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(71) Demandeurs/Applicants:
UNIVERSITE PARIS 7 - DENIS DIDEROT, FR;
CENTRE NATIONAL DE LA RECHERCHE
SCIENTIFIQUE (CNRS), FR
(72) Inventeurs/Inventors:
CZERNICHOV, PAUL, FR;
SCHARFMANN, RAPHAEL, FR;
RAVASSARD, PHILIPPE, FR;
MALLET, JACQUES, FR
(74) Agent: OGILVY RENAULT

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(57) **Abrégé/Abstract:**

The invention provides a method of regenerating pancreas function in an individual by transplantation of an effective amount of functional pancreatic cells derived from embryonic pancreatic cells not older than 10 weeks of development. Also provided is the method of producing functional animal pancreatic cell, more precisely an immortalized human beta cell line. The invention also provides a method of treatment of diabetics. Also are provided pancreatic beta cells as a medicament to treat diabetics.



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(71) Applicants (for all designated States except US): **UNIVERSITE PARIS 7 - DENIS DIDEROT** [FR/FR]; 2, place Jussieu, F-75005 Paris (FR). **CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (CNRS)** [FR/FR]; 3, rue Michel Ange, F-75016 Paris (FR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **CZERNICHOV, Paul** [FR/FR]; 24, rue du Capitaine Ferber, F-75020 Paris (FR). **SCHARFMANN, Raphaël** [FR/FR]; 3, rue Larrey, F-75005 Paris (FR). **RAVASSARD, Philippe** [FR/FR]; 7, rue des Wallons, F-75013 Paris (FR). **MALLET, Jacques** [FR/FR]; 18, rue Charcot, F-75013 Paris (FR).

(74) Agents: **MARTIN, Jean-Jacques** et al.; Cabinet Regimbeau, 20, rue de Chazelles, F-75847 Paris Cedex 17 (FR).

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(54) Title: METHOD OF PRODUCING HUMAN BETA CELL LINES

(57) Abstract: The invention provides a method of regenerating pancreas function in an individual by transplantation of an effective amount of functional pancreatic cells derived from embryonic pancreatic cells not older than 10 weeks of development. Also provided is the method of producing functional animal pancreatic cell, more precisely an immortalized human beta cell line. The invention also provides a method of treatment of diabetics. Also are provided pancreatic beta cells as a medicament to treat diabetics.



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"METHOD OF PRODUCING HUMAN BETA CELL LINES"**FIELD OF THE INVENTION**

The invention relates to the field of biology and
5 in particular to the field of cellular biology and
cellular therapy.

BACKGROUND OF THE INVENTION

Type I diabetes is due to the destruction by immune
10 mechanisms of pancreatic beta cells, resulting in the
lack of insulin production and hyperglycemia. Cell
therapy using beta cells from donors could represent
one way to cure diabetic patients. However, two main
problems have to be solved before this goal can be
15 reached. First, immunosuppressive protocols have to be
designed to provide immunologic protection of the
graft. Recent reports indicate that progress has been
made in this field (Shapiro *et al.*, 2000). The second
point to be solved concerns the small number of mature
20 beta cells from donors that are available for grafting
(Weir and Bonner-Weir, 1997). There is a need to
provide alternative sources of functional mature beta
cells. That is the problem the present invention wishes
to solve. During the last few years, it has been
25 proposed that by understanding and recapitulating beta
cell development that occurs during embryonic and fetal
life, new beta cells could be produced that could be
used for cell therapy of type I diabetes. Huge effort
and progress have thus been made to define the
30 molecular mechanisms that control prenatal pancreatic

development in rodents and the role of specific transcription and growth factors has been defined (Edlund, 1998; St Onge *et al.*, 1999; Wells and Melton, 1999; Scharfmann, 2000; Grapin-Botton and Melton, 2000; 5 Kim and Hebrok, 2001). Different tissue sources potentially rich in precursor cells are also currently tested for their ability to differentiate into mature beta cells. Such cells derive either from fetal or neonatal porcine pancreas (Yoom *et al.*, 1999; Otonkoski 10 *et al.*, 1999), or from fractions of human adult pancreas enriched in duct cells and that are thought to contain precursor cells (Bonner-Weir, 1997). So far, prenatal human pancreatic tissues (14-24 weeks) have been used unsuccessfully because all the tissues 15 derived from fetuses were at late stages of development and were already quite mature when used in different assays (Tuch *et al.*, 1984; Tuch *et al.*, 1986; Sandla *et al.*, 1985; Hayek *et al.*, 1997; Goldrath *et al.*, 1995). For instance, Tuch *et al.* (1984), Sandler *et al.* 20 (1985), Goldrath *et al.* (1995) and Povlsen *et al.* (1974) used human pancreatic fragments of 14 to 24 weeks of development that have been engrafted into immunoincompetent mice with the goal of following endocrine tissue development. After a few weeks or 25 months in recipient mice, all endocrine cell types were found when human tissues were removed (Tuch *et al.*, 1984; Sandler *et al.*, 1985). However, it is important to remember that between 14 and 24 weeks of development (the age of the tissue at the time of the 30 transplantation), endocrine cells are already present

and associated into islets of Langerhans (Bouwens et al., 1997; Stefan et al., 1983; Fukayama et al., 1986; Miettinen et al., 1992). Moreover, in these experiments using late human fetal tissues, when the quantity of insulin-expressing cells present in the graft was compared before and after transplantation, no clear increase in the beta cell mass was detected (Tuch et al.). It is consequently difficult to determine whether the human endocrine cells that were present after a few weeks or months in the mouse were newly formed endocrine cells or cells that existed before transplantation and did survive.

The present invention solves the above-mentioned problem of providing mature beta cells; indeed the inventors demonstrate that functional human beta cells can develop in NOD/scid mice from immature human embryonic pancreases not older than 10 weeks of development. More precisely, the inventors demonstrate that when human embryonic pancreases, that contained no or very few insulin-expressing cells (see Figure 4), were engrafted into immunoincompetent mice, pancreatic tissue grew, its weight increasing 200 times within six months. At the same time, endocrine cell differentiation occurred, the absolute number of human beta cells being increased by a factor of 5,000. Finally, the endocrine tissue that developed was functional, being able to regulate the glycemia of mice deficient in rodent beta cells.

This model of development of human embryonic pancreases in NOD/scid mice that seem to mimic the

ontogeny of the human pancreas that occurs *in vivo*, can now be used to study the mechanisms that control the development of the human embryonic pancreas, a question that has been partly eluded in the past due to the lack of proper experimental systems. Moreover, inventors' data do indicate that human embryonic pancreases represent a source of immature cells that can proliferate and differentiate in mass into beta cells when transplanted into an adult animal. This tissue may thus be useful as an alternative source of beta cells for transplantation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of regenerating pancreas function in an individual, the method comprising:

- (a) introducing an effective amount of animal embryonic pancreatic cells not older than 10 weeks of development, into the kidney capsule of non-obese diabetic/severe combined immunodeficiency (NOD/*scid*) animal, excepted human, wherein said NOD/*scid* is of a different species than said animal from which are obtained said embryonic pancreatic cells; and
- (b) allowing the animal embryonic pancreatic cells to develop, to differentiate and to regenerate at least a pancreatic function selected among the regulation of glycemia and the secretion of digestive enzymes ; and

(c) transplantation of an effective amount of the animal functional pancreatic cells obtained at step (b), into said individual.

The term "individual" is a vertebrate, preferably a mammal. Mammals include, but are not limited to, humans, rodents (i.e. mice, rats, hamsters, farm animals, sport animals and pets. In a preferred embodiment, the individual is a mammal and more preferably, a human.

