma membrane. The cytoplasmic protein comprises a 433-amino acid intracellular segment that contains the kinase region, an autophosphorylation site and a poorly conserved, non-catalytic, hydrophilic stretch of 77 amino acids, known as the kinase insert (KI) domain in the middle. The KI domain is involved in the binding of SH2 domains of cytoplasmic signal transduction proteins.

Mutations at the steel (sl) locus on chromosome 10 of the mouse result in phenotypes that are very similar to those seen in mice carrying W mutations (Flanagan et al., 1990; Galli et al., 1994; Toksoz et al., 1992). In 1990 several laboratories independently cloned the SLF genes from mouse, rat, and human (Zsebo et al. 1990a; Martin et al. 1990; Zsebo et al. 1990b; Anderson et al. 1990; Flanagan et al. 1990; Huang et al. 1990). They also provided biological and biochemical evidence to establish the receptor-ligand relation of c-kit and SLF.

The cDNA for mouse, rat, chicken, and human SLF encodes a growth factor which has an extracellular domain with a typical N-terminal signal sequence. Interestingly, it also has a typical transmembrane domain, and a short cytoplasmic

domain. This growth factor can be cleaved by an as yet unidentified protease. Three alternatively spliced forms of SLF have been reported in mouse: 1) the full-length form composed of about 249 amino acids; 2) a spliced form lacking exon 6, which produces a 28-amino-acid deletion; 3) an alternative form with a smaller 16-amino-acid deletion of exon 6, extending c-terminal to the c-terminal alanine residue 164 of the SLF. The deleted forms of SLF are believed to be more difficult to cleave and therefore may act as the membrane-associated form.

The original c-kit mutant, W, produces a protein without the transmembrane domain, while most other mutants comprise point mutations in the kinase domain, which result in amino acid substitutions that destroy its intrinsic kinase activity (Qiu et al., 1988; Herbst et al., 1995; Mayer et al., 1968; Nocka et al., 1990; Tan et al., 1990). The SLF mutants that have the most dramatic effects, generally resulting in death in utero or shortly after birth, are due to deletions that result in complete loss of SLF function. One interesting mutant of SLF is the sl dickie (sl^d), which represents an intragenic deletion that removes 242 bp at the 3' end of the coding sequence (Flanagan et al., 1991). The truncated SLF transcript encodes almost all of the extra cellular domain of SLF but lacks coding sequences for both the transmembrane and the intracellular domain of the wild type SLF protein. Though the truncated SLF protein was active when tested *in vitro* using mast cell proliferation assays and promoted the dispersion and differentiation of NCC into melanocytes *in vivo*, adult mice homozygous for the sl dickie exhibit a

complete lack of skin pigmentation and anemia (Steel et al., 1992; Wehrle-Haller et al., 1995).

Mechanisms of SLF's function in hematopoiesis have been studied extensively by many groups because of potential clinical values. Nishikawa et al. (1991) demonstrated that only 7.8 % of the total bone marrow cells express c-kit on their surface, in contrast to hematopoietic progenitors in adult marrow, which nearly all express c-kit. Two days after the injection of the anti-c-kit antibody, ACK2, almost all hematopoietic progenitor cells disappeared from the bone marrow, which eventually resulted in the absence of mature myeloid and erythroid cells in the bone marrow. Their data suggest that c-kit is an essential molecule for constitutive intramarrow hematopoiesis, especially for the self-renewal of hematopoietic progenitor cells at various stages of differentiation (Andre et al., 1989). Ikuta and others (Ikuta et al., 1992; Kuriu et al., 1991; Ohneda et al., 1992; Ogawa et al., 1991) found that c-kit receptor is expressed in hematopoietic stem cells from the earliest stage--i.e., pluripotent on stages. Hayman et al. (1993) showed that a relatively common progenitor expressing only c-kit transiently proliferated in response to SLF, but that a second, rarer progenitor coexpressing c-kit and c-ErbB, another tyrosine kinase receptor was induced to long-term self-renewal by the ErbB ligand TGFα, or by the c-kit ligand, SLF. In the absence of SLF or TGFα, the two progenitors underwent erythropoietin (Epo)- dependent terminal differentiation with indistinguishable kinetics. Epo induced differentiation in c-kit progenitors even in the presence of SLF, while the c-ErbB

expressing progenitors continued to self-renew when treated with Epo plus SLF, TGFα, or both. This suggested that ErbB might be a dominant determinant for sustained self-renewal of committed erythroid progenitors.

The functions and mechanisms of SLF on melanogenesis have also been studied recently. Murphy et al.(1992) reported that SLF's primary action was to keep melanocyte precursors alive. In their mouse NCC cultures, SLF alone had no effect on the final stage of melanocyte differentiation. On the other hand , they observed NCC differentiation when TPA was added. Therefore, they proposed that SLF is required for the "maintenance", but not differentiation, of melanocyte precursors in the NCC. Hirata et al. (1993) provided evidence that SLF could induce the outgrowth of c-kit positive neurites from DRG of mouse embryos, and that SLF had a tropic effect on c-kit positive neurons in the culture of dissociated DRG cells. Morrison-Graham and Weston (1993) addressed the dependence by cells of the NC-derived melanogenic lineage on SLF. They concluded that the critical period of SLF dependence lasts about four days, which began only after the second day of the dispersal *in vitro*, ending about the time when melanocytes differentiated. Lahav et.al.(1994) studied how recombinant chicken SLF affects the development of NCC. They observed that the total number of NCC, melanocytes, and their precursors was increased in the presence of SLF. They also found that SLF enhanced the differentiation rate of melanocyte precursors, by using the melanocyte early marker Mel-EM, and melanocytes. They concluded that SLF can stimulate the rate of NCC differentiation into melanocyte processes. Reid et al.(1995) labeled cultured murine trunk NCC expressing c-kit

with an anti-c-kit antibody (ACK2). They found that C-kit positive cells first appeared after 2 days in culture and subsequently expressed tyrosinase-related protein, an early marker for melanocyte lineage, and became pigmented in the presence of TPA. Elimination of the c-kit positive population by incubating the culture in ACK2 resulted in the ablation of the melanocytes. They believed that c-kit positive cells arising from the NC are melanocyte progenitors, and that SLF acts as both a survival factor and a proliferation factor for those cells. In 1995, Langtimm-Sedlak et al. did *in vitro* analysis of SLF effects on NCC differentiation. They suggested that the combination of SLF and any neurotrophin (NGF, BDNF, NT-3) is trophic for melanogenic cells, whereas SLF alone does not show a detectable effect on melanogenesis.

The goals of this project.

Mutant analysis provided strong evidence that the SLF and c-kit interaction is involved in many aspects of NCC development. However, the identification of SLF and c-kit mutants has provided little information on the precise mechanism by which defects occur in NCC and hematopoietic cell development. *In vitro* studies shed a little bit of light on the role of SLF and c-kit functions in these events, while bringing up new issues and problems at the same time.

There were a few fundamental and important questions unanswered when we started this project: 1) The time and places of expression of SLF and c-kit were only characterized at the RNA level using in-situ hybridization, whereas the distribution of SLF and c-kit proteins is not known; 2) Most *in vitro* studies were

performed in the presence of complex cell culture medium which included high concentrations of serum and chicken embryo-extract, they are known to contain many growth factors and may have positive and negative effects on the cells. Therefore, it is not known whether SLF alone can be effective on many aspects of NCC development, or SLF needs the actions other growth factors; 3) Some *in vitro* studies used TPA, which is not normally found in mammalian cells and may have complex effects that have not been characterized; 4) Many groups believe that SLF acts only as a survival factor for NCC and their derivatives, but *in vitro* studies provided little direct evidence; 5) It is not known whether SLF works in an autocrine or paracrine manner; 6) SLF can affect NCC and their derivatives in many ways, including proliferation, differentiation, and survival. The precise roles of SLF on NCC and their derivatives are not clear; 7) SLF exists as both cell-associated and cleaved, soluble forms; little has been done to address the possible distinct functions of the two different forms. The expression of the two forms in different tissues at different developmental stages has been completely ignored.

I have been studying the functions of SLF on NCC using the avian system. The goals of my thesis project are: 1) To characterize the distribution of SLF and c-kit proteins in different tissues at different developmental stages; 2) To establish a novel serum and chicken-embryo-extracts free culture medium for NCC and melanocytes in order to study the functions of SLF in a clean and sensitive background; 3) To identify the source of SLF *in vivo* and its possible autocrine actions on NCC development; 4) To dissect the precise roles of SLF on different aspects of NCC development; 5) To study the expression pattern of two forms of

SLF in different tissues at different developmental stages; 6) To explore the mechanisms of SLF functions on melanocytes survival at later stages of development and gain insight into the how the phenotypes of SLF and c-kit mutants, for example sl dickie, occur.

