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Transplantation of skin-derived precursor cells into neonatal rat brain

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Table of Content

Title: Transplantation of skin-derived precursor cells into neonatal rat brain.

We have isolated a novel source of stem cells derived from the dermis of either rodent or human skin. When induced to differentiate *in vitro*, these cells give rise to all types of neuronal lineages. In this study, we therefore investigated if naïve Skin-derived Precursor (SKPs) cells could be neuralized by the neonatal brain environment thereby generating neural cells *in vivo*. We transplanted orange cell tracker- labelled mouse SKPs into the lateral cerebral ventricule and the hippocampus of neonatal rats. Analysis of the transplants revealed that SKPs survived over the short, medium and long-term *in vivo*, with SKPs remaining viable for up to 6 weeks without forming tumors. Many cells also adopted a branched morphology, but, based on immunocytochemistry of orange cell tracker-labelled SKPs with specific markers for either neurons or glia, these morphologically complex cells did not differentiate into specific neuronal cell types. In control experiments, neural stem cells exhibited a similar in vivo pattern of survival and a lack of differentiation, thereby validating our results and that our transplantation protocol required further refinement in order to give optimal results. In conclusion, we have shown that SKPs can survive *in vivo* and do not appear to form neural lineages.

Titre: Transplantation de cellules précurseurs dérivées de la peau dans des cerveaux de rats nouveaux nés.

Nous avons isolé une nouvelle source de cellules souches dérivées du derme de rongeurs ou d'humain. Lorsque ces cellules sont induites à se différencier *in vitro*, elles adoptent les différents phénotypes de la lignée neuronale. Dans cette étude, nous voulions investiguer si les cellules souches dérivées de la peau (SKPs pour Skin-derived Precursor cells) non différentiées avaient le potentiel d'adopter un phénotype neuronal lorsque exposé à un environnement cérébrale disposant de signaux neuralisateur. Nous avons transplanté des SKPs de souris marquées avec un traceur fluorescent orange (OCT pour orange cell tracker) dans le ventricule cérébrale latérale et l'hyppocampe de rats nouveaux nés. L'analyse des cellules implantées a montré que les SKPs peuvent survivre à court, moyen et long terme *in vivo*. En effet, les SKPs sont restées viable jusqu'à 6 semaines et n'ont jamais formé de tumeurs. Plusieurs cellules ont développé des ramifications complexes, mais, basé sur les immunocytochimies pratiquées sur des SKPs marqué avec OCT et des marqueurs neuronal ou glial, ces cellules complexes morphologiquement ne semblent pas se différencier en phénotype spécifique. Dans les expériences contrôles, des cellules souches neuronales ont démontré des résultats similaires de survie et de différentiation, validant ainsi nos résultats et indiquant que notre protocole de transplantation devrait être raffiné pour donner des résultats optimaux. En conclusion, nous avons démontré que les SKPs survivent in vivo et ne se différentient pas en cellules de lignée neuronale.

1. INTRODUCTION

Stem cell biology is currently one of the most intensively studied subjects in science. Research on stem cells is important because it may help us to understand the development and function of organ systems in mammals. It may also provide insight that ultimately leads to innovative ways of treating neurodegenerative conditions such as Parkinson`s disease and spinal cord injury. What are stem cells exactly? One definition of a stem cell is that it is a cell that has the capacity to self-renew indefinitely (i.e. proliferate) while remaining in an undifferentiated state. Stem cells, however, can demonstrate pluripotency if they are induced to differentiate. Stem cells have been isolated from embryos, fetuses and adults. Adult stem cells have in turn been isolated from several different tissue sources ; bone marrow (Prockop 1997; Weissman 2000; Pittenger et al., 1999), retina (Tropepe et al., 2000), skeletal muscle (Jackson et al., 1999; Gussoni et al., 1999) and even the central nervous system (CNS) (Reynolds and Weiss, 1992; Gage 2000). These cells have been shown to have a tissue bias; in other words they will differentiate preferentially into cellular lineages similar to those that reside in their tissue of origin. For example, hematopoetic stem cells will give rise to all the different classes of blood cells while CNS stem cells will give rise to neurons and glia. However, it has been demonstrated *in vivo* that these stem cells might possess more plasticity than originally thought. Indeed, CNS stem cells transplanted into blastocysts contribute to the development of many embryonic tissues and the generation of skeletal muscle and blood cells (Bjornson et al., 1999; Galli et al., 2000; Clarke et al., 2000). Contributions to skeletal muscle and liver cell lineages have also been observed following transplantation of bone marrow stem cells into blastocyts (Gussoni et al., 1999; Ferrari et al., 1999; Petersen et al., 1999). Bone marrow stem cells are also capable of generating cells that produce neuronal markers within the brain post-implantation (Mezey et al., 2000; Brazelton et al., 2000).

While these observations suggest that adult stem cells are more versatile than originally presumed, it remains unclear whether adult CNS stem cells can be used for treating CNS disorders. At the moment, this seems highly unlikely because, unlike stem cells derived

from embryonic and fetal sources, it is almost impossible to harvest adult stem cells without having adverse effects on their functional integrity. Moreover, it is currently impossible to stimulate endogenous populations of adult CNS stem cells towards improving functional recovery. However, the impetus to develop viable transplantation protocols for adult CNS stem cells is clear given the ethical issues that currently surround the use of embryonic and fetal sources for stem cells in heterologous transplantation paradigms.

1.1 Skin-derived precursor cells

In an effort to circumvent the aforementioned transplantation and ethical issues, we attempted to isolate adult stem cells from mammalian skin. Skin is a highly accessible tissue source in which many different sensory receptor cells are found; for example, Merkel cells, Meissner's corpuscle, Ruffini's corpuscle and Pacinian corpuscle (Kandel et al., 1991). Following denervation of skin sensory receptors, the later have the capacity to repopulate the denervated area (Nurse et al., 1984). Results have shown that Merkel cells partially repopulate the damaged area of the skin within approximately 80 days (Nurse et al., 1984). These observations led us to hypothesize that skin contains precursor cells capable of generating neural cell types. We previously succeeded in generating stem cells from adult rodent skin, termed SKPs for SKin derived Precursors (Toma et al., 2001).

SKPs were isolated from a piece of skin, more specifically the dermis, taken from the abdomen of neonatal or adult mice. The piece of skin was dissociated into single cells by trituration into 10 millilitres of media (DMEM/F12 supplemented with 2% B-27, fungizone (1μg/mL) and 1% penicillin/streptomycin) containing epidermal growth factor (EGF; 20ng/mL) and fibroblast growth factor (FGF; 40ng/mL). Cells were passed through a 40 micrometre mesh filter and transferred to 50 mL culture flasks. While some cells adhered to the plastic, and others died, a small subpopulation of cells formed floating spheres after three weeks. These spheres continued to proliferate and were passaged every 4-5 days. At every passage, a fraction of the dissociated cells adhered to

the flask, while those that remained in suspension continued to proliferate into larger spheres. After 3-4 weeks of passaging, we obtained a relatively homogenous population of cells that was positive for nestin, a marker for neural precursor cells. Continual passaging of a subset of these cell lines indicated that proliferative capacity was retained for as many as 50 passages (approximately once per week for 15 months). These rather large cell aggregates differed in appearance from the neurospheres generated from the rodent or human olfactory epithelium (unpublished data; Pagano et al., 2000) or brain (Reynolds and Weiss, 1992), which are more compact and contain smaller cells.

What is the origin of SKPs *in vivo*? There are three compartments within the skin: the epidermis, dermis and nerve terminals. To determine the origin of SKPs, skin cells were cultured from epidermis, dermis and sciatic nerve. Only the dermis generated proliferating cells similar to SKPs. This population of cells isolated from the dermis may therefore play a role in regenerating elements of the damaged skin like nerve terminals and melanocytes.

SKPs can be induced to differentiate by plating them onto poly-d-lysine/laminin in the absence of growth factors. Resultant immunocytochemical analyses revealed that SKPs had differentiated into several different cell types. Some of the cells were positive for nestin, βIII-tubulin, neurofilament-M (NFM), and neuron-specific enolase (NSE), all neuron-specific markers. Other cells expressed glial fibrilary acidic protein (GFAP)(a marker for developing astrocytes), CNPase (a marker for myelinating cells) or both markers. While the expression of CNPase alone is typical of oligodendrocytes, GFAP and CNPase are co-expressed in Schwann cells. Thus, SKPs were isolated from rodent skin that, upon differentiation, were not only positive for precursor cell markers but also were capable of expressing markers specific for neuronal and glial cells (both central and peripheral).

In order to assess the multipotency of the SKPs, clonal analysis was performed. Single cells were isolated by limiting dilution, cultured for five weeks and subsequently allowed to differentiate for two weeks. The clones generated in culture gave rise to progeny that

were immunopositive for NFM, CNPase and GFAP. These data indicated that multiple neural cell types can be generated from a single SKP. Thus, SKPs are multipotent. In addition to their neural potential, SKPs can also give rise to progeny of other types of lineages. For example, when SKPs were differentiated in 3% rat serum and in the absence of growth factors, they generated a subpopulation of cells expressing smooth muscle actin (SMA) that appeared morphologically similar to smooth muscle cells. When placed in the presence of 10% foetal bovine serum, they generated cells that had the morphology and lipid droplet inclusions characteristic of adipocytes. This indicated that SKPs are multipotent and that they are not biased towards a neural fate.

Keeping in mind that the isolation of neural precursor cells from skin represents a potential therapeutic approach for autologous transplantation into the CNS, we attempted to isolate these cells from human skin. The pieces of skin that we obtained (1 mm^3) came from the scalp of neurosurgery patients, on which stereotactic instruments had been used. Cells from human skin placed in suspension with EGF, FGF and leukemia inhibitory factor (LIF) grew in spheres similar to those described for cells cultured from rodent skin (personal communication). Immunocytochemical analysis of human SKPs after several passages revealed that the cells were nestin-positive. When induced to differentiate, immunological staining revealed βIII-tubulin-positive and neurofilament-positive cells, suggesting that human skin precursor cells, like their murine counterparts, had the potential to differentiate into neurons.

Thus, our preliminary studies demonstrated that SKPs can be isolated from neonatal and adult mice as well as adult human and that they can be differentiated in vitro into neural cells. However, to address the therapeutic relevance of these findings, the true neural potential of these cells must be examined using *in vivo* models. Of all such available models, transplantation offers an attractive means of; i) definitively demonstrating the multipotency of stem cells, ii) characterizing the environmental signals that may drive cells to differentiate into a particular phenotype, and iii) assessing whether implanted cells will adopt a true CNS phenotype. Moreover, it allows us to investigate whether the

stem cells will become functional elements of the CNS, an issue that is of great importance to validating this technique as a valuable therapeutic approach.

1.2 Stem cell transplantation

Many new transplantation techniques have emerged in the past few years and they provide great hope for the treatment of neurological disorders. The progress made in the field of stem cell transplantation will be discussed in light of recent results presented in the review of the literature and in our own results section.

Cell labelling – One of the challenges of transplantation work is to be able to detect all of the cells that have been transplanted. Although many techniques are currently available, none have proven to be highly efficient and thus ideal. The most efficient techniques for cell labelling, and the most widely used, are the following ; i) isolation of cells from transgenic mouse lines, ii) Y-chromosome staining, iii) dyes, iv) adenovirally-infected cells, and v) species-specific antibodies. The best technique available for transplantation work at the moment is the use of cells derived from transgenic mouse lines. Cells prepared for transplantation are isolated from mice expressing constitutive markers such as β-galactosidase or green fluorescent protein. They are therefore visualized using fluorescent or darkfield microscopy or immunohistochemistry. One important advantage of this method over the others that have been mentioned is that all transplanted cells express the marker, even if they differentiate. However, it has been reported that the expression of the transgene can be downregulated either before or after transplantation (unpublished observations and personal communication). This introduces obvious technical issues for detecting transplanted cells during medium or long-term temporal experiments.

Transplantation of stem cells isolated from male animals into females is another option. In this situation, the transplanted cells are detected by staining of the Y-chromosome. This method labels all transplanted cells and is advantageous in that the marker continues to be expressed even if differentiation occurs. The only potential limitation of Y-

chromosome staining appears to be the specificity of the immunohistochemistry. To date, work done in the lab using this technique has been unsuccessful. However, it has not been thoroughly investigated, and since this method is used by other groups, it still remains a possible solution.

Other options include dyes and adenovirally-infected cells. These two methods have the advantage of being technically simple, accessible and result in the labelling of 100% of the cells. However, the labelling of the cells is only transient. More precisely, the dyes are diluted when the cells proliferate *in vitro* or *in vivo*. In the case of adenovially-infected cells, the marker is produced from the episome in the cytoplasm. However, the episome is also diluted during cellular proliferation and is eventually lost. Thus it is not useful for long-term studies. Moreover, the expression of the reporter gene can also be shutdown by unknown mechanisms (unpublished observations). Therefore, these techniques are ideally suited for short-term rather than long-term transplantation experiments.

Species-specific antibodies can also be useful markers for detecting transplanted cells *in vivo*. For example, in our study, SKPs isolated from mouse and transplanted into rat can be detected by mouse-specific antibodies. This technique has a number of advantages ; i) all cells are positive for the marker; ii) the marker is passed on to progeny as the cells proliferate; iii) the marker is easily detectable by immunocytochemistry. In some cases, the only limitation is that the antibodies only recognise certain cell types, as in our study, where anti-M2 and anti-M6 are specific for mouse neuronal cells. Thus, in this instance, transplanted cells that do not differentiate in the host environment will not be detected. In other words, it is impossible to detect all implanted cells. But in combination with other techniques, this approach will reveal the fate of neural stem cells that do not stain for M2 and M6 markers and allow us to examine neuronal differentiation.

Survival and environment – There is considerable variability across studies in the reported survival rates following transplantation of neural stem cells into neonates. In

general, surviving cells are found in 75 to 100% of neonatal recipients at early time points (within the first 20 weeks)(Rosser et al., 2000; Englund et al., 2002). In these animals, 10 to 30% of the cells remain viable and a proportion of 1 to 10% differentiate into neurons or glia (Yang et al., 2000; Rosser et al., 2000). One report only has shown better outcomes with numbers for survival around 50 to 65% after 65 weeks *in vivo* (Englund et al., 2002). It was therefore concluded from these studies that heterotopic neuronal integration occurred efficiently when donor cells were introduced into sites that continue to generate neurons in the brain of the newborn animal, like the subventricular zone and the hippocampus. At this point, even if these numbers appear limited, they may sometimes be sufficient for functional recovery in animal models (Svendsen et al., 1997) and scientists continue to work on various methodological changes to increase survival of cells post-transplantation.

Cell fusion in vivo – Recent papers have demonstrated that adult stem cells can fuse with ES cells *in vitro* and assume their characteristics (Terada et al., 2002; Ying et al., 2002). This suggests that transplanted stem cells can fuse with host cells and seriously bias the results obtained from such an experiment. To date, these results haven't been reproduced *in vivo*. Nevertheless, "it remains formally possible that cell fusion or alternative mechanisms of cell conversion may be significant *in vivo*, making it important to re-examine the *in vivo* data" generated in this field (Wurmser and Gage, 2002).

We will now review what exactly has been demonstrated *in vivo* with the two classes of stem cells that are of particular interest to us : embryonic and neural stem cells.

1.3 Embryonic stem cells

1.3.1 Mouse embryonic stem cells

Mouse embryonic stem (ES) cells are derived from the preimplantation blastocyst inner cell mass (Evans et al., 1981; Martin et al., 1981). The cells are cultured in the presence of leukemia inhibitory factor (LIF) or are plated on a feeder layer of mitotically inactivated mouse embryonic fibroblasts. LIF and related cytokines are required to

suppress differentiation and allow proliferation of ES cells (Svendsen and Smith, 1999). These cells are induced to differentiate if plated in the absence of LIF. When ES cells are place in suspension culture in the absence of LIF, they differentiate into structures called embryoïd bodies (EB) (Martin, 1981). Although the EB do not have the morphological organisation of embryos, they do undergo similar differentiation to generate an extra-embryonic yolk sac and the foetal germ layers; the ectoderm, mesoderm and endoderm (Doetschman et al., 1985). Depending on the factors added to the cultures, variation will occur in the differentiation spectrum. The differentiated cell types include neurogenic, cardiogenic, myogenic, hematopoietic, chondrogenic, adipogenic, endodermal, epithelial, endothelial and vascular smooth muscle cells (Wobus and Boheler 1999). EB may contain almost any cell type. Several groups have also reported that EB may contain populations of cells differentiated into all important neural cell types; however, only low levels of neuronal differentiation were found (Bain et al, 1995; Fraichard et al., 1995). If ES cells are transferred back into the blastocyst, they can colonise all lineages to produce chimaeric animals that contain a mixture of ES-cell-derived and host embryo-derived progeny in all tissues, including the germ line (Bradley et al., 1984).

1.3.2 Mouse embryonic stem cell transplants

In order to comprehensively examine ES cell transplantation potential in the central nervous system, researchers have designed in vivo experiments to assess their responsiveness to environmental cues. When undifferentiated ES cells were transferred back into the 10.5 to 15.5 days embryo, they contributed to multiple cell lineages (Martin 1981; Bradley et al., 1984; Stewart et al., 1994). However, a percentage of these cells occasionally formed tumors (Bradley et al., 1984), suggesting that perhaps the environment of E10.5 embryos must provide tumorigenic cues to ES cells. ES cells had to be differentiated *in vitro* before they could be transplanted into the nervous system to avoid tumor production.

1.3.3 Neuralization of mouse embryonic stem cells *in vitro*

In order to induce undifferentiated ES cells into becoming cells of the neuronal lineage, a variety of different techniques have been used. These different approaches will be discussed in this section.

When ES cells are aggregated in suspension to form EB, neuralization must be induced by exposure to retinoic acid (RA). Treatment for 3 days with RA led to an enhanced number of nestin-positive cells, and doubling the exposure time (around 6 days) caused cells to adopt a neuronal morphology (Fraichard et al., 1995) (Bain et al., 1995). Moreover, cells formed complex cellular networks in which some cells were found to be positive for neuron-specific antigens like βIII-tubulin, MAP2, MAP5, NFM, NF200, NF165, GAP-43, and synaptophysin (Fraichard et al., 1995; van Inzen et al., 1996; Bain et al., 1995). Some glial markers were also detected, including the astrocyte-antigen GFAP and the oligodendrocyte-antigen O4 (Fraichard et al., 1995). Some neuronal-like cells were also positive for acetylcholinesterase activity or glutamic acid decarboxylase expression, indicating that ES cells had differentiated into GABAergic and possibly cholinergic neurons (Fraichard et al., 1995). Complimentary electrophysiological studies showed the presence of voltage-dependent channels and demonstrated that action potential could be triggered by current injection (Bain et al., 1995; Fraichard et al., 1995).

Others have used lineage selection to increase the neuronal differentiation of ES cells. ES cells were allowed to form EB and were then dissociated into single cells and replated on a poly-D-lysine and laminin substrate which supports attachment of cells and development into neuronal cells (Li et al., 1998). This resulted in a homogeneous dispersion of cells, a process that terminates inductive and selective effects of cell-cell contact within the EB. This procedure showed an increase in the number of neurons and glia present in the culture. Indeed, when these cultures were immunostained for neuronal markers, like MAP2, tau, and βIII-tubulin, up to 50% of the cells were positive and appeared to have a neuronal morphology and 20% of these expressed GFAP (Li et al., 1998).

Protocols for growth factor-mediated lineage selection of neuronal cells have also been established (Okabe et al., 1996; Lee et al., 2000). Like the protocol outlined above, ES cells are isolated and grown in media until they form EB. In this case, however, no growth factors are however present during this process, resulting in the induction of neuroectodermal and mesodermal cell differentiation. The neural precursor cells are then allowed to proliferate in the presence of inductive factors like epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and sonic hedgehog (Shh). Finally, neural precursor cells are induced to differentiate into functional neurons and glia by the combined addition of neural differentiation and survival-promoting factors (for example : FGF8, Shh).

Other protocols for selective isolation of neuronal cells have been established on the basis of transgenic ES cell lines carrying tissue-specific promoters fused to selectable marker genes (Li et al., 1998). For example, neural precursors are selectively generated from ES cells transfected with the selective neomycin gene and Sox2. Sox2 is a transcription factor specific to the developing nervous system (Collignon et al., 1996). After RA induction, G418 is applied to differentiating ES cell cultures to eliminate the Sox2-negative, non-neural cells. Sox2-selected precursor cells are then further differentiated into mature neurons.

1.3.4 Mouse embryonic stem cell-derived neural precursors transplants

Efforts are now focused on using these ES-derived neuronal precursor cells in transplantation models. Brustle et al. (1997) have demonstrated that *in vitro*-generated neural precursors participate in mammalian brain development when transplanted into the ventricular area of the embryonic rat. Analysis of brain tissue revealed that the transplanted cells differentiated into neurons, astrocytes and oligodendrocytes in the telencephalic, diencephalic and mesencephalic regions. Thus, these findings suggest that neuronal precursors derived from ES cells responded to environmental signals

responsible for cell migration and differentiation and have the potential to reconstitute neuronal and glial lineages in the CNS.

Others have demonstrated the differentiation of ES cells in neurodegenerative and injury models. Differentiated mouse ES cells transplanted into the striatum of Parkinsonian rats matured into fully differentiated dopaminergic neurons and restored some of the affected behavioral function (Bjorklund et al., 2002). Treatment of ES cells with RA, which enriches for gamma-aminobutyric acid (GABA)-positive cells, and their subsequent transplantation into a rat model of Huntington's disease (quinolinic acid lesioned striatum) resulted not only in their integration into the host environment but also enhanced the survival of the animal (Dinsmore et al., 1996). Choi's group has further demonstrated the use of neurally differentiated mouse ES cells in spinal cord injury. When neural precursors were transplanted into rat spinal cord 9 days after traumatic injury and analysed after 2-5 weeks, a fraction had survived and differentiated into astrocytes, oligodendrocytes and neurons. In addition, migration as far as 8 mm away from the lesion edge was observed (McDonald et al., 1999), and functional recovery was demonstrated. Thus, ES cell-derived neural precursors have the potential to survive, integrate and differentiate *in vivo*. However, since investigators have been using many different protocols, transplanted cell populations tend to be highly heterogeneous and the resulting grafts tend to be populated by multiple cell types. More importantly, undifferentiated cells remain and their proliferation potential might lead to tumor formation. Refinement of these techniques will therefore be important, especially if we want to apply them to human embryonic stem cells.

1.4 Human embryonic stem cells

ES cells have recently been isolated from human embryos (Thompson et al., 1998). Human ES cells are derived from the inner cell mass of *in vitro* fertilized human blastocysts. When induced to differentiate *in vitro*, they have the capacity, like their murine counterparts, to form embryoïd bodies (EB) containing derivatives of all three embryonic germ layers (Thompson et al., 1998; Itskovitz-Eldor et al., 2000). mRNAs

expression studies in the various tissues of the EB revealed the presence of zeta-globin (mesoderm), neurofilament (NF) 68Kd (ectoderm) and α–fetoprotein (endoderm)(Itzkovitz-Eldor et al., 2000). Expression studies with EBs derived from human ES cells lines (Thomson et al., 1998) have also revealed that they express markers from various cell types, including; gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia and stratified sqamous epithelium (ectoderm). It has also been shown that the cells expressing myocardium marker α–cardiac actin are present as well as cells with neuronal morphologies positive for NF 68 Kd (Itzkovitz-Eldor et al., 2000).

1.4.1 Human embryonic stem cell transplants

The first model of transplantation was done by grafting undifferentiated human ES cells into mice. Derivatives of all three germ layers (ectoderm, mesoderm and endoderm) were generated in teratomas formed by injecting human ES cells into severe combined immunodeficient (SCID) mice (Reubinoff et al., 2000). Because of the presence of undifferentiated cells with proliferation potential, tumor formation was frequent. Thus the problems are the same from mice to human, and this is why the neuralizing process is an important first step in order to develop effective, non-tumorigenic transplantation therapies.