10 In a preferred embodiment, the animal embryonic pancreatic cells are human embryonic pancreatic cells. Alternatively, it could be selected among, for instance, porcines, bovines, goats, sheep, primates, rodents (i.e. a mouse, a rat, a hamster...), pancreatic
15 cells. The animal embryonic pancreatic cells of the invention are cells that are selected among cells not older than 10 weeks, not older than 9 weeks, not older than 8 weeks, not older than 7 weeks, not older than 6 weeks, not older than 5 weeks, not older than 4 weeks,
20 not older than 3 weeks, not older than 1 week of development. In a preferred embodiment, the animal embryonic pancreatic cell of the invention is of 6 to 9 weeks of development. According to a preferred embodiment, the animal embryonic pancreatic cell of the
25 invention is a human embryonic pancreatic cell that is not older than 9 weeks of development, more preferably comprised between 6 to 9 weeks of development.

The non obese diabetic/severe combined immunodeficiency (NOD/scid) animal is selected among
30 bovines, porcines, horses, sheep, goats, primates

excepted humans, rodents such as mice, rats, hamsters. In a preferred embodiment, the NOD/scid animal is a mouse. Preferably the NOD/scid mice of the invention are of any age of development, preferably sufficiently
5 old to perform a graft into the kidney capsule. Preferably, the NOD/scid mice are about of the 2 to 15 weeks of development, more preferably to 6 to 8 weeks of development. A NOD/scid animal is an animal lacking T- and B-lymphocytes and failing to generate either
10 humoral or cell-mediated immunity.

An "effective amount" is an amount sufficient to effect beneficial or desired clinical results. An effective amount can be administered in one or more applications, although it is preferable that one
15 administration will suffice. For purposes of this invention, an effective amount of embryonic pancreatic cells is an amount that is sufficient to produce differentiated pancreatic cells which are able to restore one or more of the functions of the pancreas.
20 It is contemplated that a restoration can occur quickly by the introduction of relatively large numbers of pancreas cells, for example greater than 10^9 cells. In addition, it is also contemplated that when fewer pancreatic cells are introduced, function will be
25 restored when the pancreas cell or cells are allowed to proliferate *in vivo*. Thus, an "effective amount" of pancreatic cells can be obtained by allowing as few as one pancreas cell sufficient time to regenerate all or part of a pancreas. Preferably, an effective amount
30 administered to the individual is greater than about

10¹ pancreas cells, preferably between about 10² and about 10¹⁵ pancreas cells and even more preferably, between about 10³ and about 10¹² pancreas cells. The effective amount of the animal pancreatic cells transplanted at step (c) of the method of the invention is more preferably between 10³ to 10¹² animal pancreatic cells. In terms of treatment, an "effective amount" of pancreatic cells is the amount which is able to ameliorate, palliate, stabilize, reverse, slow or delay the progression of pancreas disease, such as diabetics.

According to a preferred embodiment, the animal embryonic pancreas cells used in the methods of the present invention may be obtained from a heterologous donor (allograft), for example, an organ donor or a living donor. Alternatively, an autograft can be performed by removing a portion of an individual's pancreas at an early stage of development (prior to 10 weeks of development) or by reversing the differentiated phenotype of adult pancreas cells, and introducing the pancreas cells capable of regenerating pancreas function into the same individual. For autografts, at least about 5% of the donor individual's pancreas is removed. For allografts, at least about 5%, preferably greater than 30%, more preferably greater than 50% and even more preferably greater than 80% of the pancreas is removed. The methods of the present invention involve either allograft or autografts of pancreas cells. Each type of graft has its advantages. In particular, autografts (where pancreas cells from the same individual are used to regenerate pancreas

function) is used to avoid immunological reactions. Graft versus host reactions occur when the donor and recipient are different individuals, and the donor's immune system mounts a response against the graft.

5 Tissue typing and major histocompatibility (MHC) matching reduces the severity and incidence of graft versus host. Nonetheless, autologous introduction of pancreas cells will be especially useful in cases where the individual's pancreas is diseased. In such cases, a

10 small amount of autologous embryonic pancreas tissue will regenerate a functional pancreas. Allografts are useful in cases where the pancreas is not available, for instance if the pancreas of the individual is diseased. Various MHC matched pancreas cells can be

15 maintained *in vitro* or isolated from donors and tissue typing performed to match the donor with the recipient. Immunosuppressive drugs, such as cyclosporin, can also be administered to reduce the graft versus host reaction. Allografts using the cells obtained by the

20 methods of the present invention are also useful because a single healthy donor could supply enough cells to regenerate at least partial pancreas function in multiple recipients. Because the pancreas cells of the present invention are able to proliferate and

25 differentiate so effectively, only a small number is required to repopulate a pancreas. Accordingly, one pancreas could be divided and used for multiple allografts. Similarly, a small number of cells from one pancreas could be culture *in vitro* and then used for

30 multiple grafts. In an embodiment of the invention, the

pancreatic cells of the invention can be genetically modified in order they match all or various MHC (such cells constitute universal donor pancreatic cells). By pancreatic cells of the invention is meant either the
5 embryonic pancreatic cells or the functional pancreatic cell that have developed and differentiate into the NOD/scid animal.

Suitable techniques for isolating pancreas tissue from a donor individual are known in the art. For
10 example, extraction of pancreas cells through a biopsy needle or surgical removal of a portion or all of the pancreas tissue can be utilized.

Pancreatic tissue can be used in the methods of the present invention without further treatment or
15 modification. Modifications are described below. For both modified and unmodified cells, it is preferred that single cell suspensions are obtained from the tissue. Cell suspensions can be obtained by methods known in the art, for example, by centrifugation and
20 enzyme treatment. Pancreas tissue or cell suspensions can also be frozen and thawed before use. Preferably, the cells are fresh after isolation and processing.

Alternatively, the embryonic pancreatic cells of the present invention can be cultured long-term *in*
25 *vitro* to produce stable lines of pancreas-regenerating cells. As used herein, the term "*in vitro culture*" refers to the survival of cells outside the body. Preferably, the cultures of the present invention are "long-term" cultures in that they proliferate stably *in*
30 *vitro* for extended periods of time. These stable

populations of cells are capable of surviving and proliferating *in vitro* with an embryonic pancreatic phenotype (i.e. these cells will be "stem" cells). Methods of culturing various types of stem cells are
5 known in the art. For example, WO 94/16059 describes long-term culture (greater than 7 months) of neuronal cells. Long-term culture of other types of stem cells are also described in the art and can be applicable to the cells of the present invention. The embryonic
10 pancreatic cells cultured *in vitro* can be genetically modified to express a gene of interest, such as a therapeutic gene.

According to the present invention, the animal functional pancreatic cells transplanted at step (c)
15 are preferably introduced into the pancreas of said individual. Alternatively, such animal functional pancreatic cells are enclosed into implantable capsules that can be introduced into the body of an individual, at any location, more preferably in the vicinity of the
20 pancreas, or the bladder, or the liver, or under the skin.

As used herein, the term « introducing » means providing or administering to an individual. In the present invention, functional pancreatic cells capable
25 of regenerating functional pancreas cells are introduced into an individual. Methods of introducing cells into individuals are well known to those of skill in the art and include, but are not limited to, injection, intravenous or parenteral administration.
30 Single, multiple, continuous or intermittent

administration can be effected. The pancreas cells can be introduced into any of several different sites, including but not limited to the pancreas, the abdominal cavity, the kidney, the liver, the celiac
5 artery, the portal vein or the spleen. Preferably, the pancreas cells are deposited in the pancreas of the individual.