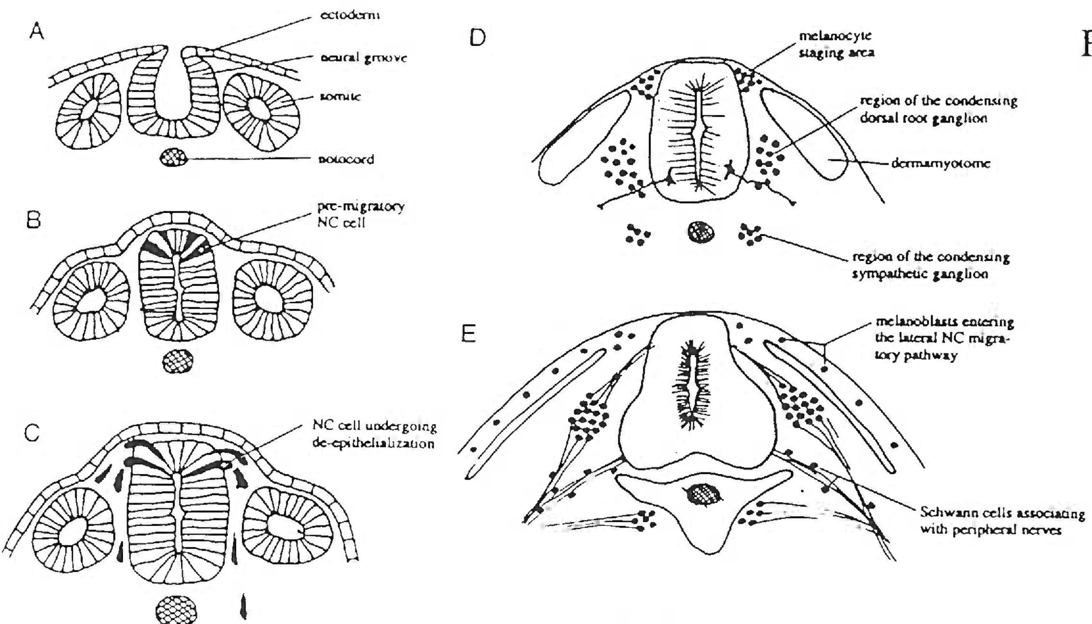

Fig. 0-1--Formation and migration of the embryonic avian NC (designated in black). **(A)** Closure of the neural groove to form the neural tube **(B)** at about E1-E2. **(C)** De-epithelialization of NC cells and their migration along the "ventral NC migratory pathway" at about E2-E3. **(D)** Condensation of NC-derived ganglia along the ventral pathway, and the formation of the "melanocyte staging area" at the entrance of the "lateral NC migratory pathway" around E4. **(E)** Opening of the lateral pathway around E5 as space-filling glycosaminoglycans are secreted by ectodermal cells, and the melanocyte-precursors migrate between the ectoderm and dermamyotome.

```
                |------------------------------SP-------------------
                 M  K  K  T  Q  T  W  I  I  T  C  I  Y  L  Q       15
GCGGTGCCTTTCCTTATGAAGAAGACACAAACTTGGATTATCACTTGCATTTATCTTCAA

---------------------------------|
 L  L  L  F  N  P  L  V  K  T  K  E  I (C) G  N  P  V  T  D       35
CTGCTCCTATTTAATCCTCTCGTCAAAACCAAGGAGATCTGCGGGAATCCTGTGACTGAT

 N  V  K  D  I  T  K  L  V  A  N  L  P  N  D  Y  M  I  T  L       55
AATGTAAAAGACATTACAAAACTGGTGGCAAATCTTCCAAATGACTATATGATAACCCTC

 N  Y  V  A  G  M  D  V  L  P  S  H (C) W  L  R  D  M  V  I       75
AACTATGTCGCCGGGATGGATGTTTTGCCTAGTCATTGTTGGCTACGAGATATGGTAATA

 Q  L  S  L  S  L  T  T  L  L  D  K  F  S  N  I  S  E  G  L       95
CAATTATCACTCAGCTTGACTACTCTTCTGGACAAGTTCTCAAATATTTCTGAAGGCTTG

 S  N  Y  S  I  I  D  K  L  G  K  I  V  D  D  L  V  L (C) M      115
AGTAATTACTCCATCATAGACAAACTTGGGAAAATAGTGGATGACCTCGTGTTATGCATG

 E  E  N  A  P  K  N  I  K  E  S  P  K  R  P  E  T  R  S  F      135
GAAGAAAACGCACCGAAGAATATAAAAGAATCTCCGAAGAGGCCAGAAACTAGATCCTTT

 T  P  E  E  F  F  S  I  F  N  R  S  I  D  A  F  K  D  F  M      155
ACTCCTGAAGAATTCTTTAGTATTTTCAATAGATCCATTGATGCCTTTAAGGACTTTATG

 V  A  S  D  T  S  D (C) V  L  S  S  T  L  G  P  E  K  D  S      175
GTGGCATCTGACACTAGTGACTGTGTGCTCTCTTCAACATTAGGTCCCGAGAAAGATTCC

 R  V  S  V  T  K  P  F  M  L  P  P  V  A  A  S  S  L  R  N      195
AGAGTCAGTGTCACAAAACCATTTATGTTACCCCCTGTTGCAGCCAGCTCCCTTAGGAAT

 D  S  S  S  S  N  R  K  A  A  K  S  P  E  D  S  G  L  Q  W      215
GACAGCAGTAGCAGTAATAGGAAAGCCGCAAAGTCCCCTGAAGACTCGGGCCTACAATGG

 |---------------------------TMS-------------------------
 T  A  M  A  L  P  A  L  I  S  L  V  I  G  F  A  F  G  A  L      235
ACAGCCATGGCATTGCCGGCTCTCATTTCGCTTGTAATTGGCTTTGCTTTTGGAGCCTTA

-----|
 Y  W  K  K  K  Q  S  S  L  T  R  A  V  E  N  I  Q  I  N  E      255
TACTGGAAGAAGAAACAGTCAAGTCTTACAAGGGCAGTTGAAAATATACAGATTAATGAA

 E  D  N  E  I  S  M  L  Q  Q  K  E  R  E  F                     270
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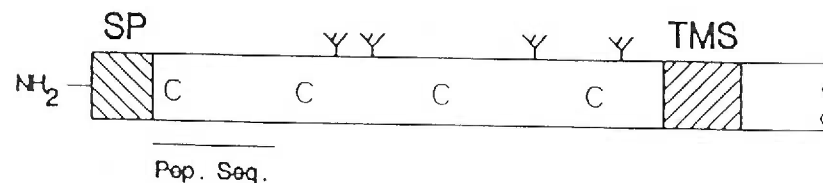

Fig. 0-2--Nucleotide sequence and predicted Amino Acid Sequence of 1.4 kb cDNA clone

The predicted amino acid sequence of the long open reading frame is shown above the nucleotide sequence using the single-letter amino acid code. The numbers at right refer to amino acids, with methionine (nucleotides 16-18) being number 1. The potential N-terminal signal sequence (SP) and the transmembrane domain (TMS) are indicated with dashed lines above the sequence, and cysteine residues in the extracellular domain are circled. A schematic of the predicted protein structure is indicated below. N-linked glycosylation sites and the location of the N-terminal peptide sequence (Pep. Seq.) are indicated.

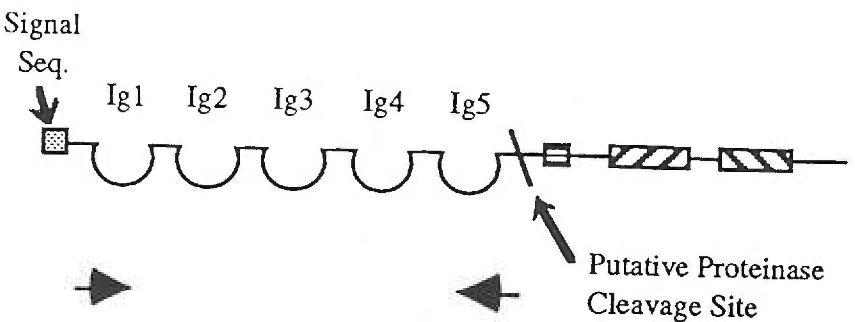

Fig. 0-3--Primary structure of c-kit. The full length protein is around 145 kDa; the processed form (following proteolysis at a putative cleavage site adjacent to the transmembrane domain) is around 60kDa. Ig#1-5 refer to the immunoglobulin-like domains found in the extracellular region. tm, transmembrane domain; kin#1-2, tyrosine kinase domains. Arrows refer to sequences correspon-ding to PCR-primers to be used to generate the 60 kDa protein (see text).

CHAPTER II--Autocrine Regulation of Neural Crest Cell Development by Steel Factor

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ABSTRACT

Steel factor [SLF] and its cognate receptor, c-kit, have been implicated in the generation of melanocytes from migrating neural crest (NC) cells during early vertebrate embryogenesis. However, the source of SLF in the early avian embryo and its precise role in melanogenesis is unclear. We report here that NC cells themselves express and release SLF protein, which in turn, acts as an autocrine factor to induce melanogenesis in nearby NC cells. These results indicate that NC cell subpopulations play an active role in the determination of their cell fate, and suggest a different developmental role for the embryonic microenvironment than what has been previously proposed.

INTRODUCTION

The neural crest (NC) is a transient structure observed during vertebrate embryogenesis as the neural epithelium folds to form the neural tube (Le Douarin, 1982; Weston, 1991). NC cells originate from within the neural tube, but soon migrate into the periphery of the embryo, traveling along two migratory pathways. NC cells in the dorsal-lateral pathway migrate through the space between the ectoderm and dermamyotome of the somites, and eventually give rise to the melanocytes of the skin. The ventral NC migratory pathway is within the mesenchyme of the forming sclerotome, as well as in the space between adjacent somites (Bronner-Fraser, 1986). NC cells migrating along the ventral pathway eventually giving rise to a wide variety of different cell types, including sensory and autonomic neurons, Schwann cells, and adrenal chromaffin cells.

Studies performed in culture and *in vivo* suggest that the fate of NC cells is regulated to a large part by the microenvironment (Anderson, 1989; Weston, 1991; Leblanc and Bronner-Fraser, 1992). These studies have led to the hypothesis that different exogenous factors (or combinations of factors) produced by different microenvironments are responsible for the generation of cell type diversity by either influencing NC cell fate in multipotent cells, or by selecting for pre-determined subpopulations. Candidates for such exogenous factors include various peptide growth factors for which migrating NC cells express receptors, including those for platelet-derived growth factor (PDGF), which has been implicated in craniofacial development (Grüneberg and Truslove, 1960; Morrison-Graham *et al.*, 1992), and the neurotrophins, which may be involved in neurogenesis (Sieber-Blum, 1991; Pinco *et al.*, 1993).

One factor which has been proposed to regulate melanogenesis in NC cells is Steel Factor [SLF; also known as c-kit ligand, mast cell growth factor, stem cell factor (Galli *et al.*, 1994)]. This glycoprotein is expressed in a variety of tissues as a transmembrane protein, which is then cleaved at a proximal extracellular site, releasing the soluble protein. Mutations of the SLF gene in mice, or of its cognate receptor, the c-kit tyrosine kinase, in mice, rats and humans, produce animals lacking pigment cells (Williams *et al.*, 1992). Injection of c-kit neutralizing antibodies into pregnant mice has been shown, moreover, to result in the hypo-pigmentation of offspring (Nishikawa *et al.*, 1991).

The simple notion of SLF as an environmental factor inducing melanogenesis in NC cells is, however, complicated by two observations. First, the SLF mRNA does not seem to be expressed by cells making up the microenvironment through which melanogenic NC cells migrate in avian embryos. *In situ* hybridization studies of chicken embryos show no detectable signal in dorsal aspects of the dermamyotome or ectoderm at the appropriate developmental stages, although there is some hybridization signal in more lateral regions of the ectoderm (Lecoin *et al.*, 1995). And second, mutant mice that express only the soluble form of SLF lack pigment cells in the skin (Flanagan *et al.*, 1991), indicating that the full length, membrane-bound form of SLF is required for melanogenesis, at least *in vivo.*

The cellular mechanism(s) by which SLF induces melanogenesis in the early embryo is also unclear. On the one hand, it has been suggested that SLF acts as a survival factor, selecting for the survival and/or proliferation of committed, latent melanoblasts in heterogeneous NC cell populations. This conclusion is supported by culture studies using mouse NC cells in which

removal of SLF from the culture medium results in the irreversible loss of pigmentation-competent cells, or cells expressing melanocyte markers (Murphy *et al.*, 1992; Morrison-Graham and Weston, 1993; Reid *et al.*, 1995). Conversely, SLF may influence the commitment of multipotent NC cells to a melanocyte fate and/or influence their subsequent differentiation.

In this paper, we show that embryonic quail NC cells themselves express both the SLF mRNA and protein *in vivo* and in culture, and that SLF acts as an autocrine factor regulating cell fate.

Materials and Methods

Cell Culture

Neural tubes were isolated as described (Loring *et al.*, 1982; Stocker *et al.*, 1991) from stages 12 and 13 quail embryos (*Coturnix coturnix japonica*) (Hamburger and Hamilton, 1951) and cultured on either 35 mm or 24-well Primaria tissue culture plates (Falcon Plastics, Ventura, CA). In each of the 24-well plates, 1 neural tube (corresponding to a 10-somite length) was plated as the source of NC cells; in each of the 35 mm dishes, 4-6 neural tubes were used. The culture medium was CCM-5 (Hyclone, Logan, UT), which is a serum- and embryo extract-free defined culture medium. It contains insulin-like growth factor type I, transferrin and bovine serum albumin as the only proteins, but lacks phorbol esters, retinoids, and other agents known to influence neural crest cell fate.

Immunocytochemistry

To generate anti-SLF antibodies, recombinant chicken SLF protein was made by subcloning a cDNA corresponding to the extracellular domain (amino acids #16-181) into the pTrcHis prokaryotic expression system (Invitrogen, San Diego, CA) and then purifying the recombinant protein over a nickel column. Rabbits were injected a total of 6 times with the recombinant protein over a period of 6 weeks. The first injection was a mixture of 150 μg protein in complete Freund's adjuvant injected sub-cutaneously; the second was similar to the first, but in incomplete Freund's adjuvant. The remaining injections were performed using recombinant SLF (300-600 μg protein) dialyzed against phosphate buffered saline and injected into the ear vein.

NC cell cultures were fixed for 30 min with 4% paraformaldehyde in Hanks' balanced salt solution supplemented with 25 mM HEPES buffer (pH 7.6). After extensive washing, the cultures were pre-incubated overnight with "Antibody Dilution Buffer" consisting of phosphate buffered (10 mM, pH 7.4) isotonic saline (PBS) containing 0.1% triton X-100 and 10% goat serum. Cultures were then incubated with primary antibody [1:300 dilution of rabbit anti-chicken SLF; 1:50 dilution of mouse anti-HNK-1 hybridoma conditioned medium (Developmental Hybridoma Studies Bank)], and then secondary antibodies [fluorescein-labeled goat anti-rabbit IgG (Vector Laboratories) and rhodamine-labeled goat anti-mouse IgM (Cappel Laboratories)]. The cultures were then washed briefly with PBS, and the central portions of each plate were cut out on a small lathe and mounted (with the cells facing up) onto a microscope slide. The surface of each disk was then covered with a 1:1 mixture of PBS with 2 mg/ml phenylenediamine and glycerol (to prevent fluorescence fading) and coverslipped. Cultures were then viewed under fluorescein and rhodamine epifluoresence optics on a Zeiss Axioplan microscope and photographed using an Olympus photomicroscopy system.

Western Blot analysis

Fifty µg protein from homogenates of E6 chickens were subjected to electrophoresis in an SDS-polyacrylamide gel, and then transferred electrophoretically onto a nitrocellulose membrane. The membrane was pre-incubated with 5% non-fat dry milk in PBS with 0.1% triton X-100 for 1 hour and then treated overnight with anti-SLF antibodies (1:2000 dilution in PBS with 0.1% triton X-100). After extensive washing, the blot was incubated with horseradish peroxidase-conjugated goat anti-rabbit antibodies (1:5000 dilution in PBS with 0.1% triton X-100), and peroxidase activity visualized

using the Enhanced Chemiluminscence (ECL) kit (Amersham, Arlington Heights, IL).

Morphometric Analyses

Morphometric analysis of the numbers of cells processes and their lengths was performed using an eyepiece reticle containing a series of calibrated, concentric circles. Cells were then picked at random and moved to the center of the reticle. The length of processes was determined by calculating the shortest distance from the proximal to the distal ends of each process. Only those cell processes of 1 cell diameter or longer were included in these calculations. Of course, this approach does not take into consideration the absolute length of those processes that do not travel along a straight line, and therefore, tends to underestimate process lengths.

***In situ* hybridization**

In situ hybridization was carried out as described previously (Wehrle-Haller and Weston, 1995). Briefly, embryos were first processed for whole mount *in situ* hybridization using digoxygenin-labeled antisense RNA probes generated by *in vitro* transcription of a cDNA corresponding to the extracellular domain of chicken SLF. Hybridization signal was then detected by immunohistochemistry using rabbit anti-digoxygenin antibodies conjugated with alkaline phosphatase. Embryos were then embedded in paraffin and sectioned at 10 μm on a microtome. Sense RNA probes served as negative controls. Attempts to perform co-label the same sections by *in situ* hybridization for SLF and immunostaining for the HNK-1 epitope were unsuccessful due to the lability of the HNK-1 epitope to the alcohol-extractions steps necessary for *in situ* hybridization.

Analysis of SLF mRNA using RT/PCR and Southern Blots

RNA was isolated from NC cell cultures using a kit (Qiagen, Chatsworth, CA) and transcribed using Superscript™ reverse transcriptase (Gibco-BRL, Gaithersburg, MD), following standard methods (Kawasaki, 1990). The cDNA was then subjected to polymerase chain reaction (PCR)-amplification (30 cycles with a annealing temperature of 50°C) using primers corresponding to the published sequence of chicken SLF (Zhou *et al.*, 1993). These primers were 5'-GCCTGCCTAATCACTGTTGGTTG-3' and 5'-AAGCCAAGTGCCTCTTTGTTACTG-3', which generated a 466 base pair reaction product corresponding to amino acid residues #64 through #218. The PCR product was transferred to Genescreen Plus™ filters (NEN, Boston, MA) and the blot was probed with a nick-translated ^{32}P-labeled cDNA (Prime-It®, Stratagene, La Jolla, CA) corresponding to a 501 base pair HindIII / PstI restriction fragment of the pTrcHis-SLF plasmid. Finally, autoradiography was performed using Dupont Reflection™ film and processed by an X-OMAT X-ray film processor.

For DNA sequence analysis, the PCR reaction product was subcloned into the pTA cloning vector (Invitrogen, San Diego, CA) and automated sequence analysis performed on an Applied Biosystems 373 sequencer.

RESULTS

Although the product of the SLF gene has clearly been implicated in melanogenesis during development (Galli *et al.*, 1994), the cellular source of SLF in the embryo is still unclear. Although some SLF mRNA signal has been reported in the lateral ectoderm of later stage embryos by *in situ* hybridization