1.4.2 Neuralization of human embryonic stem cells *in vitro*

Human ES cell differentiation needs to be controlled in order to transform this new discovery into a useful therapeutic strategy. As in the case of the murine ES cells, various cell lineages are spontaneously developed by differentiating human ES cells using techniques adapted from their murine counterpart culture protocols. Two systems were recently employed to purify a human ES-derived neural precursor population : i) lineage selection and ii) a growth factor approach. The first approach of lineage selection was developed by Reubinoff et al. (2000). To enrich the neural precursor population derived from ES cells, the ES cells were cultured on a mouse embryonic fibroblast feeder layer for a prolonged period of time (3 to 4 weeks) without changing the feeder layer. By

the end of the first week, early differentiation was observed as indicated by some changes in morphology in the centre of the colonies. The process of differentiation accelerated during the second week in culture. Neural cell adhesion molecule (NCAM)-positive cells that appeared to be neuralized could be identified in the centre of colonies. These cells were mechanically isolated and replated in serum-free medium (previously described in Svendsen et al., 1998), where they formed spherical clusters. Cellular proliferation was facilitated by supplementing the media with bFGF and EGF, a growth factor combination that is known to be effective for propagating neuroepithelial progenitors (Vescovi et al.,1999; Svendsen et al., 1998). Over the next two weeks, spheres expanded in culture with some of the cells dying. By five to six weeks, the population of proliferating spheres became relatively homogenous, and in vitro analyses revealed that approximately 95% of the cells in these spheres were neural precursors that could differentiate into all three neural lineage (Reubinoff et al., 2001).

Another group (Zhang et al., 2001) has used a different technique of lineage selection. ES cells were differentiated into EB, which were then plated in a specific medium containing FGF-2. Over a number of days in culture, plated EB generated an outgrowth of flattened cells and in the central portion developed some neural tube-like structures which was FGF-2-dependent. These neuroepithelial-like structures were then enzymatically removed and cultured as free-floating aggregates in a suspension culture. Differentiation of the neural precursors was induced by withdrawal of FGF-2 and plating on an ornithin and laminin substrate. Between 7 and 14 days of differentiation, these floating aggregates had generated all three major cell types of the CNS.

The second system established for selecting neural precursors from ES cell cultures is the growth factor approach. One group tested eight different growth factors for their differentiation capacity on human ES cells *in vitro*. They tested the potential of bFGF, transforming growth factor beta 1 (TGF-beta1), activin-A, bone morphogenic protein 4 (BMP-4), hepatocyte growth factor (HGF), EGF, beta nerve growth factor (NGF) and RA (Schuldiner et al., 2000). First, the expression of the receptors for these growth factors

was confirmed. Second, after 5 days in culture, EB were dissociated into single cells and replated in the presence of one of the eight growth factors. Differentiation of the cells was assayed by expression of 24 cell-specific molecular markers that covered all embryonic germ layers and 11 different tissues. None of the growth factors directed differentiation into single cell types; however, cells were more homogenous and displayed a larger proportion of a specific cell type. For our interest, neuralization was best enhanced by the combination of RA and NGF (Schuldiner et al., 2000). In a second study, Schuldiner et al. (2001) characterized in greater detail the neuralization effect of RA and NGF. They applied the growth factors under the same culture conditions describe above and found that they were potent enhancers of neuronal differentiation. They observed an increase in outgrowth of processes, network complexity, and the expression of neuron-specific molecules such as neurofilament heavy chain, dopamine receptor (DRD1), serotonin receptors (5HT2 and 5HT5A), and dopa decarboxylase (DDC)(Schuldiner et al., 2001). These findings therefore represented the initial steps required to direct the differentiation of human ES cells *in vitro* and provided insight into how to best adapt these techniques for transplantation.

1.4.3 Human ES cell-derived neural precursor transplants

Several groups have recently made progress examining the consequences of transplantation of ES cell-derived neural precursors. Reubinoff et al. (2001) transplanted small spheres of neural precursors into the lateral cerebral ventricles of newborn mice. They observed, by 4 to 6 weeks, dispersion of cells in various regions of the brain such as the corpus callosum, dentate gyrus and olfactory bulb. Immunocytochemical analyses of the grafted cells revealed differentiation into various cell types of all three neural lineages : GFAP-positive cells (astrocytes), NG-2 (oligodendrocyte precursors), CNPase and NFM (neurons) (Reubinoff et al., 2001). Importantly, there was no histological evidence of teratoma or non-neural tissue formation in any of the recipient animals.

Additional, independent studies also showed differentiation of human ES cell-derived neural precursors after grafting into the lateral cerebral ventricles of newborn mice

(Zhang et al., 2001). Briefly, they found cells present into various regions of the brain (cortex, hippocampus, olfactory bulb, striatum) from 1 to 4 weeks post-grafting, with cells being present up to 8 weeks post-implantation. However, cells had an affinity for two white matter tracts, the corpus callosum and hippocampal fiber tracts. Immunoreactivity for antibodies against nestin (neural precursors), βIII-tubulin and MAP2a,b (neurons) was detected in some transplanted cells. GFAP-positive cells were also found, albeit to a lesser extent. These two reports on transplantation of neural precursors derived from human ES cells led us to believe that integration and differentiation of implanted cells in animals is possible. It also pointed to the possibility of developing a therapeutic approach in which embryonic stem cells would be used as a source for transplantation.

1.5 Neural stem cells

1.5.1 Rodent neural stem cells

For many years, the concept that the different cell types in a particular area of the CNS are products of a multipotent stem cell population contained within their environment prevailed. In the late 1980s, this belief was validated when a population of multipotent neural precursors was isolated for the first time. Fredericksen et al. showed in 1988 isolation of precursor cells from embryonic rat cerebellum. These cells could also differentiate *in vitro* into neurons or glia, depending on their growth and differentiation conditions. Four years later, another group demonstrated that these cells could be transplanted back into the cerebellum of newborn mice and participate in development of that area of the brain (Snyder et al., 1992). In 1989, Temple showed that a population of neural precursors could also be isolated from embryonic day 13.5-14.5 rat forebrain and proliferated with undifferentiated features in culture. Like their cerebellar counterparts, however, they also had the potential to become different neuronal cell types (Temple 1989). Similarly, a population of precursors was isolated from embryonic rat hippocampus (Renfranz et al., 1991), optic nerve (Almazan and McKay, 1992), and cerebral cortex (Davis and Temple, 1994).

A real breakthrough in neural stem cell research then came from the studies of Reynolds and Weiss. After isolating multipotent mouse striatal embryonic progenitors that produced neurons and astrocytes (Reynolds et al., 1992), they identified adult multipotent neural precursor cells (Reynolds and Weiss, 1992). Cells were isolated from either embryonic or adult mouse striatum and grown in culture in the presence of EGF. These cells grew in aggregates called neurospheres, proliferated in suspension in the media and remained undifferentiated, as shown by their immunoreactivity for nestin, a marker for neuroepithelial stem cells. When spheres were plated on glass cover slips coated with poly-L-ornithine in the presence of EGF and the absence of serum for 21 days, the spheres adhered to the flask, cells migrated out and kept proliferating over the entire time course. Immunocytochemical analysis revealed that these cells expressed markers specific for neurons (NSE) and astrocytes (GFAP). Thus neurospheres were able to acquire the phenotype of two major cell types of the neuronal lineage. Later, clonal analysis showed that a single CNS precursor cell could give rise to spheres from which all three principal cell types of the CNS could be derived (Reynolds and Weiss 1996). This study was of particular importance in that it demonstrated the true multipotency of adult-derived neurospheres. Many subsequent studies have since isolated multipotent precursors from different areas of the adult rodent CNS like the hippocampus and even the spinal cord (Gage et al., 1995; Weiss et al., 1996).

1.5.2 Transplantation of rodent neural stem cells

The first type of experiment performed was to transplant a particular type of precursor back into the same area from which it had been isolated. For example, embryonic rat hippocampal progenitors were implanted back into the neonatal hippocampus. These cells integrated into the host tissue and acquired morphologies and gene expression profiles characteristic of host neurons and glial cells present at the implant site (Renfranz et al., 1991; Gage et al., 1995; Shihabuddin et al., 1995). Snyder et al. (1992) have isolated cerebellar mouse precursors and transplanted them into neonatal cerebellum, where implanted cells also differentiated into neurons and glia in a site-specific manner. Researchers then tried to graft the precursors outside of environment from which they

were isolated. Transplantation of neural precursors isolated from the hippocampus into neonatal or adult rat striatum showed that, in this situation the cells could adopt a neuronal fate in a site-specific manner (Lundberg et al., 1996; Catapano et al., 1999).

The key studies that demonstrated the wide differentiation potential of rodent neural stem cells were those performed in embryos. Early studies demonstrated that stem cells derived from areas such as the subventricular zone and forebrain could migrate and differentiate into various regions of the developing brain after being implanted into the embryonic ventricles (Brustle et al., 1995; Lim et al., 1997). To analyse in detail the differentiation potential of neural stem cells *in vivo*, Clarke et al.(2000) introduced adult mouse brain neural precursors into the early embryonic environment (blastocysts). Results demonstrated integration and differentiation of grafted cells into tissues of ectodermal, mesodermal and endodermal origin (Clarke et al., 2000). This clearly demonstrated that neural precursor cells have a very broad developmental capacity. An additional study by Dr. Ron McKay`s group showed for the first time that precursors implanted into E18 rat hippocampus could not only integrate and differentiate but also form functional elements in that region of the brain. Indeed, electrophysiological studies revealed that the implanted cells were electrically active and formed functionally connected neurons (Auerbach et al., 2000).

1.5.3 Human neural stem cells

The first report of the isolation of CNS precursors from human tissue came from Buc-Caron`s work (1995). In this paper, they reported the derivation of progenitor cells from 5-12 week old human fetuses. These neuroepithelial cells proliferated in culture in large aggregates and were positive for specific markers of precursor cells like nestin and vimentin. Under conditions that induced differentiation, they expressed neuron-specific antigens (MAP5,MAP2, βIII-tubulin, and NFM), astroglial antigens (GFAP), and oligodendroglial markers (A2B5)(Buc-Caron, 1995). Two years later, a group working on development of human oligodendrocyte precursors (OP) expanded neural precursors from human embryonic brain. When these nestin and PSA-NCAM positive neurospheres

were maintained in culture in the presence of bFGF, they remained undifferentiated while proliferating. When these spheres were induced to differentiate upon plating in culture dishes coated with fibronectin, cells migrated away from the spheres and formed complex neuronal-like networks. Immunocytochemical analyses of these cultures revealed the presence of βIII-tubulin, MAP2 and some GFAP positive cells (Murray and Dubois-Dalcq, 1997). Moreover, if cultured in the presence of T3, the active form of thyroid hormone, to enhance differentiation into the oligodendrocyte precursor lineage (Bass et al., 1997), a greater number of cells were immunoreactive for O4, a OP-specific protein (Murray and Dubois-Dalcq,1997). A number of additional studies described the isolation of neural precursors from human forebrain and their subsequent differentiation into neurons and glia. For example, electrophysiological studies revealed glutamate- and GABA-mediated conductance characteristic of mature neurons (Chalmers-Redman et al., 1997). In the conditions employed by Carpenter et al. (1999), tyrosine hydroxylase-positive neurons were found in culture. Clonal analysis demonstrated that human CNS precursors were multipotent and differentiated spontaneously into neurons, astrocytes, and oligodendrocytes (Vescovi et al., 1999).

1.5.4 Transplantation of human neural stem cells

The motivations behind the interest of the scientific community in the clinical use of human CNS precursors for transplantation are obvious. Over the years, many groups have thus worked on generating effective transplantation strategies for human CNS precursors. We will summarize in this section the large body of literature on the subject of human CNS precursor grafting. This section will discuss experiments using four different models : those involving the embryo and fetus, neonate, adult, and degenerative disease.

Studies using embryonic models for transplant are of great interest because they allow researchers to determine the whole range of differentiation capacity of a precursor population. One study published in 1998 (Brustle et al. 1998) demonstrated that the implantation of human neural precursors into the embryonic day 17-18 rat cerebral

ventricles, resulted in their widespread distribution throughout the brain one to eight weeks following surgery. Incorporation was observed in various grey and white matter regions (Brustle et al., 1998); for example, olfactory bulb, cortex, hippocampus, striatum, thalamus, hypothalamus, internal capsule, corpus callosum and brain stem. Cells displayed immunoreactivity to various oligodendrocyte-specific molecules (oligodendrocyte-specific glycolipids, myelin basic protein, CNPase), astrocytes (GFAP), and neurons (medium-sized neurofilament)(Brustle et al., 1998).

A recent study examined the effects of transplanting human telencephalon-derived precursors in the ventricular area of newborn mice. They showed that transplanted cells integrated into the subventricular zone (SVZ) within 48 hours and migrated during the next two weeks along normal pathways, such as the rostral migratory stream (RMS) (Flax et al., 1998). Some of the cells in the olfactory bulb were positive for NF and NeuN. Others were immunoreactive for GFAP and CNPase (Flax et al., 1998). As with *in vitro* studies, these human neural precursors gave rise to all major cell types of the neuronal lineage. Uchida et al. (2000) used the same model (except that they used SCID mice) for their human neural precursor cells and studied the sites of active neurogenesis : SVZ and the dentate gyrus of the hippocampus. In both sites, βIII-tubulin-positive cells were found as well as some Ki-67-positive cells (a marker for proliferating cells), suggesting that some cells proliferated following grafting. Some rare GFAP-positive cells were also observed. Interestingly, a certain number of precursors present in the RMS were immunoreactive for NCAM, indicating that they were committed to neuronal lineages.
New paragraph : Others have used neonatal rats for transplantation. Human cells implanted in both the hippocampus and striatum were analysed four weeks post-surgery for the presence of specific neuronal markers. Cells positive for human *tau* were observed in the hippocampus, where integration in the striatum and neocortex was apparent (Rosser et al., 2000). Another study described the most successful transplantation of human neural precursors into the striatum, hippocampus and SVZ. In this work, a high survival rate of the implanted cells was observed up to one year post-implantation. Neuronal (tau, neurofilament) and glial (GFAP) differentiation was also

demonstrated in various regions, as well as extensive migration and long distance projection of axons (Englund et al., 2002).

Transplantation in adult animals is of great importance because this model represents the challenge that researchers will have to overcome in order for human stem cells to be a useful clinical technique in humans. Rubio et al. (2000) investigated the transplantation of human neural precursors into the adult rat striatum and substantia nigra. Although no specific neuronal markers were expressed by the grafted cells, astroglial markers like GFAP were expressed by some of the transplanted cells (Rubio et al., 2000). Anders Björklund's group in Sweden performed a similar experiment with much greater success. They transplanted human neural precursor cells into the SVZ, RMS, hippocampus and striatum of adult rats. Their results showed differentiation in a site-specific manner. For example, cells migrated from the SVZ via the RMS to the OB where they were positive for markers like NeuN, tyrosine hydroxylase and GAD_{67}, similar to the dopaminergic and GABAergic cells normally present in that region. NeuN and calbindin-positive cells were found in the dentate gyrus where granular cells normally express these markers. This site-specific differentiation occurred not only in the neurogenic areas, but also in non-neurogenic regions. In the striatum, grafted cells expressed GAD_{67} and calbindin just like normal cells that populate the area. It is important to note that glial cells were also observed in the host animals. This study demonstrated that neural precursors derived from humans can be transplanted into adult rats where they can differentiate and integrate in a site-specific manner.

Finally, the ultimate clinical test in animals is the degenerative disease model. Many teams have tried to investigate the use of human neural precursor cells in models of Parkinson's and Huntington's disease. In 1996 and 1997, Clive Svendsen published two papers describing the transplantation of human neural precursors derived from the mesencephalon, into the chemically injured striatum. These cells survived and a limited number of them differentiated into tyrosine hydroxylase-positive neurons (Svendsen et al., 1996; Svendsen et al., 1997; Ostenfeld et al., 2000). Some cells also adopted an

astroglial fate. Moreover, a couple of animals demonstrated behavioral amelioration of their symptoms. Others have transplanted, in the same model, dopaminergic neurons derived from precursors in vitro prior to grafting. Numerous tyrosine hydroxylase neurons were found to populate the injured striatum up to 6 weeks after implantation (Sanchez-Pernaute et al., 2001). In a model of Huntington's disease in rat, transplantation of human neural precursors has shown that a large proportion of surviving cells undergo differentiation. Indeed cells expressing neuronal antigens, including DARPP-32, were identified indicating a mature striatal phenotype (Armstrong et al., 2000). Collectively, these studies show that these cells represent a potential, alternative source of tissue for neural transplantation in degenerative diseases.

1.6 Plasticity of adult stem cells

Embryonic stem (ES) cells are the most versatile type of cell that can be isolated from mammals. Detailed studies have demonstrated that they are multipotent and even totipotent since they possess the potential to differentiate into any cell type of their organism of origin. As described in previous sections, their multipotency has been shown both *in vitro* and *in vivo*. In contrast, the different populations of adult stem cells isolated from various sources were originally thought to be restricted in their differentiation potential. Accordingly, initial studies revealed that stem cell populations derived from hematopoietic, muscle, or neural tissue had a differentiation potential that was limited to their tissue of origin. As discussed in detail here, however, these hypotheses are being questioned in light of recent experiments in the field of adult stem cell research.

The first demonstration of the transdifferentiation potential of adult stem cells was achieved by intra-germ layer conversion. Ferrari et al. (1998) demonstrated that when bone marrow-derived stem cells, which have been shown to develop into hematopoietic and mesenchymal lineages, are transplanted into damaged muscles (chemically induce with cardiotoxin), they not only migrate but also differentiate into mature myocytes, thereby regenerating damaged areas. In a similar paradigm, Gussoni et al. (1999)

transplanted marrow-derived precursors into *mdx* mice (a model for Duchenne's muscular dystrophy.). Intravenous injection revealed that, in addition reconstituting the hematopoietic compartment, cells also had the capacity to incorporate into host muscles and differentiate. Transplanted cells partially restored function in the affected muscle and re-established dystrophin expression (Gussoni et al., 1999). These experiments suggested that hemapoietic stem cells can differentiate not only into all major blood cells but also into other cells of the mesodermal lineage like muscle cells. Others have also shown that upon injection into irradiated mice, murine skeletal muscle-derived cells are able to differentiate into all major hematopoietic cells; B cells, T cells, granulocytes/macrophages and myeloid cells (Jackson et al., 1999). This shows that precursors isolated from muscle can give rise to cells normally originating from hematopoietic stem cells. Thus, muscle cell precursors are yet another example of an adult stem cell population that has broader differentiation potential than originally believed.

Intra-germ layer conversion has also been demonstrated with cells other than hematopoietic or muscle-derived stem cells. After validating the isolation of precursors cells from the pancreas, Shen et al. (2000) demonstrated that these cells could be differentiated in vitro into hepatocytes when treated with a synthetic glucocorticoid (dexamethasone)(Shen et al., 2000). *Ex-vivo* studies in pancreatic buds of mouse embryos showed that these pancreatic precursors could also adopt hepatocyte phenotypes *in vivo*. Another group (Zuk et al., 2001) demonstrated that adipose tissue-derived stem cells can differentiate *in vitro* into adipogenic, chondrogenic, myogenic and osteogenic cells in the presence of lineage-specific induction factors (described in details in Zuk et al., 2001). These results collectively show that different adult stem cell population are not restricted to their normal biological repertoire of differentiation.

The second significant type of experimental manipulation that further confirmed the broad differentiation potential of adult stem cells involved their successful extra-germ layer conversion. Recent findings, both *in vitro* and *in vivo*, have shown, for example,

that bone marrow stem cells differentiate into endodermal lineages. When implanted into either injured liver or a relevant liver disease model, grafted bone marrow cells gave rise to hepatocytes (Petersen et al., 1999; Lagasse et al., 2000; Theise et al., 2000), and even participated in the regeneration of the organ. Krause et al. (2001) further demonstrated that these marrow cells not only generated hepatocytes but also migrated and differentiated in other organs or tissues of endodermal origin such as the lung, the gastrointestinal tract and the skin. These findings were corroborated by another group that showed that bone-marrow-derived stem cells can be converted into endothelium, ectoderm and endoderm at the single cell level both *in vitro* and *in vivo* (Jiang et al., 2002).

Other reports have also shown the differentiation of bone marrow stem cells into neuroectodermal lineages. *In vitro* treatment of marrow stem cells with β-mercaptoethanol for 24 hours induces the differentiation of cells that exhibit a neuronal morphology (Woodbury et al., 2000), and markers including NSE, NeuN, NF-M and tau-positive. Moreover, for the first six days following treatment, the cells expressed TrkA, the nerve growth factor receptor. These *in vitro* experiments therefore suggested that bone marrow stem cells can also give rise to cells of the neuronal lineage. Many groups have also shown that bone marrow stem cells migrate into the brain of recipient mice and express neuron-specific antigens (Kopen et al., 1999; Brazelton et al., 2000; Mezey et al., 2000). Analysis of newborn mice with bone marrow precursors transplanted into their lateral cerebral ventricles revealed that 12 days following grafting, cells had migrated into the forebrain and the cerebellum (Kopen et al., 1999). Similar findings were found when adult mouse bone marrow stem cells were delivered intravascularly into irradiated adult mice. Cells survived for up to 6 months, migrated from the site of injection and differentiated (Brazelton et al., 2000). Marrow cells that migrated into the brain expressed neuronal antigens like NF-M, Neu N, and βIII-tubulin (Brazelton et al., 2000; Mezey et al., 2000). These findings further confirmed the capacity of bone marrow stem cells to adopt a neuroectodermal lineage fate despite their mesodermal origin.

The literature on extra-germ layer conversion also includes studies of neural stem cells. Two years ago, a report suggested that mouse and human neural stem cells had myogenic potential. In culture, adult neural stem cells from either mouse or human co-cultured with adult murine C2C12 myoblasts for 4 to 7 days generated muscle cells (Galli et al., 2000). Differentiated cells expressed myosin heavy chain and MyoD, markers characteristic of myogenic cells. When transgenic neural precursor cells were injected into damaged tibialis anterior muscle of adult immunodeficient mice, they integrated into the muscle fiber structure and expressed lacZ (which had been placed under the muscle-specific mouse myosin light chain 3F promoter). These findings demonstrated that both murine and human neural precursors are capable of undergoing myogenic differentiation *in vitro* and *in vivo*.

During the same year, Clarke et al. (2000) demonstrated that adult neural stem cells have an even wider differentiation potential. Upon transplantation of adult ROSA26 mouse neural precursors into either chick embryos or mouse blastocysts, grafted lacZ-positive cells were observed in various structures of the developing chimeric embryos. Cells were not only found in the nervous system, but also in mesodermal and endodermal derivatives where they expressed markers specific for the tissue of integration. In chick chimeras, implanted cells integrated and differentiated into mesonephros and notochord (mesodermal derivatives), as well as liver and intestine (endodermal derivatives). In mice chimeras, grafted cells had incorporated and differentiated in heart, liver, intestine and other tissues. These results demonstrated that adult neural stem cells can contribute to the formation of chimeric chick and mouse embryos and differentiate into derivatives of all three germ layers.

One finding that was not reported by Clarke et al. (2000) is the possibility that adult neural stem cells can give rise to hematopoietic cells, something that has been reported by others (Bjornson et al., 1999; Shih et al., 2001). In these studies, adult neural stem cells isolated from ROSA26 mice were injected into irradiated adult mice. Analysis of these animals after 20-22 weeks showed that injected cells populated the hematopoietic

system of the recipient mice, giving rise to granulocytes/macrophages, myeloid cells, B and T lymphocytes (Bjornson et al., 1999). Although this suggested that neural stem cells also have hematopoietic potential, a study by Morshead et al. (2002) in which a lack of contribution of adult neural stem cells to the hematopoietic system in a total of 128 animals argues that hematopoietic competence is a rare property of neural stem cells. While They suggested that long-term passaging of neural stem cells may affect the outcome of the transplantation, their results exclude hematopoietic competence as a consistent property of intravenously injected neural stem cells (Morshead et al., 2002).