The term "pancreas" refers to a large, elongated yellowish gland found in vertebrates. The pancreas has
10 both endocrine and exocrine functions, producing the hormones insulin and glucagon and, in addition, secreting digestive enzymes such as trypsinogen, chymotrypsinogen, procarboxypeptidase A and B, elastase, ribonuclease, desoxyribonuclease
15 phospholipase A, pancreatic lipase, pancreatic α -amylase. The term "pancreas cells" or "pancreatic cells" refers to cells obtained from the pancreas. In a preferred embodiment, the pancreatic function according to the invention is the regulation of glycemia.

20 The present invention also provides a method wherein said individual is an insulin-dependent diabetic. Therefore, the invention also contemplated to provide a method of treatment of diabetes in a human patient in need of such treatment, the method
25 comprising the steps of:

(a) introducing an effective amount of human embryonic pancreatic cells not older than 10 weeks of development, more preferably from 6 to 9 weeks of development, into the kidney capsule
30 of non-obese diabetic/severe combined

immunodeficiency (NOD/*scid*) animal, excepted human; and

- 5 (b) allowing the embryonic pancreatic cells to develop, to differentiate and to regenerate at least the pancreatic function; and
- (c) transplantation of an effective amount of the human functional pancreatic cells obtained at step (b), into said patient,
- 10 (d) and treating diabetics, wherein said treatment is effected by the regeneration of said pancreatic function of regulation of glycemia.

The previously described method is more specifically dedicated to the treatment of diabetes in a human patient.

- 15 As used herein, the term « regeneration of said pancreatic function » refers to the growth or proliferation of new tissue. In the present invention, regeneration refers to the growth and development of functional pancreas tissue. In most instances, the
- 20 regenerated pancreas tissue will also have the cytological and histological characteristics of normal pancreas tissue. For example, the pancreas cells introduced in to the individual and allowed to generate functional pancreas tissue are expected to express
- 25 insulin and glucagon, and digestive enzymes along with other markers indicative of pancreas, such as Nkx6.1, Pax6, or PC1/3. Functions of the pancreas can be challenged by measures and tests known in the art, such as insulin or glucagon expression. According to a

preferred embodiment, the non-obese diabetic/severe combined immunodeficiency animal is a mouse.

The present invention also provides a method of producing functional animal pancreatic cell wherein
5 said method comprises the steps of:

- (a) introducing an effective amount of animal embryonic pancreatic cells not older than 10 weeks of development, more preferably from 6 to 9 weeks of development, into the kidney capsule
10 of non-obese diabetic/severe combined immunodeficiency (NOD/scid) animal, excepted human, wherein said NOD/scid is of a different species than said animal from which are obtained said embryonic pancreatic cells; and
- 15 (b) allowing the animal embryonic pancreatic cells to develop, to differentiate and to regenerate at least a pancreatic function selected among the regulation of glycemia, and the secretion of digestive enzymes. In a preferred
20 embodiment, said pancreatic function is the regulation of glycemia; and
- (c) collecting animal pancreatic cells obtained at step (b), and
- (d) optionally *in vitro* culturing the cells
25 obtained at step (c).

The invention encompasses the functional animal pancreatic cell obtainable or obtained by the method according to the invention. Preferably said cell is selected among pancreatic alpha cell, pancreatic beta
30 cells, pancreatic delta cells. Preferably, said cell is

a pancreatic beta cell. Such Pancreatic beta cell preferably expresses insulin in response to glucose. Moreover, the present invention provides a functional pancreatic beta cell that expresses glucagon in response to glucose. Additionally, said functional pancreatic cell expresses and secretes digestive enzymes. Said cell is preferably a human cell or a rat cell.

The pancreatic cells of the present invention, (i.e. the embryonic pancreas cells or the functional pancreatic beta cells) can be modified, for example, using particular cell culturing conditions or by genetic engineering techniques. This latter modification includes the introduction of a transgene into said cell, either integrated into the genome of said cell, or present as an extrachromosomal replicon. In one embodiment, the method of producing functional animal pancreatic cell of the invention further comprises the step of genetically modifying the the cells obtained at step (d) and/or further comprises the preliminary step of genetically modifying said animal pancreatic cells not older than 10 weeks of development. By "genetically modifying" it is meant, introducing at least one transgene (i.e. an exogenous nucleic acids molecule) in said cell, said transgene being stably transmitted to the cell progeny. As used herein, by transgene it is means any nucleic acids molecule, more preferably any genomic DNA or RNA, recombinant DNA or RNA, or cDNA sequences, either single or double stranded.

Preferably, the transgene comprises at least a sequence of interest operatively linked to a promoter sequence to form an expression cassette that directs the expression of the gene of interest into pancreatic
5 cells. Said transgene or expression cassette is either present in a linear form, but preferably said transgene or DNA cassette is part of a nucleic acid fragment or is cloned into a cloning and/or expression vector. "operatively linked" as used herein, includes reference
10 to a functional linkage between a promoter and a second sequence (i.e. the sequence of interest), wherein the promoter sequence initiates and mediates the transcription of said DNA sequence corresponding to the second sequence. A "promoter" or a "promoter sequence"
15 is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription downstream (3'direction) coding sequence. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains
20 responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contains TATA boxes and CAT boxes. Additional responsive elements can be found in the promoter sequence or in its vicinity, such as activating
25 sequences ("enhancers"), inhibitory sequences ("silencers") and upstream sequences. Various promoters, including ubiquitous or tissue-specific promoters, and inducible and constitutive promoters may be used to drive the expression of the gene of
30 interest. Preferably, the promoter is able to drive the

expression of the gene of interest in at least the pancreatic cells, or in pancreatic cells at specific stage of development, such as in pancreatic cells not older than 10 days of development, or in mature beta
5 cells. More preferably, the promoter is the insulin gene promoter and more preferably is selected among the rat insulin gene promoter (Genbank accession N°GBJ00748), the mouse insulin gene promoter (Genbank accession N°GBX04724), the human insulin gene promoter
10 (Genbank accession N°GBAP001994). Alternatively the promoter is inducible by an antibiotic, such as tetracycline. In another embodiment, the promoter drives the expression of the gene of interest in a temperature dependent manner; in such case, the
15 promoter is preferably the one of the gene coding for temperature sensitive mutant form of large T antigen of SV40.

According to the present invention, a "vector" is a replicon in which another polynucleotide segment
20 is attached, so as to bring the replication and/or expression to the attached segment. A vector can have one or more restriction endonuclease recognition sites at which the DNA sequences can be cut in a determinable fashion without loss of an essential biological
25 function of the vector. Vectors can further provide primer sites (e.g. for PCR), transcriptional and/or translational initiation and/or regulation sites, recombinational signals, replicons, selectable markers, etc. Examples of vectors include plasmids, phages,
30 cosmids, phagemid, yeast artificial chromosome (YAC),

bacterial artificial chromosome (BAC), human artificial chromosome (HAC), virus, virus based vector, such as adenoviral vector, lentiviral vector, and other DNA sequences which are able to replicate or to be
5 replicated *in vitro* or in a host cell, or to convey a desired DNA segment to a desired location within a host cell. In a preferred embodiment the vector is a viral based vector, more preferably a lentivirus-based vector such as a HIV-1 based vector. Such a lentivirus-based
10 vector is preferably used to transfect embryonic pancreatic cell of the invention.

The recombinant DNA technologies used for the construction of the expression vector according to the invention are those known and commonly used by persons
15 skilled in the art. Standard techniques are used for cloning, isolation of DNA, amplification and purification; the enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases are carried out according to the manufacturer's
20 recommendations. These techniques and others are generally carried out according to Sambrook *et al.* (1989).