(Matsui *et al.*, 1990; Keshet *et al.*, 1991; Lecoin *et al.*, 1995; Wehrle-Haller and Weston, 1995), these sites and stages are different from those where melanoblasts first appear. This suggests that other sources of SLF may also be present in the embryo. One formal possibility, of course, is that NC cells themselves express SLF and that this growth factor acts in an autocrine fashion to influence melanogenesis. To address this possibility, we immunostained cultured NC cells with monospecific antibodies generated against the extracellular portion of this growth factor. Fig.1 shows that the vast majority of quail NC cells (approximately 98%) cultured for 48 hours in a serum- and embryo extract-free defined medium expressed SLF-immunoreactivity (see also Table 1). The immunostaining pattern showed a punctate appearance within the cytoplasm, as well as some cell surface staining. Similar staining patterns and proportions of SLF^+ cells were also observed in NC cultures grown for periods as short as 12 hours or in the presence of 10% fetal bovine serum (data not shown). Studies with antisera against the chicken c-kit gene product showed a similar pattern of staining to that of SLF, with about 96% of HNK-1^+ NC cells expressing c-kit immunoreactive material (not shown). Although we were unable to immunostain cultures simultaneously for both c-kit and SLF (since both antisera were generated in rabbits), the high proportions of SLF^+ or c-kit^+ cells indicate that most NC cells in these cultures express both the growth factor and its receptor.

As a control for antibody specificity, western blot experiments were performed. Fig.2 is a protein immunoblot showing that this anti-recombinant SLF antibody recognizes only a single band at around 23 kDa in homogenates of various tissues dissected from E14 chickens, including liver,

brain, heart, kidney, and lung. This molecular size corresponds to that of the soluble form of SLF as predicted by the sequence of the chicken SLF cDNA (Zhou *et al.*, 1993), and these tissues correspond to locations in which SLF has been observed in mouse (Matsui *et al.*, 1990). This immunoreactivity was not present when the antibodies were preincubated with recombinant SLF (data not shown).

To determine whether this immunoreactivity in NC cultures was *bone fide* SLF or a related gene product, RNA was prepared from similar cultures and processed for reverse transcription/ polymerase chain reaction (RT/PCR)-amplification using primers corresponding to SLF. Fig.3 is a Southern blot of this amplification reaction probed for SLF, and shows a band at the appropriate size in the lane prepared from NC cell cultures (lane 4). This band in the NC cell lanes was not present when the reverse transcriptase step was omitted (lane 5), indicating that the original template was RNA and not contaminating genomic DNA. Sequence analysis of these bands confirmed that they were highly homologous to the published sequence of quail SLF (Genbank accession #U43078), with only two conservative amino acid substitutions, which probably correspond to polymorphisms present in this outbred species.

To determine whether NC cells express SLF at the relevant ages *in vivo*, *in situ* hybridization studies were performed. Fig. 4 shows the earliest observed expression of SLF hybridization signal in the dorsal NC migratory space of the upper cervical regions of an E4 chicken (at the arrow), corresponding to the region described as the migration "staging area" (Wehrle-Haller and Weston, 1995). This region contains NC cells which remain in close physical association for a period of 24-36 hours until the space

between the dermamyotome and ectoderm opens to allow migration under the presumptive skin (Weston *et al.*, 1978). Fig.4 also shows SLF hybridization signal in the surface ectoderm at ventral locations, as had been shown earlier (Lecoin *et al.*, 1995), but not in the neural tube nor in the sclerotome. Hybridization signal was not observed in the dorsal NC migratory space at more caudal levels of these embryos, nor at any level in younger embryos (data not shown). These results suggest that NC cells localized within the staging area express the SLF gene product *in vivo*, and are consistent with observations that NC cells emigrating from the staging area have become committed to a melanogenic fate by these developmental stages (Vogel and Weston, 1988; Erickson *et al.*, 1992; Rogers *et al.*, 1992).

One important remaining question, however, is whether SLF acts by supporting the survival of melanocyte precursors or by influencing the commitment of multipotent NC cells. To distinguish between these possibilities, we performed a series of culture experiments. In the first set of studies, NC cells were grown in serum- and embryo extract-free defined medium in the presence of either SLF or bFGF, as a positive control for melanogenesis (Stocker *et al.*, 1991). Fig.5A shows that SLF was more potent than bFGF at inducing pigmentation in these cultures, and that antibodies against SLF partially neutralized the effects of SLF. Similar proportions of pigmented cells were also observed when SLF and bFGF were added to the medium 24 hours after the neural tubes were placed in culture. This suggests that the effects of these growth factors were not due to initial differences in the types of NC cells that migrated onto the culture surface.

Interestingly, the morphology of the pigmented cells induced by SLF or bFGF also differed in our cultures. Figs.5C and 5D show that NC cells

cultured for 14 days in the presence of SLF display a more mature melanocyte morphology with a larger somata and more extensive cell processes, as compared with bFGF-treated cells (Table 1). This difference in morphology may indicate that SLF induces a more complete stage of cytodifferentiation than does bFGF. In any case, the finding that SLF increased the numbers of pigmented cells in defined medium is consistent with those of Lahav *et al.* (1994) using serum- and embryo extract-containing culture medium.

To test whether this effect of SLF was due to the survival of melanocyte precursors, highly enriched cultures of NC cells were grown in defined medium in the presence or absence of SLF for 5 days, and then the medium was changed with fresh medium. Presumably, if SLF were a survival factor, then the lack of SLF during the initial 5 day period should result in the irreversible loss of most SLF-dependent cells. Fig.5B shows, however, that cultures lacking SLF during the initial 5 days ("ø:SLF") showed only a 2-day delay in the onset of pigmentation, as compared with cultures treated with SLF for the entire 14 day period ("SLF:SLF"), and that the final extents of pigmentation were similar. These results indicate that SLF probably does not act as a survival factor for NC-derived melanocyte precursors, but instead acts to induce their differentiation into melanocytes. However, these data do not exclude the possibility that low levels of endogenous SLF may be sufficient for the survival of melanocyte precursors. Such low levels of SLF produced by NC cells during the initial 5 days may, in fact, explain why pigmented cells appeared in the untreated cultures (panel A) and in the "ø:SLF" cultures only one day following growth factor addition (panel B). In any case, we also found that NC cultures treated with SLF-neutralizing antibody during the entire 14 period (i.e., "Ab:Ab") gave rise to fewer pigmented cells than those in

defined medium alone (i.e., "ø:ø"), indicating that the spontaneous pigmentation was due to the presence of SLF, and not some other NC-produced protein.

DISCUSSION

In this paper, we show that NC cells express both the c-kit receptor as well as its ligand, the SLF growth factor. *In vivo*, SLF *in situ* hybridization signal was found in cells proximal to the neural tube at a development stage when NC cells are known to be migrating in this location (Bronner-Fraser, 1986). In culture, we found that NC cells express an mRNA whose sequence is highly homologous to the published sequence of the chicken and quail SLF gene products, and that approximately 98% of the cells expressed SLF immunoreactivity. The onset of SLF expression is likely to occur sometime during early stages of NC cell emigration from the neural tube. *In vivo*, for example, we first observed SLF *in situ* hybridization signal in the dorsal NC migratory space of the upper cervical spinal cord at E4, which is at least 24 hours after NC cell emigration has begun at this axial level (Bronner-Fraser, 1986). In culture, on the other hand, SLF-immunoreactive NC cells were observed as early as NC cells first began to emigrate onto the culture surface at about 12 hours. This small difference in the timing of appearance of SLF in NC cells *in vivo* and in culture can be explained by either differences in the sensitivity of the methods used for detection or by the possibility that these culture conditions somehow induce precocious SLF expression.

The conclusion that SLF is actually being released by NC cells is based, however, more indirect data. We found, for example, that SLF neutralizing

antibodies could partially inhibit the spontaneous appearance of melanocytes in NC cells cultured in a serum-free and embryo extract-free medium lacking exogenous SLF. This would suggest that at least some SLF is either being released by NC cells, or at least, is being translocated to an extracellular compartment accessible to the neutralizing antibodies (i.e., is present on the cell surface). On the other hand, attempts to directly demonstrate SLF immunoreactive material in western blots of concentrated medium from NC cell cultures have been less successful (data not shown). There are, however, at least two possible explanations for these negative results. First, the numbers of NC cells used in these studies was necessarily low due to the limitations of the microsurgical methods used to isolate NC cells, and we may have simply been below the limits of detection. Second, it is possible that NC cells don't process membrane-bound SLF to its soluble form under our culture conditions.

Regardless of which form of SLF is actually present, there are at least two non-mutually exclusive mechanisms by which SLF could act -- as a survival factor for a subpopulation of latent melanoblasts, or as a factor influencing the commitment of multipotent NC cells to a melanocyte fate. Our evidence, however, would seem to favor the latter possibility. We found, for example, that depriving NC cells of SLF during the initial 5 days in culture did not prevent their subsequent pigmentation induced by late additions of SLF. Presumably, this 5-day period is sufficiently long for cell death to have occurred (e.g., Greene, 1977), suggesting that SLF is not necessary for survival, at least with cultured avian NC cells. On the other hand, these data do not rule out the possibility that committed melanoblasts become dependent on SLF for their survival at later stages of development. This latter possibility

is consistent with studies performed using mouse NC cells. In these experiments, SLF factor was found to increase the numbers of c-kit positive cells in NC cultures, suggesting that SLF was acting either as a survival or mitogenic signal (Murphy *et al.*, 1992). It should be noted, however, that these studies involved the use complex culture medium containing serum, embryo extract, and phorbol esters, complicating the interpretation of which effects of SLF were direct and which involved combinations of factors. It is also possible, however, that the NC cells of mice and birds utilize SLF in different ways for inducing the appearance of melanocytes. Perhaps mice do indeed require SLF as a survival factor (and perhaps a differentiation factor, as well), whereas birds utilize SLF at an earlier step in the process of melanogenesis to influence cell fate decisions among pluripotent cells.

In any case, the possibility that SLF acts to influence such cell fate decisions is also consistent with earlier observations by Weston and colleagues. They showed that the ability of cultured avian NC cells to give rise to melanocytes depended on the period of time in which the cells were in close physical apposition to each other (Glimelius and Weston, 1981; Vogel and Weston, 1988). Whereas NC cells that formed tight clusters for periods of at least 36 hours gave rise to numerous pigmented cells, NC cells that were either prevented from forming clusters, or in which the clusters were dissociated within the 36 hour period, contained significantly fewer melanocytes. Although these studies did not identify the factor(s) involved, they are consistent with the view that some endogenous factor influences NC cell fate, possibly including SLF. Interestingly, these effects of prolonged association had the opposite effect on the appearance of NC-derived cells expressing a neuronal marker (Vogel and Weston, 1988). This suggests that

the endogenous factor or factors may actually change the fate of multipotent NC cells from a neuronal to a melanocyte fate. Although our data are consistent with the possibility that this endogenous factor is SLF, it remains to be determined directly whether SLF actually suppresses neurogenesis in multipotent NC cells.

If the SLF/c-kit axis is indeed involved in melanogenesis, then our finding that most NC cells express both the SLF and c-kit receptor proteins in culture might have predicted that melanocytes should comprise the largest population of NC-derived cells. This is, however, not the case *in vivo*. Lineage tracing studies, in which individual NC cells were injected *in vivo* with a marker and then the fates of their progeny monitored, clearly show that only a small subpopulation of NC cells actually giving rise to melanocytes (Fraser and Bronner-Fraser, 1991). Together with the studies reported here, this may indicate that negative factors or conditions play a major role in the determination of cell fate. One likely condition acting in such a negative fashion may be the extent to which NC cells are dispersed during early stages of their migration *in vivo*. NC cells that form melanocytes of the skin, for example, are initially tightly clustered in a space adjacent to the neural tube for a period of about 36 hours, until the space between the dermamyotome and ectoderm opens up allowing dispersion of these cells. This clustering of NC cells has been termed the "migration staging area" and has been suggested to play a critical role in the determination of melanogenesis by NC cells (Weston, 1991). Consistent with this notion are observations that NC cells migrating along the dorsal-lateral pathway seem already to be committed to a melanocyte fate (Erickson and Goins, 1995). Perhaps the relatively high concentrations of SLF expressed by neighboring NC cells in the migration

staging area specifies a melanogenic fate for those cells that subsequently enter this pathway.

In contrast to the dorsal migratory pathway, NC cells that migrate along the ventral pathway soon become mixed with non-NC cells in a loose mesenchyme (Bronner-Fraser, 1986), which may not be permissive for melanogenesis. If, for example, SLF requires a relatively high concentration and/or direct contact between responding cells, then the mixed mesenchyme of the ventral pathway would not be expected to produce melanocytes. In addition, it is possible that there are other factors acting in a redundant fashion to inhibit ectopic melanogenesis along the ventral NC migratory pathway. One such factor may be transforming growth factor type-ß (TGFß), which has been shown to inhibit melanogenesis by NC cells in response to other peptide growth factors, as well as factors present in the substratum (Stocker *et al.*, 1991; Rogers *et al.*, 1992). Various TGFß isoforms have been shown to be expressed, moreover, by cells adjacent to the ventral NC migratory space of chicken embryos at the appropriate developmental stages *in vivo* (Jakolew *et al.*, 1992; 1994). It is interesting to note, therefore, that cranial neural crest cells, which undergo melanogenesis to a far lesser extent than those of the trunk (Leblanc *et al.*, 1995), have been shown to express and process TGFß (Brauer and Yee, 1993).

In any case, the observations that NC cells express growth factors that are capable of influencing their own development would suggest that NC play a more active role in the determination of their cell fate than has previously been hypothesized.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Fig.1 Presence of SLF immunoreactivity in embryonic quail NC cells in culture. NC cells were isolated as described (Loring *et al.*, 1982; Stocker *et al.*, 1991) from stages 12 and 13 quail embryos (Hamburger and Hamilton, 1951) and cultured in serum- and embryo extract-free defined medium (CCM-5; Hyclone, Logan UT). After 2 days, the cultures were fixed and double-immunostained for SLF and HNK-1 as a marker of NC cells (Bronner-Fraser, 1986). **(A)** SLF immunostaining of NC cells, **(B)** the same field of cells as in A immunostained for HNK-1, **(C)** pre-immune serum substituted for anti-SLF serum, **(D)** the same field of cells as in C immunostained for HNK-1. The bar at the upper right in panel B corresponds to 10 μm.