However, in April of this year, two papers published in Nature cast doubt on the body of literature supporting the general plasticity of adult stem cells. Two groups, working independently, suggested that some of the surprising plasticity of adult stem cells might be explained by simple cell fusion rather than transdifferentiation. This suggestion was based on the fact that while both groups intended to show that ES cells can induce bone marrow cells (Terada et al., 2002) and neural stem cells (Ying et al., 2002) to transdifferentiate into embryonic-type stem cells when cultured together *in vitro*, they observed that the adult stem cells fused spontaneously with the ES cells and adopted their characteristics. This therefore raises the possibility that previous work describing transdifferentiation may in fact be attributable to, at least in part, to simple cell fusion.

Despite the fact that the relative contribution of transdifferentiation and simple cell fusion is still subject to intense debate, adult neural stem cells clearly demonstrate a differentiation potential greater than originally thought. The reports discussed above definitively demonstrated that neural stem cells are not necessarily biased to give rise to neuronal progeny. In fact, if provided with the right cues in their environment, they can adopt the phenotype of all three primary germ layers.

These new findings prompted us to ask what is the relationship between ES cells and neural stem cells? As we discussed before, ES cells can give rise to any cell type in mammalian organisms. Neural stem cells are derived from ES cell progeny at some point

during embryonic development. Recent studies have demonstrated that neural stem cells can actually be derived directly from ES cells (Tropepe et al., 2001). They also demonstrated that ES cells have an autonomous tendency to differentiate into neural cells, but *in vivo*, this tendency is inhibited by intercellular signals that suppress neuronal differentiation (Tropepe et al., 2001). This demonstrates that these cell populations are closely related but remain distinct *in vivo*.

1.7 Summary

In light of the results described in the above review of the literature, it is clear that the developing nervous system contains the appropriate environmental cues to differentiate either uncommitted ES or neural stem cells into neural derivatives. In this study, we therefore wanted to investigate if naïve SKPs could be neuralized by the neonatal brain environment, thereby resulting in the generation of neural cells *in vivo*. In order to test our hypothesis, we transplanted mouse SKPs into the lateral cerebral ventricle and hippocampus of neonatal rats.

2. RESULTS

To isolate precursors from skin (SKPs), we dissociated a piece of abdominal skin from neonatal mice into single cells and cultured them in uncoated flasks in serum containing the growth factors EGF and FGF. Although many of the cells adhered to the bottom of the flask and a great number also died, small floating spheres of cells formed after a couple of days. Floating spheres were subsequently dissociated and passaged. During the first few passages, a fraction of the cells continued to adhere to the flask. However, cells that remained in suspension continued to proliferate in floating spheres that continued to increase in size. Over the course of 3-4 weeks, in which the cells were passaged every 5 days, a purified population of floating spheres was obtained. To compare the pluripotency of SKPs with the neural stem cells (NS) derived from brain, we also followed the protocol described above to isolate NS from E15-17 mouse forebrain. These cells were used as a positive control in all subsequent experiments.

2.1 Comparison of SKPs versus neurospheres in culture

Before initiating work *in vivo* with SKPs and neurospheres, we characterized the two types of cells. In order to qualitatively compare them on a morphological level, the individual cells and the spheres were examined. Cultures of SKPs were dissociated into single cells and seeded in new flasks. The diameter of single cells was then measured with a micrometer ocular on an inverted microscope. SKP cells were approximately 15-20 μm wide while cells from neurospheres were roughly 3-5 μm wide (Figure 1). In order to compare the gross morphology of the spheres themselves, we simply observed different lines in culture every day from one passage to the next. The relative size of the two sphere types was similar, based on observation with the micrometer ocular. However, the density of the spheres differed; SKPs formed diffuse aggregates while NS were more compact (figure 1). Both populations were also clearly distinct with respect to their morphology.

We then compared the relative rates of proliferation of SKPs and NS, since accurate quantitation of this parameter is critical prior to conducting labelling and transplantation

experiments. To precisely determine the doubling time of SKPs and NS, both cell types, which proliferate in suspension cultures either as large cellular aggregates or spheres, were passaged by dissociation into single cells and subsequently proliferated to generate new spheres. Doubling time was then determined by counting cells at the time of plating and then every 2 days thereafter over 8 days. This method was used on two adult SKP lines and revealed a doubling time of 1.75 days. Interestingly, SKPs from early (24 passages; line #1) and late (91 passages; line #2) passages demonstrated similar doubling times. This rate of proliferation was greater than NS, which doubled every 2.5 days.

In order to measure the percentage of total SKPs and NS that were proliferating, we employed an antibody, Ki-67, which recognizes a protein specific to proliferating cells. Early passage SKPs and NS (two different lines of passage #4 to 10 for each type of cells) were taken from proliferation media and induced to differentiate by plating them on a poly-D-lysine/laminin substrate in media devoid of growth factors. The spheres were dissociated into almost single cells and plated under these conditions for 12-24 hours. During this time period, cells adhered to the culture wells but kept proliferating. Immunostaining of early passage SKPs and NS with Ki-67 revealed that 92 ± 3.3% of the skin precursors were positive for the marker compared with only 10 ± 6.1% of the neural precursors (Figure 2), thus confirming the higher relative rate of proliferation of SKPs.

Another important variable to consider prior to labelling and transplantation of SKPs is to identify the cell types contained within the sphere. The importance of this point is emphasized by prior work using ES cells, in which it was demonstrated that neuralized multipotent stem cells tend to integrate and differentiate better than undifferentiated cells. Neurospheres are known to contain multipotent stem cells and neural precursors, but also a variety of committed neural precursors and terminally differentiated neural cells. Undifferentiated multipotent stem cells represent only 5% of the content of NS (Reynolds and Weiss,1996). SKPs, in contrast, are a relatively homogenous population of cells composed of 95% of undifferentiated multipotent stem cells (Toma et al., 2001). The remaining 5% is compose of neural precursors and committed neural precursors

However, after long-term passaging, the spheres tend to be highly enriched in neural precursors (personal communication). We therefore compared the proportion of neural precursors present in cultured SKPs and NS. SKPs and NS (two different lines at passage between 4-10) were dissociated into almost single cells and plated under conditions that stimulate differentiation for 12-24 hours. Cells were subsequently immunostained for nestin, an intermediate filament specific to neural precursors (Lendahl et al., 1990)(figure 3). Close to 100% of the cells in the neurospheres were nestin-positive while only 30-60% expressed the marker in the SKP spheres. Related studies in our laboratory have shown that the variable nestin content of SKPs is attributable to at least two factors: the efficiency of initial dissociation in culture and the more efficient dissociation of the cells from skin tissue of neonatal relative to adult dermis. The proportion of neural precursors present in NS (100%) and SKPs (30-60%) observed prior to transplantation therefore confirmed previously published observations that NS are more committed than SKPs to the neuronal lineage.

2.2 Markers for transplantation

Although many different techniques can be used to label cells for transplantation, we chose to work with six of them; transgenic mice, adenovirally-driven marker proteins, Hoechst, 5-bromo-2-deoxy-uridine, species specific antibodies, and orange cell tracker.

2.2.1 Transgenic mice

We evaluated the potential applicability of this technique by attempting to isolate SKPs from two transgenic mouse lines (GtRosa26 and GFP).

GtRosa26 - Mice homozygous for the ROSA26 retroviral insertion do not differ phenotypically from their wild type littermates (Friedrich and Soriano, 1991). LacZ is expressed in all tissues of the developing embryo and in most tissues of the adult mouse. Thus, almost all cells express the lacZ marker gene. Skin of neonatal mice was dissected and cultured into stable SKPs. After 3 to 5 passages, ROSA26-SKPs were plated 24 hours in differentiation media, and immunostained for lacZ to verify that SKPs expressed the transgene. All cells expressed the marker gene. Because various groups working on

transgenic lines have expressed concern about transgene silencing over time in culture, we decided to test transgene expression on later passage ROSA26-SKPs cultures. As with earlier passaged lines, late passage ROSA26-SKPs (6-10 passages) were plated for 24 hours in differentiation media and immunostained for lacZ. None of the cells expressed the transgene. Given that continued lacZ expression is critical for successful identification of SKPs following transplantation, we concluded from these experiments that ROSA26-SKPs were ultimately not the best starting material for transplantation experiments.

GFP - A second transgenic mouse line with an "enhanced" GFP cDNA under the control of a chicken β-actin promoter and cytomegalovirus enhancer, which results in expression of the marker in all tissues, with the exception of erythrocytes and hair (Hadjantonakis et al., 1998) was subsequently tested. Skin of these mice (neonates) was dissected and stable SKPs cultures were established. After 3 to 5 passages, GFP-SKPs floating green spheres could be visualized under fluorescent light. However, as with the ROSA 26-SKPs expression of the transgene stopped after roughly 8 passages. Therefore, neither of the two transgenic mouse lines represents a viable strategy for the labelling SKPs prior to initiating transplantation studies.

2.2.2 Adenovirally-driven marker proteins (β-gal, GFP)

An alternative strategy to transgenic mouse lines is the use of adenoviral vectors. Adenovirally-infected cells contain the relevant DNA construct as an episome, which expressed by the cells own transcriptional and translational machinery. We therefore tested if SKPs could be efficiently labelled with either β-gal or GFP by incubating them for 24 hours with 50 MOI of each adenovirus. After 24 hours, media containing the virus was removed and replaced with fresh media. SKPs were then kept in culture for an additional 48 hours. To verify expression of the marker and assess the labelling efficiency, GFP-infected SKPs were simply observed under fluorescent light while those infected with β-gal were immunostained with an anti-β-gal monoclonal antibody. Both markers were present in 50-75% of the cells. Thus, even shortly after infection, the

labelling efficiency is not sufficiently high to use an adenoviral strategy for pre-implantation labelling of SKPs.

2.2.3. Hoechst

Previous reports that used Hoechst (Baron Van Evercooren et al., 1991; Franklin and Blakemore, 1995), prompted us to evaluate whether it could be used to label SKPs for transplantation. Bisbenzimide Hoechst 33342 is a dye that binds DNA at AT base pairs. Under fluorescent light, the nucleus of labelled cells fluoresces blue. To test Hoechst's potential, cells from a 10 mL flask were resuspended in roughly 50 μL of media containing 10 μg/mL of Hoechst, and incubated for 30 minutes. Following this incubation period, Hoechst-containing media was washed and cells were resuspended in fresh media. A fraction of these SKPs were plated in four well chamber slides coated with poly-D-lysine/laminin, and were allowed to attach to the bottom of the well for 2-3 hours prior to their visualization under fluorescent light. All cells were positively labelled, suggesting that this technique had a very high labelling efficiency.

After following the same labelling protocol, Hoechst-labelled SKPs were transplanted into six neonatal rats. SKPs were injected into either the hippocampus or the lateral cerebral ventricle (three animals for each location), and 7 days after the procedure, the brain of each animal was sectioned and the sections were observed under an ultraviolet filter. While many cells had survived the transplantation procedure, and Hoechst-labelled nuclei were found in large numbers in the dentate gyrus and the ventricular area (Figure 4 A and B), The blue fluorescence produced by Hoechst also seemed to be present in host cells. In fact, a cloud of marker could be seen around the area where the grafted cells were most likely present (figure 4B). Others have also made similar observations (Iwashita et al., 2000). Thus, while Hoechst is a valuable technique to quickly verify if the implanted cells have been transplanted at the right location, it is not suitable for investigating the differentiation potential of the transplanted cells because grafted cells cannot (one word) be clearly distinguished from host cells.

2.2.4. 5-bromo-2-deoxy-uridine (BrdU)

BrdU labelling has also been widely used in transplantation studies (Brown and Stanfield, 1989). BrdU is a nucleotide analog that can be incorporated into DNA (it replaces thymidine) during proliferation of cells *in vitro* and *in vivo* (through systemic injection). Cells, which have incorporated BrdU into their DNA, can be detected using an anti-BrdU antibody. To evaluate whether BrdU could be used to label SKPs for transplantation, cultured SKPs were incubated with BrdU for 48 hours. During this period, proliferating cells incorporated the analog, after which they were plated for 12-24 hours in differentiation media on coated four well chamber slides. Cells were then immunostained with anti-BrdU(figure 5). As with many other markers, 100% labelling efficiency was not attained. Moreover, the presence of green fluorescence in the nuclei of a fraction of the cells suggested that the high proliferation rate of the SKPs resulted in the dilution of BrdU (figure 5). We were therefore concerned that if cells kept proliferating *in vivo*, dilution of the BrdU would results in the inability to track even the fraction of cells that were BrdU-positive as they differentiated. Compounding the problems associated with dilution effects and poor labelling efficiency, a recent paper also showed that BrdU, like Hoechst, might be taken up by host cells around the transplant area (Englund et al., 2002), thereby making it impossible to distinguish between implanted and host cells.

2.2.5. Species specific antibodies

An alternative method that can be used to identify implanted cells is to transplant mouse cells into rat brain. All transplanted mouse cells surviving in the neonatal rat brain environment may then be identified by using antibodies raised against specific mouse antigens. Mouse-specific antibodies raised against a mouse astrocyte antigen (M2) and a mouse neuronal and glial antigen (M6) were generated and characterized (Yan et al., 1996; Lagenaur and Schachner, 1981). In order to evaluate the efficacy of this approach, neurospheres were isolated from mice and plated for 12-24 hours in differentiation media as small spheres in four well chamber slides coated with poly-D-lysine/laminin. Cells were then immunostained with anti-M2 and anti-M6 antibodies (figure 6). As expected,

the spheres were positive for both antigens. However, since these antibodies only label differentiated cells, and neurospheres are composed of not only differentiated cells but also neuronal progenitors, only those implanted cells undergoing differentiation could be identified using this technique. Nevertheless, these markers remain a useful diagnostic tool for post-transplantation studies that are concerned with long-term differentiation (i.e. later time points).

2.2.6. Orange cell tracker

The last labelling technique we used was an orange fluorescent dye, Cell Tracker^{TM}. While it is a probe that freely passes through cell membranes, once inside the cell it undergoes a chemical modification that produces an impermeant by-product. To test its ability to effectively label SKPs, SKPs were resuspended in a flask containing 5 mL of media, to which 0.2 μL per mL of orange cell tracker was added. Cells were incubated for 30 minutes at 37°C, washed with fresh media and resuspended for plating on coated chambers slides in differentiation media for 12-24 hours. After this time period, slides were visualized by fluorescence microscopy, and all cells fluoresced bright orange (figure 7). This technique was thus efficient at labelling all SKPs; however like other dyes, it is important to keep in mind that dilution of the marker during proliferation represents a potential problem. Nevertheless, of all the markers that were evaluated, orange cell tracker was the best because of its high labelling efficiency, fluorescence intensity and reproducibility. It was therefore chosen as the method to be used for all transplantation experiments described hereafter.

2.3 Transplantation

In the introduction, we detailed the rationale for our *in vitro* experiments. Briefly, SKPs have been shown to be multipotent, particularly with respect to culture conditions where they can differentiate into all major neuronal cell types : neurons, astrocytes, and oligodendrocytes. Of particular interest then, is whether SKPs demonstrate the same differentiation potential *in vivo*. In other words, can SKPs be neuralized by the environmental cues contained within the central nervous system? To address this

question, we chose the neonatal rat brain as a model since it possesses the environmental signals required for neuralization. Furthermore, it does not require immunosuppression (Rosser et al., 2000), and it is technically greatly reliable. As site of injection, we chose the granular cell layer in the hippocampal dentate gyrus, a well described neurogenic area, and the lateral cerebral ventricle, where others have demonstrated migration and incorporation of cells into various regions of the brain regions. Approximately one hour before transplantation, SKPs were labelled with orange cell tracker (OCT). One μL of a cell suspension (50000 OCT-positive cells) was injected into either the hippocampus or lateral ventricle of post-natal day one rats. As a positive control, neural precursors isolated from E14-15 mouse forebrain were transplanted in the same location using identical labelling and injection protocols. As a negative control, either culture media from the last wash of the labelling protocol or freeze-thawed, killed OCT-labelled SKPs were injected in the same locations. In all, 27 animals were injected with SKPs, 20 animals with neural precursors, and 25 control animals were included. Animals were sampled at three temporal phases following injection; phase 1 (1-7 days), phase 2 (7-14 days), and phase 3 (14-42 days). At all time points analysed, none of the negative controls showed OCT-positive cells.

2.3.1 Phase 1 (1-7 days)

Several important experimental questions were addressed during the first phase following grafting. To evaluate how the cells were adapting to their new environment, whether they were proliferating and/or dying, four animals were sampled daily : two animals transplanted with SKPs, and 1 positive and negative control.

OCT-positive SKPs and NS were identified under fluorescent light. Cells transplanted into the hippocampus mostly remained in a dense core and as expected, no apparent migration or morphological differentiation was observed (figure 8). Failure to inject cells directly into the hippocampus resulted in their localization in the ventricular area either above or below the hippocampus, or in the lateral ventricles. Under such circumstances, the cells could be found on more sections of the processed brain and seemed to be more

spread out. However, Two important points should be noted : a) the presence of cells in the ventricular area does not imply migration and b) the majority of the transplants were within the area above or below the hippocampus. In the case of neurospheres and, in some instances, SKPs, migration out of the core was observed within the first week (figure 8F). When injected directly into the hippocampus, some cells also adopted a morphologically complex phenotype between 5 and 7 days post-operation, with the cell body being either elongated or with processes extended around it (figure 8F). To verify if the morphologically complex cells were expressing specific neuronal markers, we immunostained SKPs and NS transplanted animals from 5, 6 and 7 day time points with anti-GFAP, anti-M2, and anti-M6. None of the morphologically complex OCT-positive cells colocalized with any of the three markers. We therefore concluded that transplanted SKPs and NS : i) survived the transplantation procedure, ii) survived in vivo, iii) migrated out of the graft core, and iv) adopted a differentiated morphology. However, lack of expression of any of the selected markers made it impossible to characterize the specific nature of the observed differentiation.

Since SKPs and NS are actively proliferating upon their isolation for transplantation, dilution of the OCT label could account for the inability to colocalize the implanted cells with specific neuronal markers. As a result, we immunostained all animals sacrificed during the first week following transplantation to detect, visualize and analyse the nuclear antigen Ki-67. The presence of OCT- and Ki-67 labelled cells was confirmed using confocal microscopy (figure 9). As expected, positive signals from host cells were observed throughout the brain and very few OCT-labelled SKPs and NS expressed Ki-67 (figure 9). This suggests that the majority of the grafted cells were not proliferating.

To evaluate what fraction of transplanted cells died either during or following the surgical procedure, TUNEL staining was conducted on every animal from 1 to 7 days post-implantation. We analysed specimens by confocal microscopy to determine what percentage of OCT-positive cells were also TUNEL-positive. In the first three days post-transplantation, large areas of the core of the grafted SKPs were TUNEL-positive (figure

10 A to C). In the NS transplant, the same massive cell death at the graft core was not observed although it was clear that some OCT-positive cells were dying. For both types of transplants, no TUNEL-positive cells were observed between 4 and 7 days. These observations suggest that cell death occurs rapidly in the first week following grafting, in both cell types, but that SKPs are more susceptible than NS to apoptosis. To confirm the results obtained using TUNEL staining, parallel experiments were performed using an antibody that recognizes cleaved caspase-3, one of the key executioners of apoptosis (figure 10 D to F). Visualization of activated caspase-3 by confocal microscopy revealed a distribution that was identical to that observed following TUNEL staining (figure 10). These results collectively confirm the initial temporal pattern of cell death observed in both cell types post-transplantation.

The objective of the next series of experiments was to further characterize the grafted cells and to gather more information on the host environment surrounding the transplant area. Two distinct approaches were used to address these objectives : i) fibronectin staining for the characterization of the transplant area, and ii) GFAP staining for the characterization of the host. First, fibronectin immunohistochemistry was performed on the same 28 animals described above. We used confocal microscopy to examine the localisation of OCT-positive cells and fibronectin-positive fibers in the specimens. Our observations demonstrated that fibronectin immunoreactivity co-localised with the graft core of OCT-positive cells. Fibers of fibronectin were found at the same location but also exhibited a pattern of expression analogous to the morphology of the graft core (figure 11). In the surrounding host area, only endogenous blood vessels were fibronectin positive. In contrast, fibronectin fibers were not co-localised with OCT-labelled NS. From the observations made of the SKPs transplants, it is impossible to conclude if the fibronectin networks were produced by the SKPs forming a microenvironment or produced by the host sending new blood vessels to and inside the graft. However, the absence of fibronectin immunoreactivity in NS transplanted animals leads us to believe that fibronectin was produced by the SKPs, and, moreover, that the SKPs might be creating their own microenvironment and not integrating into the host at this point.

However, another mutually exclusive explanation is that only the fibronectin-positive SKPs survive transplantation. Indeed, SKPs initially express both nestin and fibronectin, but when they differentiate, they segregate into two distinct populations. Thus, *In vivo*, it is possible that only the fibronectin-positive population survives while the nestin one dies.

Second, GFAP immunostaining was performed on the same animals and results were analysed using the same protocol. The experiments demonstrated a strong GFAP signal in the host environment both in SKPs and NS transplants (figure 12). Indeed, positive fibers could be seen in the vicinity of the transplant, suggesting a reaction to cell implantation. This signal was observed only during the first week. Positive fibers were numerous for the first four to six days and then slowly disappeared. This suggests a reaction of the host towards the implanted cell, which is a well-known phenomenon.

The experiments performed in phase 1 provided a significant amount of relevant information about the events that occurs during the first week post-transplantation. We confirmed that a percentage of SKPs and NS survived for up to 7 days *in vivo* and were potentially migrating from the implantation site. In the case of SKPs, the apparent production of fibronectin suggests that the cells were not only surviving, but were also functional and adapting to their new environment. Other findings, such as the fact that some cells are lost after implantation, that proliferation does not occur and that the host is reacting to the operation, were expected.

2.3.2 Phase 2 (7-14 days)

Although in the second week post-transplantation, this phase still represents an early time point. Our objective during this phase was to address whether the surviving cells were evolving in their new environment. Two animals transplanted with SKPs, 2 with NS and 1 control were sacrificed at 7 and 14 days following grafting. In order to visualize OCT-positive SKPs and NS on a morphological level, analysis of all animals was conducted under fluorescent light. At 7 days, both types of transplants showed similar gross

morphology. Cells remained in a dense graft core where it was virtually impossible to observe single cells. As previously mentioned, a small percentage of neurospheres as well as SKPs migrated out of the core after 7 days (figure 13). NS, and in certain cases SKPs, injected in the dentate gyrus adopted a morphologically complex phenotype (figure 13D). At 14 days, the size of the graft core was reduced relative to that observed at 7 days. Furthermore, less debris was present at the implantation site. Thus, at this point, we were able to easily detect single cells (figure 14). Remaining cells, located in the granular cell layer of the dentate gyrus, showed signs of migration and differentiation (figure 14). In fact, both SKPs and neurospheres were in the process of adopting a complex morphology. involving elongation and branching. Differentiation was more frequent and elaborate in the case of CNS precursor transplants. To test if the morphologically complex cells were expressing specific neuronal markers, we immunostained SKPs and NS transplanted animals (14 days time point) for GFAP, M2, and M6. None of the complex OCT-positive cells co-localized with any of these three markers. We therefore concluded that OCT-positive SKPs and NS located in the granular cell layer of the dentate gyrus, while able to show signs of migration and morphological differentiation, were not expressing specific neuronal markers.