The transgene and the vector according to the invention is introduced either into functional
25 pancreatic beta cells or into pancreatic cells not older than 10 weeks of development, by using known techniques, such as for example transfection by calcium phosphate precipitation, lipofection, electroporation, heat shock, microinjection into a pronucleus, "gene
30 gun", use of artificial viral envelopes, viral

infection. Such transgene or vector is either integrated by homologous recombination into the host genome or by random integration, or is present as an extra-chromosomal replicon.

5 As used herein, "sequence of interest" means any double stranded or single stranded DNA or RNA molecules. It can either be a genomic DNA fragment, such as gene(s), intron(s), exon(s), regulatory sequence(s) or combinations or fragments thereof, or a
10 recombinant DNA molecule such as a cDNA gene for instance. When, the sequence of interest may encode a protein of interest, said sequence of interest may be a selection gene, a reporter gene, a therapeutic gene, an immortalizing gene, a transforming gene. Such sequence
15 of interest may comprises any of the genetic elements necessary for the correct expression of this protein of interest in the host cell.

In one embodiment such "sequence of interest" encode for a protein of interest having for example a
20 diagnostic or a research interest. Examples of such proteins having a research interest are the selection proteins. As used herein, "selection gene" means a gene that encodes a protein or a peptide (i.e. a selectable marker) that allows one to select for or against a
25 molecule or a cell that contains it, often under particular conditions i.e. in presence of a selective agent. These selection proteins include but are not limited to products which provide resistance against otherwise toxic compounds (e.g., antibiotics). For
30 example, the ampicillin or the neomycin resistance genes

constitute genes encoding for selection marker of the invention. Those selection markers can be either positive or negative (see Capecchi et al., US 5 631 153). According to a preferred embodiment, said
5 selection marker protein is a positive selection marker protein encoded by a gene selected in the group consisting of antibiotic resistance genes, hisD gene, *Hypoxanthine phosphoribosyl transferase* (HPRT) gene, guanine-phosphoribosyl-transferase (Gpt) gene. Said
10 antibiotic resistance gene is selected in the group consisting of hygromycin resistance gene, neomycin resistance genes, tetracyclin resistance gene, ampicillin resistance gene, kanamycin resistance gene, phleomycin resistance gene, bleomycin resistance gene,
15 geneticin resistance gene, carbenicillin resistance gene, chloramphenicol resistance gene, puromycin resistance gene, blasticidin-S-deaminase gene. In a preferred embodiment, said antibiotic resistance gene is a hygromycin resistance gene, preferably an *Escherichia*
20 *coli* hygromycin-B phosphotransferase (hpt) gene. In this case, the selective agent is hygromycin. In another preferred embodiment, said antibiotic resistance gene is a neomycin resistance gene. In this case, the selective agent is G418. In another embodiment, the positive
25 selection marker protein of the invention is His D; in that case, the selective agent is Histidinol. In another embodiment, the positive selection marker protein of the invention is *Hypoxanthine phosphoribosyl transferase* (HPRT) or *Hypoxanthine guanosyl phosphoribosyl*
30 *transferase* (HGPR); in that case, the selective agent

is Hypoxanthine. In another embodiment, the positive selection marker protein of the invention is guanine-phosphoribosyl-transferase (Gpt); in that case, the selective agent is xanthine. It is also in the scope of the invention to use negative selection marker proteins. For example, the genes encoding for such proteins are the HSV-TK gene; in that case the selective agent is Acyclovir-Gancyclovir. For example, the genes encoding for such proteins are the Hypoxanthine phosphoribosyl transferase (HPRT) gene or the guanine-phosphoribosyl-transferase (Gpt) gene; in these cases, the selective agent is the 6-Thioguanine. For example, the gene encoding for such proteins is the cytosine deaminase; in that case the selective agent is the 5-fluoro-cytosine. Other examples of negative selection marker proteins are the viral and bacterial toxins such as the diphtheric toxin A (DTA).

Examples of such proteins having a research interest are the reporter proteins. By "reporter gene" is meant any gene which encodes a product (i.e. the reporter protein) whose expression is detectable. A reporter protein may have one of the following attributes, without restriction: fluorescence, enzymatic activity, or an ability to be specifically bound by a second molecule (e.g., biotin or an antibody-recognizable epitope). Examples of reporter proteins are autofluorescent proteins such as the green fluorescence protein (GFP), the enhanced green fluorescence protein (EGFP), the red fluorescence protein (RFP), the blue fluorescence protein (BFP), the yellow fluorescence

protein (YFP) and the fluorescent variants of these proteins. In a preferred embodiment, the gene of interest is a gene coding GFP. Examples of reporter proteins having enzymatic activity are β -galactosidase, 5 β -glucoronidase, alkaline phosphatase, luciferase, alcohol deshydrogenase, chloramphenicol-acetyl transferase, peroxydase.

In another embodiment, the sequence of interest is a therapeutic gene. A "therapeutic gene" is a gene that 10 corrects or compensates for an underlying protein deficit or, alternately, that is capable of down-regulating a particular gene, or counteracting the negative effects of its encoded product, in a given disease state or syndrome. For example, a therapeutic 15 gene of the invention is a transcription factor or a hormone, a growth factor that will enhance pancreatic cells insulin secretion.

In another preferred embodiment, the sequence of interest is a transforming gene. By "transforming gene" 20 it is meant gene that gives to the cells that contain it and in which it is expressed, the ability to have a transformed or cancerous phenotype. Transformed cells preferably presents the following characteristics: immortality (or unlimited growth), the lost of contact 25 inhibition, the independance toward growth factors, tumorigenicity, the lost of the anchoring. Examples of transforming genes are cellular genes *ras*, *met*, *NGL* (ex-neu), *bcl-X*, telomerase gene, or viral gene, such as large T antigen of SV40, or its temperature 30 sensitive mutant form. According to a preferred

embodiment the gene of interest is a temperature sensitive mutant of the large T antigen of simian virus 40 (SV40).

In a preferred embodiment, the sequence of interest
5 is an immortalizing gene. By "immortalizing gene" it is meant gene that gives to the cells that contain it and in which it is expressed, the ability to grow (almost) indefinitely. The cells transfected by an immortalizing gene are able to divide (almost) indefinitely, unlike
10 normal cells which are programmed to divide a limited number of times (for example less than 50 divisions for an adult fibroblast). Among immortalizing genes, one can recite *myc* and *myb* cellular genes, DNA virus gene, such as large T antigen, or a temperature sensitive
15 mutant form of large T antigen E1A. Immortalizing genes generally encode for nuclear proteins that bind to DNA, either directly or indirectly via protein factors.

Beside the immortalization of the pancreatic cell of the invention by introducing a transgene encoding for an
20 immortalizing gene into the cell, the present invention also encompasses the immortalization of the pancreatic cells by transfection with a compound selected in the group comprising a natural virus, a recombinant virus, or a fragment thereof, a virus based vector that
25 comprises an immortalizing gene. Consequently the method of producing functional animal pancreatic cell of the invention further comprises the step of immortalizing said animal embryonic pancreatic cells not older than 10 weeks of development, and/or further comprises the step
30 of immortalizing the cells obtained at step (d). Such

virus may be selected among lentivirus, simian virus SV40, Epstein-Bahr virus, Moloney leukemia virus. Preferably, the cell of the invention are transfected with a lentivirus based vector, more preferably a HIV-1
5 based vector.

The virus based vector of the invention more preferably, the HIV-1 based vector of the invention comprises an expression cassette as previously described that comprises a gene of interest operatively linked to
10 a promoter, such as a rat insulin promoter or a human insulin promoter. In one embodiment, the HIV-1 based vector comprises a reporter gene as a gene of interest, and more preferably the GFP gene operatively linked to the rat insulin gene promoter. In another embodiment the
15 HIV-1 based vector of the invention comprises an expression cassette comprises an incogene as a gene of interest, operatively linked to the rat insulin promoter.