Fig.2 Protein immunoblot showing SLF-immunoreactivity in various tissues dissected from E14 chickens. Note the single immunoreactive band at 23 kDa (at the arrow). Lane 1, brain; Lane 2, heart; lane 3, kidney; lane 4, liver; lane 5, muscle.

Fig.3 Southern blot of an RT-PCR reaction using SLF primers indicating expression of a 466 base pair SLF reaction product from RNA extracted from cultured embryonic quail NC cells (lane 4). Positive control for the Southern blot involved electrophoresis of a restriction digest of the SLF plasmid (lane 1); positive controls for the RT-PCR reaction involved substituting total quail brain RNA for NC RNA (lane 2). Negative controls involved omitting the reverse transcriptase steps in reactions with total brain RNA and NC RNA (lanes 3 and 5, respectively).

Fig.4 *In situ* hybridization showing SLF mRNA in the NC "migration staging area" (Wehrle-Haller and Weston, 1995) of an E4 chicken. **(A)** Bright-field photomicrograph of a tissue section through the cervical region of a stage 22 embryo (Hamburger and Hamilton, 1951); **(B)** the same section viewed through Nomarski optics. Sense strand RNA served as negative controls in these studies (not shown).

Note the presence of SLF hybridization signal in the migration staging area adjacent to the dorsal neural tube (at the arrow), as well as some hybridization signal in the ventral ectoderm (at the asterisk). The bar at the upper right in panel B corresponds to 100 µm. nt, neural tube; dm dermamyotome; no, notocord.

Fig.5 Effects of chicken SLF on melanogenesis **(A)** and survival **(B)** of cultured embryonic quail NC cells. **(A)** NC cells were cultured in CCM-5 defined medium in the presence or absence of SLF (100 ng/ml), basic fibroblast growth factor (25 ng/ml), SLF plus bFGF, or SLF plus SLF-neutralizing antiserum (1:100 dilution). Cultures were observed daily for the presence of pigmented cells, and the proportions of cultures containing such cells were calculated. These experiments were performed three times with qualitatively similar results, and these data represent the average of these studies. Note that SLF induced robust pigmentation (>90%) by 8 days and that the presence of SLF-neutralizing antibodies reduced this by more than half. The small decreases in the percentages of cultures containing pigmented cells at later times is probably due to some cell death beginning around 8-10 days in culture. However, these decreases were more apparent in those cultures treated with bFGF, rather than SLF. **(B)** Medium-switch experiments in which NC cells were cultured in the presence or absence of SLF or SLF-neutralizing antiserum for 5 days, and then the medium was changed for the remaining 9 days. Again, these experiments were performed three times with qualitatively similar results, and the results here represent the average of these studies. "Ø:Ø" refers to cultures treated with defined medium for the entire 14 days; "Ø:SLF" refers to cultures treated with defined medium alone for 5 days followed by 9 days in SLF-containing medium; "SLF:SLF" refers to cultures treated with SLF for the entire 14 days. (**C** and **D**) Morphology of NC-derived cells cultured for 14 days in the presence of 100 ng/ml SLF (C) or 25 ng/ml bFGF (D). Note that SLF induced a more mature melanocyte morphology in NC cells than did

bFGF, with cells displaying significantly longer processes. The bar in the upper right corner of panel D corresponds to 10 μm.

Table 1. Effects of SLF and bFGF on the morphology and differentiation of cultured neural crest cells.

	Number of processes per cell	Mean length of each process	Proportion of pigmented NC cells
Control cells	0.45 ± 0.12	15.6 ± 5.8 μm	0.3 ± 0.7
bFGF-treated cells	0.54 ± 0.19 (n.s.)	18.2 ± 2.6 μm (n.s.)	14.0 ± 5.4 ($p < 0.001$)
SLF-treated cells	2.69 ± 0.25 ($p < 0.02$)	122.4 ± 27.1 μm ($p < 0.02$)	95.2 ± 6.9 ($p < 0.001$)

Neural crest cells were cultured in serum-free and embryo extract-free CCM-5 culture medium in the presence or absence of growth factor for 14 days and then fixed and processed for microscopic examination. Morphometric analysis was performed on 300 cells in each of 3 control, 3 bFGF- and 3 SLF-treated cultures. The percentage of pigmented cells was determined by counting pigmented and unpigmented cell in 12 control, 12 bFGF-treated, and 12 SLF-treated cultures. Statistical comparisons between the growth factor-treated cultures and the control cultures were made using ANOVA tests. Note that SLF induced statistically significant differences in the numbers of processes per cell, the mean length of each process, and the proportions of pigmented cells.

n.s., not significant (i.e., $p > 0.05$).

Figure 1. NCC express both SLF and c-kit

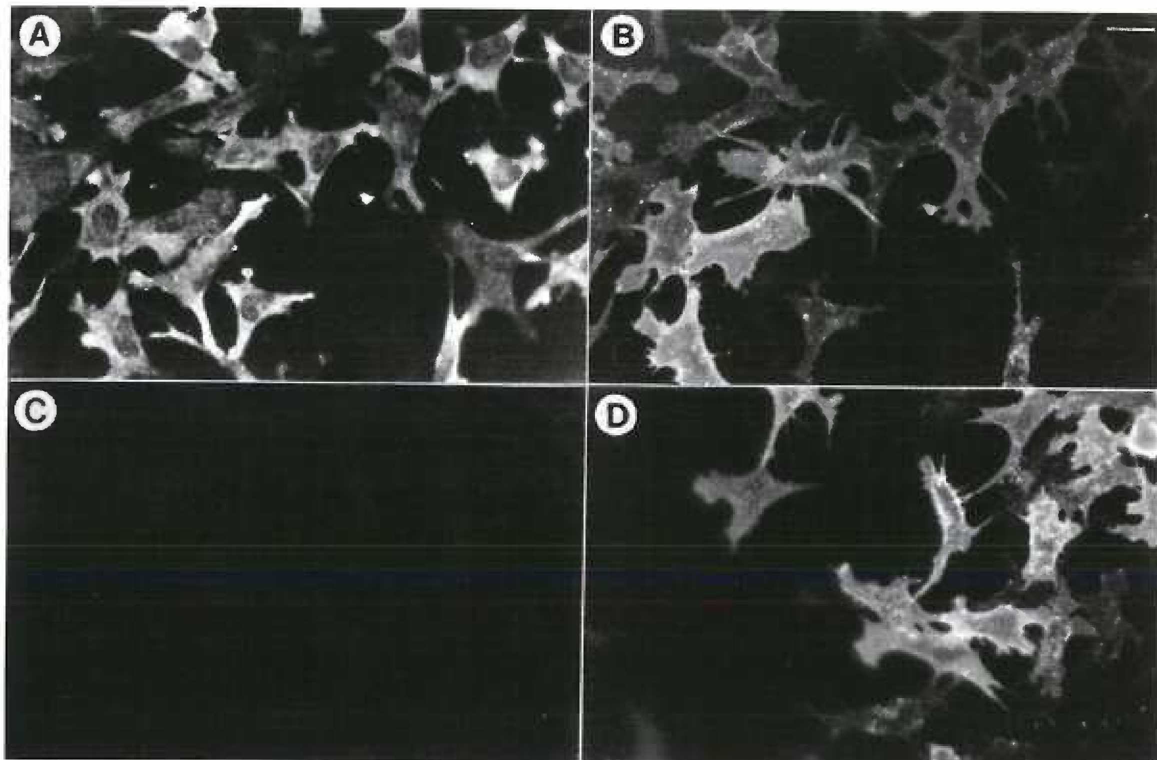


Figure 2. SLF expression in different tissues

1 2 3 4 5

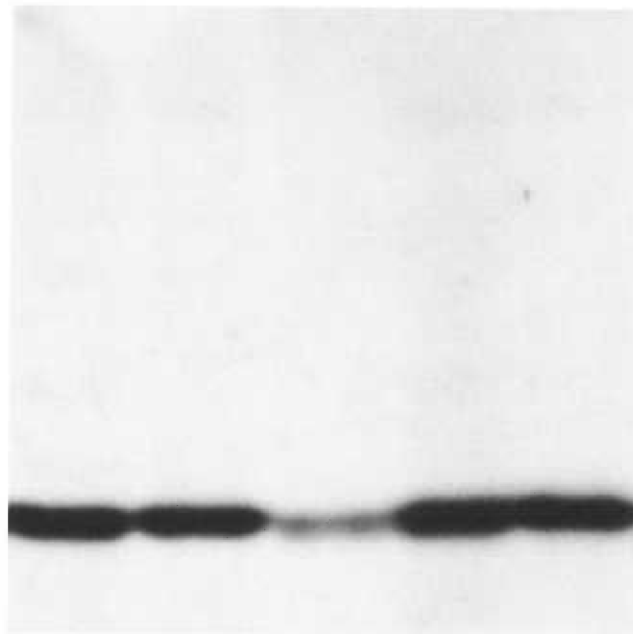


Figure 3. RT-PCR analysis of SLF Expression in vitro

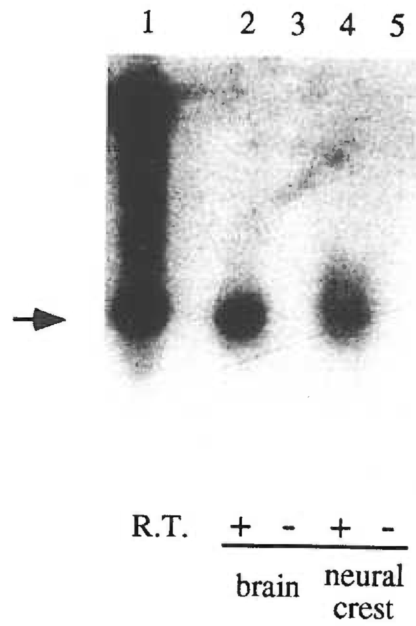


Figure 4. In-situ hybridization on E4 chicken

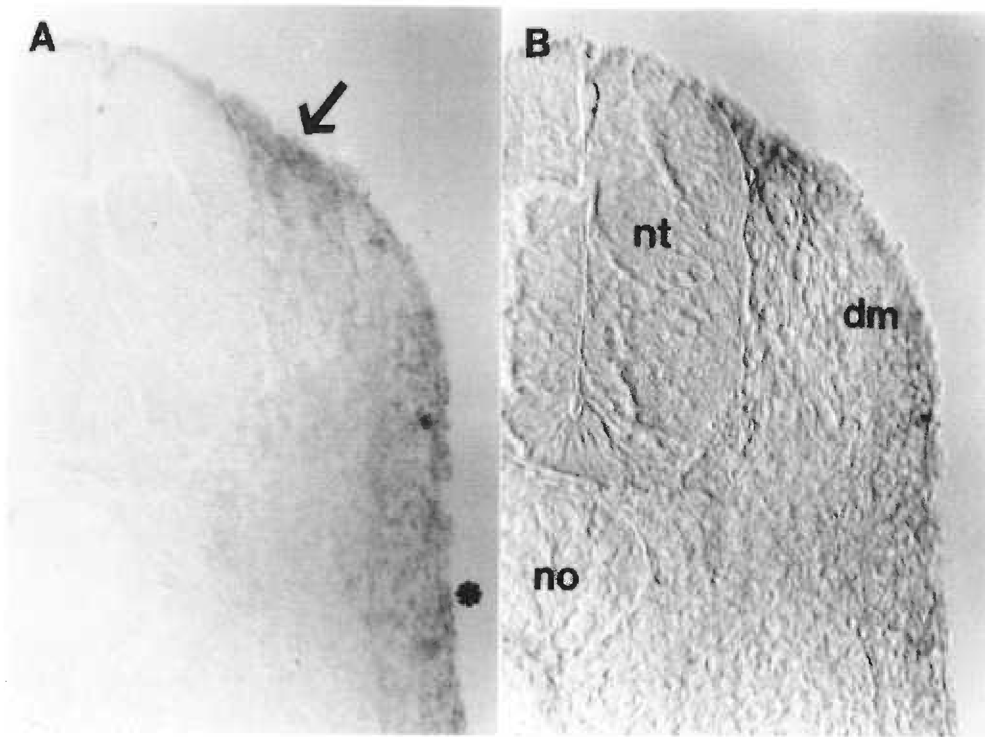


Figure 5
Guo et al.

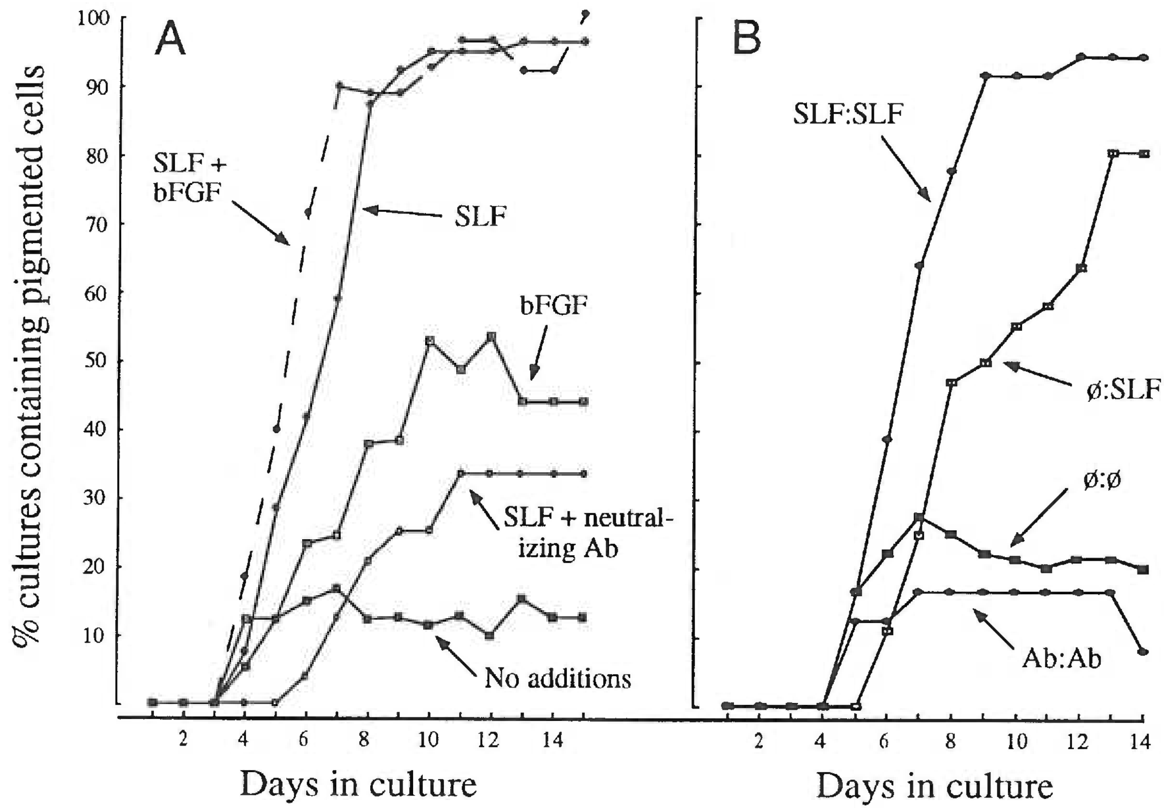
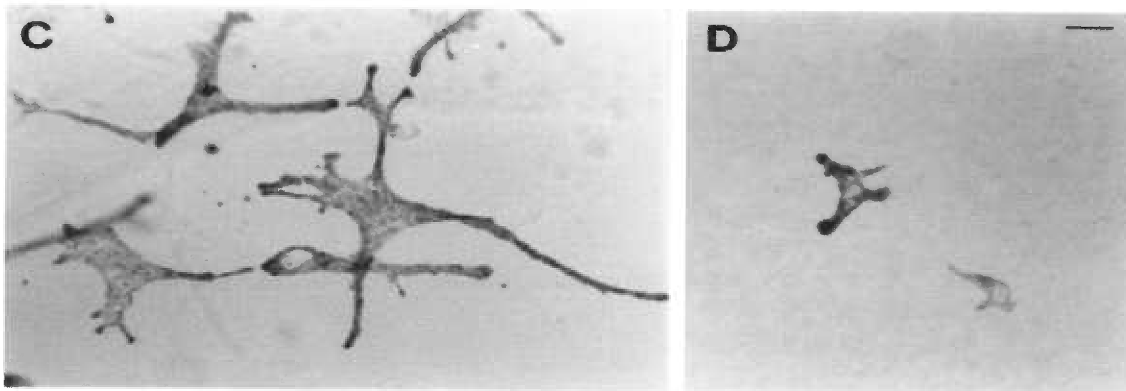


Figure 5C-D. Morphology of melanocytes induced by different factors



AN APPENDIX TO CHAPTER II

Determination of the specificity of the antibodies by western-blot analysis.

To demonstrate whether the antibodies are specific to the fusion proteins, western blot analysis was performed. Figure 0-6a shows that the anti-SLF antibodies recognized a single band of SLF fusion protein at 29 KDa. A good way to show the specificity of an antibody is to pre-absorb the antibody with the fusion protein to see whether the pre-absorption can abolish the activity of the antibody. As shown in lane 2 of figure 6a, the activity of SLF antibody was abolished when pre-absorbed with SLF protein. Pre-absorption with c-kit protein, however, could not abolish the activity. This indicates that the anti-SLF antibody is specific. Similar results using the c-kit antibody are shown in figure 6b .

Expression patterns of SLF and c-kit are consistent with its playing a role in melanogenesis.

The western blot analysis showed the specificity of the antibodies to the fusion proteins, but it did not address the reactivity and specificity to the native SLF and c-kit in chicken and quail tissues. So western blot studies were performed using E6 whole embryo extracts to determine the specificity of the anti-SLF and anti-c-kit antibodies to avian tissues. Figure 0-7b shows that the anti-SLF antibody recognized specific bands at 23 KDa, that was abolished when pre-absorbed with the SLF fusion protein. The specificity of the anti-c-kit antibody is shown in figure 0-7a. To determine whether SLF was expressed at the developmental stages consistent with its playing a role in melanogenesis, chicken and quail whole embryo extracts from E2 to E8 were prepared, separated on SDS-gel, transferred to a nitro-cellulose membrane and probed with anti-SLF. It is believed that

melanogenesis starts at E4 and pigmentation becomes obvious at E8 in quail and chicken. Figure 0-7c shows that SLF was expressed from E2 to E8, consistent with SLF playing a role in melanogenesis. The expression of SLF in different tissues is demonstrated by western blot as well (0-7D) . Expression of SLF in many different tissues indicates that SLF may have other functions unrelated to its role in melanogenesis. Figures 0-7e and 0-7f shows c-kit expressions in whole embryos from E2 to E8 and in different tissues, this pattern of expression is consistent with what has been shown in mouse.

FIGURE LEGENDS

Figure 0-6. Western-blot analysis of anti-SLF and anti-c-kit antibodies with purified fusion proteins.

Figure 6a shows SLF fusion proteins probed with anti-SLF antibody (lane 3), with anti-SLF pre-absorbed with c-kit fusion protein (lane 2) and with anti-SLF pre-absorbed with SLF fusion protein (lane 1). The single band is approximately 29 KDa.

Figure 6b shows the results with the c-kit fusion protein probed with anti-c-kit (lane 3), with anti-c-kit antibody pre-absorbed with SLF fusion protein (lane 2) and anti-c-kit pre-absorbed with c-kit fusion (lane 1). The single band in figure 4b is approximately 68 KDa.

Figure 0-7. Western-blot analysis of chicken and quail tissues. 0-7a shows the E6 chicken tissues probed with anti-c-kit antibody (lane 1), anti-c-kit pre-absorbed with non-related proteins (lane 2), anti-c-kit pre-absorbed with c-kit fusion proteins (lane 3), and pre-immune serum (lane 4).

0-7b shows the E6 quail whole embryo extract probed with anti-SLF (lane 1), anti-SLF pre-absorbed with SLF fusion protein (lane 2).

0-7c shows the SLF expression in E2 to E8 chicken (lane 1-7) and quail (lane 8-14) embryos.

0-7d shows the expression of SLF in different tissues, E6 chicken whole embryo extract was used as a control (lane 1), E8 quail brain, heart, kidney, lung and muscle (lane 2-6), E 14 chicken brain, heart, kidney, liver and muscle (lane 7-11), E14 quail brain, heart, liver and muscle (lane 12-15).

0-7e shows the c-kit expression in E2 to E8 chicken (lane 1-7) and quail (lane 8-14) embryos.

0-7f shows c-kit expressions in E6 whole embryo extracts (lane 1), E 8 quail brain, heart, kidney (lane 2-4), lung and muscle (lane 6-7), E 14 chicken brain, heart, kidney, lung and muscle (lane 8-12), E14 quail brain, heart, lung and muscle (lane 12-16).

Figure 0-6. Western-blot analysis of anti-SLF and anti-c-kit antibodies.

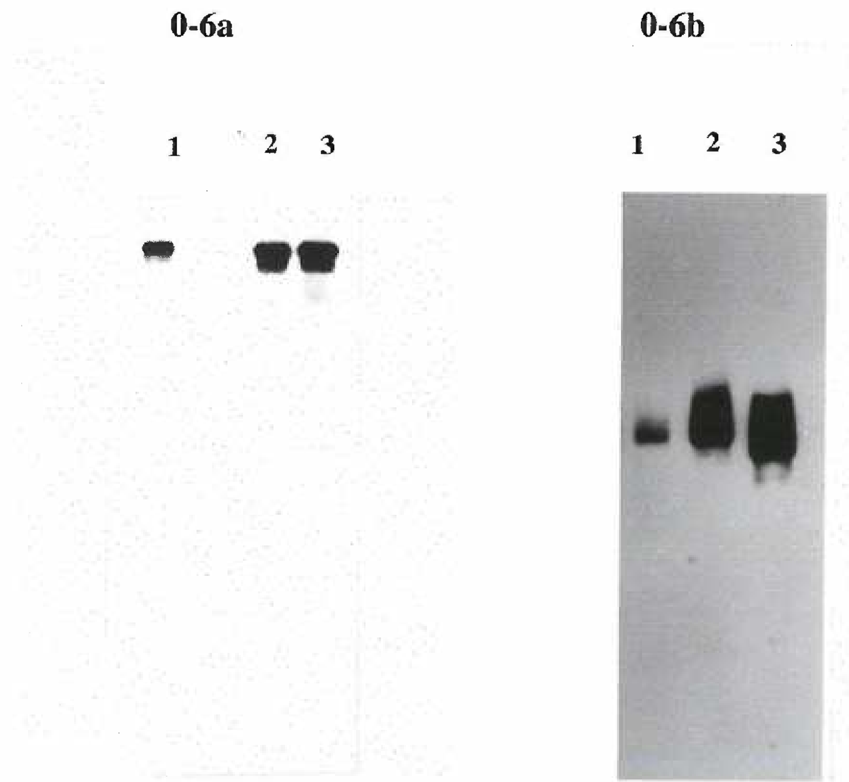


Figure 0-7. Western-blot analysis of tissues

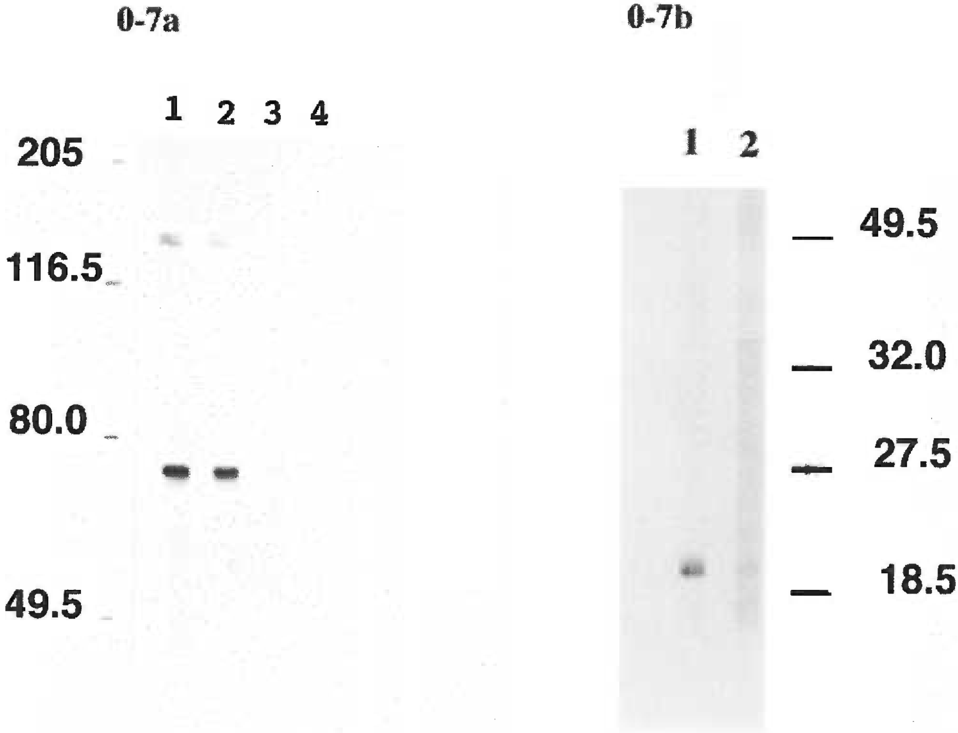


Figure 0-7c

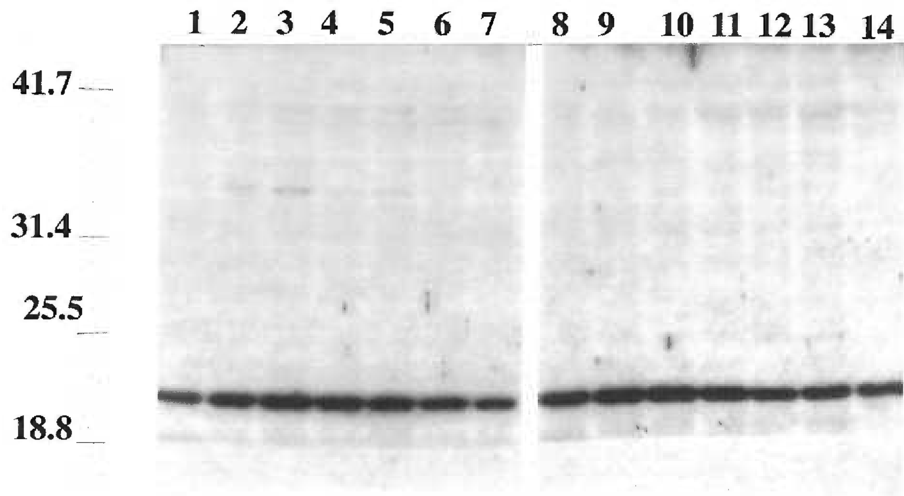


Figure 0-7d

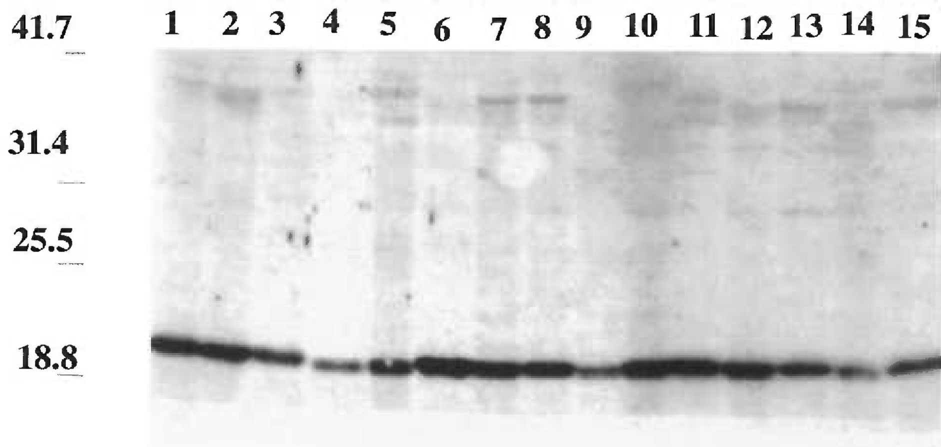
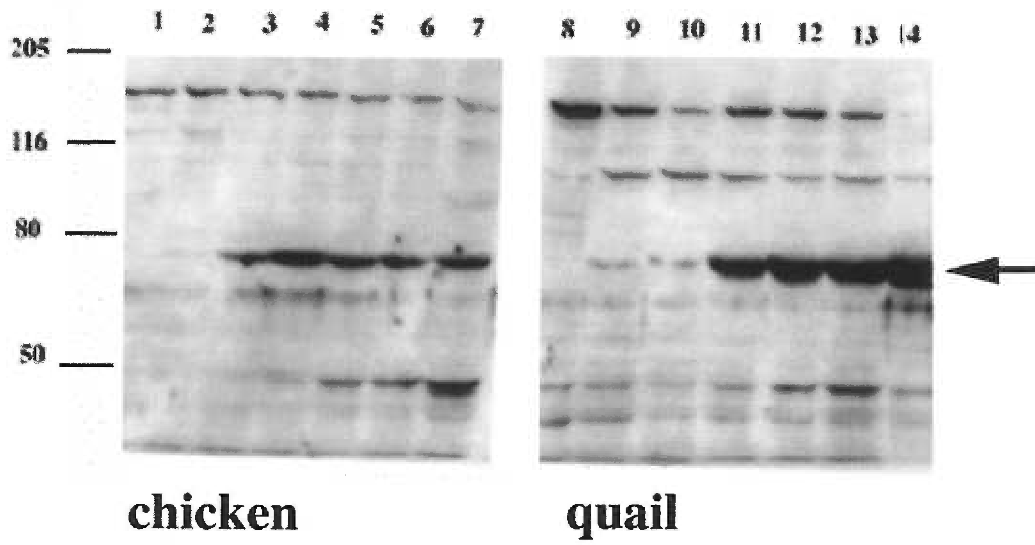
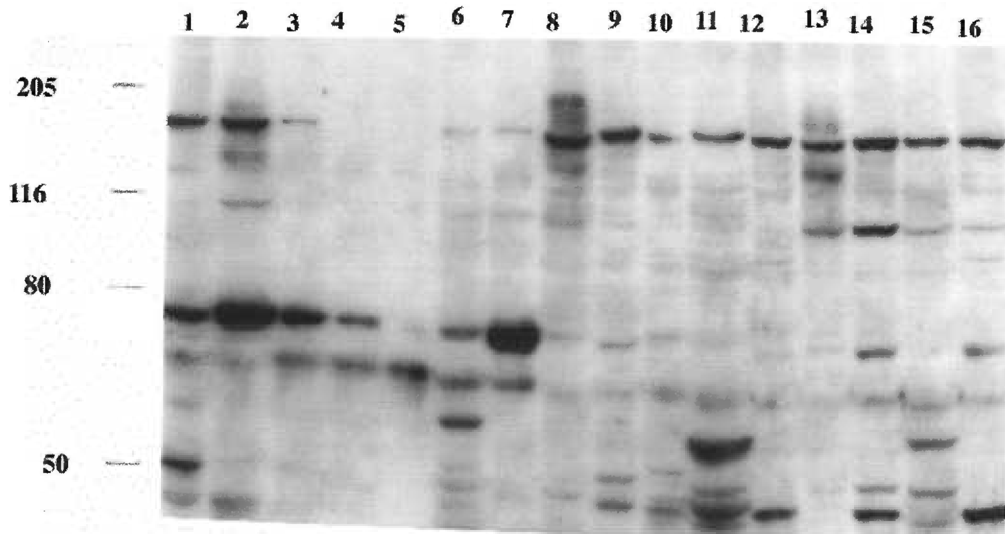


Figure 07-e. C-kit expression in E2 to E8 embryos



0-7-f



CHAPTER III--Melanocytes Depend on Autocrine Action of Membrane-bound Form of SLF for Their Survival

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This part is in preparation for publication

Abstract

Neural crests give rise to diverse cell types during development and has been an ideal system to study the mechanism of cell fate determination. It is suggested that the environment neural crest cells face during migration as well as at their final targets plays an instructive role in their fates. Steel growth factor is one of the environmental cues identified recently. Natural, soluble SLF proteins have been purified from many tissues and have been assumed to be the prominent active form. However, structural analysis of the deduced amino acid sequence of SLF revealed that it is a membrane-associated protein with a typical transmembrane domain. Recent studies suggest that the membrane form of SLF may have important functions as well. To study the functions of the two forms of SLF, I first examined the expression of them by western-blot analysis. I observed that soluble SLF is the major form in the very early embryo. At later stages, soluble SLF is still the dominant form in brain and muscle, while membrane SLF becomes the dominant form in skin. To distinguish the function of different forms of SLF, I generated stable CHO cell lines which express a mutant form of SLF which can not be cleaved and therefore remains associated with the cell surface. An inhibitor for SLF cleavage and an inhibitor for c-kit kinase activity were also applied to dissect the differential roles of soluble/released and membrane associated forms of SLF. I demonstrated that soluble SLF promotes NCC differentiation into melanocytes in a dose-dependent manner, while the membrane bound SLF expressed by CHO cells has no effect. The melanocyte survival assay demonstrated that SLF is required for the survival of melanocytes, and that membrane SLF is sufficient for the basal level of survival of cultured melanocytes.

Furthermore, I provided evidence that melanocytes may use membrane SLF in an autocrine fashion via direct cell-cell contact for their survival. The data presented here provides evidence that a growth factor in two different forms play distinct roles in the development of NCC.

Introduction.

One of the fundamental questions in developmental biology is how the tremendous diversity of cells that comprise different structures of the adult organism is generated from a single fertilized egg. Neural crest cells (NCC) are one of the systems widely used to address how cells differentiate and follow different fates in the early development of vertebrates (Le Douarin, 1982). The neural crest is a transient structure in development arising between presumptive neural and epidermal ectoderm at the point where the neural folds meet to form the neural tube. Once the neural crest structure is formed, cells within it start to migrate along distinct pathways. The cells migrate along two distinct routes in the trunk region. Some cells start to migrate immediately along a ventral pathway, through the somitic mesenchyme, while others stall at the melanocyte staging area (MSA), then migrate along the dorsolateral pathway, between the dorsal somite and the epidermis. The cells migrating along the ventral pathway form the dorsal and sympathetic ganglia, as well as, the adrenal medulla. However, the late migrating NCC choose a different fate and become melanocytes in the skin.

It is believed that most NCC are multipotent and become gradually restricted while migrating through different pathways and interacting with different factors in the changing environment. Steel growth factor (SLF) is considered one of the factors in the environment which is critical for the development of NCC, especially those taking the dorsolateral pathway. Analysis of mice and human mutants in SLF and its receptor c-kit suggests an important role for SLF in NCC development. The mechanisms of SLF function have been explored extensively after the cloning of SLF genes in 1990 (Anderson et al., 1990; Copeland et.al.,

1990; Huang et.al., 1990; Martin et.al., 1990; Williams et.al., 1990; Zsebo et.al., 1990.).

Structure analysis of the deduced amino acid sequence of SLF revealed that it is a membrane bound protein with a transmembrane domain and cytoplasmic tail. SLF also has a typical signal sequence at the N-terminus and a putative protease cleavage site near the transmembrane domain. The fact that SLF has both a signal sequence for secretion and a typical transmembrane domain seems contradictory. On the one hand, the signal sequence and the putative cleavage site suggest that this protein can be secreted; on the other hand, the existence of a transmembrane domain implies that the molecule is associated with the cell surface. Experimental results on the biological functions of SLF from different laboratories are also confusing, and sometimes contradictory. Natural soluble SLF proteins have been purified from many tissues, but expression of the SLF gene in many cell lines failed to produce soluble SLF. Another contradiction is that while some groups claim SLF is only a survival factor for NCC and their derivatives, the mutant phenotypes in humans and mice and in-situ analysis suggest that SLF has more extensive roles (Besmer et.al., 1993; Motro et.al., 1991.) . For example, many laboratories have demonstrated that SLF has mitogenic effects on NCC and their derivatives and is able to induce NCC to differentiate into melanocytes (Guo et.al., 1997; Lahav et.al., 1994.) . For years, the soluble SLF has been assumed to be the active form utilized in the embryo. In fact, most biological studies were done with the recombinant soluble SLF. However, although soluble SLF has multiple and potent activities *in vitro* on NCC and melanocytes, but absence of the membrane bound SLF in SLF dickie mice causes severe melanocyte defects (Flanagan et.al., 1990; Steel et.al., 1992.) . In the SLF dickie mice, the SLF gene is