The presence of fewer cells at 14 days relative to day 7 suggested that there was additional cell death following the initial burst of apoptosis during phase 1. While it was thus of interest to quantify cell death during this second phase, we could not use either the TUNEL assay or activated caspase-3 immunostaining because the signal is generally too weak after 3 days in the animals. Moreover, we could not count cells because of the dense nature of the graft core, which made it impossible to reliably count individual cells. The results we could obtain were therefore qualitative in nature. For both SKPs and NS, cells were lost but to a lesser extent than during phase 1 with the process being more gradual and less abrupt. Comparing the number of cells present at day 14 to day 1, cell loss in the NS transplant was less than in the SKPs transplant. In conclusion, although we could not precisely quantitate cell numbers after two weeks *in vivo*, it remains clear that cells were still undergoing apoptosis. However, it was also clear that NS continued

to exhibit a better survival rate and adapted more readily to their new environment than SKPs. The experiments realized in phase 2 confirmed that a percentage of the total SKPs and NS that were grafted survived up to 14 days *in vivo*, and that the surviving cells migrated from the implantation site as well as differentiated.

2.3.3 Phase 3 (14-42 days)

This phase represents the later time points of this study. Our objective during this phase was to assess whether surviving cells could be neuralized by the neonatal rat brain environment. Two animals transplanted with SKPs, 2 with NS and 1 control were sacrificed at 21, 28, 35, and 42 days following grafting to formally address this possibility.

To determine the precise number of surviving OCT-positive cells, randomly chosen series of sections from each animal were visualized using fluorescence microscopy. All cells present on these sections were counted and the cell count obtained for each series was multiplied by the total number of series for every animal to obtain whole animal cell number approximations (Table #1).

TABLE #1

Weeks	**SKPs**			**Neurospheres**		
	Animal #	*Cell number*	*% of survival*	*Animal #*	*Cell number*	*% of survival*
3	53	≅600	10	70	≅2000	32
	54	≅600	10	71	≅2000	32
4	56	0	0	73	≅1500	24
	57	271	4.3	74	≅1500	24
5	59	115	1.8	76	≅1000	16
	60A	150	2.4	77	≅1000	16
6	60C	61	0.97	79	≅800	13
	60D	67	1.1	80	≅800	13

For NS, total cell numbers and the percentage of cell survival agree with previously published reports (Rosser et al., 2000 and Englund et al., 2002). For SKPs, fewer cells survived relative to NS, with neurospheres demonstrating a greater relative rate of survival (figure 15). To rule out that greater rates of cells death in SKPs were not attributable to a greater sensitivity to a lack of certain nutrients provided by the culture media, we tested the effect of nutrient deprivation on the survival of SKPs and NS. Both cell types were plated on coated four well chamber slides in DMEM/F12 alone without growth factors or serum, conditions in which cultured SKPs and NS normally die due to the lack of survival factors such as FGF and EGF. TUNEL labelling at 12, 24 and 48 hours was done to assess, based on viability, which population was more sensitive to a lack of essential nutrients. At 12 hours, 23% of the SKPs were TUNEL-positive compared to 90% of the NS (figure 16). At 24 and 48 hours, the percentage of TUNEL-positive SKPs gradually increased to levels initially observed for NS. This ruled out the possibility that the differential survival of SKPs observed in our initial series of experiment was due to a greater sensitivity to nutrient content of the culture environment. However, the lower survival rate of SKPs *in vivo* may be due to other, yet to be determined factors. Additional experiments will therefore be required in order to establish the precise mechanism by which SKPs are differentially susceptible to higher mortality rates post-implantation.

To address whether those cells that survived were migrating, integrating and differentiating after long post-implantation periods, in the developing CNS, we identified OCT-positive SKPs and NS by fluorescence microscopy. Since this phase corresponds to a long period (four to six weeks) in which the graft area is stabilized, it was almost all clear of debris and only viable cells remained (figure 17 and 18). The vast majority of the surviving OCT-labelled SKPs and NS observed at these time points possessed a complex morphology. As depicted in figures 17 and 18, some cells were elongated and had extended numerous processes around their cell body. These Morphologically differentiated cells were found in greater numbers in the following locations;

hippocampus, subventricular zone, and corpus callosum. In contrast, cells in the midbrain area and the neocortex rarely showed differentiated features.

Series of immunohistochemistry were subsequently performed on all phase 3 animals to investigate whether these morphologically complex cells had differentiated into neurons or glia. A variety of protein markers detecting the neuronal or glial antigens most likely to be present in these cells after long-term grafting were used; NG2, for detection of glial cells within the developing and mature central nervous system that have the properties of oligodendrocyte precursor cells ; GFAP, for detection of developing astrocytes ; M2, for detection of mouse astrocyte antigens; and M6, for detection of mouse neuronal and glial antigens. None of these neuronal markers co-localized with OCT-positive SKPs or NS, which led us to conclude that although cells had adopted a morphologically complex appearance, no specific differentiation had occurred. In other words, the developing rat brain environment was not capable of neuralizing the SKPs or the NS. However, earlier work done with NS and neonatal transplants suggests that at least glial differentiation should have occurred. One possible explanation for these discordant results is that the orange cell tracker either blocks the signals of the immunostaining or perhaps more importantly, blocks normal differentiation of SKPs or NS. This issue will be elaborated upon later.

Collectively, experiments performed in phase 3 demonstrate : i) SKPs and NS can survive for up to 6 weeks, ii) the survival rate of NS is similar to that previously reported in the literature, iii) the NS survival rate is better than that of SKPs and iv) SKPs and NS are possibly, although we have no direct evidence, migrating and differentiating in brain structures such as the hippocampus, the subventricular zone and the corpus callosum.

2.4 Differentiation of SKPs in presence of orange cell tracker *in vitro*

In order to validate our *in vivo* results, we needed to verify whether SKPs could differentiate normally in the presence of OCT. SKPs were plated in differentiation media supplemented with 20% fetal bovine serum, EGF (10 ng/mL), and FGF (10 ng/mL) for 7

days. The cells were labelled with OCT at the beginning of the experiment and one fifth of OCT concentration was left in the culture media in order to prevent OCT dilution effects (In culture, OCT-labelled cells remain positive for about 72 hours [laboratory observations and product information]). Following the 7 day differentiation period, cells were also labelled for nestin and βIII-tubulin (figure 19). Results demonstrated that both markers were present and that the percentage of positive cells was in accordance with previously published studies (Toma et al., 2001). Therefore, OCT does not block immunostaining or differentiation of SKPs *in vitro*.

In parallel, Nao Kobayashi in the lab performed differentiation experiments with other dyes like PKH-26 and DiI. In these preliminary experiments, SKPs were plated and allowed to proliferate in the presence of FGF and EGF for 3 days. They were then grown in differentiation media supplemented with FBS for 4 days, after which, they were labelled (PKH or DiI), trypsinized and replated for 4 additional days prior to performing immunocytochemical analyses. This protocol mimics that done for her *ex-vivo* studies on hippocampal slice cultures. Immunostaining for neurofilament (NFM), Hu C/D, and synaptophysin was performed to determine whether the dyes co-localised with specific neuronal markers. Results indicated, however, that only a minor population of PKH or DiI-labelled cells co-expressed NFM, Hu C/D and synaptophysin (figure 20). This suggest that, unlike OCT, dyes like PKH-26 and DiI can block normal differentiation of cells. In the future, these preliminary results need to be completed with certain experiments; i) the markers (OCT, PKH-26 and DiI) have to be compare using the same differentiation protocol, and ii) a control experiment demonstrating what is the percentage of cells that differentiate into NFM, HuC/D, and synaptophysin positive cells in the absence of dye. Taken together, these two independent sets of experiments suggest not only that OCT does not block differentiation of SKPs and NS but also, that it represents a valid experimental technique that requires further optimization for implantation studies.

3. DISCUSSION

Both *in vitro* and *in vivo* data are needed to complete the characterization of a new source of stem cells. In the case of our novel source of stem cells, some *in vitro* work had been done previously in the laboratory, but foundations had yet to be laid *in vivo*. Stem cell transplantation performed in this study has thus provided considerable information regarding SKPs. For example, SKPs isolated from mouse skin can survive for up to 6 weeks in neonatal rat brain. Moreover, we suggest that the transplanted cells retained their biological functions, considering the production of a fibronectin network *in vivo*. In regions like the hippocampus, the subventricular zone and the corpus callosum, SKPs migrated from the implantation site and adopted a complex morphology suggesting differentiation into a specific phenotype.

Where does this work stand with respect to related work done within the stem cell community? As for survival, the numbers obtain with the NS transplants are comparable to those that others have obtained when transplanting neural precursors into neonatal rat; Approximately 10 to 30% of the cells remain viable for up to 20 weeks post-transplantation (Rosser et al., 2000 and Englund et al., 2002). While the survival rate of SKPs cannot be directly compared to any data in the literature, survival of even 1% of the transplanted cells for up to 6 weeks is very positive. Indeed, survival of cells derived from skin inside the central nervous system environment is exceptional, especially since the cells showed signs of biological activity by their expression of fibronectin filaments.

As for site-specific differentiation, both SKPs and NS demonstrated signs of migration and differentiation in our experiments. These results are encouraging considering that, in general, when multipotent stem cells are transplanted, only a fraction of the cells (1 to 10%) differentiate in a site-specific manner. However, some reports have demonstrated exceptionally encouraging results. Yang et al. (2000), 14 days after transplanting rat spinal cord neuronal restricted precursors into the neonatal rat anterior subventricular zone, observed extensive migration and integration in various regions of the brain; the olfactory bulb, frontal cortex, and occipital cortex. They also reported that all

transplanted neuronal precursors expressed neuronal markers such as MAP-2, βIII-tubulin, GABA, and glutamate. An additional study by Rosser et al. (2000) reported that four weeks after the transplantation of human CNS precursors into either the striatum or hippocampus of neonatal rats, some of the implanted cells were positive for human-specific tau antibodies, and had differentiated in a site-specific manner. Accordingly, human tau-specific fibres were found to project along pathways appropriate for the site of neuronal integration. Only one report has shown extensive survival and differentiation in a long-term experiment. Englund et al. (2002) observed a range of 75 to 100% survival in recipients in the 2- to 20-weeks groups and 50-60% in recipients in the 40- to 65-weeks groups. In addition, extensive integration and differentiation of human neural precursors from the embryonic forebrain into the striatum, hippocampus, and the subventricular zone was observed. It is important to stress, however, that the results described in these papers are exceptional in the field of transplantation.

The facts that our experiments did not reproduce the results describe above makes it difficult to evaluate the multipotency of SKPs. Considering that SKPs and NS OCT-positive cells did not colocalize with a specific neuronal marker, we had to conclude that the developing rat brain environment did not neuralize the transplanted cells. In the case of the SKP-transplant, it is possible that they were not neuralized because of their non-neuronal origin. In contrast, various studies have demonstrated that NS or CNS precursors have the capacity to survive, integrate and differentiate in various CNS environments. Multiple factors can account for the inability of NS to differentiate *in vivo* in our studies compared to previously published work. Like we described above, success in transplanting CNS precursors varies, even in the literature. Which differences can account for the disparity between our results and those of others? Our experience with manipulation and transplantation of NS is limited. We established our protocols based on studies available in the literature and reproduced their manipulations in our work. However, while these experiments appear straightforward, they are actually very sensitive to a multitude of influencing parameters; i) the labelling technique, ii) the

preparation of the cells in culture prior to implantation, iii) the environment in which the cells are transplanted, and iv) the properties of the transplanted cells themselves.

The first parameter is the labelling technique. We decided to investigate if some technical aspects could be interfering with the proper differentiation of implanted cells. We investigated whether OCT was the reason why differentiation into the neuronal phenotype was impaired. We tested *in vitro* if normal differentiation of SKPs occurred in the presence of OCT and *ex vivo* if differentiation of SKPs and NS occurred in the presence of other dies like PKH-26 and DiI. These experiments showed; i) that *in vitro*, OCT does not block differentiation; ii) and that *ex vivo*, differentiated cells rarely colocalize with PKH-26 or DiI-positive cells. Even if these results are contradictory, they demonstrate that OCT did not block differentiation. Although the presence of certain dyes during cell differentiation might be modifying their ability to do so, we were unable to find any evidence that OCT negatively affected the differentiation potential of our transplanted cells. In contrast, these experiments demonstrated that OCT represents a good method for cell labelling in the context of our transplantation work.

To this day, there is not a consensus on the second parameter, which is the ideal method of preparing cells prior to transplantation. The most important question is how long should NS be passaged in culture before they are used for transplantation? When passaged over time, the composition of the spheres changes. For example, the percentage of terminally differentiated neuronal cells tends to increase. In the literature, NS were transplanted as early as possible following their isolation. While we adopted the same strategy, we cannot be sure that these are the optimal conditions for the cells to be implanted *in vivo*.

The third parameter that can play a major role in influencing transplantation results is the environment in which the cells are implanted. At a given time, different areas of the brain are undergoing different stages of development. In other words, the time point at which the cells that constitute the various regions of the brain terminally differentiate

differs in a cell-type specific manner. For the transplantation to be effective then, the cells have to be implanted at the right place at the right time. Indeed, precursors have to be injected in an area undergoing a differentiation step to which they can respond. This parameter cannot be dissociated from the fourth one; the inherent properties of the transplanted cell themselves. Even, if the cells are transplanted at the appropriate site in the developing brain, most cells remain undifferentiated upon transplantation. One possible explanation for this lack of differentiation is that the cells do not receive the appropriate cues from their new environment. Indeed, most of the cells don't grow fibers after implantation, a process that enables them to find the cues required for growth, migration, and differentiation. A second possibility is that the precursors are destined to generate cells specific to the area from which they were isolated. It is possible that cells like the NS in our experiments, isolated from the forebrain, are only responsive to molecular signals/cues of the developing forebrain. When transplanted in the developing hippocampus they are only partially able to respond to cues present in this environment. In summary, all of these parameters have to be taken into account when evaluating our transplantation results with NS.

In the next section, we will discuss the experiments we want to perform in the future to optimize the transplantation of SKPs. Ultimately, these experiments will enable us to optimize our protocols and direct our efforts toward more clinically oriented experiments that relevant to disease or injury models of transplantation.

Labelling

Finding the best approach to mark the cells for transplantation has been tedious. Like we described in the results section, many techniques were considered and analysed but none were optimal. This suggests that the field of transplantation could still be improved by the development of better labelling techniques. At present, when assessing transplantation studies, every result obtained needs to be put in perspective with the advantages and disadvantages of the specific technique that was used. In the future, we would like to repeat our results using different markers. In order to do so, we will

explore alternative labelling methods. Alternatives for labelling have already been used in certain research projects and some appear to be promising. For example, a research group in the U.S. has used the transferrin receptor as an intracellular delivery device for magnetic nanoparticles. Once tagged with the magnetic particles, the oligodendrocytes were transplanted into myelin-deficient rats (Bulte et al., 1999). Cells were analysed using magnetic resonance microscopy. Although not widely used at the moment, magnetic labelling could be promising in the future, both for laboratory and clinical experiments, especially because it would allow for the analysis of a transplantation model throughout the whole period of the experiment instead of at fixed time points.

For the moment, we are planning to focus on three options. First, the use of the Y chromosome staining. This technique has been widely used and is very efficient. Male cells are transplanted into a female host and a DNA labelling technique is used to label the male-specific Y chromosome. In this situation, 100% of surviving transplanted cells are labelled, proliferation cannot affect labelling efficiency, and the staining is highly specific. In our situation, we would isolate SKPs from male animals and perform the transplantation into female neonatal rats. Second, xenotransplantation is an option. We have transplanted murine cells into rat; however, few mouse-specific markers are available. In contrast, numerous human-specific markers are detectable with specific antibodies. We therefore think that human SKPs should be transplanted into rat. To date, work in our laboratory in which SKPs have been isolated from human skin has been promising. SKPs were successfully isolated from human skin and their differentiation potential appears to be similar to that of murine SKPs. Human SKPs would improve the efficacy of our current labelling technique, and at the same time, they would be more relevant starting material with which to study questions with future clinical applications. Third, we would like to repeat the isolation of SKPs from transgenic mouse lines. Even if the technique hasn't proven to be reliable to date , it still remains an important option for these experiments.

To test labelling techniques with mouse SKPs, we want to perform *ex-vivo* transplantations. In this experimental model, slices of the hippocampus would be put in culture conditions where the slice of tissue remains alive and transplantation is possible in a very specific location. These experiments are shorter in time than transplantation, technically simple and allow tracking of the implanted cells on a daily basis. Nao Kobayashi has already implemented the system in the laboratory and it has proven to be very effective in testing different techniques for SKPs transplantation.

Transplanted cells

Like we discussed in a previous section, spheres of SKPs contain differentially committed cells. SKPs are a relatively homogenous population of cells composed of 95% of stem cells and uncommitted precursors (Toma et al., 2001). In the introduction, we reviewed data in the literature that demonstrated that undifferentiated ES cells do not integrate as well as *in vitro*-predifferentiated ones upon transplantation into the CNS. Because of the high content of stem cells within the SKP-containing spheres, it is possible that they require a predifferentiation step in vitro prior to transplantation. This predifferentiation into neuronally committed precursors or terminally differentiated neural cells could increase survival and integration within the host. This possibility is currently being formally addressed in the laboratory. Experiments on predifferentiation of SKPs *in vitro* with molecules like retinoic acid and growth factors are also being performed. We would like to predifferentiate SKPs into committed precursors or terminally differentiated cells and transplant them into specific region of the neonatal recipient to test if in this situation, the implanted cells are more responsive to environmental cues present in the transplant area.

Transplantation

We discussed above the different parameters that can interfere with the proper site-specific differentiation of transplanted SKPs and NS. The environment in which they are implanted plays a major role in influencing the ability of the grafted cells to adapt to it. In the future, we want to analyze in detail which period of neonatal rat development is

best suited for the integration and differentiation of SKPs. We will perform embryonic transplantation of SKPs at various developmental time points to address this question. Simultaneously, we will further investigate which site of implantation is optimal for the transplantation of SKPs by grafting cells into different regions of the brain.

Our findings demonstrate that SKPs can be used for transplantation work. They survive pre-operation manipulation, injection and implantation into host tissues. Moreover, they show signs of migration and morphological complexity. Of course, the technique itself needs to be improved before SKPs demonstrate *in vivo* their multipotentiality that has already been shown *in vitro*. Our project was based on trying to show this potential. Unfortunately, the limitations of our transplantation technique prevented us from fully addressing their potential versatility. Future experiments will enable us to show the great potential of SKPs for transplantation. Indeed, once technical improvements are made and multipotentiality is proven *in vivo* both in normal and pathological animal models, SKPs could potentially be brought to the next step; therapeutic transplantation. SKPs as a therapeutic approach would offer a number of advantages; they are an accessible and abundant source for transplantation, they represent an autologous source for implantation, and they are an easily expandable population of cells (low population doubling). SKPs would also circumvent two major problems associated with the current technique ; source of tissue and immunological response. For the moment, many aspects of the biology of SKPs need to be characterized before we are able to perform experiments on animal models, and ultimately, on human models. However, our results and those with human SKPs, theoretically show promising results. We therefore believe that SKPs may eventually turn into a promising therapeutic approach, especially since we also believe that transplantation is the most promising approach for the treatment of neurological diseases or injuries.

4. CONCLUSION AND SUMMARY

In this study, we wanted to investigate if naïve SKPs could be neuralized by the neonatal brain environment thereby generating neural cells *in vivo*. After performing the required experiment, we had to conclude that they are not neuralized by neonatal brain environment. However, SKPs appear to be very promising has an alternative source for transplantation, but further experiments are required to explore their full potential.

5. FIGURES

Figure 1 : Phase micrographs of spheres of cells isolated from E14-15 mouse cortex (neurospheres) and cultured for 1 month or from P3 mouse skin and cultured for 3 weeks.

Figure 2 : Photomicrographs of cells immunoreactive for Ki-67 (green) after 24 hours of plating in differentiation condition. Cells are counterstained with Hoechst (blue) to show nuclei.

Figure 3 : Photomicrographs of cells immunoreactive for Nestin (green) after 24 hours of plating in differentiation condition. Cells are counterstained with Hoechst (blue) to show nuclei.

Figure 4 : Representative photomicrographs of SKPs labelled with the fluorescent dye Hoechst. (A and B) Fluorescent photomicrographs of Hoechst-labelled cells (blue) in the ventricular area of a 3 days post-transplantation neonatal rat (A)and in ... of a 7 days post-transplantation neonatal rat (B) viewed with the UV filter. The Hoechst dye is detectable in the nuclei.

Figure 5 : Representative photomicrographs of SKPs labelled with 5-bromo-2-deoxy-uridine (BrdU). Cells were labelled 48 hours prior to plating for 24 hours in differentiation media and immunostain with monoclonal anti-BrdU (green). Cells are counterstained with Hoechst (blue) to show nuclei.

Figure 6 : Representative photomicrographs of NS immunostain with M2 and M6 monoclonal mouse specific antibody. Cells were plated 24 hours in differentiation media and immunostain with M2 and M6 (green). In parallel, phase micrographs of the same images to show the spheres.

Figure 7 : Representative photomicrographs of SKPs labelled with the fluorescent dye, orange cell tracker (OCT). Cells were labelled with OCT (red) 30 minutes prior to plating for 24 hours in differentiation media. Cells are couterstained with Hoechst (blue) to show nuclei.

Figure 8 : Photomicrographs of OCT-labelled SKPs and neurospheres during the first week post-transplantation. [A (2 days), B (3 days), C (4 days) and E (7 days)] Sagittal section showing a dense graft core of SKPs in the hippocampus. [D (3 days) and F (7 days)] Sagittal section showing a dense graft core of neurospheres in the hippocampus. In *F*, it is possible to see cells away from the transplant that adopted complex morphology.

Figure 9 : Confocal images of OCT-labelled cells (red), Ki-67-labeled cells (green) and double-labelled cells (yellow) transplanted in the hippocampus. These are typical images of labelling with Ki-67 during the first week following transplantation. These images are taking at 3 (A and D), 4(B) and 5(C) days. A to C are SKPs and D are NS.

Figure 10 : Confocal images of OCT-labelled cells (red), Tunel-labelled or cleaved caspase-3-labeled cells (green) and double-labelled cells (yellow) transplanted in the hippocampus. These are typical images of labelling with tunnel (A to C) and caspase-3 (D to F) during the first 3 days following transplantation. These images are taking at 3 days. Green-labelled cells are transplanted cells that are dying and have lost the OCT markers.

Figure 11 : Confocal images of OCT-labelled cells (red) transplanted in the hippocampus. Fibronectin is shown in green. These are typical images of labelling with fibronectin during the first week following transplantation. These images are taking at 3 days. SKPs: A to E; NS: F to H.

Figure 12 : Confocal images of OCT-labelled cells (red) transplanted in the hippocampus. Glial acidic fibrillary protein (GFAP) is shown in green. These are typical images of labelling with GFAP during the first week following transplantation. These images are taking at 3 days. SKPs: A to E; NS: F to H.

Figure 13 : Photomicrographs of OCT-labelled SKPs and neurospheres at 7 days post-transplantation. A) Sagittal section showing a graft core of SKPs in the hippocampus. B) SKPs located in the granular cell layer of the dentate gyrus show signs of migration and differentiation C) Sagittal section showing a graft core of neurospheres in the hippocampus. D) Neurospheres located in the granular cell layer of the dentate gyrus show signs of migration and differentiation.

Figure 14 : Photomicrographs of sagittal section of OCT-labelled SKPs and neurospheres at 14 days post-transplantation. A)Section showing a graft core of cells in the ventricular area. B and C) sections showing cells in the hippocampus. Higher magnification of section showing the morphological complexity of cells in the hippocampus (D and E) and in the ventricular area (F). SKPs: A to E; NS: F.