Multi-transgenic cells are also in the scope of the
20 invention. Moreover, a pancreatic cell of the invention may be immortalized by the above mentioned compound and genetically modified to express another gene of interest.

The isolated functional pancreatic cells of the
25 invention can be cultured *in vitro* prior to introduction into the individual. Suitable culture media are well known to those of skill in the art and may include growth factors or other compounds which enhance survival, proliferation or selectively promote the

growth of certain sub-types of pancreatic cells such as alpha, beta, delta pancreatic cells.

It is another embodiment of the present invention to provide a pancreatic cell of the invention as a
5 medicament. More precisely, the present invention relates to the use of a pancreatic cell of the invention for preparing a medicament to treat diabetics, hypoglycemia, or pathologies associated to a dysfunction of the digestive enzymes. In a preferred
10 embodiment, the invention relates to the use of a pancreatic cell of the invention for preparing a medicament to treat diabetics. The present invention also provides the use of a pancreatic cell of the invention for cell therapy.

15 The invention also encompasses treatment of diseases or amelioration of symptoms associated with disease, amenable to gene transfer into pancreas cell populations obtained by the method disclosed herein. Diseases related to the lack of a particular secreted
20 product including, but not limited to, hormones, enzymes, interferons, growth factors, or the like can also be treated by genetically modified pancreas cells.

It is also a goal of the present invention to use a pancreatic cell of the invention for studying the
25 physiopathological development of diabetes. Such a cell *in vitro* cultured or engraft into an individual as an allograft or an autograft, would be highly useful to study molecular, biological, biochemical, physiological and/or physio-pathological mechanisms of glycemia
30 regulation and/or also digestive enzyme expression,

secretion and regulation. For such applications, immortalized functional beta cells are greatly appreciated. Indeed, the present invention allows to generate human beta cell lines.

- 5 The present invention also provides a method of producing animal pancreatic cell at different stages of development wherein said method comprises the steps of:
- 10 (a) introducing an effective amount of animal embryonic pancreatic cells not older than 10 weeks of development, into the kidney capsule of non-obese diabetic/severe combined immuno-deficiency (NOD/*scid*) animal, excepted human, wherein said NOD/*scid* is of a different species than said animal from which are obtained said embryonic pancreatic cells; and
 - 15 (b) allowing the animal embryonic pancreatic cells to develop, optionally to differentiate and optionally to regenerate at least a pancreatic function selected among the regulation of glycemia and secretion of digestive enzymes ;
 - 20 and
 - (c) collecting animal pancreatic cells obtained at step (b) at different periods of time, and
 - (d) optionally *in vitro* culturing the cells
 - 25 obtained at step (c).

Such pancreatic cells obtained by the method of the invention are useful for studying pancreas development. Such pancreatic cells and the embryonic pancreatic cells used in the above method may be genetically

modify. Preferably, the pancreatic cells obtained are immortalized.

Another embodiment of the present invention is the NOD/*scid* animal in which the embryonic pancreatic cells
5 have been engrafted. Such NOD/*scid* animal comprises at least one functional pancreatic cell of the invention at any stage of development, which is derived from the engrafted embryonic pancreatic cell.

The present invention relates to the use of the
10 NOD/*scid* animal of the invention to study and understand the development and the functioning of healthy or pathologic pancreas. Such animal constitutes an excellent model to understand and study pancreatic development, mainly human pancreatic development.
15 Moreover, such animal would be useful to screen compounds able to modulate pancreas development or to modulate the regulation of glycemia, by modulating or by acting, for instance, on the insulin or glucagon expression, or on the expression of any targeted gene
20 or protein involved in glycemia regulation. Such animal would be also useful to screen compounds able to modulate the expression of digestive enzymes. By "modulate", it is meant "enhance", "decrease", or "cancel".

25

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of the skill in the art to which this invention belongs.

The figures and examples presented below are provided as further guide to the practitioner of ordinary skill in the art and are not to be construed as limiting the invention in anyway.

5

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Development of the human pancreas in NOD/scid mice. (A) a pancreas at 8 weeks of development
10 before transplantation. (B-E) the pancreases were grafted under the kidney capsule of NOD/scid mice and analyzed 7 days (B), 2 months (C), 6 months (D), and 9 months (E) later.

15 **Figure 2: Evolution of the weight of the transplanted pancreas.**

Grafts were removed at different time points after transplantation, finely dissected to remove the fat, and weighed. A total of 22 grafts were analyzed.

20

Figure 3: Histological analysis. Human pancreas at 8 weeks of development before transplantation (A), and one month (B) and six months (C) after transplantation stained with an anti-pan cytokeratin antibody (revealed
25 in green) or with an anti-vimentin antibody (revealed in red).

Figure 4: Development of the pancreatic endocrine tissue in NOD/scid mice. Eight-week pancreas before
30 grafting (A), and 7 days (B), one month (C), 2 months

(D), 6 months (E) and 9 months (F) after transplantation.

Insulin (revealed in green) and glucagon (revealed in red) immunostainings. The arrows in (A) represent 2 cells that stain positive for both insulin and amylase. In G and H are shown representative in hybridizations of a proinsulin probe on sections from 8-week human pancreas before grafting (G) and after 6-months engraftment (H).

10

Figure 5: Evolution of the endocrine cell mass during the transplantation period.

The absolute mass of insulin-expressing cells is presented in arbitrary units. A total of 16 grafts were analyzed.

15

Figure 6: Cell proliferation analysis.

Double immunostaining for BrdU (red) and pancytokeratin (green) (A, C) or BrdU (red) and insulin (green) (B, D) on sections of human embryonic pancreas that developed in *scid* mice during 1 month (A and B) or during 3 months (C, D). Mice were sacrificed 2 hours after BrdU injection.

20

Figure 7: Human endocrine cells developed in mice resemble mature endocrine cells.

25

Sections of a human embryonic pancreas 6 months after transplantation. (A). Insulin (revealed in green) and Pax 6 (revealed in red); (B). Insulin (revealed in green) and Nkx6.1 (revealed in red); (C, D). Insulin

30

(revealed in red) and PC1/3 (revealed in green); (E). Insulin (revealed in green) and Cytokeratin-19 (revealed in red); (F). Cytokeratin 19 alone (revealed in red).

5

Figure 8: Functional development of the human pancreas graft.

(A) Three months after transplantation, *scid* mice (red lines) were injected with alloxan. Non-grafted 10 mice (blue line) also received alloxan. While the glycemia of the non-grafted mice increased rapidly, that of the grafted mice remained stable. When grafts were removed by nephrectomy at day 7 or day 43, glycemia increased rapidly.

15 (B) Mouse pancreas before alloxan treatment and at the end of the experiments (C) (day 43 after alloxan) stained for insulin (revealed in red) and glucagon (revealed in green), indicating that alloxan has destroyed the vast majority of host-insulin-expressing 20 cells. (D). Section of the human graft at the end of the experiment (day 43 after alloxan) stained for insulin (revealed in red) and glucagon (revealed in green), indicating that alloxan had no effect on human beta cells that developed.

25

Figure 9: Development of rat embryonic pancreases engrafted in Scid mice and search for conditions to transduce insulin-expressing cells using recombinant lentiviruses.

5 E16 rat pancreases were dissected. In **A.**, they were immediately stained for glucagon (green) and insulin (red). In **B.** and **C.**, they were infected with lentiviruses expressing GFP under the control of the insulin promoter and grafted during 7 days. **B.** staining
10 for insulin (red) and glucagon (green). **C.** No GFP-expressing cells could be detected under such conditions. In **D.** and **E.**, E16 pancreases were dissociated and next infected with lentiviruses expressing GFP under the control of the insulin
15 promoter. **D.** staining for insulin (red) and glucagon (green). **E.** GFP-expressing cells could be detected under such conditions.