truncated before the cleavage site and produces only the soluble SLF. This soluble protein was active when tested in proliferation assays on mast cells and fibroblasts *in vitro* and showed some activity in the embryo (Anderson et.al., 1990; Steel et.al., 1992; Wehrele-Haller.) . In the SLF dickie mutant embryos, melanocytes are detected at early stages of development in the skin, but the adult mice have no pigmentation in their coats.

These inconsistent phenomena raise some very important questions: 1) If soluble SLF is the prominent active form, why is it released from the cell surface instead of being secreted directly? 2) If soluble SLF is indeed as effective as demonstrated *in vitro*, why does lack of the membrane-bound form in the SLF dickie mice result in such a severe defect on pigmentation in adults despite the presence of soluble SLF? 3) Which form of SLF is actually used in the embryos, soluble, membrane-bound, or both? 4) If both forms of SLF are active *in vivo*, do they have similar functions, and where and when do they work?

We wanted to determine the functions of the two different forms of SLF. We established stable CHO cell lines expressing the membrane-bound form by mutating the cleavage site and performed co-culture studies with NCC and melanocytes. We also applied a neutralizing antibody against SLF, a protease inhibitor which can block the cleavage of SLF, and kinase inhibitor to block c-kit receptor to distinguish between the activities of the two forms of SLF on NCC proliferation, differentiation, and on melanocyte survival. We studied the expression of the two forms of SLF in different tissues and at various stages of development.

We provide evidence that different forms of SLF are used in different tissues at different developmental stages. The NCC differentiation at early stages is

mediated by the soluble form of SLF, while the membrane-bound form of SLF is necessary at later stages of development to maintain the viability of melanocytes. We previously showed that NCC express both SLF and c-kit and suggested that SLF may work in an autocrine fashion. Here we demonstrate that melanocytes express membrane-bound SLF on the cell surface and suggest that SLF and c-kit interaction, through direct contact among melanocytes, is critical for the survival of melanocytes. Our results provide an example of a growth factor with a transmembrane domain, active in both soluble and membrane-bound forms, each with tissue and stage specific function. Our results also provide some biological significance for producing a soluble growth factor through cleavage of the membrane-bound-form instead of direct secretion. The membrane bound form itself has unique biological functions rather than simply providing an extra level control of its activity.

MATERIALS AND METHODS.

Construction of the membrane-bound SLF.

Membrane bound SLF mutant was generated by PCR. Two sets of primers were used(5'- GCAC AAGCTT ATG AAG AAG GCA CAA ACT TGG- 3', 5'- CAGG AGG AAA CGA AAT TGT TTT TGT GAC AGC-3'; 5'- AAA ACA ATT TCG TTT CCT CCT GTT TCT TCC AGC TCC CTT AGG AAT GAC-3', 5'- CGTG GAATTC CTA TGC CTT GTC GTC GTC GTC CTT GTA GTC CAT CAC TTG TAG ATG TTC TTT TTC-3'), one with point mutations introduced, to generate two pieces of DNA fragments with 21 bp nuclei acids overlaping. The two pieces were purified and mixed as new templates for the final round of PCR to produce

the construct encoding membrane-bound SLF. The mutant construct includes the entire SLF cDNA with coding sequence for cleavage VAAS in wild type replaced by VSSS, and a flag sequence introduced at the N-terminus. The PCR products were cloned to TA cloning vector (Invitrogen, San Diego, CA) and sequenced with an automatic sequencer (Applied Biosystem 373 sequencer) to make sure proper mutation was introduced. PCR machine: COY Tempcycler II Model 110S, COY laboratory products Inc., Ann Arbor, MI.

Stable transfection of the membrane-bound SLF construct.

The membrane-bound SLF construct was subcloned into pCDNAIII mammalian expression system and transfected into CHO cells using a kit from Boehringer Mannheim. Calcium phosphate-DNA precipitates were prepared by mixing DNA solutions and HBS(added 0.5 ml DNA solutions into 15 ml tubes with 0.5 ml HBS)with air bubble forming by pipette aid pump attached with a 2 ml pipette. The DNA precipates were vortexed before adding to plates and incubated at 37° C for five hours. Glycerol shock was done by treating cells with 25% glycerol in 1X Hanks/Hepes for two minutes. 10 ug DNA was used for each CHO cell plate, and a total of three plates were transfected. The pCDNAIII vector without insert was also transfected as a control. Cells transfected were selected in the presence of G418.

Western-blot analysis.

Whole cell lysates of CHO cells expressing membrane bound SLF or vector alone were collected with RIPA buffer in the presence of PMSF, leupeptin and aprotinin. Protein concentrations were measured using bio-rad assay. Equal

amount of protein were loaded onto 12% SDS-gel, and run at 180 volts for 6 to 8 hours. Then the separated proteins were transferred onto nitrocellulose transfer membrane (MSI Micron Separatioins Inc., Westborough, MA)at 500 mA over night in a cold room. The membrane was blocked with 5% dry milk in PBS for one hour at RT, probed with anti-flag antibody at 1:1000 for two hours at RT, reacted with G-R IgG conjugated with HRP (Vector, CA) at 1: 5000 for one hour at RT. After ECL reaction for 2 minutes (Pierce, Rockform, Illinois), film was exposed (Kodak film OX-Omay Blue XB-1) with the membrane, developed with a Kodak M35A X-MAT processor. Tissues from Japanese quail embryos were prepared for Western-blot analysis as well. Whole embryo extracts from E2 to E7, as well as skin, muscle and brain tissue extracts from E8 to E16 were prepared in the same RIPA buffer. Western-blot was performed under the conditions described, except the primary antibody used was anti-SLF antibody made from rabbit at 1:2000.

Immunocytochemistry studies.

Immunocytochemistry (ICC) under non-permeablizing conditions was performed on CHO cells with membrane-bound SLF to show the SLF expression on cell surface. The experiment was carried out without any detergent through out the whole process. CHO cells were fixed with 4% paraformaldehyde in PBS for 8 min at RT, blocked with 10% goat serum in PBS for 25 minutes, probed with anti-SLF antibody 405 at 1: 200, RT, 40 min, then incubated with G-R IgG biotin labeled (Vector, CA) at 1:200, RT, 40 min, Avidin-Texas-Red at 1:300 for 30 min. Washed with PBS twice, mounted with Vectorshield (Vector, CA) solution and cover-slid. ICC on melanocytes under non-permeablizing conditions was

carried out on melanocytes to determine the expression of SLF on the cell surface. Melanocyte culture were fixed with 4 % paraformaldehyde for 8 minutes, RT. Then the cultures were incubated with 10 % goat serum for 25 minutes, anti-SLF antibody made from chicken at 1:50 for 40 minutes, then probed with 1:150 biotin-G-C IgY for 40 minutes, and avidine-FITC for 30 minutes.

ICC on E14 quail sections was performed using the anti-SLF antibody from chicken. E14 legs of quail were quickly frozen in O.C.T in liquid nitrogen, sections were prepared using cryostat 2800 Frigocut-N(Leica). Tissue slides were fixed for 8 minutes in 4 % paraformaldedyde at RT, then blocked with 10 % goat serum for one hour, 1:50 chicken anti-SLF antibody or 1:50 Chicken IgY as control for three hours. G-C IgY at 1:150 were added for two hours, followed by an incubation with 1:400 Avidin-FITC for one hour at RT.

Cell proliferation assay.

Neural tubes were cultured in HyQ-CCM5 for 24 hours to let NCC migrate out, then removed. NCC were dispersed by dissection needles and co-cultured with CHO cells expressing membrane-bound SLF, CHO cells expressing vector alone, HyQ-CCM5, or HyQ-CCM5 with rSLF at 20 ng /ml for 24, 48, or 72 hours. Co-cultures were incubated with BrdU labeling solution in fresh HyQ-CCM5 medium for 50 minutes, fixed with 70% ethanol in glycine buffer at -20° C for 40 minutes, and probed with diluted anti-BrdU antibody from Boehringer Manheim Biochemica. For each experiment, at least six areas were counted and the percentage of BrdU positive NCC was averaged. Results shown represent

average of four 72 hour co-culture experiments. The CHO cells were treated with mitomycin C at 5 ug/ml for four hours before co-culture to prevent their proliferation and BrdU interaction.

Melanogenesis assay.

Neural tubes were cultured for 24 hours in HyQ-CCM5 and removed by dissection needles. NCC were dispersed by dissection needles to provide more contact with the added CHO cells. To prevent CHO cells from over-proliferation and make to them stay attached to the plates longer, the CHO cells were treated with 5 ug/ml mitomycin C for two hours before the co-culture. NCC were co-cultured with 10^4 CHO cells, or rSLF at 5, 10, 20, 40, or 80 ng/ml in each well of 24-well-plate. At day four of co-culture, melanocytes were apparent and numbers were recorded for six to eight subsequent days. Results shown here were the average of three experiments, each representing at least six wells containing melanocytes cultured for six days.

Melanocytes survival test.

NCC cultures were prepared as above and cultured in 5% serum and 50 ng/ml rSLF for seven to eight days. During the period of incubation, most NCC differentiated into melanocytes. The cultures with melanocytes were washed with HyQ-CCM5-5, then co-cultured with HyQ-CCM5, CHO cells expressing vector, CHO expressing membrane-bound SLF, or 20 ng/ml rSLF for four days. The total number of melanocytes and dead melanocytes was determined daily. The percentage of melanocyte death

was averaged for each experiment with about nine wells for each experimental condition. The final figure represents the average of three experiments.

RESULTS.

Construction and Characterization of Cell Lines expressing the Membrane-bound of SLF.

To compare the functions of two forms of SLF, we generated a stable cell line expressing a membrane bound form of SLF. The membrane-bound SLF was generated by PCR with the cleavage site VAAS in the wild type replaced with VSSS, and a flag sequence introduced at C-terminus (Fig. 2-1a). The PCR products were cloned into TA cloning vector, and sequenced to make sure the correct point mutation was introduced. This was subcloned into pCDNAIII, and transfected into CHO cells. Stably transfected CHO cell lines were characterized by Western-blot and ICC. Western-blot with anti-flag antibody showed that the CHO Cells expressing the membrane-bound SLF produced a protein of 31 KDa, which was not detected in cells expressing pCDNAIII vector or in the parental CHO cells (figure 2-1b). To demonstrate that the membrane-bound SLF is indeed associated with cell surfaces, ICC was performed under non-permeablizing conditions. Figure 2-1c represents the ICC results visualized under confocal microscopy. The CHO cells expressing the membrane SLF were immunoreactive to anti-SLF antibody, while the cells expressing the vector did not react to the antibody. This suggests that the membrane-bound SLF is indeed expressed on the cell surface. To make sure that expressed membrane-bound SLF was not cleaved, the supernatants of the cultures were collected, and concentrated about 50

times with centriprep concentrators (Amicon, Inc., Beberly, MA). There was no SLF detected in the supernatant when tested by western-blot analysis (data not shown).

Both the membrane-bound and soluble forms of SLF have mitogenic effects on NCC.

One interpretation for melanocyte defect in SLF dickie is that the membrane form is more potent than soluble SLF at maintaining the viability of melanocytes, perhaps by activating the receptor more efficiently and keeping it active longer. To compare the functions of the two forms side by side, we performed a series of *in vitro* co-culture experiments. First, cell proliferation assays were performed to test the possible roles of the two different forms of SLF on NCC proliferation. NCC were co-cultured with CHO/membrane-bound SLF or in the presence of rSLF at 20 ng/ml for 24, 48, or 72 hours. The total number of NCC and NCC which were BrdU positive were counted in randomly selected fields. Results shown in figure 2-2 are the average of four 72 hour co-culture experiments. The data suggest that both the rSLF and membrane-bound SLF expressed by the CHO cells increased the percentage of BrdU positive NCC to a similar degree. Co-cultured NCC for 24 and 48 hours gave similar results (data not shown). This suggests that both forms have mitogenic effects on NCC. rSLF is generally used at 100 ng/ml *in vitro,* thus SLF at as low as 20 ng/ml working as well as the membrane-bound SLF suggests that the membrane form SLF is not more potent that the soluble form.

Soluble form of SLF is responsible for NCC differentiation into melanocytes.

We have previously shown that soluble SLF at high concentration (100 ng/ml) caused over 90% of NCC in culture to differentiate into melanocytes. Here we tested the possible function of membrane-form SLF on NCC differentiation (Fig. 2-3). Generally, melanocytes appear in the NCC culture treated with soluble SLF. However, CHO/membrane SLF failed to promoted melanogenesis in NCC after 6 days of co-culture. On the other hand, soluble SLF at as low as 5 ng/ml induced melanogenesis. With the increase of SLF concentration, the number and proportion of melanocytes in the cultures increased. This suggests that soluble SLF promotes NCC differentiation in a dose-dependent and inductive manner. The membrane form of SLF could not promote melanogenesis either because it did not provide SLF at a concentration high enough, or it requires more than six days to do that. It is clear that the membrane form of SLF is not more potent than the soluble on promoting NCC differentiation. We have shown previously that SLF at 100 ng/ml induces NCC differentiation instead of just supporting the multipotent NCC or melanoblast survival. Western-blot analysis (Figure 2-5) shows that soluble SLF is the prominent form in early stages when melanogenesis occurs. The data presented here

suggest that soluble SLF may be the prominent form responsible for NCC differentiation into melanocytes *in vivo.*

Soluble and membrane-form SLF both support melanocyte survival *in vitro.*

The SLF dickie phenotype in mice suggests that soluble SLF is either unavailable or available but unable to promote melanocyte survival *in vivo.* It is important to distinguish between the absence of soluble SLF for

melanocyte survival and the inability of available SLF to promote the survival melanocytes. To test the different possibilities, we decided to test directly whether soluble SLF can support melanocyte survival. Melanocytes were co-cultured with either rSLF or the CHO cells/membrane-bound SLF for four days, the total number of melanocytes and the number of dead melanocytes were counted daily. Figure 2-4, representing the average results of three experiments, shows that there is a basal level of melanocyte death when melanocytes are cultured in serum-free HyQ-CCM5 or co-cultured with CHO/vector. However, the melanocyte death was reduced when co-cultured with CHO/membrane SLF. Surprisingly, soluble SLF at as low as 20 ng/ml reduced melanocyte death to similar extent. This, and the proliferation assay, rule out the possibility that membrane form is more potent than the soluble SLF in any aspect.

Different forms of SLF are expressed in different tissues at different developmental stages.

The above experiments suggest that different form of SLF have similar effects on NCC proliferation and melanocyte survival *in vitro*. The similar functions of two forms of SLF implies that if the soluble form of SLF were available in the skin at later stages of development as assumed, the soluble SLF would be able to support the survival of melanocytes. This directed us to test the availability of soluble SLF by studying the expression of the two forms in various tissues at different developmental stages, especially at later stages. We studied the expression of SLF in different tissues at different developmental stages via western-blot. Figure