Figure 15: Photomicrographs of sagittal section of OCT-labelled SKPs and neurospheres at 21 days post-transplantation. A and E) sections showing a graft core of cells in the ventricular area. B to D) Section showing cells in the hippocampus. SKPs: A to D; NS: E.

Figure 16: Representative photomicrographs of Tunel positive SKPs and neurospheres. Tunel immunoreactivity was assessed 12 hours after plating in DMEM/F12 alone. SKPs are showing 25% of positivity compare to 90% in the case of neurospheres. Cells are counterstained with Hoechst (blue) to show nuclei.

Figure 17: Photomicrographs of sagittal section of OCT-labelled SKPs and neurospheres at 28 days post-transplantation. A)Section showing a graft core of SKPs in hippocampal area. B and C) Section showing NS in the ventricle. Higher magnification of section showing the morphological complexity of cells in the ventricular area (C).

Figure 18 : Photomicrographs of sagittal section of OCT-labelled SKPs and neurospheres at 35 and 42 days post-transplantation. A to C) Section showing SKPs in hippocampal area at 35 days post-transplantation. D) Section showing NS in the hippocampus at 35 days. E and F) Section showing SKPs in the hippocampal area at 42 days.

Figure 19 : Photomicrographs of 7 days differentiated OCT-labelled SKPs. Cells were labelled with OCT during the week of differentiation *in vitro* and then labelled for nestin and βIII-tubulin. Cells immunoreactive for nestin (above, green) and βIII-tubulin (bottom, green).

Figure 20 : Confocal images of SKPs labelled with PKH or DiI and immunostain for neuron-specific markers after two period of 4 days of differentiation separated by a replating step. Panel A shows PKH-labelled SKPs (red) stain for neurofilament (green); for this panel and the others, cells in yellow are double-labelled. Panel B shows DiI-labelled SKPs (red) stain for HuC/D (green). Panel C shows DiI-labelled SKPs (red) stain for synaptophysin (green).

Figure 1

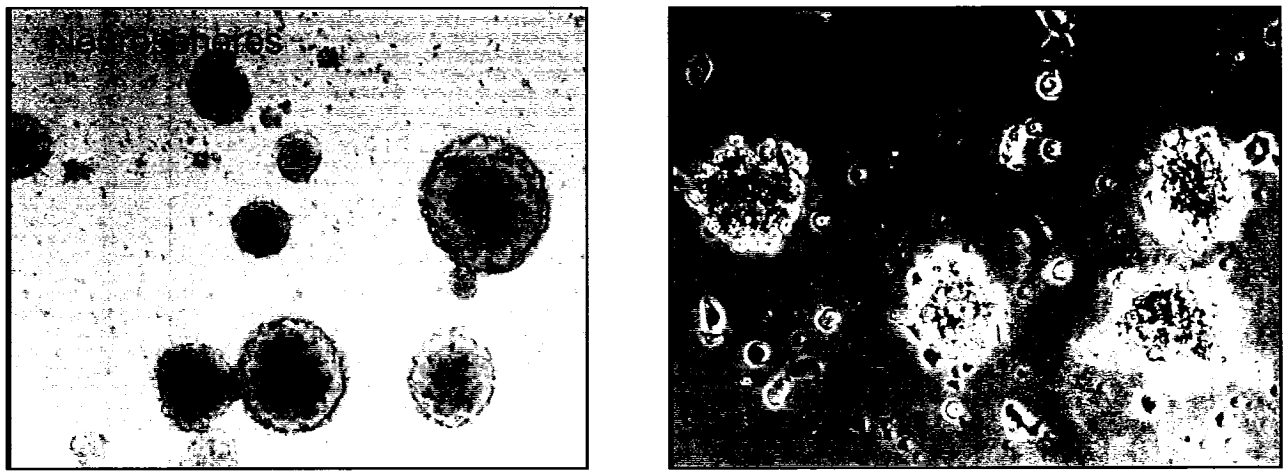
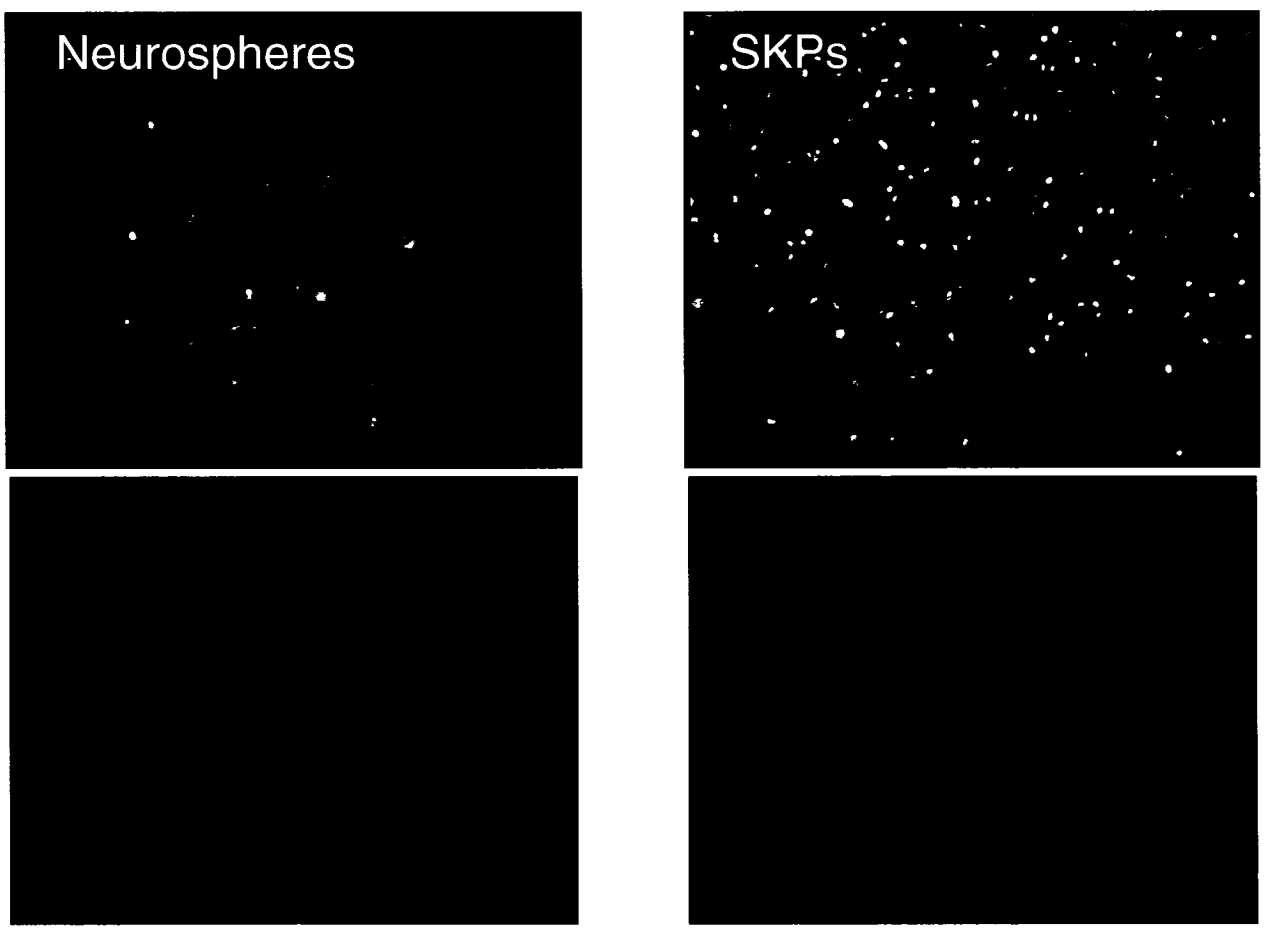


Figure 2



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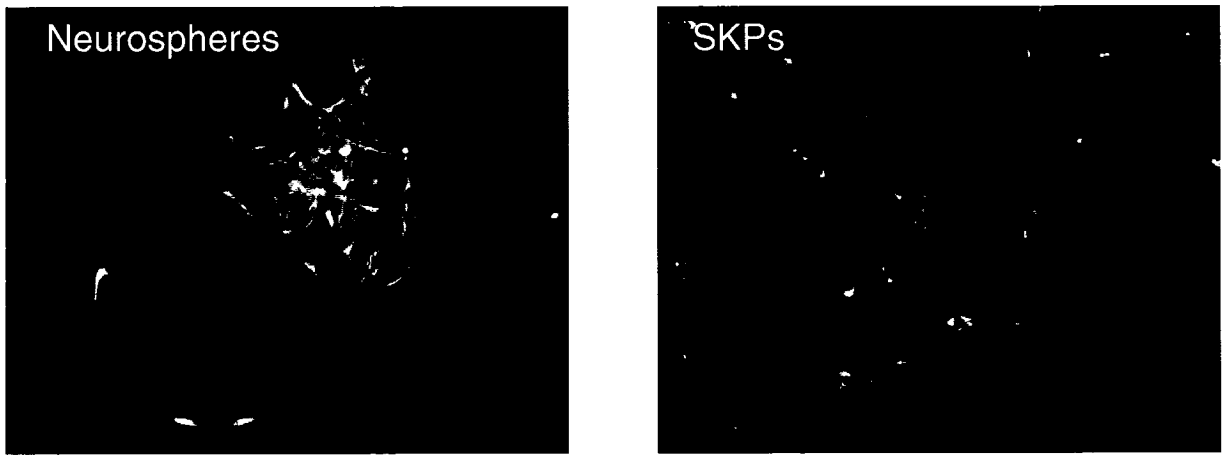

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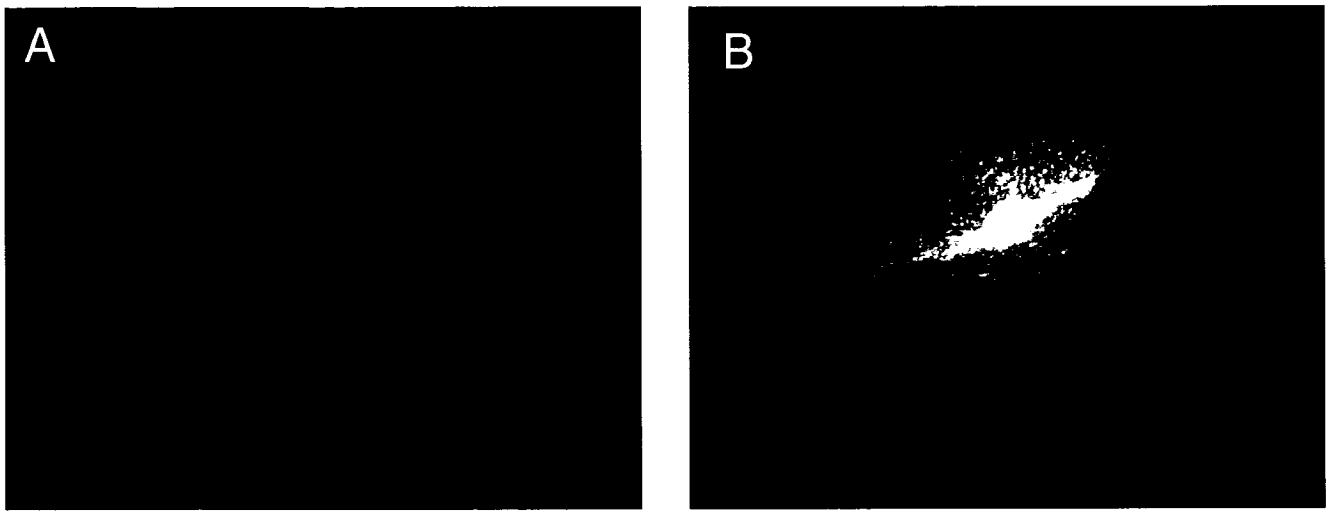

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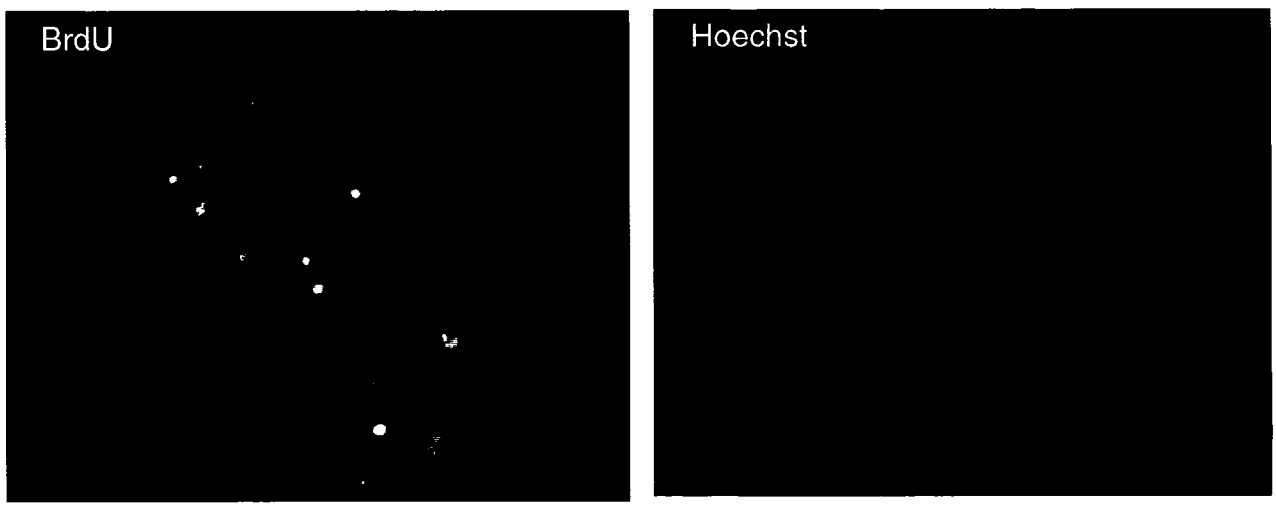

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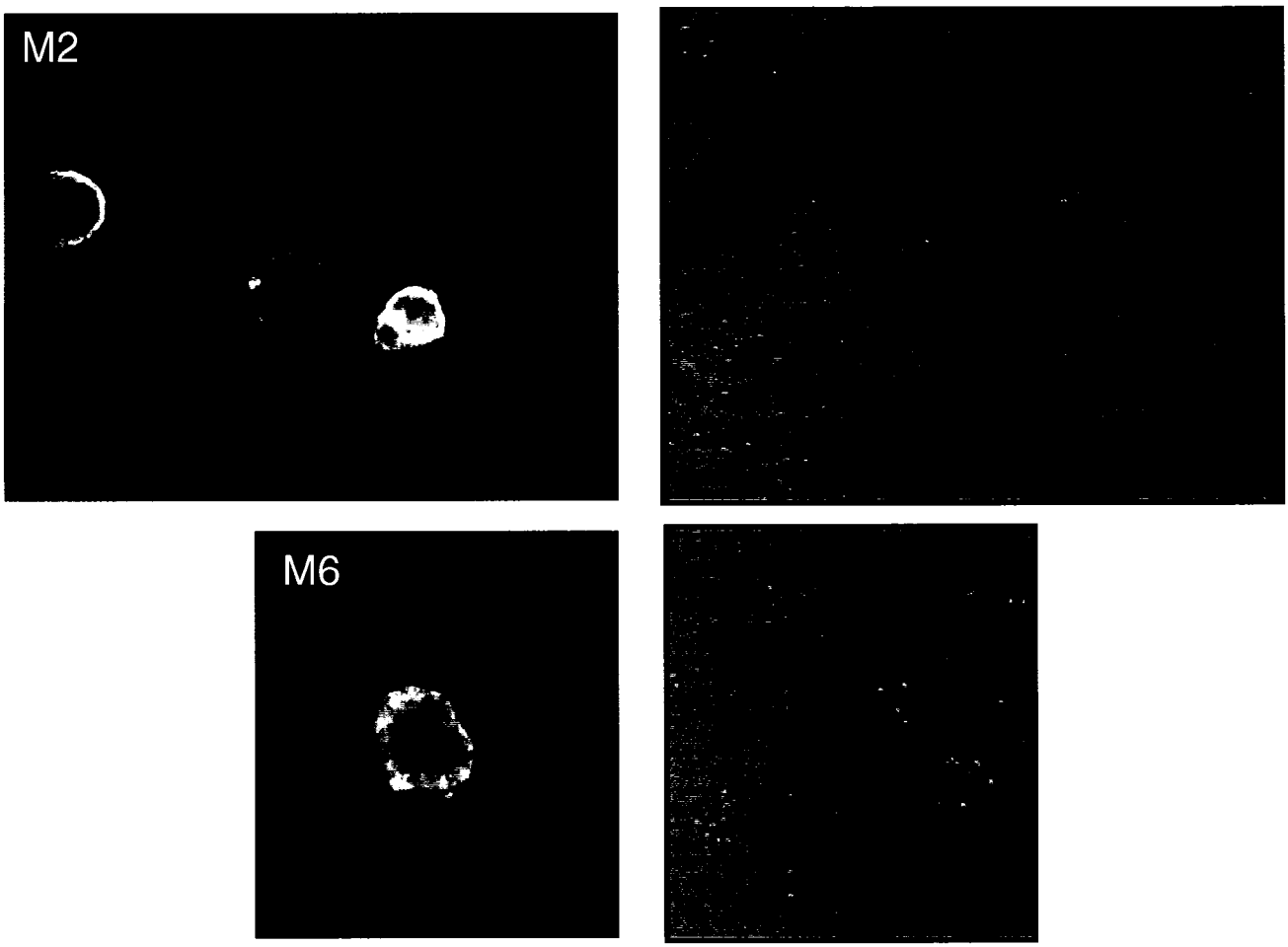

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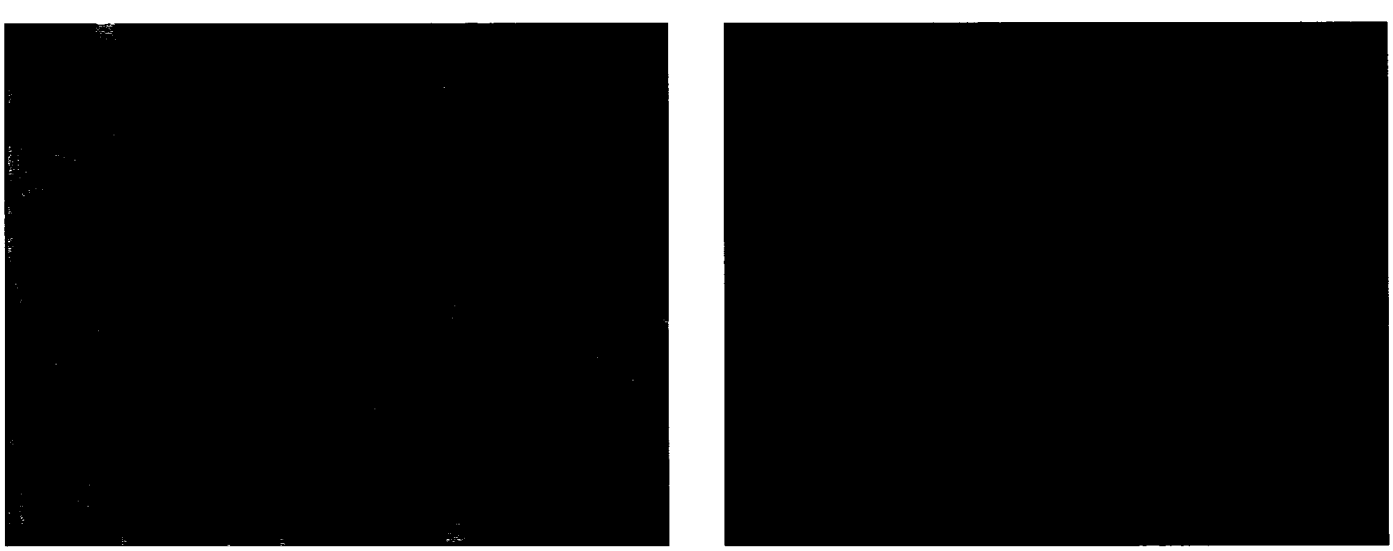

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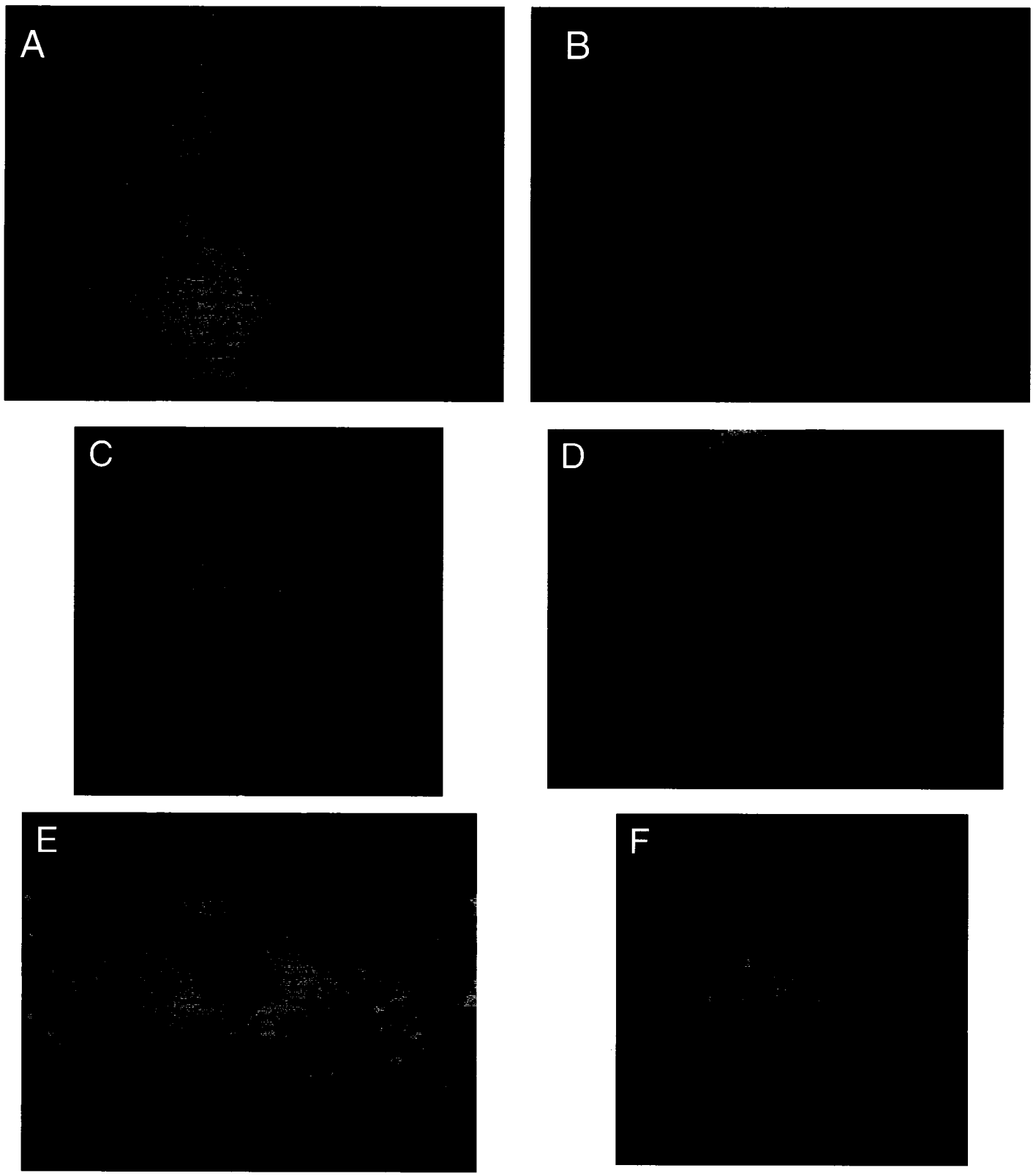