Figure 10: Expression of GFP in insulin-expressing
20 **cells.**

E16 pancreases were dissociated and next infected with lentiviruses expressing GFP under the control of the insulin promoter and grafted during 7 days. **A.** GFP staining; **B.** insulin staining; **C.** Panels A and B were
25 superimposed. **D.** Staining for glucagon (red) and GFP (green); **E.** Staining for Cytokeratin (red) and GFP (green).

Figure 11: Long term expression of GFP in insulin-expressing cells.

E16 pancreases were dissociated and next infected with lentiviruses expressing GFP under the control of the insulin promoter and grafted during 1 Month. **A, D.** GFP staining; **B, E.** insulin staining; **C, F.** Panels A+B and D+E were superimposed.

Figure 12: GFP⁺/INS⁺ cells develop from infected progenitor cells.

A. Insulin content in E16 pancreases before (day 0) or following a 7 day-graft (Day 7). **B.** Pregnant rats at E16 were injected with BrdU. On the left panel, the tissues were stained for BrdU (in red) and insulin (in green). On the right panel, the tissues were stained for BrdU (red), insulin (blue) and glucagon ((green). Note that the endocrine cells do not proliferate. **C.** E13 pancreases were dissociated and next infected with lentiviruses expressing GFP under the control of the insulin promoter and grafted during 7 days. **Left.** GFP staining; **Middle.** insulin staining; **Right.** Left and right panels were superimposed.

EXAMPLES

1 - RESEARCH DESIGN AND METHODS

1.1 Human tissues

Human pancreases were dissected from embryonic tissue fragments obtained immediately after voluntary

abortions performed between 6 and 9 weeks of development (WD), in compliance with the current French legislation and the guidelines of our institution. The warm ischemia time was less than 30 min. Gestational
5 ages were determined from several developmental criteria: duration of amenorrhea; crown-rump length measured by ultrasound scan; hand and foot morphology. Pancreases were dissected and either fixed and embedded in paraffin or grafted to non-obese diabetic/severe
10 combined immunodeficiency (NOD/*scid*) mice as described below.

1.2 Animals and transplantation into NOD/*scid* mice.

15 NOD/*scid* mice were bred in isolators supplied with sterile-filtered, temperature-controlled air. Cages, bedding and drinking water were autoclaved. Food was sterilized by X-ray irradiation. All manipulations were performed under a laminar flow hood. Embryonic
20 pancreases (6-9 weeks of development (WD)) were implanted, using a dissecting microscope, under the left kidney capsule of 6- to 8- week-old NOD/*scid* mice that had been anaesthetized with Hypnomidate (Janssen-Cilag). At different time points after the
25 graft (7 days - 9 months), mice were sacrificed and the grafts were removed, weighed, fixed in formalin 3.7 % and embedded in paraffin. For cell proliferation analysis, mice were injected with Bromo-deoxy Uridine (BrdU) (50mg/kg) 2 hours before sacrifice.

1.3 Immunohistochemistry.

Four μm -thick sections were cut on gelatinized glass slides. For immunostaining, sections were deparaffinized in toluene, rehydrated, microwaved in
5 citrate buffer 0.01 M, pH 6, and permeabilized for 20 min in Tris-Buffered Saline (TBS) containing 0.1 % Triton. Non-specific sites were blocked for 30 min in TBS containing 3% BSA and 0.1 % Tween 20 and sections were incubated overnight at 4°C with primary antibodies.
10 The sections were then washed and incubated 1h at room temperature with the appropriate secondary antibodies, labeled with 2 different fluorochromes. The primary antibodies were: mouse anti-human insulin (Sigma Aldrich, 1/1000); guinea pig anti-pig insulin (Dako;
15 1/2000); mouse anti-human glucagon (Sigma Aldrich, 1/2000); rabbit anti-human pan-cytokeratin (Dako, 1/500); mouse anti-human cytokeratin. 19 (Dako; 1/50); mouse anti-pig vimentin (Dako; 1/30); rabbit anti-proconvertase 1/3 (gift from Dr Steiner, 1/200);
20 rabbit anti-Pax6 (gift from Dr S. Saule); rabbit anti-rat Nkx6.1 (gift from Dr Serup); Mouse anti-BrdU (Amersham). Fluorescent secondary antibodies were: fluorescein-anti-guinea pig antibodies (Dako, 1:500); fluorescein-anti-rabbit antibodies (Immunotech, 1/200);
25 fluorescein-anti-mouse antibodies (Immunotech, 1/200); Texas-red-antimouse antibodies (Jackson, 1/200); Texas-red-anti-rabbit antibodies (Jackson, 1/200).

1.4 Surface quantification and statistical analysis.

All images were numerized using a Hamamatsu C5810 cooled tri-CCD camera. Pictures were made at the same magnification, and analyzed with the IPLab software (version 3.2.4, Scanalytics Inc.). For each transplanted tissue, sections were taken at regular intervals throughout the graft and stained for insulin. Three to four sections and 3-5 views per section were analyzed. The evolution of the beta cell mass during the transplantation period was calculated as the product of the surface that stained positive for insulin by the corresponding graft weight.

1.5 In situ hybridization.

For *in situ* hybridization, sections were deparaffinized, rehydrated and permeabilized in PBS containing 1% Triton X-100. Prehybridization was done at 70°C in hybridization buffer (50% formamide, 5 X SSC, 5X Denhardts' solution, 250~µg/ml yeast RNA, 500µg/ml herring sperm DNA). RNA probes were labeled with DIG-UTP by *in vitro* transcription using the DIG-RNA labeling kit (Boehringer Mannheim). Hybridization was initiated by addition of fresh hybridization buffer containing 1pg/ml probe and continued overnight at 70°C. Thereafter, the slides were washed with decreasing concentrations of SSC. Revelation was processed by immunohistochemistry. Non-specific sites were blocked with 2% blocking reagent (Boehringer Mannheim) in Tris 25mM pH7.5, NaCl

140mM, KCl 2.7mM Tween 20 0.1 % for 30 min at room temperature. Slides were then incubated overnight at 4°C with alkaline phosphatase-conjugated polyclonal sheep anti-DIG antibody (diluted 1:1000, Boehringer Mannheim). The reaction product was visualized by an enzyme-catalyzed color reaction using nitro blue tetrazolium and 5-bromo-4chloro-3-indolyl-phosphate medium (Bohringer Mannheim). Sections were incubated until the colored reaction product developed at the sites of hybridization. The slides were washed in H₂O, mounted and visualized on a Leitz DMRD light microscope (Leica). The probe used here corresponded to human proinsulin.

1.6 Test of induction of diabetes.

To determine the capacity of the graft to regulate the glycemia of the mouse, grafted and nongrafted NOD/scid mice were injected intravenously (i.v.) with alloxan (Sigma-Aldrich, 90mg/kg body weight), that is known to destroy rodent, but not human, beta cells (Eizirik *et al.*, 1994). Glucose levels were measured on blood collected from the tail vein every day during one week, using a portable glucose meter (GlucoMen, A. Menarini diagnostics, Firenze, Italy). To confirm the contribution of the graft to the normalization of blood glucose values in the host, grafts were removed by unilateral nephrectomy at different time points (7 days or 42 days) after the injection of alloxan and blood glucose levels were measured.

1.7 Animals and dissection.

Pregnant rats were purchased from the Janvier breeding center (CERJ, Le Genet, France). The morning of the discovery of the vaginal plug was designated as embryonic day 0.5 (E0.5). Pregnant rats were killed with CO₂, the embryos were harvested and the pancreases were dissected. For cell proliferation analysis, pregnant rats were injected with BrdU (100 mg/kg) 30 minutes before killing.