2-5 shows the expression of SLF in whole embryo tissues at early stages, and in skin and brain at later stages. In early stages from E2 to E8 SLF is the major form in the whole embryo. This is the period critical for NCC migration and melanogenesis. From E8 to E12 in the skin, we found that the soluble form is the most prominent. However, at E13 the level of the membrane form is much higher and becomes the prominent form from E14 on. Soluble SLF becomes undetectable after E13. In the brain, however, the soluble form is the major form from early stages to E16. This suggests that soluble SLF is not available in skin at later stages of development and therefore can not support the survival of melanocytes. This also suggests that the two forms of SLF are expressed in a variety of tissues at different stages and perhaps the two forms of SLF may perform distinct functions.

Melanocytes express the membrane-form SLF and use it in an autocrine fashion for their survival.

Our western-blot analysis here, and melanocytes defects in SLF dickie mutant suggest that membrane-bound form SLF is the prominent form in skin available for melanocyte survival. We have previously shown that multipotent NCC express both SLF and its receptor c-kit and SLF may work in an autocrine manner on NCC development. It is likely that melanocytes, which are differentiated from NCC, are still expressing SLF and use the SLF on cell surface to maintain their viability. To test this hypothesis, we performed immunocytochemistry studies under non-permeablizing conditions to determine whether melanocytes indeed express membrane SLF.

Figure 2-6 shows immunoreactivity of cultured melanocytes with anti-SLF antibody (Fig. 2-6a). This suggests that melanocytes *in vitro* are

expressing SLF on their surface. To see whether melanocytes express membrane SLF *in vivo*, we performed ICC on E14 sections. According to Western-blot data, membrane-bound form SLF is the major form in skin at this stage, therefore the background should be very low, and the cells which are reactive to anti-SLF antibody should be the cells expressing membrane-bound SLF. Since melanocytes account for less that 1 % of total cells in skin, we expect to see scattered signals if melanocytes are the source of SLF. Indeed, we observed that melanocytes in the hair follicles are positive when probed with ant-SLF antibody, and the SLF signals are scattered (Fig. 2-6b). This suggest that at this stage melanocytes express SLF, perhaps the membrane form *in vivo* as well. The data we present in this study and melanocyte defects in SLF dickie mutant suggest that melanocytes express membrane SLF and use it in an autocrine fashion for their survival via direct contact. Figure 2-6c shows that morphology and distribution of melanocytes in vivo make the direct cell-cell contact possible.

Membrane-SLF is sufficient for melanocyte survival.

To demonstrate that SLF signal is required for melanocyte survival, melanocytes were treated with a neutralizing anti-SLF antibody at 1 : 100, c-kit inhibitor at 10 uM, or a protease inhibitor for SLF cleavage at 10 uM (figure 2-7a). When melanocytes were cultured in serum-free medium HyQ-CCM5 for four days, there was about 20% cell death. Anti-SLF antibody and c-kit inhibitor both increase cell death to about 60%. However, the protease inhibitor which blocks the cleavage of SLF did not change the basal level of cell death. This suggests that the SLF signal is required for melanocyte survival and that the membrane form is sufficient for the basal level of survival. To further demonstrate that membrane-SLF

is responsible for the basal level of melanocyte survival *in vitro,* we cultured the melanocytes at a lower density in HyQ-CCM5, and compared the cell death of melanocytes which are associated with other cells and with that of the single cells which are not in contact with other cells. Cell death for single cells is about twice as high as in cells which are associated with others(Fig. 2-7b). This suggests that melanocytes associated with others may survive through signals provided by membrane SLF due to direct contact.

DISCUSSION.

Hydrophilicity analysis of the deduced amino acid sequence of SLF suggests that it is a membrane bound protein with a transmembrane domain (Anderson et al., 1990). However, SLF has been assumed to be a growth factor which is secreted and soluble. The soluble form of SLF has been purified as a natural protein from tissues, used to test the biological functions of this growth factor *in vitro*, and considered an active form *in vivo*. However, several groups have suggested that the membrane-form of SLF may have some functions as well. Anderson et al. proposed that SLF, both in soluble and membrane-bound form, is active based on their mast cell proliferation assay. The analysis of SLF dickie mutants by Steel et al. in 1992 and Wehrle-Haller et al. in 1995 provided strong *in vivo* evidence that membrane SLF may be active *in vivo* as well. Using a probe for TRP-2 mRNA, a marker for early melanocyte precursors, Steel et al. and Wehrle-Haller et al. performed in-situ hybridization studies on SLF dickie mutant mice. They both observed TRP-2 positive cells at about E11, and noticed that the number of these cells decreased after E12. These data suggest that NCC in the

SLF dickie mutant mice migrate to the right targets, differentiate into melanocytes perhaps through the soluble SLF, but either fail to mature, or mature but do not survive due to a lack of membrane-bound SLF.

While these studies established a role for the membrane-bound form of SLF in melanocyte survival, they provided no clue as to why this form is required for survival even though the mutant mice still produce the soluble SLF. There are several ways to interpret the melanocyte defect in SLF dickie mutant mice. First, although one would assume that a soluble protein like SLF can diffuse to many places including the skin where many melanocytes are located, the actual presence of soluble SLF has not been observed. Thus, the soluble form of SLF may not be present in the skin at later stages of development for melanocytes survival. Second, the membrane form may be somehow more biologically potent, and hence could support melanocyte survival longer. In this case, the functions of membrane-bound SLF could not be replaced by the soluble form even it were available. Third, the survival of melanocytes may require both forms of SLF to work at the same time in a synergetic manner.

To check the expression pattern of SLF in various tissues at different stages, we performed Western-blot analysis. We found that soluble SLF is the major form in the whole embryos in early stages from E2 to E8, when NCC are migrating into their targets and undergoing melanogenesis. The SLF dickie phenotype suggests that melanocyte dependence on membrane-bound SLF may occur at very late stages of development. Our western-blot analysis on skin tissues at later stages demonstrated that membrane-bound SLF becomes prominent after E13. This suggests that the soluble SLF is not available in the skin at later stages of

development, and that the membrane form is the major form used in the skin for melanocyte survival.

To find out whether the membrane form is more potent than the soluble SLF, we compared the biological activities of the two forms on NCC proliferation and melanocyte survival. Our co-culture studies showed that both the soluble and membrane forms of SLF increased NCC proliferation to similar degrees. Next, we compared the effect of the two forms on the survival of melanocytes directly by culturing the melanocytes either with soluble SLF or CHO cells expressing the membrane bound SLF. We found that soluble form at as low as 20 ng/ml, reduced melanocyte death as effectively as the membrane SLF. These results suggest that membrane and soluble SLF have similar functions in general and that the membrane form is not a more potent promoter of melanocyte survival. Based on the Western-blot analysis, the timing of NCC migration and differentiation, and the phenotypes of the SLF dickie mutants, we propose that the soluble form is critical for NCC proliferation, migration, and differentiation at early stages, whereas the membrane form is responsible for the survival of melanoblasts or melanocytes later in development.

We previously showed that soluble SLF at high concentrations caused over 90% of NCC to differentiate into melanocytes at the end of 14 days of culture (Guo et.al., 1997). Our data suggested that soluble SLF is a differentiation factor and induce the fate of multipotent NCC, instead of being only a survival factor for them. This notion is consistent with our Western-blot results in this study, which shows that the soluble form of SLF is the dominant form at early stages when NCC are undergoing differentiation into melanocytes. To further demonstrate the role of SLF on melanogenesis and test the possible involvement

of membrane SLF in this process, we co-cultured NCC with CHO cells expressing membrane bound SLF, or soluble SLF at different concentrations. We found that soluble SLF promoted NCC differentiation into melanocytes in a dose-dependent manner, while membrane SLF expressed by CHO cells showed no effects on NCC when co-cultured for 6 days. Considering the fact that we could apply CHO cells at certain densities, and that CHO cells cultured under our experimental conditions became difficult to adhere to the surface of plates after four days in the co-culture, our negative results with membrane bound SLF can not exclusively rule out the possibility of involvement of this form *in vivo* in melanonegesis.

Several groups have suggested that SLF is a survival factor for melanocyte precursors based on their *in vitro* culture studies (Murphy et.al., 1992; Morrison-Graham et.al., 1993; Reid et al., 1995.). On the other hand, it has been demonstrated by many groups that SLF has mitogenic effect on NCC and melanocyte precursors. Unless clonal analysis is performed in a serum-free medium, it is hard to distinguish SLF's roles on proliferation and survival on NCC and melanocytes. We previously showed that melanocyte precursors could survive in serum-free medium without exogenous SLF for five days, and that melanogenesis still occurred with only two days delay after NCC were cultured in the presence of anti-SLF antibody in the medium for five days (Guo et.al., 1997). Analysis of SLF dickie mutant suggests that SLF is required at later stages of development for mature melanocyte survival. This notion is also supported by experiments performed by Nishikawa et.al. (1991). In their studies, an anti-c-kit antibody was injected into wild type adult mice, and coat color defects were observed in the injected mice. However, more direct evidence that SLF is required for the survival of mature melanocytes is lacking. We took the

advantage of the serum- and chicken-embryo-extract-free medium we established and tested the function of SLF on the survival of melanocytes. We found that most melanocytes can survive for four days in the medium. The addition of anti-SLF antibody and c-kit inhibitor each increased melanocyte death significantly, while the inhibitor for SLF cleavage had no effect. This argues that SLF is required for melanocyte survival, and that the membrane bound SLF is sufficient for the basal level of viability of melanocytes. To further demonstrate that membrane bound SLF is sufficient for melanocyte survival, we compared the cell death of melanocytes which were associated with others and those which were isolated in the culture. At the end of four days, the percentage of cell death for the single melanocytes were about twice that of the cells which are associated with others.

We have previously shown that NCC express both SLF and its receptor and SLF may work in an autocrine fashion(Guo et al., 1997). The conclusions, based on this study and SLF dickie mutant analysis, that membrane bound SLF is required for melanocyte survival, raises the question of whether melanocytes survive through autocrine action of membrane SLF. To test the possible autocrine action of membrane bound SLF on melanocytes survival, we first checked the expression of SLF by melanocytes *in vitro*. ICC on melanocytes under non-permeablized conditions demonstrated that melanocytes express membrane SLF on the cell surface. We observed that the staining of SLF antibody is in a clustered pattern with stronger labeling at the tips of processes in many cells. We next performed ICC on E14 sections to study the possible expression of SLF by melanocytes in *vivo*. According to our Western-blot data, membrane bound SLF is the dominant form at this stage in skin, while the soluble

SLF is not detected. This means that the signal we can detect in skin tissues of E14 sections should come from cells expressing membrane SLF. ICC showed that melanocytes, especially those in the hair follicles, are immunoreactive to anti-SLF antibody. Although melanocytes account for less than one percent of total cells in skin, we noticed that distribution and morphology of melanocytes provide their ability to contact each other directly. We propose that membrane bound SLF works in an autocrine fashion, through direct contact via their process to maintain the viability of melanocytes, and that lack of membrane SLF in the SLF dickie mice results in the failure of the direct interactions between SLF and its receptor.

Most growth factors, which are well characterized and familiar to us, are secreted and soluble. Two classic examples are nerve growth factor and epidermal growth factor. Many soluble growth factors have been produced and applied to both *in vitro* and *in vivo* studies; however, exceptions were found when the fibroblast growth factors (FGF) were cloned and characterized. Structure analysis revealed that members of this growth factor family have no classical signal sequence for secretion (Mason, 1994; Miyamoto, 1993; Abraham,1986; Eckenstein et al., 1993). Over the years, there has been severe debate on whether FGFs are actually secreted. Members of the Delta (Struhl et al., 1998; Schroeter et al., 1998), which play important roles in neuronal development, are transmembrane proteins and have not been found to be cleaved or released as soluble molecules. Recent studies on semaphorins, a group of proteins involved in axon guidance, found that some of these molecules are secreted, while others are membrane bound (Tessier-Lavigne and Goodman, 1996). Studies by several groups suggest that both soluble and membrane semaphorins play critical roles in axon guidance (Yu et. al., 1998; Matthes et.al.,

1995; Puschel et.al.). Our data provide the first example of a growth factor with a transmembrane domain, which is both membrane associated and soluble, is active in both forms at different developmental stages. The fact that the membrane form of SLF is used, perhaps in an autocrine fashion, for melanocyte survival is quite interesting. We believe that this autocrine action of membrane SLF occurs through direct contacts between melanocytes, and has at least two advantages over the soluble form acting in paracrine fashion. First, the autocrine-like activity could be more efficient, in that the protease or proteases which cleave the SLF do not need to be active and SLF can be locally concentrated while expressed at low levels. Second, this may provide specificity to the SLF and c-kit interactions in certain tissues at certain stages in the development.

FIGURE LEGENDS

Fig. 2-1. Characterization of SLF mutant expressed in CHO cells. 2-1a) shows that the cleavage site VAAS in the wild type was mutated in the membrane bound SLF to VSSS by PCR. 2-1b) Western-blot results of whole CHO cell lysates: lane 1) CHO/membrane-bound SLF, lane 2) CHO/wild type SLF, lane 3) CHO/vector, lane 4) CHO parental cell lysates. CHO cells expressing membrane SLF shows a protein at about 31 KDa, the predicted size of the mature SLF molecule (arrow), which is also expressed by the CHO cells with wild type SLF. 2-1c) ICC results on non-permeablized CHO cells. CHO cells/membrane-bound SLF on the left are positive to anti-SLF antibody, the CHO cells/vector control on the right are negative.