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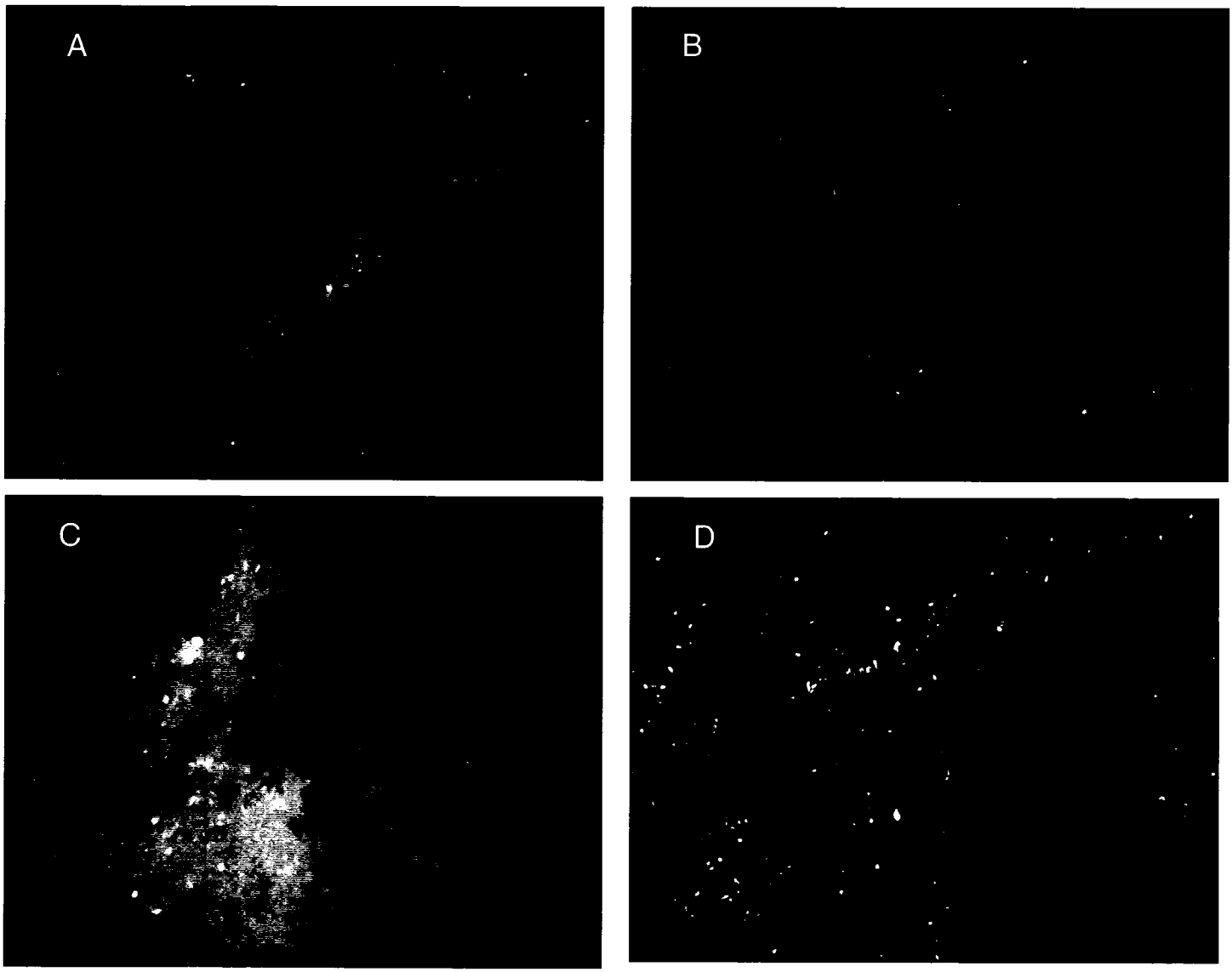

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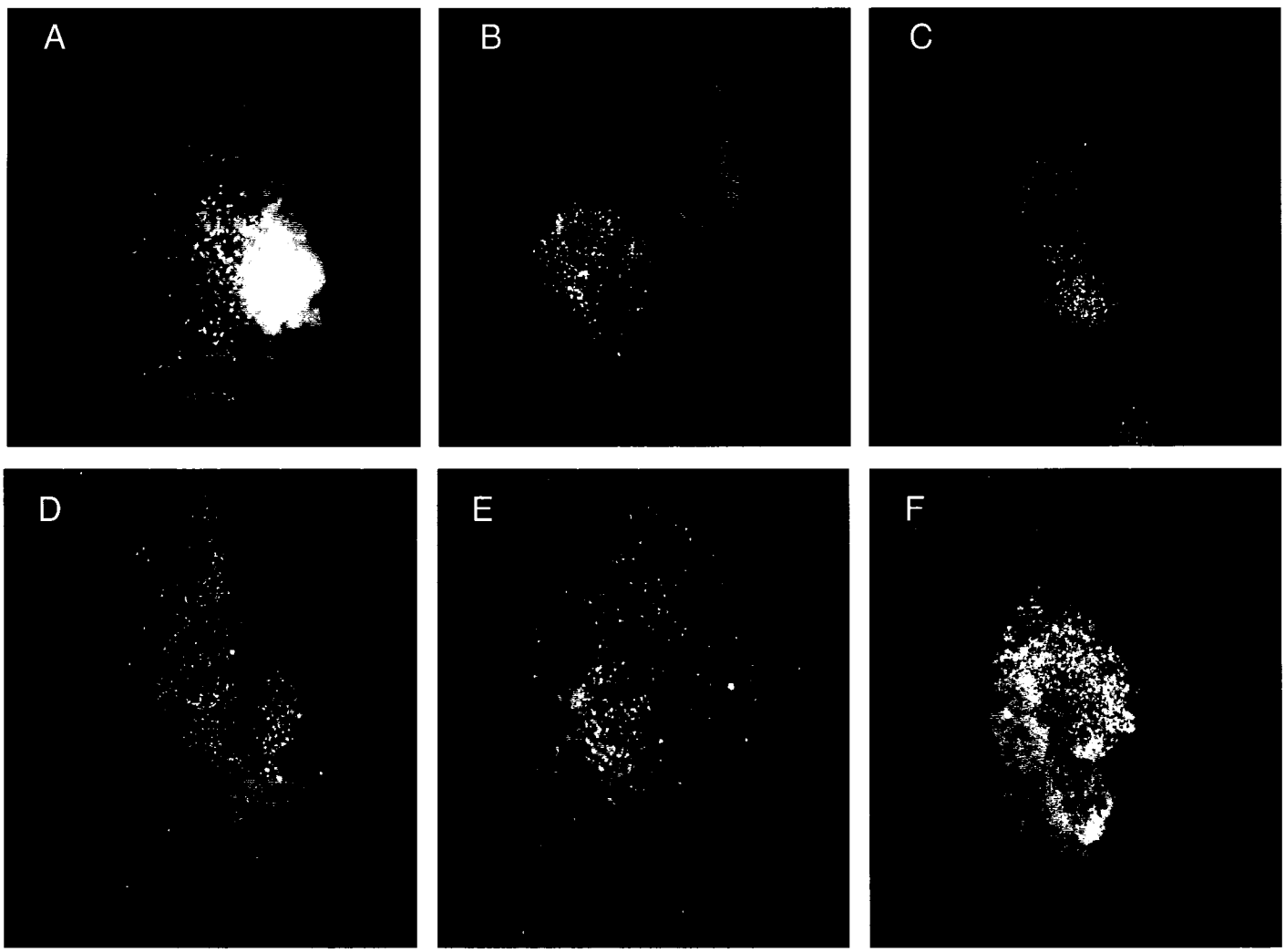

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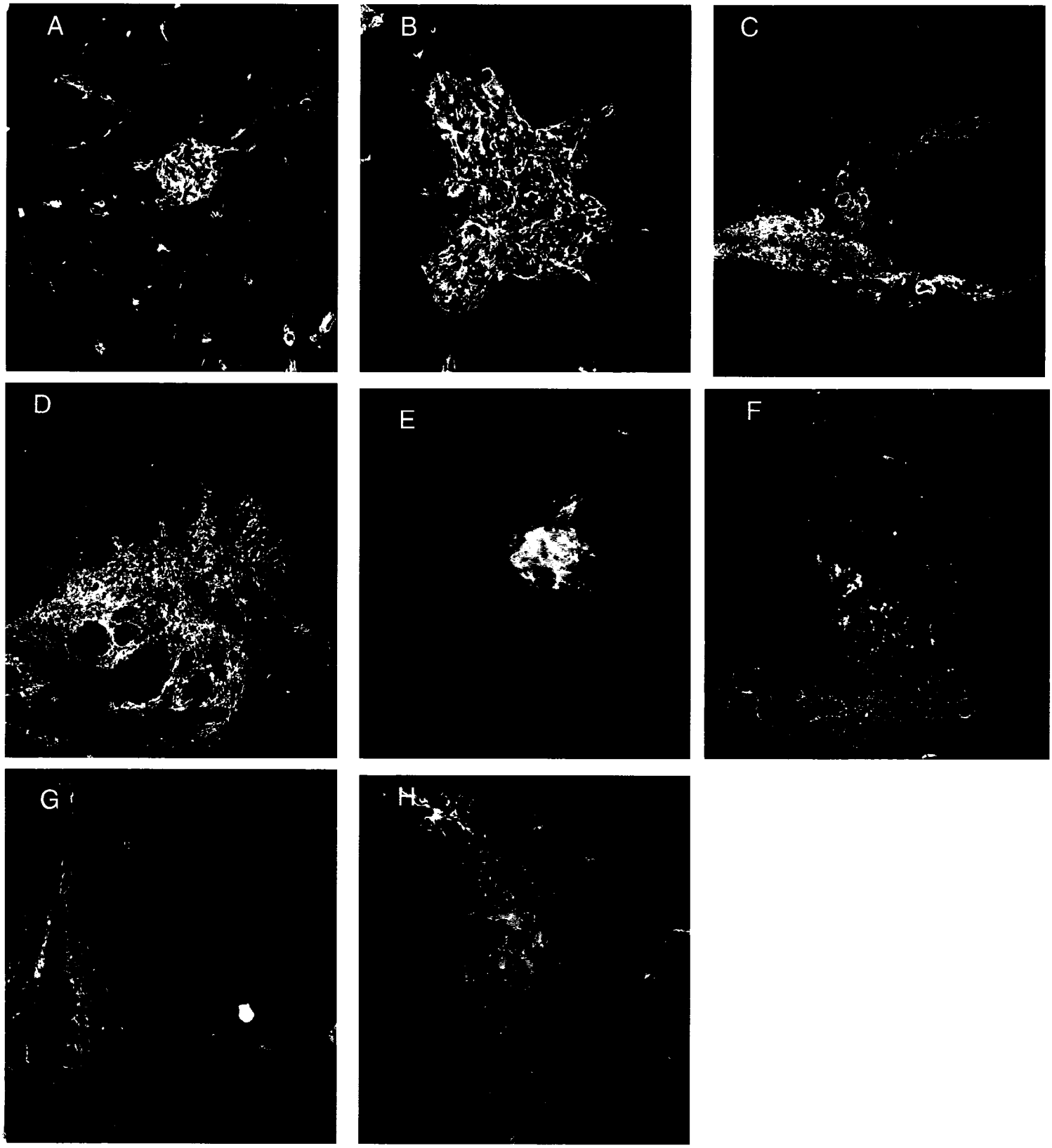

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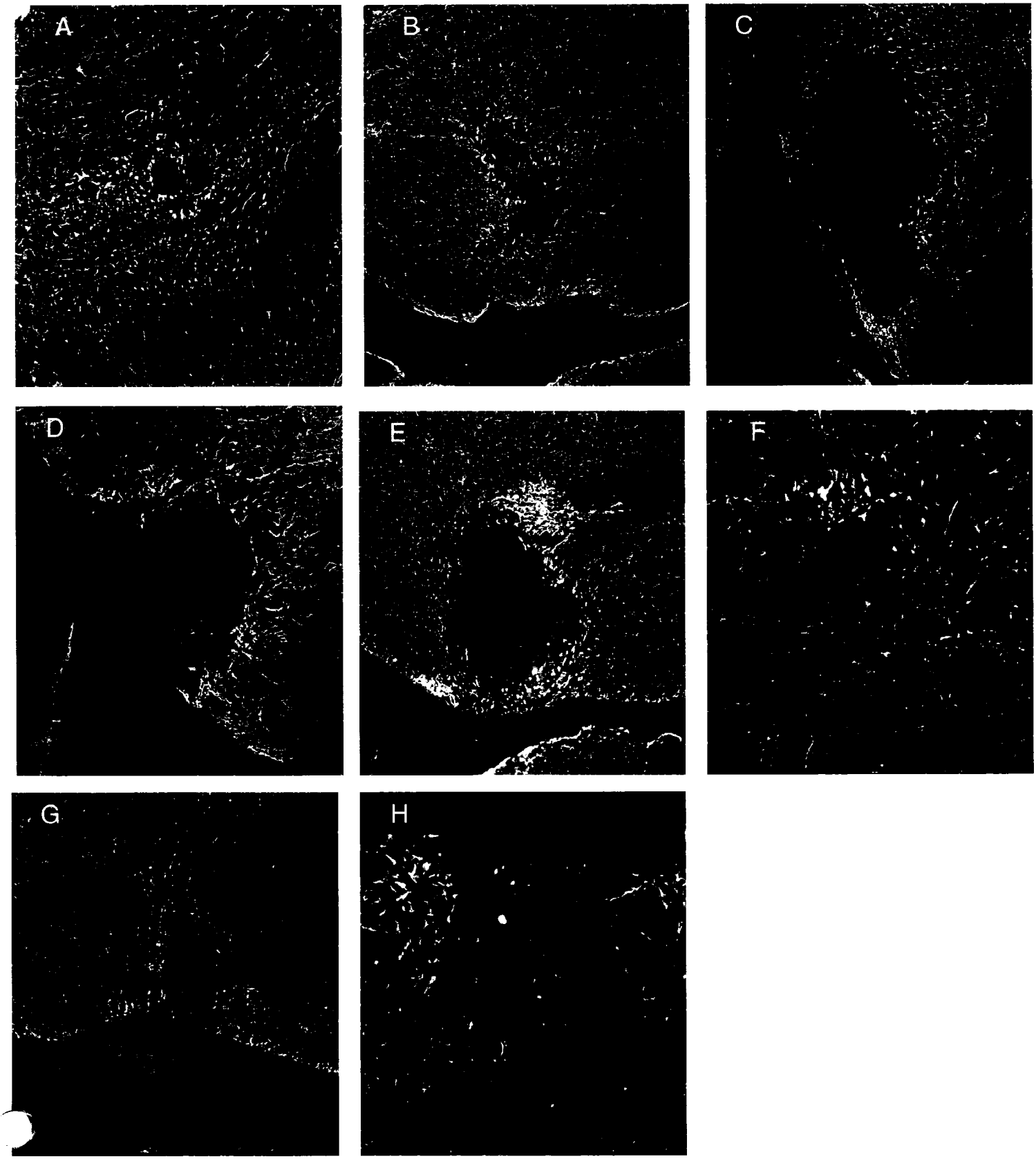

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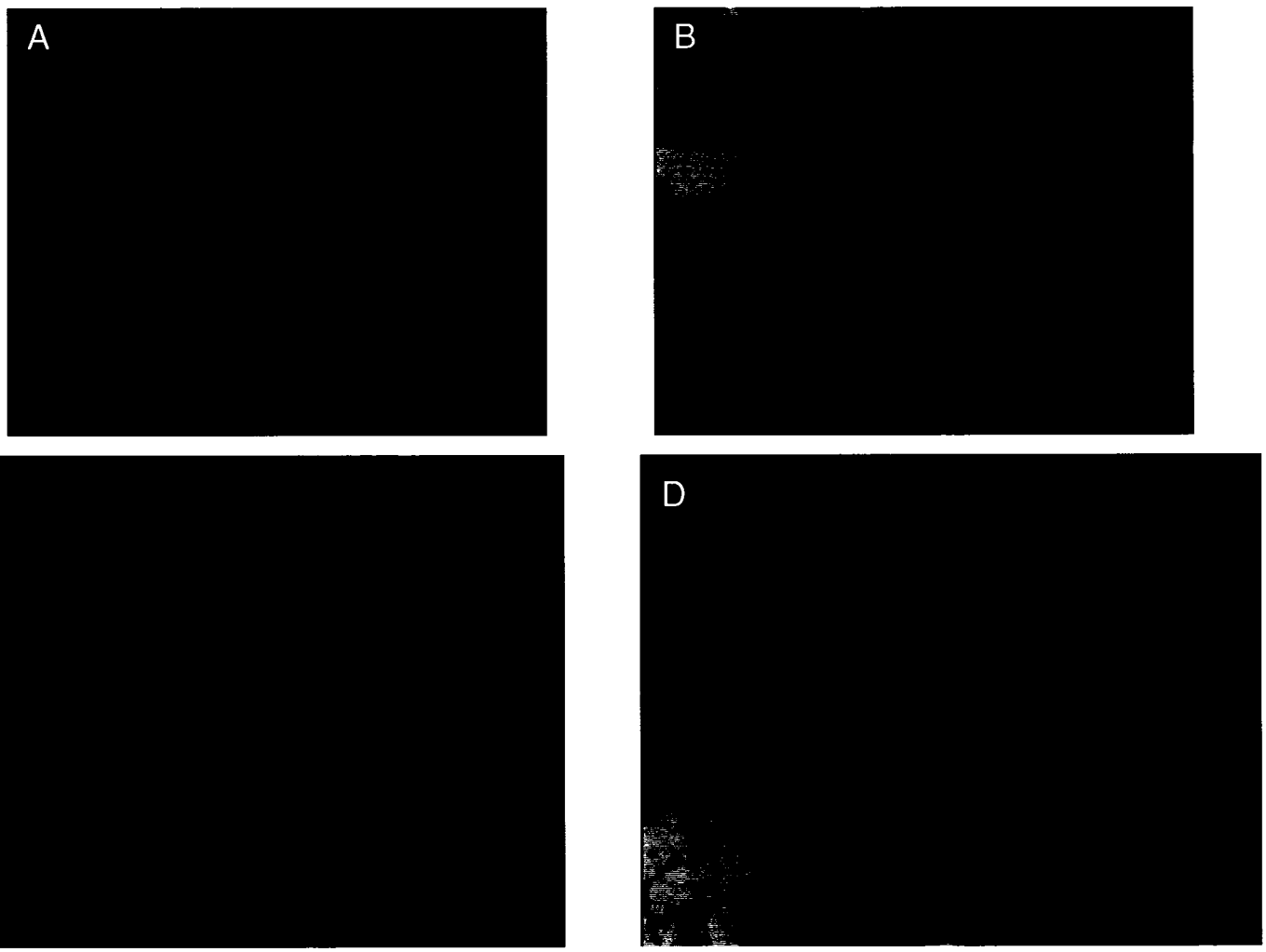

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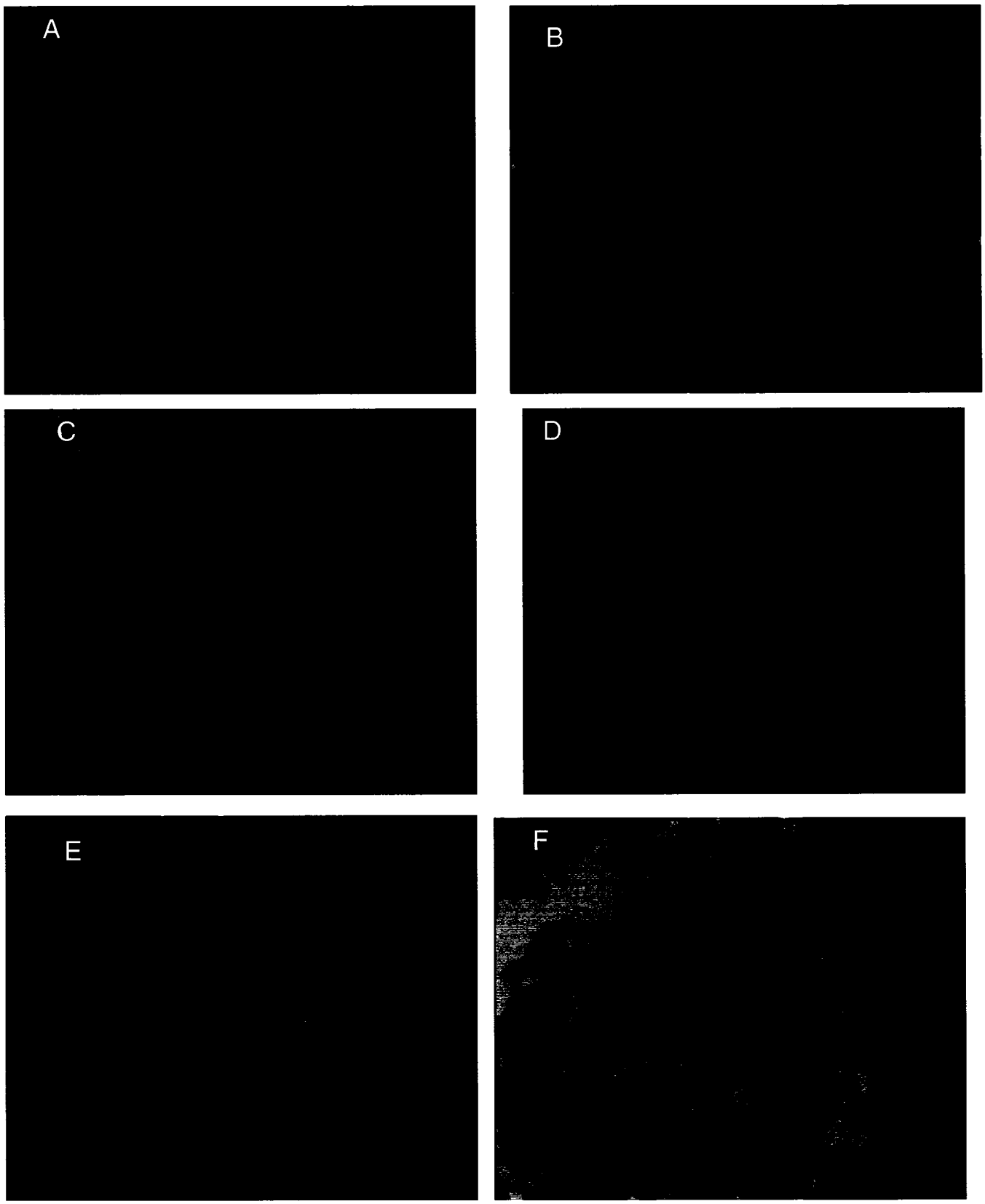