10

1.8 Construction and preparation of HIV-RIP-GFP vector: DNA constructs and vector production.

The promoterless pTRIP GFP has been described previously (Zennou *et al.*, 2000). A 408 bp fragment of the Rat insulin promoter (RIP408) was produced from the pBS-RIP-beta globin plasmid (kindly provided by P. Herrera, (Sanvito *et al.*, 1995)) by PCR using the expand system (Roche) with the following primers :

MluI RIP sens = 5' cgacgcgtGGACACAGCTATCAGTGGGA 3' (SEQ ID N°1)

BamHI RIP antisens = 5' cgggatccTAGGGCTGGGGGTTACT 3' (SEQ ID N°2).

The resulting PCR product was subcloned into the pGEMT-easy vector (Promega). The MluI-BamHI fragment was then inserted into a MluI-BamHI linearized promoterless pTRIP GFP vector. To rule out point mutations induced by PCR the inserted RIP408 was entirely sequenced.

Stocks of vectors were prepared by transitory co-transfection of 293T cells with the p8.7 encapsidation plasmid (Δ Vpr Δ Vif Δ Vpu Δ Nef) (Zufferey *et al.*, 1997),

30

pHCMV-G encoding the VSV envelope and the pTRIP RIP408-GFP vector (Yee et al., 1994), as described previously (Zennou et al., 2000). The supernatants were treated with DNase I prior to ultracentrifugation then
5 resuspended in PBS and frozen (-80°) until use.

1.9 Infection of embryonic pancreases:

Recombinant lentiviruses were used to infect either intact or dissociated pancreases. For dissociation,
10 pancreases were incubated with 0.16 mg of Dispase I (Roche Bohringer) for 5 to 30 min depending of the size of the tissue and mechanically dissociated. For infection, the intact or dissociated tissue were incubated for one hour at 37°C with 200 µl of RPMI
15 medium 1640 containing penicillin (100 units/ml), streptomycin (100 mg/ml), L-glutamin (2mM), and 10µl of viral particles (12 µg/ml of p24). To increase the rate of viral infection, DEAE-dextran was added at the concentration of 10 µg/ml.

20

1.10 Transplantation:

Scid mice were kept in isolators supplied with sterile-filtered, temperature-controlled air. Cages, bedding and drinking water were autoclaved. Food was sterilized
25 by X-ray irradiation. All manipulations were performed under a laminar flow hood. Embryonic dissociated pancreases were implanted using a dissecting microscope under the left kidney capsule as previously described (Castaing et al., 2001), with the following
30 modifications. The left kidney was exteriorized and a

small transverse incision was made through the capsule on the ventral surface of the kidney, near the inferior pole. A pocket was created under the capsule, and a small silicon ring was partially pushed into the pocket under the capsule to provide a chamber for the transplanted tissue (Thomas et al., 1997). The dissociated cells were then introduced into the cylinder using a 50 μ l Hamilton syringe with a blunt 22 gauge needle, and the ring fully introduced into the pocket, so that the capsule on the top and the kidney parenchyma on the bottom formed a sealed space contained the pancreatic cells. This method confines the cells to a single position so that the development could be easily studied. At different time points after transplantation, the mice were killed with CO₂, the kidney removed, and the grafts were fixed in formalin 3,7% and embedded in paraffin.

1.11 Immunohistochemistry:

Four micrometer-thick sections were collected and analyzed by immunohistochemistry as described elsewhere (Cras-Meneur et al., 2001; Miralles et al., 1998). The antisera were used in the following dilutions: mouse anti-Human insulin (Sigma), 1/2,000; mouse anti-Human glucagon (Sigma), 1/2000; mouse anti-human pancytokeratin (Sigma), 1/100; mouse anti-BrdU (Amersham), 1/4; rabbit anti-human pancytokeratin (Dako), 1/500; rabbit anti-glucagon (DiaSorin), 1/2000; rabbit anti-porcine insulin (DiaSorin), 1/2000; and rabbit anti-GFP (Clontech), 1/200. The fluorescent

secondary antibodies from Jackson ImmunoResearch Laboratories were FITC anti-rabbit antibodies, 1/200; and Texas red anti-mouse antibodies, 1/200. For double-labeling immunofluorescence, antibodies raised in
5 different species were applied to the sections and revealed by using antispecies antibodies labeled with two different fluorochromes. Single-labeled sections incubated with mismatched secondary antibodies showed no immunostaining, confirming the specificity of the
10 secondary antisera. Photographs of the sections were taken using a fluorescence microscope (Leica, Leitz DMRB). They were digitized by using a Hamamatsu (Middlesex, NJ) C5810 cooled 3CCD camera.

15 **1.12 Insulin content:**

For insulin content determination, embryonic pancreatic buds or grafted pancreases were homogenized. Insulin was extracted overnight in 10 ml of cold acidified ethyl alcohol (1,5% HCl, 75% EtOH) at -20°C. The
20 extracts were stored at -20°C until assayed for insulin.

Immunoreactive insulin was measured with an Radioimmunoassay, using monoiodated ¹²⁵I-labeled porcine insulin (Sorin Biomedica, Sallugia, Italy) as a tracer,
25 guinea-pig anti-insulin antibody kindly provided by Dr Van Schravendijk (Brussels, Belgium) and purified rat insulin (Novo Nordisk, Boulogne, France) as standard. Charcoal was used to separate free from bound hormone. The sensitivity of the assay was 0.25 ng/ml.

2 - RESULTS

2.1 Human embryonic engraftment in NOD/*scid* mice.

In the present study, 48 embryonic pancreatic tissues (6-9WD) were grafted to NOD/*scid* mice. Mice
5 were sacrificed at different time points after transplantation (7 days-9 months), and the grafts were dissected. Thirty-nine grafts were recovered. Among the 9 non-recovered grafts, one grafted mouse died, but the graft was present. In 8 cases, mice died for unknown
10 reasons and graft growth was not analyzed. The evolution of grafted tissues in terms of volume and mass was next followed. As shown in Fig.1, the human embryonic tissue developed massively when grafted under the kidney capsule of the *scid* mice. While at 7-9 weeks
15 of gestation, before grafting, the volume of embryonic pancreas was not more than 4 mm³ (Fig. 1A), it increased with time in the mouse host and reached a volume of a few cm³ 6 months later (Fig. 1B-E). The evolution of the grafted tissue in term of mass was
20 also followed. While, after 1 week in the mouse, graft weight was less than 10 mg, in the same range of the ungrafted tissue, it next increased rapidly with time to reach 100 mg after 8-12 weeks and 1000 mg after 33-38 weeks (Fig. 2). Immunohistochemistry using
25 anti-cytokeratin and anti-vimentin antibodies was performed to follow the evolution during the grafting period of the epithelial and mesenchymal cells present in the human embryonic pancreas before transplantation. As shown in Fig. 3A, before grafting, an 8WD pancreas
30 is composed of epithelial cells forming ducts and

mesenchymal cells. One month and 6 months after transplantation, the tissue was also composed of epithelial cells that stained positive for cytokeratin and mesenchymal cells positive for vimentin, indicating
5 that both cell types did develop during the graft (Fig. 3B and C).

2.2 Development of the endocrine tissue.

Before transplantation, only a few endocrine cells
10 were detected by immunohistochemistry that stained positive either for glucagon, or for both insulin and glucagon. Such cells were dispersed in the pancreatic tissue and were not associated into islets of Langerhans (Fig. 4A). Once transplanted, the endocrine
15 tissue started to develop and the number of endocrine cells increased with time (Fig. 4B-F). In panels G and H are shown representative hybridizations for proinsulin before and after transplantation for 6 months of an 8-week old pancreas. Huge increase in the
20 number of cells that express proinsulin mRNA can be clearly visualized. Evolution of the insulin-positive cell mass was quantified after immunohistochemistry. As shown in Fig. 5, the absolute surface occupied by insulin-expressing cells was multiplied by 300 after
25 8-12 weeks in the mouse and by 5,000 after 21-28 weeks.