Fig. 2-2. SLF increases NCC proliferation. BrdU incorporation assay on NCC co-cultured for 72 hours with membrane or soluble SLF. rSLF at 20 ng/ml (bar 2) and SLF mutant (bar 4) both increased the percentage of BrdU positive NCC over control: HyQ-CCM5 control (bar 1), vector control (bar 3).

Fig. 2-3. Melanogenesis assay. Soluble SLF promotes melanogenesis in a dose-dependent manner, while membrane SLF has no effect. Shown here are the average of three experiments. The Y axis is the average number of melanocytes in each culture at the end of six days co-culture. Bars 1 through 8 respectively are: CHO/vector control, CHO/membrane-bound SLF, HyQ-CCM5 control, 5, 10, 20, 40, and 80 ng/ml rSLF in HyQ-CCM5.

Fig. 2-4. Soluble SLF supports melanocyte survival as effectively as the membrane form. Shown here are the average of 3 experiments. For each experiment, the total number of live and dead melanocytes were counted daily for four days. Each condition had about nine individual cultures. About 20% of melanocytes cultured in HyQ-CCM5 or with CHO/vector are dead. However, CHO cells expressing membrane SLF and rSLF at 20 ng/ml reduced the melanocytes death to about 10 %.

Figure 2-5. SLF expression in different tissues at different stages. The antibody used was anti-SLF at 1:2000. The lower band at 23 KDa (arrow

heads) is the soluble form of SLF, the bands at about 40 KDa (arrows) represent the membrane forms. Lane 1, E10 brain, lane 2, E10 skin, lane 3, E12 brain, lane 4, E12 skin, lane 5, E14 brain, lane 6, E14 skin, lane 7, E14 skin prepared without the presence of protease inhibitors, lane 8, E15 brain, lane 9, E15 skin.

Figure 2-6. Melanocytes express the membrane-form of SLF *in vitro* and *in vivo*.
2-6a shows ICC on cultured melanocytes under non-permeablizing conditions. 1) SLF antibody probed cells are positive. 2) chicken IgY negative control. In E14 sections (2-6b), melanocytes at the edges of hair follicles are SLF positive. 1) SLF antibody probed E14 sections, 2) normal IgY control. 2-6c shows the distribution of melanocytes *in vivo*.

Figure 2-7. Membrane SLF is sufficient for melanocyte survival. 2-7a) Melanocyte death increases when cells are treated with an anti-SLF antibody or c-kit inhibitor. The inhibitor for SLF cleavage did not affect the cell death.
2-7b) Cell death in cell-cell associated melanocytes is one half the level of isolated melanocytes.

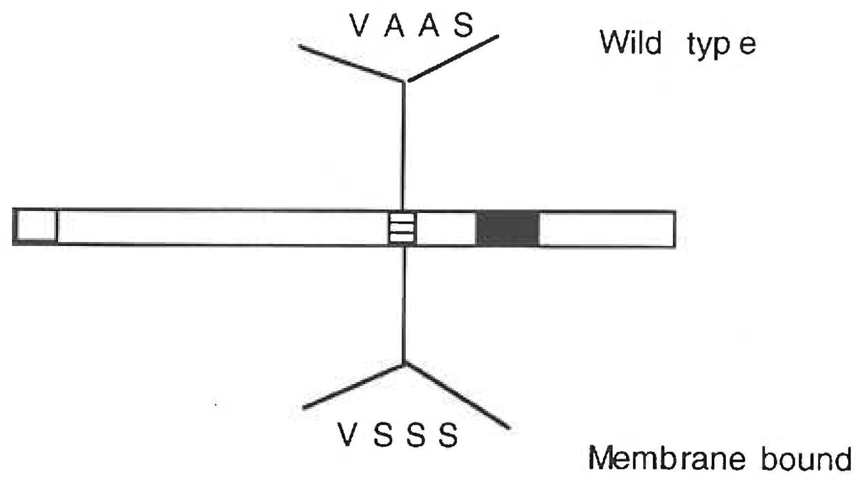


Figure 2-1a. Primary structure of SLF. ▭ , signal sequence; ■ , transmembrane domain; ☰, preteinase cleavage site.

Figure 2-1b. Western-blot analysis of CHO cell lines

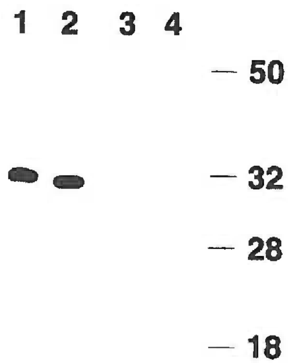
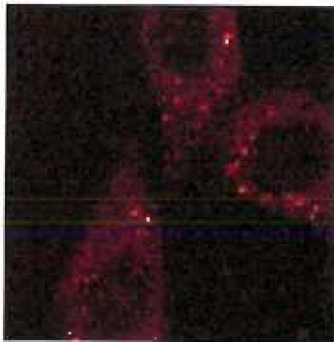
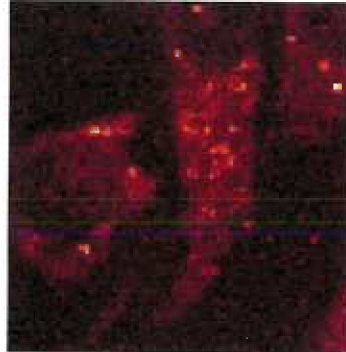


Fig. 2-1c Immunocytochemistry analysis of CHO cell lines

2-1c1



2-1c2



2-1c3

Figure 2-2. SLF promote NCC proliferation

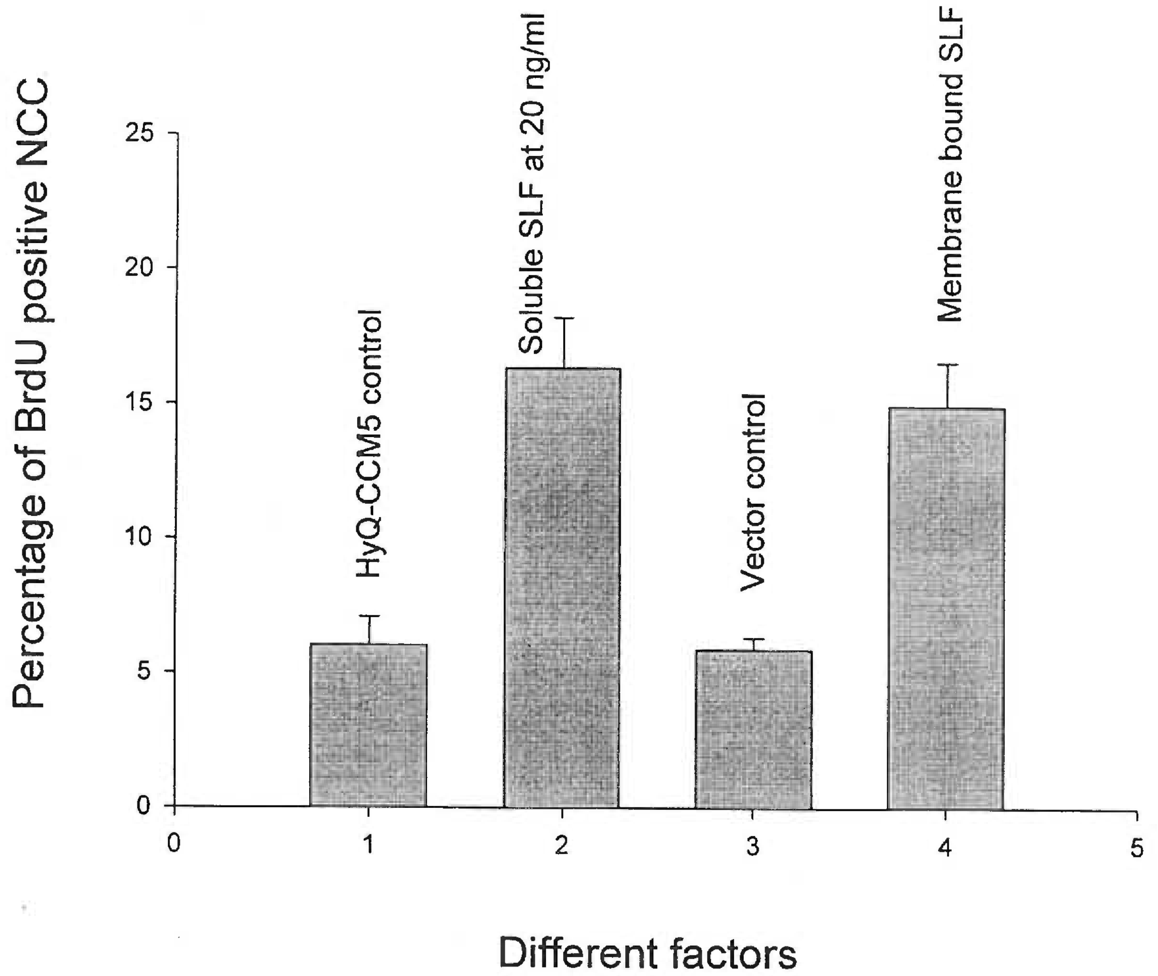


Figure 2-3. SLF induced melanogenesis

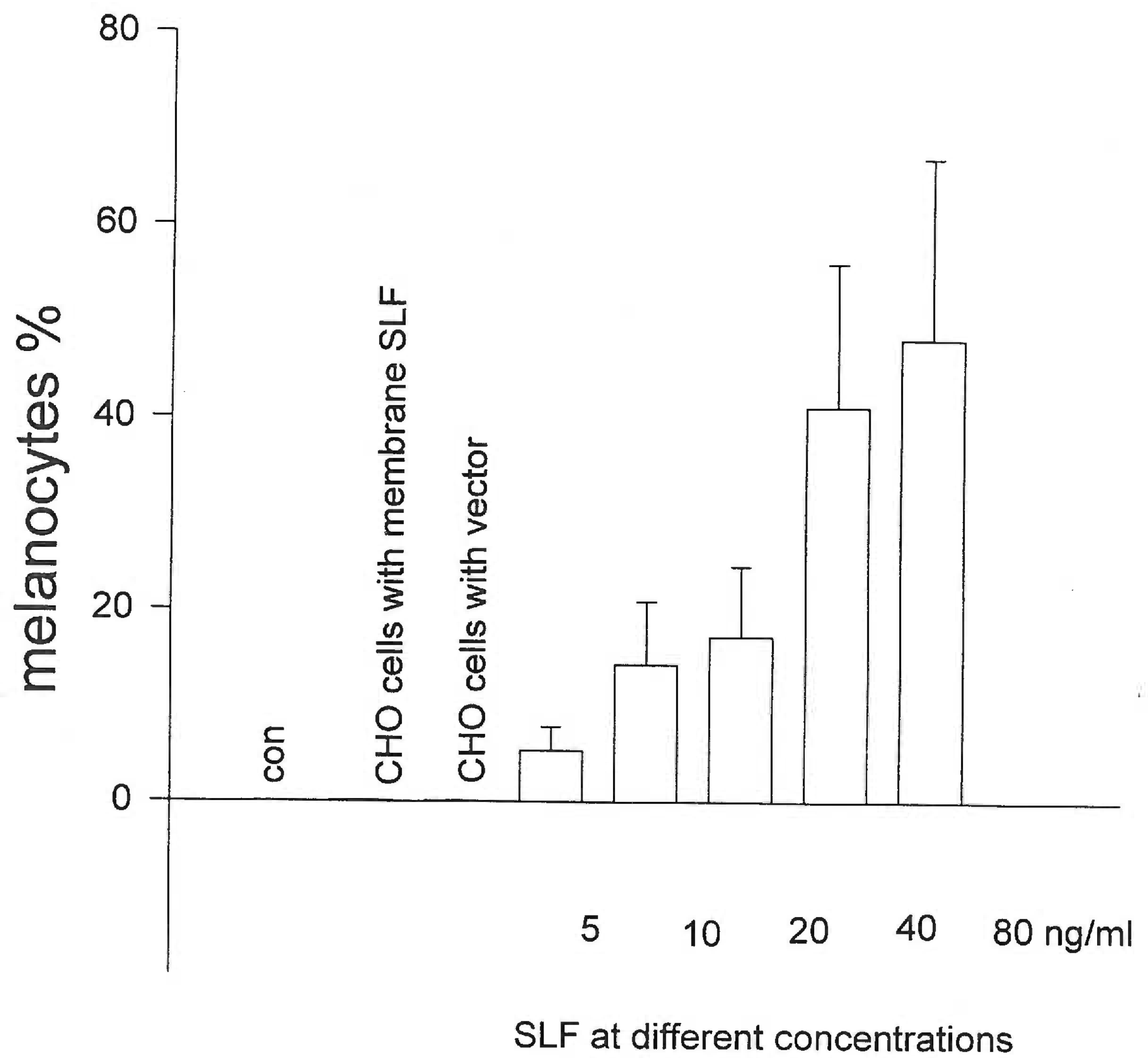


Figure 2-4.

Soluble SLF support melanocytes survival in vitro

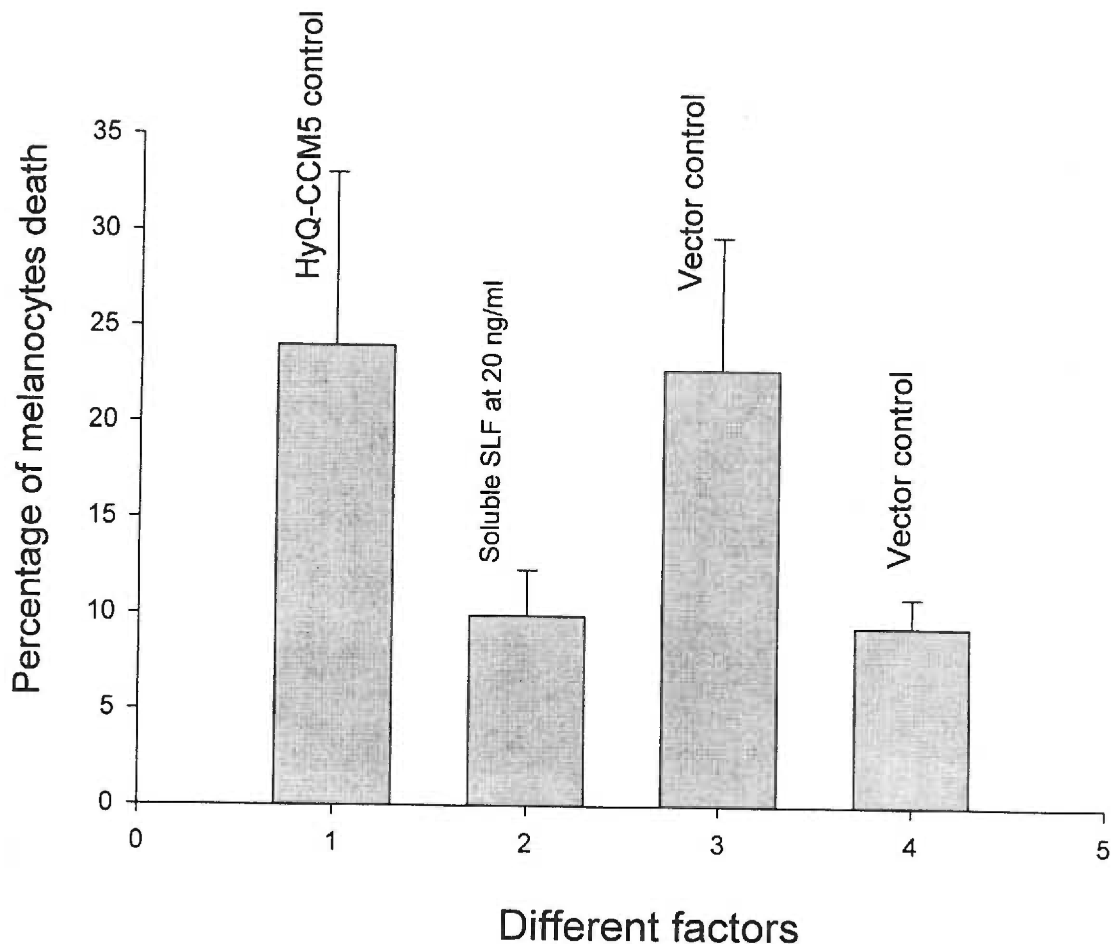


Figure 2-5. Expression of two forms of SLF in different tissues

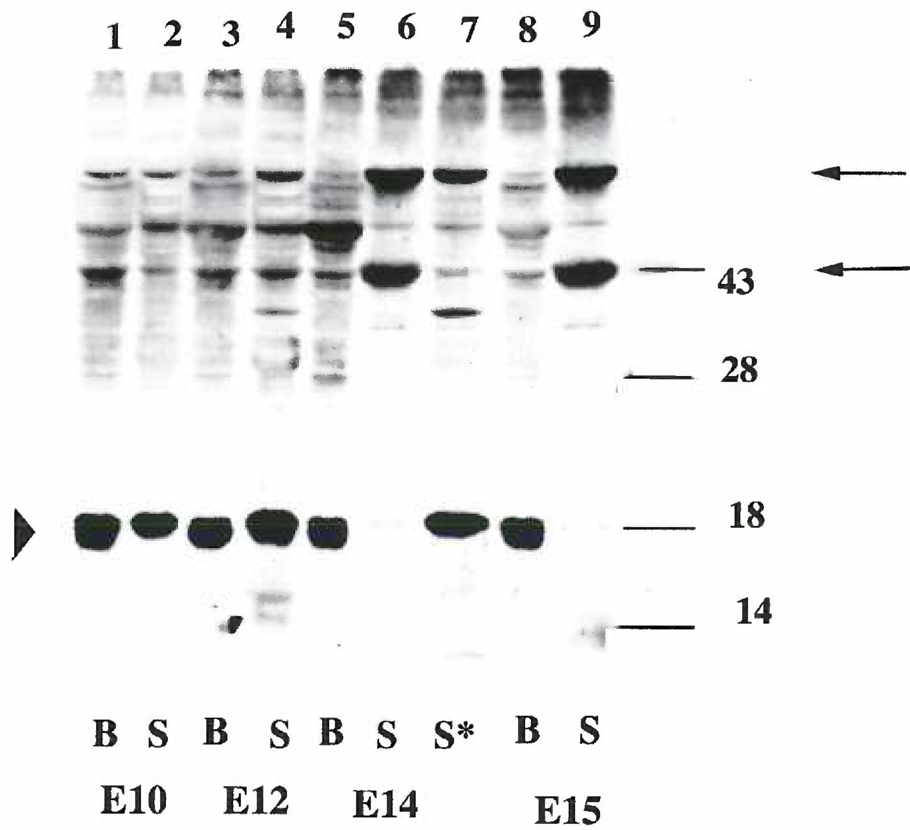
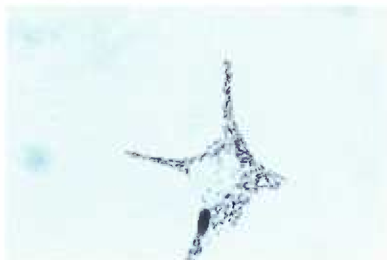


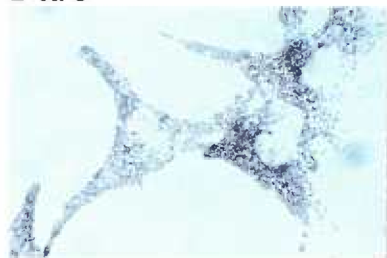
Fig. 2-6a ICC analysis on melanocytes in vitro

2-6a-1



2-6a-2

2-6a-3



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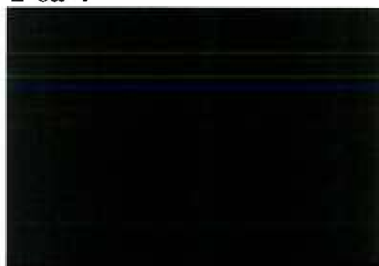
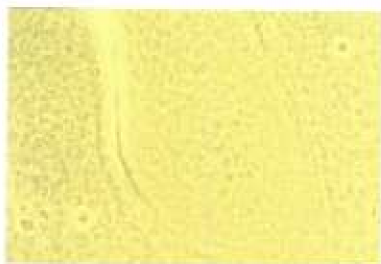


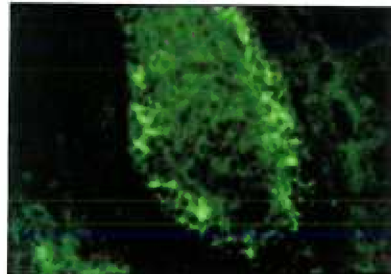
Fig. 2-6b ICC analysis on melanocytes in vivo

2-6b-1



2-6b-2

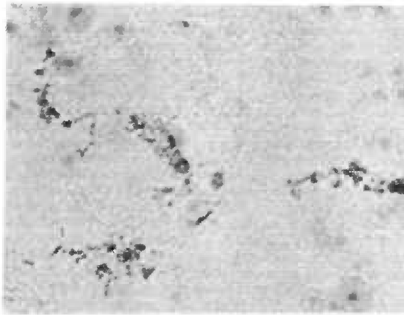
2-6b-3



2-6b-4

Figure 2-6c. Melanocytes distribution in vivo

6c1



6c2

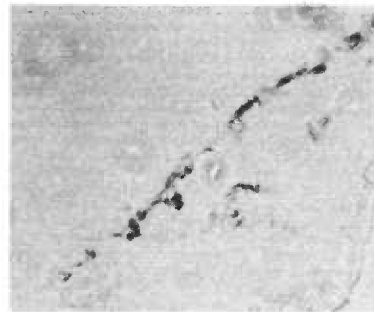


Figure 2-7a. SLF is required for melanocytes survival.

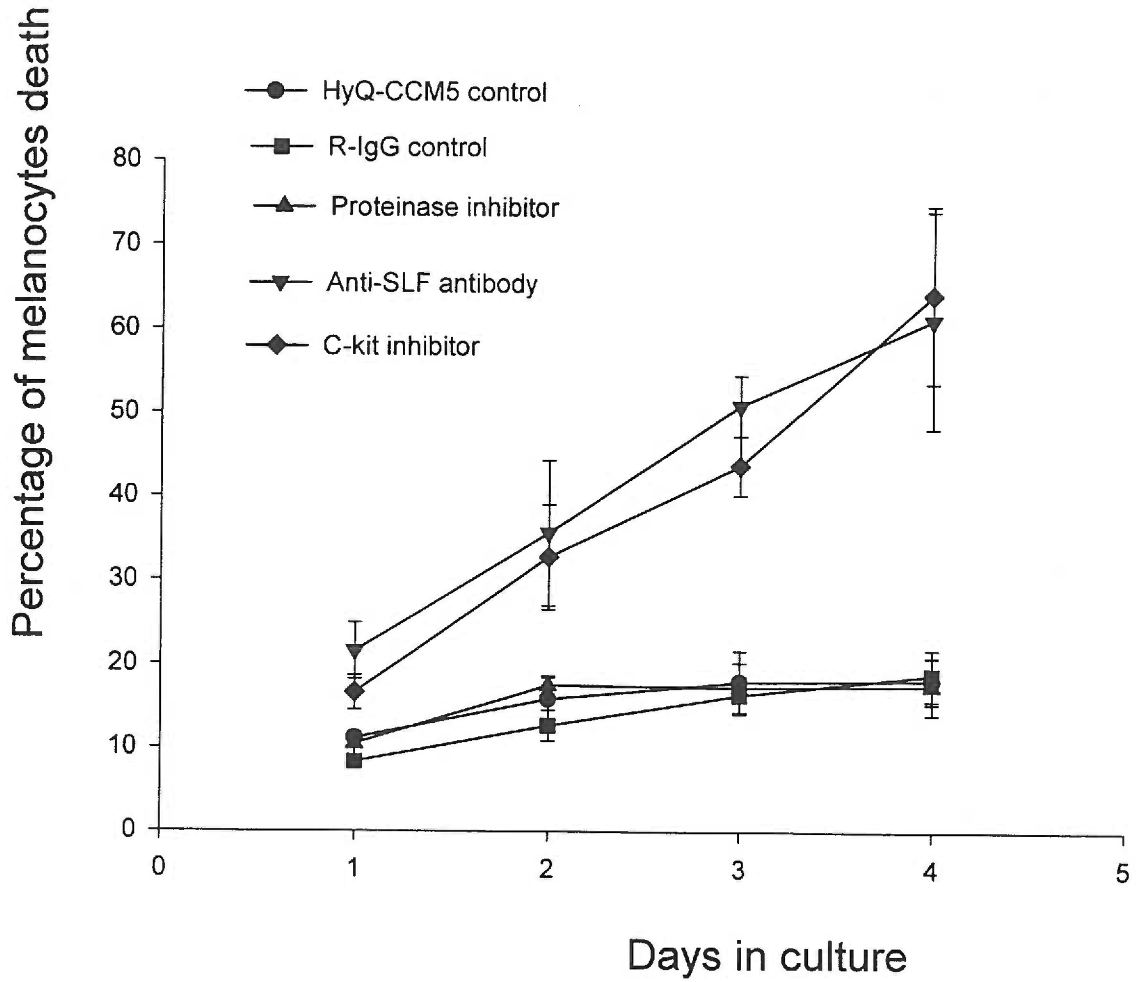
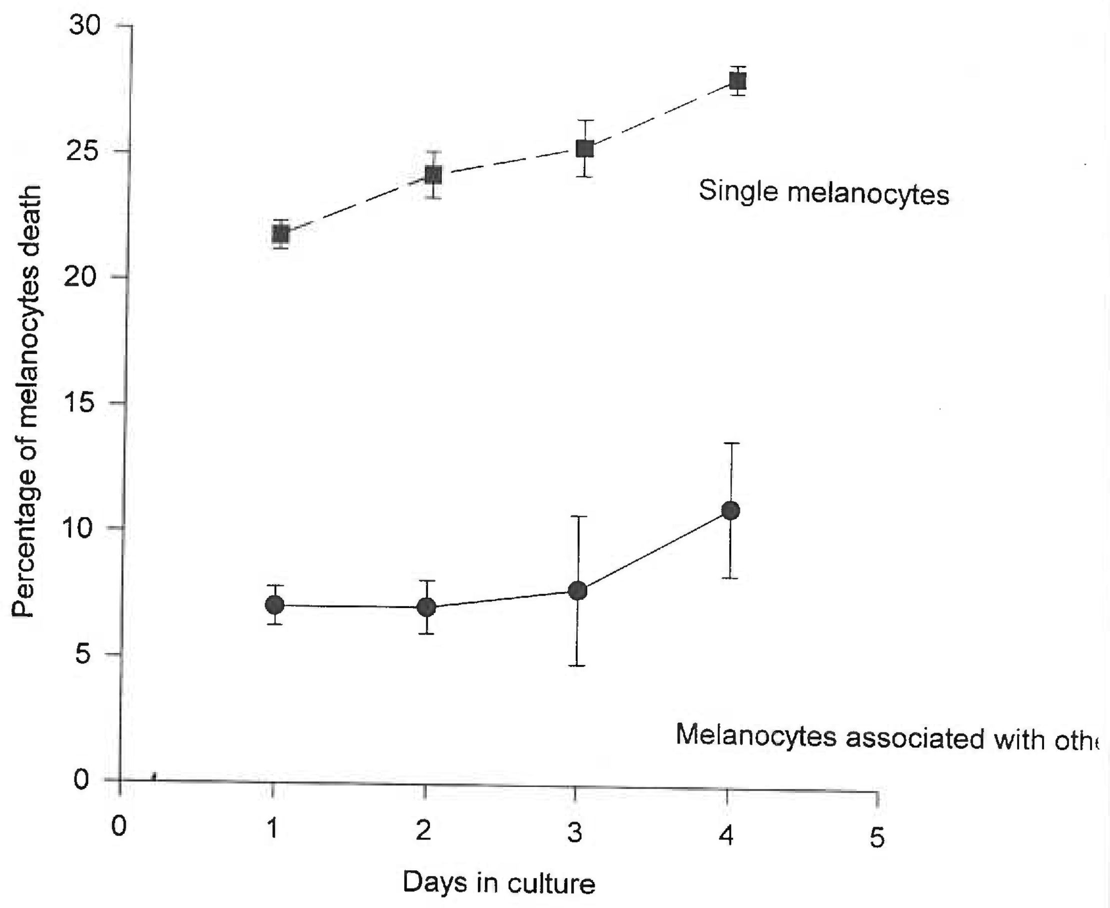


Figure 2-7b. Membrane SLF is sufficient for melanocytes survival



CHAPTER IV--Discussion.

Summary of experimental results.

This dissertation represents my studies of the biological functions of SLF in NCC development. I analyzed the expression of SLF and its receptor in different tissues at different developmental stages utilizing antibodies generated from rabbits and chickens. These analysis showed that the temporal and spatial expressions of SLF and its receptor c-kit are consistent with their roles in NCC development. I demonstrated that NCC express both c-kit and SLF, and that SLF may work in an autocrine fashion. The expression of SLF by NCC was demonstrated *in vitro* with immunocytochemistry and RT-PCR on cultured NCC. The SLF expression by NCC was also demonstrated *in vivo* by in-situ hybridization on E4 embryo sections. The data in this project suggest that SLF is inductive to NCC development, promoting their differentiation into melanocytes. The SLF effect on NCC differentiation is blocked when a neutralizing antibody against SLF is added. The fact that NCC express SLF, a key regulator of their development, indicate that NC cells play an active role in determining their cell fates and suggest a different developmental role for the embryonic microenvironment than what has been previously proposed. SLF and c-kit were found to be expressed in many other tissues in addition to the migration pathways and targets of NCC and their derivatives, suggesting that SLF is involved in the development of many different cell types.

Western-blot analysis revealed that soluble SLF is the major form in the very early embryo. At later stages, soluble SLF is still the prominent form in brain

and muscle, while membrane SLF becomes the dominant form in skin. Western-blot data directed us to study the different roles of different forms of SLF at different developmental stages. To study the biological functions of SLF *in vitro*, I established a novel serum and chicken embryo extract-free medium for culturing NCC and melanocytes. This culture system allowed us to study the roles of SLF in NCC development in a sensitive and less complex background. To study the function of different forms of SLF, I generated CHO cell lines which stably expressed a mutant form of SLF which can not be cleaved and therefore remained associated with the cell surface. I also applied an inhibitor for SLF cleavage and an inhibitor for c-kit kinase activity to dissect the differential roles of soluble/released and membrane associated forms of SLF. I also demonstrated that soluble SLF promoted NCC differentiation into melanocytes in a dose-dependent manner, while the membrane bound SLF expressed by CHO cells had no effect. We showed that SLF is required for the survival of mature melanocytes, but not their precursors. We provided evidence that melanocytes use membrane SLF in an autocrine fashion for their survival. ICC analysis on melanocytes under non-permeablizing conditions showed that melanocytes express SLF on their surfaces. ICC on E14 sections of quail demonstrated that melanocytes express membrane SLF *in vivo* as well. Melanocyte survival assay demonstrated that SLF is required for the survival of melanocytes, and that membrane SLF is sufficient for the basal level of survival of cultured melanocytes. Western-blot analysis on tissues, ICC *in vitro* and *in vivo*, phenotype of SLF dickie mutant, and our melanocyte survival test all support

the notion that melanocytes survive through autocrine action of membrane SLF via direct cell-cell contacts.

NCC play active roles in development.

In the present project, the source of SLF and the possible autocrine regulation of SLF on NCC development was explored. SLF was purified as a soluble protein and has been assumed to work in the soluble form in a paracrine fashion on NCC. However, there is some evidence suggesting that SLF might work in an autocrine manner on NCC and their derivatives. Weston's laboratory found that NCC formed clusters and some cells in the clusters differentiated into melanocyte. If the clusters were dispersed, however, the melanogenesis decreased. This implies that there might be some interactions among NCC themselves which are partially responsible for the melanogenesis. According to Weston, NCC migrating along lateral pathways form clusters at the melanocyte staging area and the close association is critical for melanogenesis *in vivo*. However, Weston did not identify the endogenous factor or factors in the NCC. Here, we provide evidence that NCC express SLF *in vivo* and *in vitro*. We showed that cultured NCC are immunoreactive to anti-SLF antibodies. RT-PCR on cultured NCC produced similar conclusions. We also detected SLF signal at the melanocyte staging area at E4. Our data suggest that SLF may be the factor or one of the factors which influenced melanogenesis in these *in vitro* experiments. Erickson et al. showed that NCC migrate along the lateral path only if they are specified and pre-determined to become melanocytes. It is possible that the interactions among NCC at the melanocyte staging area through SLF are responsible for

the specification and predetermination. Another piece of evidence implying SLF works in an autocrine fashion is the SLF dickie mice. These mice produce a truncated, soluble SLF which does not have the transmembrane and cytoplasmic portions. This truncated form is active when tested *in vitro* on mast cell proliferation assay and promote initial dispersion and differentiation of NCC into melanocytes in the mutant embryos. The homozygous mice have melanoblast detectable at early stages but show no coat pigmentation in the adult. This suggests the possible autocrine interactions among melanocytes through membrane SLF is required in the wild type mice for melanocyte survival.

To test the notion that SLF may work in an autocrine fashion for melanocyte survival, I performed immunocytochemistry on melanocytes under non-permeablizing conditions and showed that melanocytes express SLF on the cell surface. This is consistent with our Western-blot analysis, which demonstrated that the membrane-bound form is the major form of SLF in skin at the later stages in development. Our data suggest that SLF may work in an autocrine fashion for the survival of melanocytes in the skin.

Growth factors which are important for NCC fates used to be found only in the environment. The fact that NCC themselves express SLF, a key regulator in the development of NCC, really changes our view on the roles the environment play during NCC development. Presumably, some environmental factors favor the expression of SLF. So the environment can influence the development through regulating the expression of determinative factors expressed by the multipotent cells.

SLF is inductive to NCC differentiation into melanocytes.

SLF has been claimed as only a survival factor for NCC and melanocytes. The data presented in this study suggest that SLF plays instructive and multiple roles on the development of NCC. Mutants of SLF and c-kit in mice and human suggest that SLF is required either for melanogenesis or for melanocyte survival, or both. SLF may act in the following ways in melanogenesis: 1) SLF acts in an inductive manner to induce melanogenesis in cells that would otherwise not differentiate into melanocytes; 2) SLF acts to promote the survival of pre-committed, but latent pre-melanocytes within heterogeneous NCC populations; 3) SLF acts as a selective mitogen specifically for latent pre-melanocytes, allowing these cells to proliferate significantly; 4) it may involve a combination of the above possibilities.

Many studies suggested that SLF is only a survival factor for melanoblast, and that SLF can not change the fates of multipotent NCC.
Reid et al.(1995) suggested that SLF worked as a survival and proliferative factor for c-kit positive cells in the NCC. Based on studies on cultured NCC, Murphy et al. proposed (1992) that "SLF is required for maintenance, but not differentiation, of melanocyte precursors in the NC." However, TPA and 10% serum were used in both studies. We found that either TPA or serum alone could induce melanogenesis in NCC cultures. TPA is not a molecule that exists in living cells and its effects may not reflect the physiological cellular response to endogenous molecules. The presence of TPA and serum in this system creates a high basal level of melanogenesis, making it insensitive and complex to study the effects of SLF.

We used the serum-free medium HyQ-CCM5 to study the possible role of SLF in melanogenesis. We performed the experiments in the absence of TPA, chicken embryo extracts and serum. Under our conditions, NCC migrated from neural tube, and attached to the plate well, with morphology similar to those cultures in serum containing medium. The basal level of melanogenesis was very low (less than 0.1% of total cells, less than 1 melanocyte each NT). We demonstrated that about 90% of cells in the NCC cultures were melanocytes at the end of 14 days treatment with SLF at 100 ng/ml. We found that SLF induced melanogenesis of NCC in a dose dependent manner. Our data clearly demonstrate that SLF is an inductive factor on the differentiation of NCC into melanocytes. Our data is consistent with results from Le Douarin's laboratory also using quail NCC. They found that SLF could enhance the differentiation rate of melanocyte precursors in NCC cultures. The different conclusions from different labs can be due to one or a few of the following factors: 1) there might be some difference between mice and chicken, for example SLF might have different roles in the two systems; 2) the mice embryos used may be older, so the NCC used were more restricted in development; 3) the high background and complex mediums in these experiments made it difficult to observe the effects of SLF on melanogenesis.

SLF is not required for the survival of melanocyte precursors.

We performed a series of experiments to test directly whether SLF is required for the survival of NCC and melanocyte precursors. In one set of experiments, we cultured NCC in defined medium in the presence or absence

of SLF for five days, and then switched to medium with SLF for the rest of experiment. Presumably, if SLF was a survival factor for melanoblasts and NCC, the absence of SLF during the first 5-day period should result in dramatic and irreversible loss of melanocyte precursors. However, the lack of SLF for 5 days only caused a 2-day delay of melanogenesis in these experiments. Generally, it takes four days under our culture conditions to observe appearance of melanocytes. The 2 day delay of the onset of pigmentation suggests that these melanocytes came from the melanoblasts which survived the 5 days culture in the absence of SLF, and that these cells did not come from a population which would normally become other cells. To make sure that these melanocytes which survived the 5 day culture without exogenous SLF did not utilize the low level of SLF released by NCC or membrane SLF for their survival, we cultured NCC in the serum-free medium in the presence of anti-SLF antibodies for the initial 5 days, then switched to mediums with SLF. Again, the presence of anti-SLF antibody caused only 2 day delay of the onset of melanogenesis. Our data suggest that SLF is not required for NCC and melanoblasts survival. Our result is consistent with data from Maya Sieber-Blum's lab (1995). Langtimm-Sedlak et al. studied the influence of SLF on NCC development by an *in vitro* colony assay in a serum-free culture medium. They found that SLF is trophic for early NCC only if SLF is present throughout the culture period, and it was not observed when a neurotrophin was present in addition to SLF. They also observed that the combination of SLF and any neurotrophin tested (NGF, BDNF, NT-3) is trophic for melanogenic cells, whereas SLF alone does not affect melanogenesis. They suggested that melanogenic cells become dependent on