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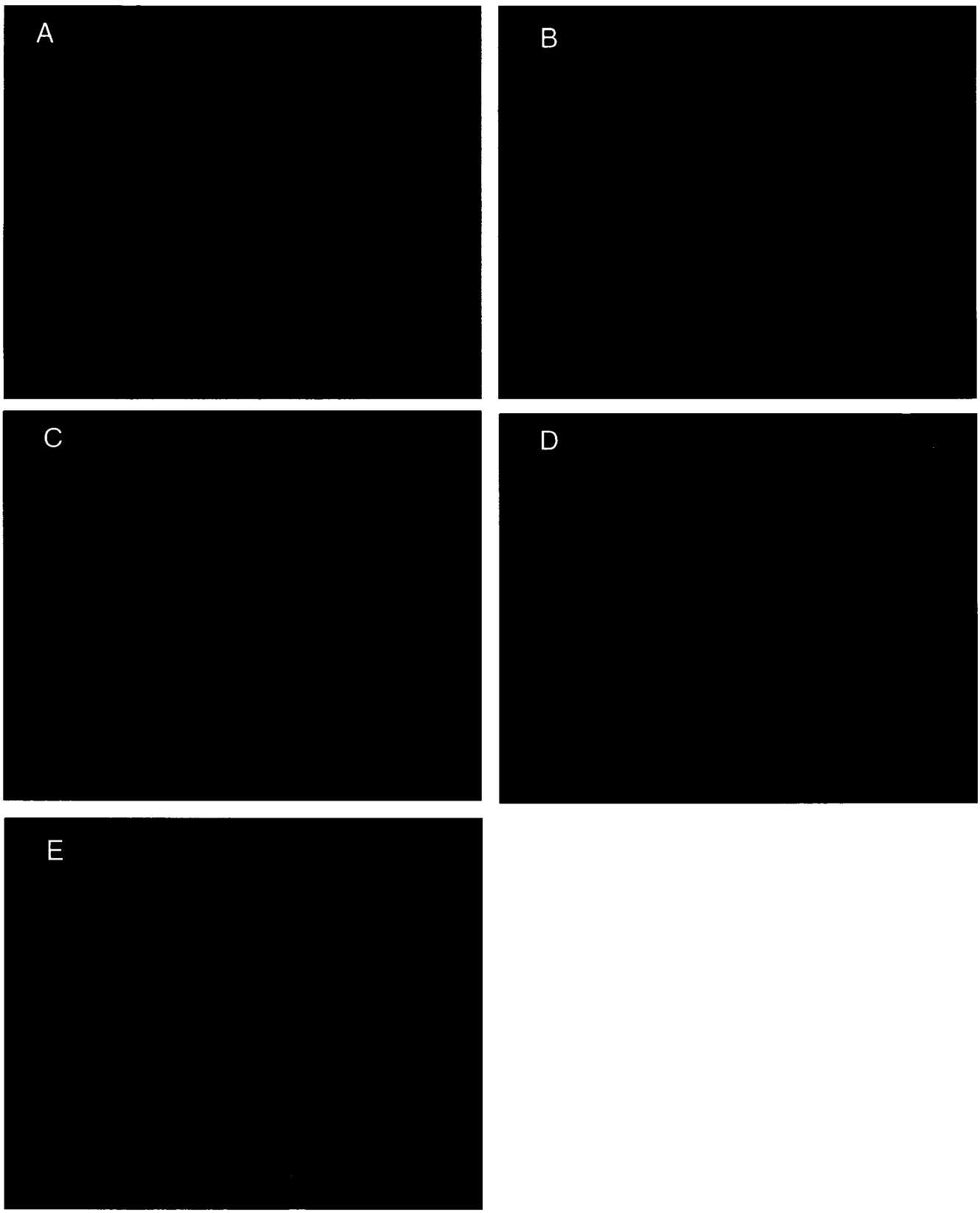

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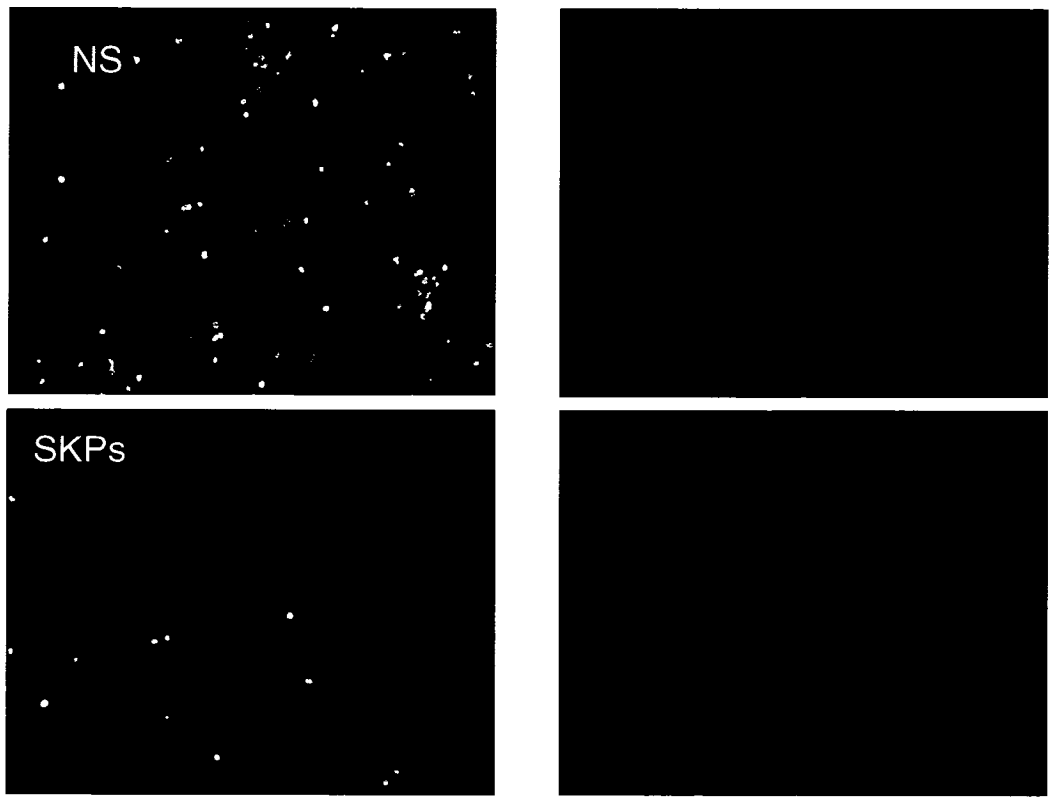

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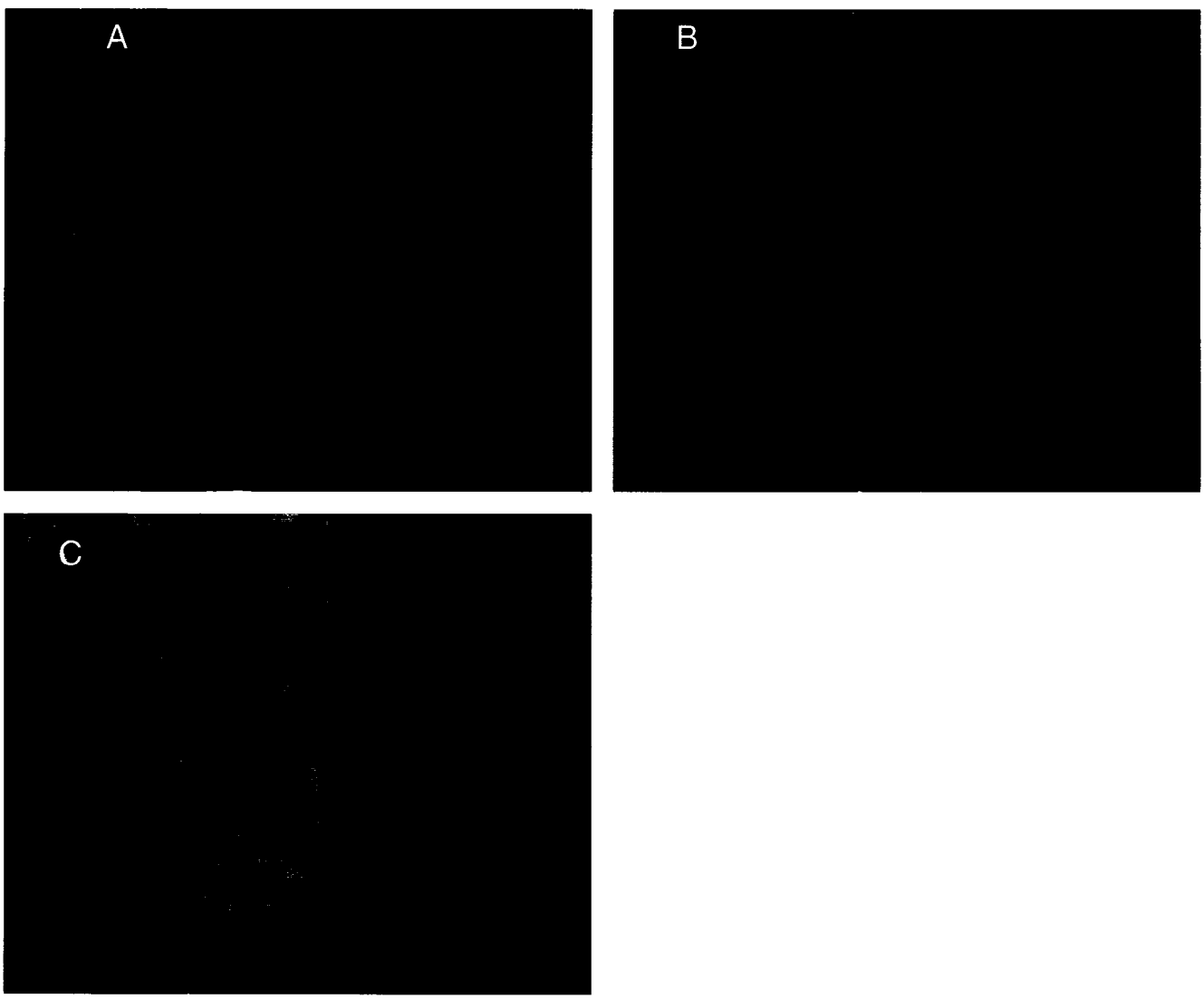

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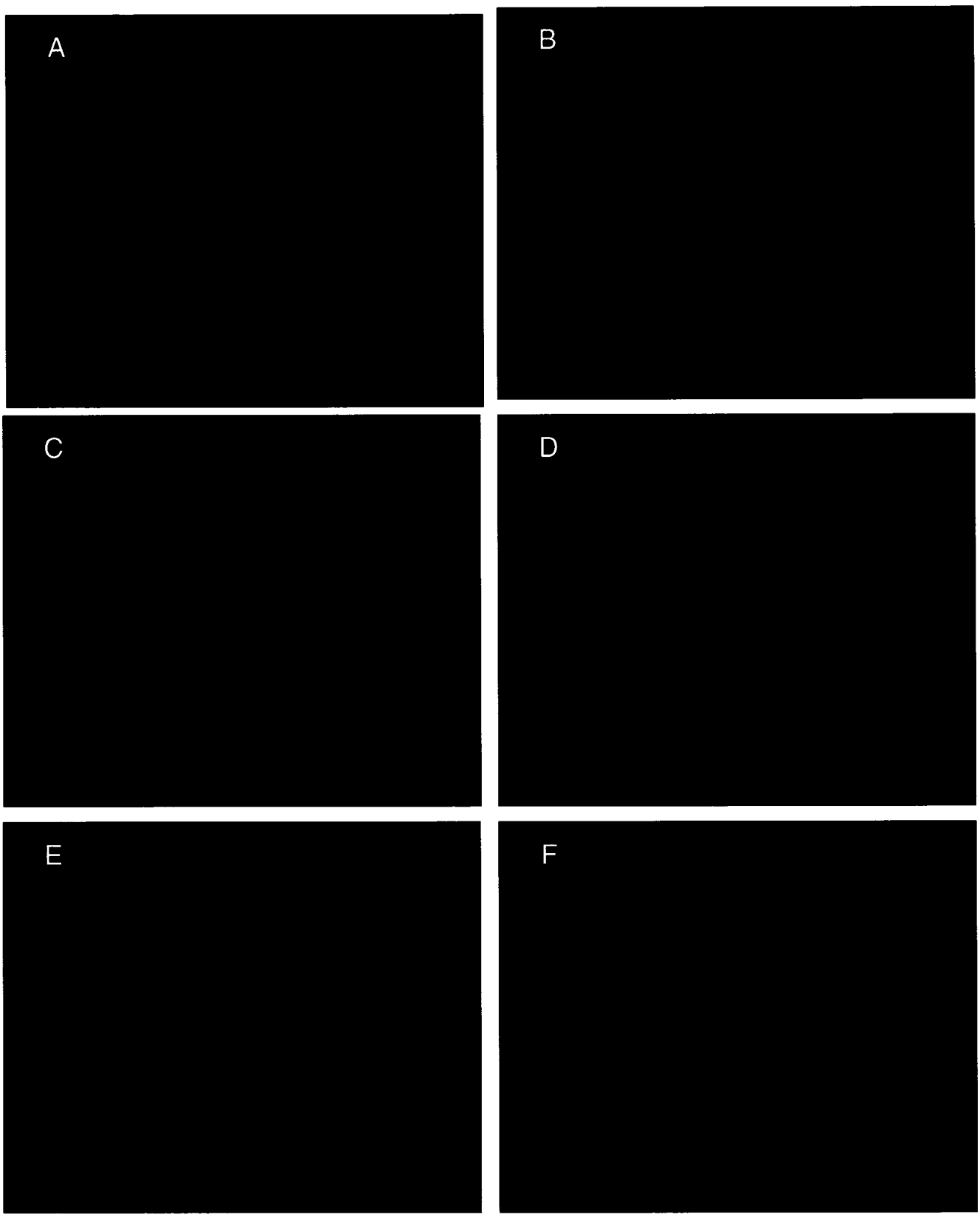

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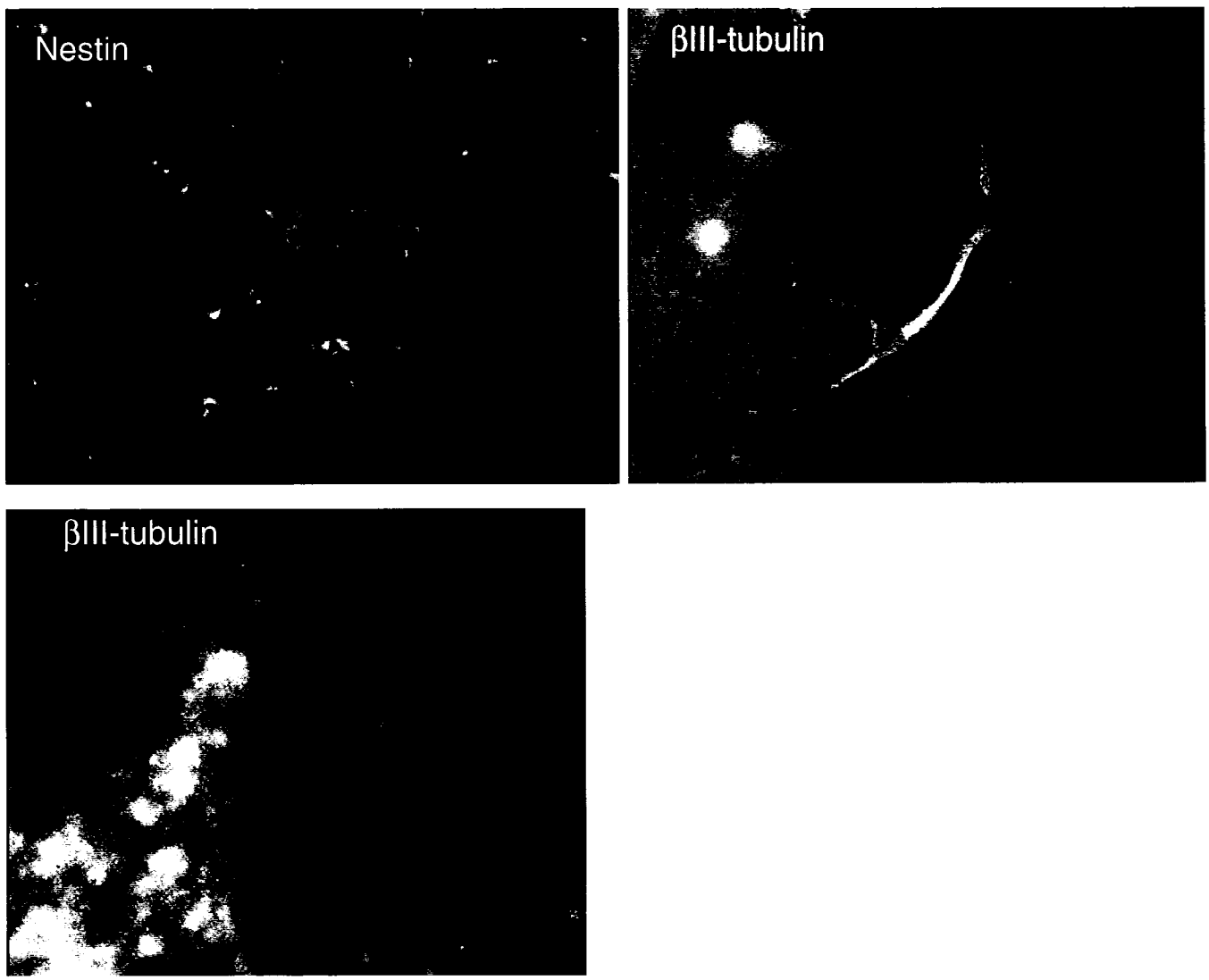
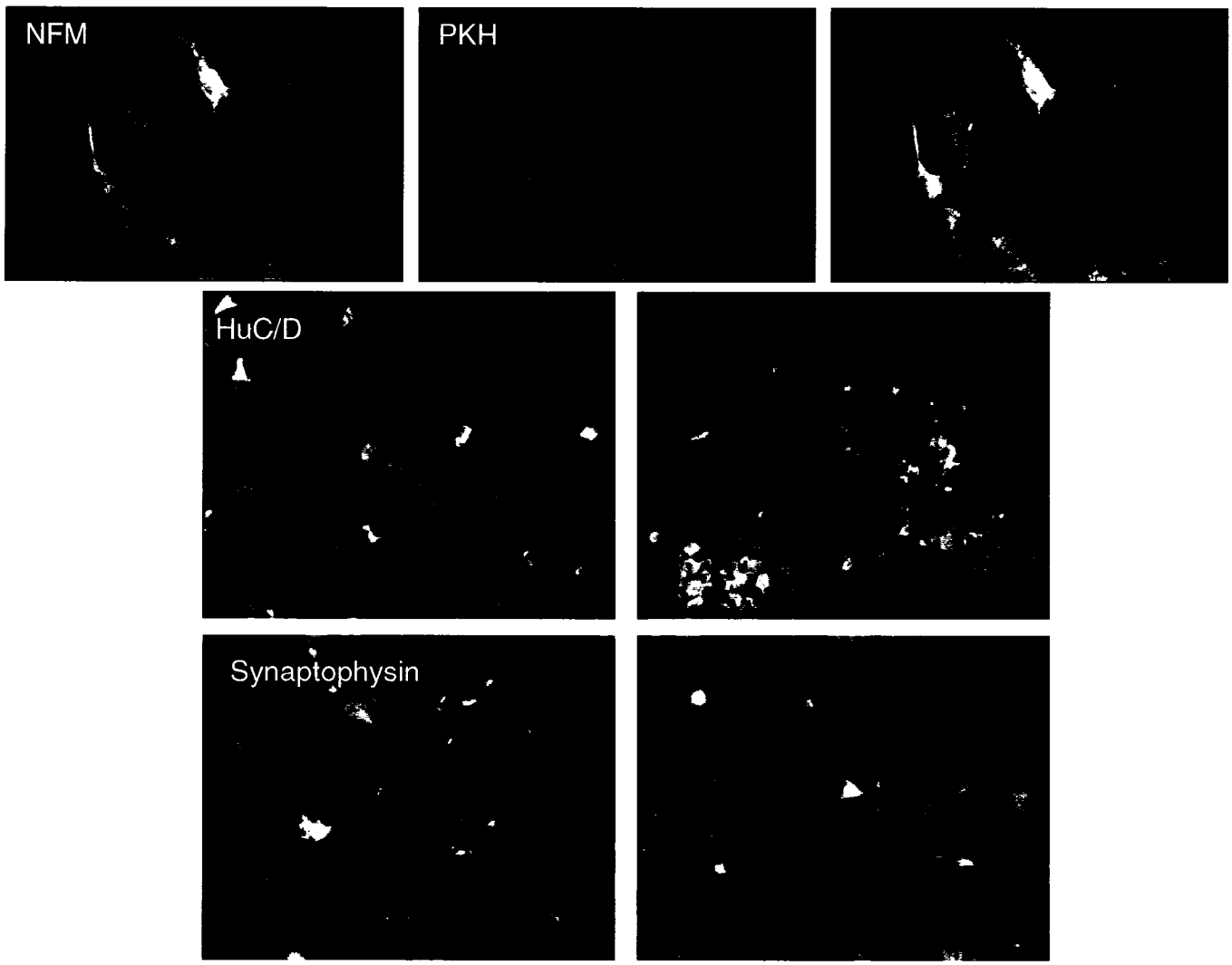


Figure 20



6. MATERIALS AND METHODS

6.1 Isolation of neural precursors from embryos

The preparation of forebrain neural stem cell from mouse embryos has been previously described in detail (Daadi et al., 1998). Briefly, cortical tissue, obtained from E15-17 CD1 mice, was dissected in ice-cold Hank's balanced salt solution (Gibco BRL) and then transferred into 37°C media (DMEM/F12 supplemented with B-27, fungizone and penicillin/streptomycin) containing epidermal growth factor (EGF) and fibroblast growth factor (FGF). The tissue was mechanically triturated with a plastic pipet into almost single cell suspension. Cell were grown in flask and were passaged every 5-6 days. For differentiation, cells were plated in 4 well chamber slides precoated with laminin and poly-D-lysine (both Collaborative biomedical products). Cell density was 10 spheres of about 15000 cells per well. Cultures were maintained at 37°C in a 5% CO_2 incubator.

6.2 Immunocytochemistry

Cells grown in 4 well culture slides (Nunc) were washed gently twice with phosphate-buffered saline (PBS) to remove any media left. Cells were fixed with a 4% paraformaldehyde solution for 20 minutes followed by two washes with PBS (5 minutes). They were then permeabilize 5 minutes using a solution of 0.2% NP-40 in PBS followed by three PBS washes (5 minutes each). Blocking solution (6% normal goat serum [Jackson Immunoresearch Laboratories Inc.] and 0.5% bovine serum albumine (Fischer Scientific) in distilled water) was applied for 1h½ to 2 hours. Primary antibody diluted in PBS was incubated overnight at 4°C followed by three washes with PBS. Ki-67 (1:200 monoclonal;BD PharMingen), nestin (1:400 monoclonal; PharMingen), β-gal (1:800 monoclonal;Sigma-Aldrich), m2 (1:10 polyclonal;the kind gift of Dr. K. Lagaher), m6 (1:10 polyclonal;the kind gift of Dr. K. Lagaher), and βIII-tubulin (1:500 monoclonal;Tuj1 clone;BABCO) were the primary antibodies used during these in vitro studies. Secondary antibody diluted in PBS was incubated 1 hour at room temperature followed by three washes in PBS. Secondary antibodies were cy3-conjugated goat anti-mouse (1:200), cy3-conjugated goat anti-rabbit (1:400), FITC-conjugated goat anti-mouse (1:50), and FITC-conjugated goat anti-rabbit (1:200)(all from Jackson

Immunoresearch Laboratories). Cells were incubated in Hoechst (1:3000) for 1 minutes followed by three washes in PBS. Slides were mounted using geltol (Immunon).

6.3 5-bromo-2-deoxy-uridine (BrdU) labelling in vitro

BrdU was added to cells in culture (10 μM) for 48 hours. Cells were fixed with ethanol (EtOH) 70% for 30 minutes. EtOH was remove and cells are allowed to air dry for approximately 10 minutes. Cultures were then treated with 2N HCl for 10 minutes followed by a 10 minutes neutralization with 0.1M borate buffer ($Na_2B_4O_7 \bullet H_2O$ - pH 8.5). Cells were then washed three times (5 minutes each) with 0.5% Tween 20 and 1% BSA in PBS followed by an incubation with mouse monoclonal anti-BrdU (1:40 monoclonal (Becton Dickinson) in 1% BSA in PBS) overnight at 4°C. Cells were then washed three times with PBS and incubated with FITC (1:400 in PBS) or cy3 (1:200 in PBS) conjugated anti-mouse secondary antibody (all from Jackson Immunoresearch Laboratories) for 1 hour at room temperature. After, cells were washed three times with PBS, counter stained with Hoechst (1:3000 in PBS) for 1 minute, and washed again. Slides were mounted using geltol.