neurotrophins only after exposure to SLF. We found that NCC cultured in the presence of bFGF grew very well, proliferated robustly, and differentiated into melanocytes. This suggest that at least bFGF can keep NCC and melanoblast alive and that SLF is not required for their survival. Multipotent NCC may be able to respond to a number of the many growth factors expressed in early stages of development. It is unlikely that NC cells would depend solely on SLF for their survival, because NCC can response to many growth factors.

SLF acts as a survival factor for melanocytes.

The potential role of SLF on melanocyte survival was studied in this thesis project. In-situ hybridization analysis of SLF dickie mice revealed that melanoblasts were detected in the early stages of development in the skin, but the adult mice lost coat pigmentation, suggesting that SLF is required to maintain the viability of melanocytes. Nishikawa (1991) reported that injection of the antibody ACK2 to adult, normally pigmented wild-type mice resulted in partial loss of coat color. These experiments indicate that SLF/c-kit interactions are required to maintain the melanocyte survival both in early development and the adult. We demonstrated that most melanocytes could survive in the defined medium. Antibody against SLF and a kit inhibitor increased the death of melanocytes, while the inhibitor for SLF cleavage did not affect the basal survival of melanocytes. The melanocyte death rate was reduced when co-cultured with CHO cells expressing membrane-bound form of SLF or cultured in the presence of recombinant SLF. We also showed that

melanocytes which are associated with others in the defined medium had a lower cell death rate compared with that of isolated, single melanocytes. These data suggest that SLF is required for melanocyte survival, and that the membrane form of SLF is sufficient for the basal level of melanocytes survival *in vitro.*

We propose that melanocyte survival is mediated by interactions between membrane SLF and c-kit via direct cell-cell contact among melanocytes. Western-blot analysis of quail skin and brain tissues showed that soluble SLF is the dominant in brain tissues from early stages to E16. In the skin, the membrane bound form of SLF become dominant at later stages from E14. ICC on cultured melanocytes suggest that SLF is expressed on the cell surfaces of melanocytes. On E14 tissue sections, cells near the edges of hair follicles, where most melanocytes localized, were immunoreactive to the anti-SLF antibody. It is apparent that most melanocytes, both inside or outside the hair follicles, are close enough to make direct contact among themselves. We noticed that membrane-bound SLF was expressed in clusters on the melanocyte surface, and that many melanocytes have high level of SLF on the tips of their processes. The above data and sl dickie phenotype suggest that, the membrane-bound form of SLF is the form used for melanocytes survival *in vivo*, and it works via direct contact among the melanocytes. The direct interaction between membrane-bound SLF and c-kit among melanocytes provides a more specific and efficient way to maintain the viability of melanocytes in the adult.

Our data extend our understanding of the melanocyte defects in the sl dickie mice. The soluble SLF in the embryos is sufficient for NCC to migrate

into the skin and differentiate into melanocytes. That is why at E11.5 melanoblasts were detected with in-situ hybridization with anti-sense of TAP2 (Wehrle-haller et al., 1995), an early melanocyte marker. However, in absence of membrane form of SLF, melanocytes can not contact one another and promote survival. As a result, there is no coat pigmentation in the adult. There might be a very low level of soluble SLF in the skin in adult mice. However, melanocytes which account for less than 1% of total cells have to compete with other cells in the skin, and the level of soluble SLF may be too low to activate c-kit and maintain the viability of melanocytes.

Soluble and membrane bound forms of SLF play different roles at different developmental stages.

The possible distinct functions of two forms of SLF were dissected in this thesis study. When we think about growth factors, we generally consider them as soluble, secreted proteins such as EGF and NGF. However, many secreted growth factors have transmembrane domains and are localized on the cell surfaces before cleavage by proteases. It is not clear why secreted proteins are targeted to the cell surface, and cleaved instead of being released directly. It has been suggested that the cleavage process controls the release of these growth factors into the extracellular space. There are many other ways to regulate the level of growth factors and their activities. The transcriptional of the gene can be regulated. The stability of mRNA can be manipulated. The rate of translation and post translation modification can be regulated. The activities of growth factors can be controlled in many ways as well. For example, binding efficiency of the receptor can change. The

internalization via their receptors can be regulated. The activities of proteases which can degrade the growth factors can be adjusted. It is not efficient to produce a soluble molecule by synthesizing a bigger molecule first, then cleaving it. It is certainly not necessary if it is only for the regulation, since many molecules are synthesized and released directly to the extracellular spaces and are well regulated. There is accumulating evidence that some growth factors are active in the membrane form and are never released. Delta, the ligand for Notch which is involved in neurogenesis, has not been found to be cleaved into the soluble form. In another example Semaphorin SemaI, a transmembrane protein required in Drosophila for embryonic motor and CNS axon guidance, is believed to work through the membrane form. Now we provide evidence that SLF, a growth factor with a transmembrane domain, works in both the cell surface associated and the soluble forms.

Our data suggests that soluble form of SLF promotes NCC differentiation into melanocytes, while the membrane form of SLF is critical for the survival of melanocytes. We found that the soluble form of SLF induced melanogenesis in NCC in a dose-dependent manner. However, in the defined medium, even the NCC at relatively high density failed to differentiate into melanocytes; NCC failed to differentiate even when co-cultured with CHO cells expressing membrane form of SLF. Presumably, the membrane form SLF could not provide SLF at a concentration high enough to cause NCC to differentiate into melanocytes. In vivo, at the stages when melanogenesis occurs, soluble form of SLF is the major form throughout embryo and is available to promote NCC differentiation into melanocytes. Although both membrane and soluble forms of SLF can promote melanocytes survival *in vitro*, our Western-blot

analysis showed that the membrane form of SLF is the major form in the skin at later stages of development. The SLF dickie phenotype and SLF expression analysis we performed suggest that the membrane form of SLF is the form responsible for the maintenance of melanocytes.

SLF has multiple functions in many cell lineage.

Our studies and data from other labs suggest that SLF has multiple functions in NCC development. First, SLF is a mild mitogen for mast cells and NCC (Copeland et al., 1990; Huang et al., 1990; Martin et al., 1990; Williams et al., 1990; Zsebo et al., 1990). This is consistent with our findings that both soluble SLF and CHO cells expressing membrane form SLF increased the percentage of BrdU positive NCC in the culture at the end of 24, 48, and 72 hours. We also demonstrated that SLF can induce for NCC differentiation into melanocytes in a dose dependent manner. We believe that SLF is a differentiation factor instead of a survival factor for melanocyte precursors. Our *in vitro* studies showed that SLF is not critical for the melanocyte precursors survival. However, our data suggest that the membrane form of SLF is required for maintaining the viability of mature melanocytes. Carnahan et al (1994) showed that SLF is a neurotrophic factor for NCC-derived chicken sensory neurons. SLF has been shown to play important roles in the development of other cell lineages as well. The hematological phenotype of mice with mutations at the steel locus is characterized by a reduced number of hematopoietic stem cell, macrocytic anemia, and a reduction of tissue mast cells. Several studies have shown that SLF is a potent stimulator of early events in hematopoiesis. deVries et al. reported (1991) that SLF stimulated the

proliferation murine spleen colony-forming cells *in vitro*. These cells are considered to be closely related to the hematopoietic stem cells. Toksoz et al. reported that stromal cells expressing the membrane-bound form SLF supported the maintenance of human hematopoietic progenitors in culture for up to four weeks.

NCC are multipotent.

We were not able to test directly whether NCC are multipotent with clonal analysis, because the process of dispersing NCC into single cells caused many cells to die. Our melanogenesis test showed that over 90% of the NCC differentiated into melanocytes at the end of 14 days in culture media containing exogenous SLF, while only a small proportion of NCC become melanocytes *in vivo*. Our data implies that NCC are multipotent, and their fates can be changed when treated with different factors. Fraser and Marianne-Fraser (1989) addressed this question by intracellular microinjection of vital fluorescent dye into premigratory and migrating NCC. They demonstrated that many premigratory trunk NCC give rise to descendants with distinct phenotypes in multiple NC derivatives. Their results are consistent with the notion that NCC are multipotent prior to their emigration from the NT and become restricted in phenotype after leaving the NT either during their migration or at their final targets. They also demonstrated that migrating trunk NCC can be multipotent, giving rise to cells in multiple NC derivatives, including both neural and non-neuronal elements within a given derivatives. They suggested that the restriction of NCC fate occurs relatively late in migration or at the final sites of NCC localization. So far, no studies

have excluded the possibility that small number of NCC are heterogeneous and predetermined before their migration. The *in vitro* clonal analysis could miss a small population at the beginning of the culture or the small population could die during the clonal cultures. Our differentiation experiments did not convert 100% of NCC into melanocytes even when SLF was added at high concentrations.

Negative regulation of melanogenesis.

Our data showed that over 90% of NCC differentiated into melanocytes at the end of 14 days in culture. We also showed that over 95% of NCC express c-kit and SLF. In vivo, however, only the late migrating NCC along lateral pathways differentiate into melanocytes. The NC-derived dorsal root ganglion (DRG) cells undergo cellular condensation at roughly the same time period of development in which the melanocyte staging area appears in the lateral pathway, and yet this neural structure normally contains no pigmented cells. Our western-blot analysis showed that SLF was expressed at high levels at these early stages. This indicates that nascent DRG might contain inhibitory factors that can supersede the inductive effect of SLF. This hypothesis is supported by observations that, when early DRG cells were dissociated in culture, there was a spontaneous appearance of melanocytes (Nichols and Weston, 1977; Ciment et al., 1986), presumably due to the disruption of cellular associations that normally would block the appearance of melanocytes *in vivo*. Previous work from our lab demonstrated that various isoforms of TGFβ were expressed in chicken embryos at stages when they might influence melanogenesis, including high levels of expression within the

ventral NC migratory pathway (Jakolew et al., 1992,1994). Stocker et al. found that TGFβ1 could directly inhibit melanogenesis in NC cultures induced by bFGF (Stocker et al., 1991). There are reports that cranial NCC, which remain largely unpigmented in culture, synthesize and release their own TGFβ1 (Gatherer et al., 1990; Roger et al., 1992).

Western-blot analysis of c-kit expression in different tissues and at different stages revealed that there are bands of 100 and 60 KDa proteins besides the mature 150 KDa c-kit. The 100 KDa is similar to the molecular weight of the extracellular domain of the c-kit, and has been observed by other groups (Broudy et al., 1994; Lev et al., 1992; Wypch et al., 1995). This extracellular domain contains the immunoglobulin-like domains responsible for SLF binding and therefore may act to sop-up SLF, neutralizing its activity at the places and times when SLF is not needed. Our preliminary evidence suggest that this domain might serve as a signal to down-regulate the level of soluble SLF, and flip the switch from the soluble to the membrane form of SLF in the skin at the later stages of development. This truncated c-kit protein may be one of the factors capable of blocking the melanogenesis of DRG cells.

Future Directions.

NCC migrate along two different pathways over long distance, and differentiate into a varieties of cell types during development. So far, little is known about the mechanisms and molecules that guide NCC migration. In-situ hybridization (Keshet et al., 1991) analysis demonstrated that melanocyte precursors migrate along a gradient of SLF in a manner consistent with a

chemotactic role for SLF. To date, however, there is no direct evidence that SLF can direct melanoblast migration.

Although a few groups have dissected the signal pathways of SLF/kit, our understanding of the pathways is still fragmentary (Heldin, 1995; Herst et al., 1995; Hess et al., 1988; Miyazawa et al., 1991; Ikuta et al., 1991; Schlessinger et al., 1992). Walter Englaro et al. studied mitogen-activated protein (MAP) kinase pathway in melanogenesis using B-16 melanoma cells. They showed that cAMP-elavating agents stimulated MAP kinase, induced a translocation of MAP kinase, and an activation of the transcription factor AP-1. We found that SLF's ability to induce NCC differentiation into melanocytes was blocked by PC compound, an inhibitor for MAP kinase, suggesting that SLF/c-kit works through MAP kinase. So far, the pathways of SLF induced activation of c-kit has not been performed on NCC directly, and nothing is known about the relationship of SLF and hormones (like endothelin, melanocyte stimulating hormone) or transcription factors.

Our Western-blot analysis of early embryos with antibodies against c-kit suggests the c-kit can be cleaved before its transmembrane domain. However, little is known about the mechanisms of the cleavage, the possible functions of the cleaved fragment Complex mechanisms are required to regulate cellular proliferation and differentiation promoted by growth factors. Down-regulation of receptor protein is one of the mechanisms involved in the modulation of growth factor-mediated signaling. Several mechanisms involved in this negative regulation has been described such as: 1) acceleration of receptor internalization and degradation; 2) modulation of the ligand binding affinity of the receptor; 3) inhibition of receptor

phosphorylation; and 4) activation of a proteolytic cleavage of the extracellular domain of the receptor. James R. Downing et al (1989) showed that PKC activated a protease which specifically cleaves the CSF 1 receptor and release its ligand-binding domain from the cell.

The soluble protein has been shown to retains the ability to specifically bind SLF, and was able to block the mitogenic effect of SLF on cultured SLF dependent cells, and it may function as a physiological antagonist of SLF. However, no one has sequenced the 100 KDa and conform its identity. The cleavage site is not known and the protease involved in the process is not yet characterized. Much work needs to be done to demonstrate that the soluble c-kit can antagonize SLF's function *in vivo* when SLF is not needed.

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