6.4 Tunel labelling *in vitro*

Cells were fixed in 4% paraformaldehyde solution for 20 minutes followed by two (5 minutes each) washes in PBS (pH 7.2). A solution of 0.2% NP-40 in distilled water was used for permeabilization during 5 minutes followed by two washes in PBS (5 minutes each). 70 μL per slide of the following Tunel mixture was applied : terminal deoxynucleotidyl transferase (TdT)(Promega) 1.5 μL, biotin dUTP 1.0 μL, 5X buffer 20 μL, and H_2O 77.5 μL. Cells were incubated in Tunel mixture for 45 minutes to 1 hour at 37°C followed by two (5 minutes) washes with buffer (NaCl-tris) and one with PBS. Streptavidin-conjugated DTAF (Jackson Immunoresearch Laboratories Inc.) was diluted in water (1/2000) and applied for 1 hour at room temperature. Cells were counter stained with Hoechst (1/3000) for 1 minute followed by three washes with PBS (5 minutes each). Slide were coverslip using geltol.

6.5 Cell labelling

Adenovirally-driven marker proteins - Floating SKPs were subcultured into a 10 mL flask from a 20 mL one 72 hours before transplantation. That day, the cells were infected with 50 MOI of GFP or β-gal virus (Exogen Neurosciences, Montreal, Quebec, Canada and Dr. F. Graham, McMaster University, Hamilton, Ontario, Canada, respectively (Mazzoni et al., 1999)) in 5 ml of media. At this point, the flask contained about $10X10^6$ cells. After 24 hours, the cells were washed (3 times 5 minutes with fresh media) from the virus and placed in 10 mL of fresh culture media. 48 hours later, they were spun down and resuspended in 50 μL of media and injected in animals.

Hoechst – Floating SKPs were subcultured into a 10 mL flask from a 20 mL one 48 hours prior to labelling. On the day of the surgery, floating SKPs were incubated 30 minutes with Hoechst (10 μg/mL). Cells were then placed in 10 mL of fresh media for 30 minutes, during that period, they were stirred every 5 minutes. Finally, SKPs were resuspended in 50 μL of fresh media and injected in animals.

5-bromo-2-deoxy-uridine (BrdU) – SKPs were subcultured into a 10 mL flask from a 20 mL flask 72 hours before transplantation procedures. 24 hours after subculturing, 2 μM of BrdU was added to the culture media. After 48 hours, the cells were washed (3 times 5 minutes with fresh media) of BrdU and placed in 10 mL of fresh culture media. They were spun down and resuspended in 50 μL of media and injected in animals.

Orange cell tracker (Molecular Probes) - Floating SKPs were subcultured into a 10 mL flask from a 20 mL one 48 hours prior to labelling. On the day of the surgery, floating SKPs were incubated 30 minutes with orange cell tracker (10 μM). Cells were then placed in 10 mL of fresh media for 30 minutes, during that period, they were stirred every 5 minutes. Finally, SKPs were resuspended in 50 μL of fresh media and injected in animals.

PKH26 red fluorescent cell linker – Cells in suspension were labelled like described in detail in the Sigma product information sheet "methods for general cell labelling". In brief, cells were washed from the original media and resuspended in diluent C (supplied with the kit). Cells were incubated with the dye during 2-5 minutes at 25°C. The staining reaction was stopped with serum. Cells were washed with media, resuspended in limited volumes of fresh media and transplanted.

Lipophilic tracer – DiI (Molecular Probes) – Cells were spun down and the media was removed. The cells were resuspended in sucrose. 10 μL of stock dye solution was added per 1 mL of cell suspension. The dye was left for 10 minutes. An equal volume of PBS was then added to the sucrose solution containing the cells. Cells were spun down, the supernatant was removed and cells were resuspended in media for transplantation.

6.6 Transplantation

The cells were utilized for transplantation approximately 5 days following the last passage to obtain small spheres of about 40-50 cells. We spun down the content of one 10 mL flasks containing approximately 5 X 10^6 cells and resuspended the pelate in about 50 μL of DMEM/F12. About 1 μL (approximately 50000 cells) of that cell suspension was injected into post-natal day 1 Sprague-Dawley rats (Charles River Canada). The rats were cryoanesthetized before being placed onto the stereotactic instrument. The skin precursor cells were injected in the dentate gyrus of the hippocampus or anterior lateral cerebral ventricle at the following coordinates: anterior/posterior –0.9mm, lateral 1.1mm and ventral/dorsal –1.5 from bregma for the hippocampus and +0.5, 1.5 and –2.5 from bregma for the ventricular area. The cells were injected with a 2 μL Hamilton syringe and the following procedure was observed: 1 μL over 2 minutes (0.5 μL every 30 seconds), needle remained 1 minute in position after the last 0.5 μL injection and finally the needle remained 1 minute 1mm dorsal of the injection before complete removal.

Two controls were performed for transplantation : i) we injected cells that have been killed by a process of freeze-thawing to confirm that the OCT is not taken up by resident brain cells from the dying transplanted cells; ii) we injected media taken from the last

wash of the labelling protocol to confirm that no OCT is taken up by brain cells from the media containing the precursor cells.

6.7 Tissue processing

At the different time point, rats were terminally anaesthetized with somnotol (65 mg/mL;MTC Pharmaceuticals) and transcardially perfused with 0.1M PBS followed by rapid fixation with ice-cold 4% paraformaldehyde solution. Brains were removed and placed in PFA overnight, before being transferred to 30% sucrose in PBS. Sagittal sections were cut on a cryostat at thickness of 30 μm. Depending of the age of the animal, 8-12 series were collected for further processing.

6.8 Tunel labelling *in vivo*

Brain slices were fixed in 4% paraformaldehyde solution for 5 minutes followed by two (5 minutes each) washes in PBS (pH 7.2). Slides were then incubated for 5 minutes in solution of ethanol and acetic acid (2:1) at –20°C followed by two washed with PBS (5 minutes each). 100 μL per slide of the following Tunel mixture was applied : TdT 1.5 μL, biotin dUTP 1.0 μL, 5X buffer 20 μL, and H_2O 77.5 μL. Slides were incubated in the Tunel mixture for 45 minutes to 1 hour at 37°C followed by two (5 minutes) washes with buffer (NaCl-tris) and one with PBS. Slides were then incubated in a solution of 10% BSA and 0.3% Triton X-100 in PBS for 1 hour at room temperature followed by three (5 minutes each) washes in PBS. Streptavidin-conjugated DTAF or Cy3 (Jackson Immunoresearch Laboratories Inc.) was diluted in water (1/2000) and applied for 1 hour at room temperature. Cells were counter stained with Hoechst (1/3000) for 1 minute followed by three (5 minutes) washes with PBS. Slide were coverslip using geltol.

6.9 Immunohistochemistry

For Ki-67 (1:200 monoclonal;BD PharMingen), caspase-3 (1:500 polyclonal;Cell Signaling Technology), fibronectin (1:400 polyclonal;Sigma), GFAP (1:200 polyclonal;DAKO), NG2 (1:150 polyclonal;Chemicon international), neurofilament (1:200 polyclonal; Chemicon), Hu C/D (1:20 monoclonal; Molecular Probes), and

synaptophysin (1:400 monoclonal; Chemicon) the protocol was performed with first doing a quick fixation with 4% PFA solution during 10 minutes. The slides were then incubated in 10% BSA and 0.3% Triton-X 100 (Fischer Scientific) in PBS for 1 hour. Then incubated overnight with the primary antibody diluted in 3% BSA and 0.3% TX-100 in PBS at 4°C. Specimens were washed three times with PBS (5 minutes each) and then incubated for 1 hour at room temperature with the secondary antibody diluted in 3% BSA and 0.3% TX-100 in PBS. Secondary antibodies were FITC-conjugated goat anti-mouse (1:50) and FITC-conjugated goat anti-rabbit (1:200)(both from Jackson Immunoresearch Laboratories). Three washes with PBS (5 minutes each) followed. Sections were coverslip using geltol.

The same protocol was used for the primary antibodies M2 and M6 but no steps needed TritonX-100 because permeabilization wasn't needed.

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8.APPENDIX

12. Potential Hazards to Personnel and Animals It is the responsibility of the investigator to obtain the necessary Biohazard and/or Radiation Safety permits before this protocol is submitted for review. **A copy of these certificates must be attached, if applicable.**

No hazardous materials will be used in this study: ☒

a) Indicate which of the following will be used in animals:

☐ Toxic chemicals ☐ Radioisotopes ☐ Carcinogens ☐ Infectious agents ☐ Transplantable tumours

b) Complete the following table for each agent to be used (use additional page as required).

Agent			
Dosage			
Route of administration			
Frequency of administration			
Duration of administration			
Number of animals involved			
Survival time after administration			

c) After administration the animals will be housed in:

☐ the animal care facility
☐ laboratory under supervision of laboratory personnel

Please note that cages must be appropriately labeled at all times.

d) Describe potential health risk (s) to humans or animals:

) Describe measures that will be used to reduce risk to the environment and all project and animal facility personnel:

13. Reviewer's Modifications (to be completed by ACC only): The Animal Care Committee has made the following modification(s) to this protocol during the review process. Please make these changes to your copy. You must comply with the recommended changes as a condition of approval.

Dr. LYNN MATSUMIYA'S RECOMMENDATIONS: (D.V.M.)

10a:

- should state that newborn pups will be decapitated w/o anesthesia

10f:

- only needs 120 mg/kg IP as anesthetic overdose with pentobarbital

5. Summary (In language that will be understood by members of the general public)

a) Rationale: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge.

The use of stem cells to replace or augment damaged tissues has tremendous therapeutic potential. However, the current source of stem cells is embryonic tissue, an approach that raises serious ethical issues. We have recently demonstrated that we can isolate a pluripotent stem cell from adult skin and olfactory epithelium. This stem cell can give rise to both neural and mesenchymal cell types. In this renewal, we propose to continue our study of this potentially autologous source of adult stem cell.

b) Specific Objectives of the Study: Summarize in point form the primary objectives of this study.

The specific objective of this research is to isolate and study neural precursors from brain, olfactory epithelium or skin tissue. The stems cells we have isolated have the potential to become many different kind of cells. It is our hope to be able to isolate these cells and transplant them into areas of damaged tissue. When transplanted, these precursor cells are given the correct signal cues and can thereby be incorporated into the tissue and heal the damaged area. In order to do this, we first have to understand how these precursors can be induced to generate differenciated cell types. This is done using a variety of methods. Cell culture allows us to visualize living neural precusor cells; they can be exposed to various factors and then the results can be examined and compared. Biochemistry allows the quantification of data, as well as the opportunity to explore processes at a "gene and molecule" level. Finally the use of animals (rodents) provides a unique and irreplaceable model system to examine what the individual cells cannot tell us: specifically, how neurons or these neural precursor cells of the brain and nervous system interact with each other and with their normal, complex, biological environment.

c) Progress Report: If this is a renewal of an ongoing project, **briefly** summarize what was accomplished during the prior approval period and indicate if and how the current goals differ from those in the original application.

When we first obtained approval for this work, we had only just begun to demonstrate that skin derived precusors (SKPs) could become neurons and glia. Since that time, we have characterized the stem cells and their neural progeny much more extensively. Moreover, we have demonstrated that SKPs can generate mesodermal progeny, including smooth muscle, adipocytes and bone cells. We propose to continue our investigation of SKPs to determine how pluripotent they are and whether or not we can regulate their differentiation.

d) Summary of Procedures for Animal Use Report to the CCAC : Using **key words,** describe the procedures used (e.g. anaesthesia, breeding colony, injection IP, gavage, drug administration, major survival surgery, euthanasia by exsanguination, behavioural studies). Refer to Appendix 1of the Guidelines for a more complete list of suggested key words.

Keywords: research, primary cell culture, tissue collection, breeding

6. Animals To Be Used

a) Purpose of Animal Use (Check one):

1. ☒ Studies of a fundamental nature/basic research
2. ☒ Studies for medical purposes relating to human/animal diseases/disorders
3. ☐ Regulatory testing
4. ☐ Development of products/appliances for human/veterinary medicine

b) Will the project involve breeding animals? NO☐ YES ☒
Will the project involve the generation of genetically altered animals? NO ☒ YES ☐
Will field studies be conducted? NO ☒ YES ☐

c) Description of Animals

	Species 1	Species 2	Species 3	Species 4	Species 5	Species 6
Species	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse
Supplier/Source	Charles River	Charles River	Charles River	in house	in house	in house
Strain	CD1	CD1	CD1	CD1X transg.	CD1X transg.	CD1X transg.
Sex	m/f	m/f	m/f	m/f	m/f	m/f
Age/Wt	adult	newborn	embryo	adult	newborn	embryo
# To be purchased	75	50	25	0	0	0
# Produced by in-house breeding	0	0	0	75	50	25
# Other (e.g.field studies)	0	0	0	0	0	0
#needed at one time	1-3	1 litter	1-2 litter	1-3	1 litter	1-2 litter
# per cage	1-5	1 litter	N/A	1-5	1 litter	N/A
TOTAL# /YEAR	**75**	**50 litters**	**25 litters**	**75**	**50 litters**	**25 litters**

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals.

7. Justification of Animal Usage

a) Please justify the number of animals requested for each species described above, **based on the experimental objectives of the project**. Include information on experimental and control groups, # per group, and failure rates. Also justify in terms of tatistical requirements, product yield, etc. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. Use the table below when applicable (space will expand as needed).

In order to study and understand neural precursor cells the experiments performed are unfortunately terminal. This is because crucial tissues must be removed and studied. Cultures from the animal will be performed weekly in order to ensure that there is sufficient amount of cells from different age time points (adult, newborn or pre-natal) needed to do the many different immunocytochemistry experiments. There are a lot of "immunos" to be done because little is known about how these precusor cells behave and immunocytochemistry shows which proteins are involved giving insight into their fonctions. If we do weekly cultures from the animals then the total number breakdown is as follows: using 1-2 adult animals (CD1 and/or transgenic) a week gives an approximate yearly total of 75 mice/strain, for newborns (CD1 and/or transgenic) we would use 1 litter/week, equaling about 50 litters total/year and we will be using embryos (CD1 and/or transgenic) once every two weeks, equaling 25 litters total/year. These numbers of animals to be used will give us a sufficient amount of tissue to work with enabling us to understand more about neural precursor cells.

Test Agents or Procedures	# of Animals and Species Per Group	Dosage and/or Route o Administration	# of endpoints	Other variables (i.e. sex, weight, genotypes,etc.)	**Total number of animals**
e.g. 2 Drugs	6 rats	.03, .05 mg/kg - IM, IP (4 variables)	1, 7, 10 days (3 variables)	Male, Female groups (2 variables)	2 x 6 x 4 x 3 x 2 = 288
Species #1	**1-2 mice**	**N/A**	**50**	-	**1.5 X 50 = 75**
Species #2	**1 litter**	**N/A**	**50**	-	**1 X 50 = 50**
Species #3	**1 litter**	**N/A**	**25**	-	**1 X 25 = 25**
Species #4	**1-2 mice**	**N/A**	**50**	-	**1.5 X 50 = 75**
Species #5	**1 litter**	**N/A**	**50**	-	**1 X 50 = 50**
Species #5	**1 litter**	**N/A**	**25**	-	**1 X 25 = 25**

b) Please justify the need for live animals versus alternate methods (e.g. tissue culture, computer simulation).

We perform tissue culture using both primary tissues (directly from the animal), and transformed cell lines (cells that have been passed through several generations of growth). Live animals provide the (as yet) best model for a functional, intact nervous system. Furthermore, trangenic animals allow for the examination of specific genetic factors and their impact on biological processes.

c) Describe the characteristics of the animal species selected that justifies its use in the proposed study (consider characteristics such as body size, species, strain, data from previous studies or unique anatomic/physiological features)

Mice: All of our transgenic work is done in mice; this is a common practice in today's research. They are reliable fast breeders, easy and safe to handle and house.

8. Animal Husbandry and Care

a) Special cages NO ☒ YES ☐ Specify:

Special diet NO ☒ YES ☐ Specify:

Special handling NO ☒ YES ☐ Specify:

b) Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function (e.g. stress, radiation, steroids, chemotherapeutics, genetic modification of the immune system)?

NO ☒ YES ☐ Specify:

c)

Multiple institution facility housing: NO ☒ YES ☐

Indicate all facilities where animals will be housed: Building: MNI Room No: 866

Indicate area(s) where animal use procedures will be conducted: Building: MNI Room No: BT-112

If animal housing and animal use are in different locations, briefly describe procedures for transporting animals:

The animals are only transported within the MNI from the animal quarters to our laboratories, in designated transport cages, each cage being loosely covered with a dark plastic bag to keep it hidden from view.

9. Standard Operating Procedures (SOPs)

Complete this section if you plan to use any of the UACC SOPs listed below. **It is UACC policy that these SOPs be used when applicable.** Any proposed variation of the SOPs must be described and justified. The Standard Operating Procedures can be found at the UACC website at www.mcgill.ca/fgsr/rgo/animal/ . The completed and signed SOP form must be attached to the protocol.

Check all SOPs that will be used:

Blood Collection (UACC#1)	☐	Production of Monoclonal Antibodies (UACC#7)	☐
Anaesthesia (rodents) (UACC#2)	☐	Production of Polyclonal Antibodies(UACC#8)	☐
Analgesia (rodents/larger species) (UACC#3)	☐	Collection of Amphibian Oocytes (UACC#9)	☐
Breeding (transgenics/knockouts) (UACC#4)	☒	Rodent Surgery (UACC#10)	☐
Transgenic Generation (UACC#5)	☐	Neonatal Rodent Anaesthesia and Euthanasia (UACC#11)	☐
Knockout/in Generation (UACC#6)	☐	Euthanasia of adult and neonatal rodents (UACC #13)	☒

10. Description of Procedures

a) **For each experimental group, describe all procedures and techniques in the order in which they will be performed** - surgical procedures, immunizations, behavioural tests, immobilization and restraint, food/water deprivation, requirements for post-operative care, sample collection, substance administration, special monitoring, etc. **If a procedure is covered by an SOP, no further detail is required.** Appendix 2 of the Guidelines provides a sample list of points that should be addressed in this section.

CULTURE OF NEURAL PRECURSOR CELLS

Adult, newborn pups or embryos from CD1 or CD1 X transgenic mice will be euthanized (Somnotol or CO2) olfactory epithelium, brain or skin will be removed. The tissue will then be cultured to isolate neural precursor cells. These cells will then be used to better understand neural precursor cells and how they function by doing immunocytochemistry on them. When embryonic tissue is required, the mother animal will be euthanized with CO2 or with Somnotol, along with cervical dislocation. Embryos are obtained from the euthanized mother animal (as above) and the cells are cultured.

Endpoint of Project: When the function of neural precursor cells from peripheral tissue is better understood.

b) **Field Studies –** Provide all relevant details. Procedures to be conducted (e.g. surgery, blood collection, tagging etc.) should be described above.

Method of capture/restraint, duration of captivity, potential injury/mortality, monitoring frequency:

Transportation and /or housing of animals in the field:

Special handling required:

Capture of non-target species, potential injury/mortality:

Will captured animals be released at or near the capture site YES ☐ NO ☐
If not, specify if they will be relocated to other locations and/or populations.

Describe any potential ecological disruption this study may cause:

It is the responsibility of the investigator to obtain all necessary permits for work with wildlife. Copies of these permits must be forwarded to the Research Ethics Officer (Animal Studies) when they are obtained.

c) Pre-Anaesthetic/Anaesthetic/Analgesic Agents: List all drugs that will be used to minimize pain, distress or discomfort Table will expand as needed.

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency

d) Administration of non-anaesthetic substances: List all non-anaesthetic agents under study in the experimental component of the protocol, including but not limited to drugs, infectious agents, viruses (table will expand as needed).

Species	Agent	Dosage (mg/kg)	Total volume (ml) per administration	Route	Frequency

e) Endpoints : 1) Experimental – for each experimental group indicate survival time .
2) Clinical - describe the conditions, complications, and criteria (e.g. >20% wt.loss, tumour size, vocalizing, lack of grooming) that would lead to euthanasia of an animal before the expected completion of the experiment (specify per species and project if multiple projects involved).

Endpoint of animal's life: When the animal is at the appropriate age to be experimented upon, i.e. embryonic, early post-natal or adult.

Specify person(s) who will be responsible for animal monitoring and post-operative care
Name: Jean Toma **Phone#: 398-3334**
Mahnaz Akhvan **398-3334**

f) Method of Euthanasia – According to CCAC guidelines, justification must be provided for use of any physical method of euthanasia without prior use of anaesthesia (justify here):

Specify Species	
mouse	☒ anaesthetic overdose, list agent/dose/route:Somnotol 400mg/kg IP
	☐ exsanguination with anaesthesia, list agent/dose/route:
mouse pups	☒ decapitation without anaesthesia ☐ decapitation with anaesthesia,list agent/dose/route:
mouse	☐ cervical dislocation
mouse adult	☒ CO_2 chamber
	☐ other (specify)
	☐ not applicable (explain)

11. Category of Invasiveness: B ☒ C ☐ D ☐ E ☐

Categories of Invasiveness (from the CCAC *Categories of Invasiveness in Animal Experiments).* Please refer to this document for a more detailed description of categories:

Category A: Studies or experiments on most invertebrates or no entire living material.

Category B: Studies or experiments causing little or no discomfort or stress. These might include holding animals captive, injection, percutaneous blood sampling, accepted euthanasia for tissue harvest, acute non-survival experiments in which the animals are completely anaesthetized.

Category C: Studies or experiments involving minor stress or pain of short duration. These might include cannulation or catheterizations of blood vessels or body cavities under anaesthesia, minor surgery under anaesthesia, such as biopsy; short periods of restraint, overnight food and/or water deprivation which exceed periods of abstinence in nature; behavioural experiments on conscious animals that involve short-term stressful restraint.

Category D: Studies or experiments that involve moderate to severe distress or discomfort. These might include major surgery under anaesthesia with subsequent recovery, prolonged (several hours or more) periods of physical restraint; induction of behavioural stresses, immunization with complete Freund's adjuvant, application of noxious stimuli, procedures that produce pain, production of transgenics (in accordance with University policy).

Category E: Procedures that involve inflicting severe pain, near, at or above the pain threshold of unanaesthetized, conscious animals. Not confined to but may include exposure to noxious stimuli or agents whose effects are unknown; exposure to drugs or chemicals at levels that (may) markedly impair physiological systems and which cause death, severe pain or extreme distress or physical trauma on unanaesthetized animals. **According to University policy, E level studies are not permitted.**

12. Potential Hazards to Personnel and Animals It is the responsibility of the investigator to obtain the necessary Biohazard and/or Radiation Safety permits before this protocol is submitted for review. **A copy of these certificates must be attached, if applicable.**

No hazardous materials will be used in this study: ☒

a) Indicate which of the following will be used in animals:

☐Toxic chemicals ☐Radioisotopes ☐Carcinogens ☐Infectious agents ☐Transplantable tumours

b) Complete the following table for each agent to be used (use additional page as required).

Agent			
Dosage			
Route of administration			
Frequency of administration			
Duration of administration			
Number of animals involved			
Survival time after administration			

c) After administration the animals will be housed in:

☐ the animal care facility
☐ laboratory under supervision of laboratory personnel

Please note that cages must be appropriately labeled at all times.

d) Describe potential health risk (s) to humans or animals:

e) Describe measures that will be used to reduce risk to the environment and all project and animal facility personnel:

13. Reviewer's Modifications (to be completed by ACC only): The Animal Care Committee has made the following modification(s) to this protocol during the review process. Please make these changes to your copy. You must comply with the recommended changes as a condition of approval.

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