2.3 Human undifferentiated epithelial cells, but not endocrine cells, proliferated during the engraftment period.

To define whether the increase in the absolute number of endocrine cells was due to the differentiation of precursor cells or to the proliferation of the few endocrine cells that were present before grafting, engrafted mice were injected with BrdU 2 hours before sacrifice and immunohistological analysis was performed. As shown in Fig. 6, after both 1 and 3 months of development in the mouse, while cells that stained positive for both cytokeratin and BrdU were frequently detected, cells positive for both insulin and BrdU were very rarely found. These results strongly suggest that increase in the endocrine cell mass was due to the differentiation of precursor cells, rather than to the proliferation of rare preexisting endocrine cells.

2.4 Human endocrine cells developed in mice resemble mature endocrine cells.

To define whether human endocrine cells that developed in NOD/*scid* mice express markers known to be present in human beta cells developed *in vivo*, a series of antibodies were tested by immunohistochemistry. As shown in Fig. 7, beta cells developed in NOD/*scid* mice express specific transcription factors such as Nkx6.1 and Pax6 (Panels A, B), as well as PC 1/3, an enzyme necessary for the processing of proinsulin into insulin (panels C, D). Moreover, endocrine cells in the grafts

are frequently associated in islets of Langerhans, with a core of insulin-expressing cells surrounded by glucagon-expressing cells (Fig. 4, panels E, F). Finally, while as described previously (Bouwens *et al.*, 1997), the first endocrine cells found in human embryonic pancreas stain positive for cytokeratin, the human insulin-expressing cells that developed in NOD/*scid* mice did not express cytokeratin 19 (Fig. 7, panels E, F).

10

2.5 Functional development of human pancreas grafts.

To define whether the human beta cells that developed in the grafts were functional, 15 NOD/*scid* mice were engrafted with human embryonic pancreas. Three months later, grafted or non-grafted NOD/*scid* mice were injected with alloxan, a drug known to be toxic for murine, but not for human beta cells (Eizirik *et al.*, 1994). Before alloxan injection, blood glucose levels were not statistically different in the transplanted and non-transplanted mice. After alloxan treatment, the glycemia of all non-grafted mice increased up to 6g/l. Conversely, the glycemia remained stable in 12 out of 15 engrafted mice. To demonstrate that glycemia regulation in engrafted mice injected with alloxan is indeed due to the development of the graft, unilateral nephrectomies were performed to remove the grafts in 6 mice and blood glucose levels were monitored. As shown in Fig. 8A, after removal of the graft by unilateral nephrectomy, either 7 or 43

30

days after alloxan injection the mice became hyperglycemic. Murine pancreases and grafts were also analyzed for the presence of insulin- and glucagon-expressing cells before alloxan injection, or
5 at the end of the experiments when the animals were sacrificed. As shown in Fig. 8, very few insulin-expressing cells were detected in the murine pancreas of grafted NOD/*scid* mice that had been injected with alloxan, compared to NOD/*scid* mice that
10 were not treated with alloxan. On the other hand, a huge amount of insulin-producing cells was present in the human graft after alloxan treatment.

The inventors demonstrate that human early embryonic pancreas can develop when engrafted under the
15 kidney capsule of NOD/*scid* mice. The size and weight of the grafts increased considerably and endocrine cells differentiated which were organized into islets of Langerhans and showed numerous criteria of maturity. Finally, the human endocrine pancreatic tissues that
20 developed could reverse diabetes in mice, indicating its functionality.

In the present study, the inventors used NOD/*scid* mice as recipients for transplantation. NOD/*scid* mice were generated by crossing the *scid* mutation from
25 C.B-17-*scid/scid* mice onto the NOD background. These animals are lacking T- and B-lymphocytes, (Shultz et al., 1995), and fail to generate either humoral or cell-mediated immunity. Because of the absence of xenograft rejection in *scid* mice, they were previously
30 used as recipients for human or fetal hematolymphoid

tissues and cells (Roncarolo *et al.*, 1995). Non hematopoietic human tissues such as ovarian cortex (Weissman *et al.*, 1999), thyroid (Martin *et al.*, 1993), skin (Levy *et al.*, 1998) and airway (Delplanque *et al.*, 5 2000) were also successfully transplanted in this model. However, the capacity of these tissues to develop functional properties and to replace a physiological function has been demonstrated only rarely. One example of physiological function 10 replacement is the demonstration that adrenocortical tissue can form by transplantation of bovine adrenocortical cells and replace the essential functions of the mouse adrenal gland (Thomas *et al.*, 1997). However, in this work, the transplanted tissue 15 was not of human but of bovine origin. Moreover, the tissue had been expanded from a primary culture of bovine adrenocortical cells. Donor cells were thus already fully differentiated at the time of grafting.

In the present invention, the inventors demonstrate 20 that immature human embryonic pancreas can develop and acquire functional properties in *scid* mice.

By grafted immature rudiments that contained undifferentiated epithelial cells and mesenchymal tissue and almost no insulin-expressing cells, the 25 inventors demonstrate that the absolute mass of insulin-expressing cells was multiplied by nearly 5,000 after 6 months in the mouse. The fact that very few endocrine cells were present before transplantation, while a massive amount of endocrine cells was detected

a few weeks later, clearly indicates that neoformation of endocrine cells occurred in the present model.

Theoretically, the observed increase in the human beta cell mass could be due either to the proliferation
5 of rare preexisting insulin-expressing cells, or to the differentiation of precursor cells. It is thought that during prenatal life, increase in the beta cell mass is mainly due to the differentiation of precursor cells rather than to the proliferation of preexisting beta
10 cells. This is quite clear in rodents where a large number of experiments have been performed that indicate that the increase in the endocrine cell mass observed during fetal life cannot be explained by the proliferation of preexisting endocrine cells (Swenne,
15 1992)). While less information is available, this seems to be also the case in humans, where, during embryonic/fetal life, insulin-expressing cells stain rarely positive for Ki67, and hence are rarely or not cycling (Potak *et al.*, 2000; Bouwens *et al.*, 1997). The
20 inventors' data indicate that when chimeric mice are injected with BrdU, a large number of cytokeratin-positive cells present in the graft stain positive for BrdU, while no or very rare insulin-positive cells do. Thus, it can be postulated
25 that in the grafts newly formed beta cells derived from precursor cells present in the duct epithelium that did proliferate and differentiate during engraftment rather than from proliferation of the few endocrine cells present in the rudiment, a mechanism that does
30 recapitulate normal development.

Different arguments indicate that the human beta cells that did develop *in vivo* in NOD/*scid* mice are mature. First, these insulin - expressing cells did not coexpress glucagon and are thus different from the first insulin - expressing cells detected in the human pancreas at early stages of development (Polak *et al.*, 2000 ; Larsson and Hougaard, 1994 ; De Krijger *et al.*, 1992). Next, it has been shown that the insulin - expressing cells present in the pancreas before 16 weeks of development express cytokeratin 19, while the insulin-expressing cells found later during development stain negative for this marker (Bouwens *et al.*, 1997). The inventors' data indicate that the human beta cells that develop in NOD/*scid* mice stain negative for cytokeratin 19 and do thus resemble adult mature beta cells. Next, human beta cells that develop in NOD/*scid* mice express the prohormone convertase PC 1/PC3, an enzyme that is necessary for the processing of proinsulin into insulin (Kaufman *et al.*, 1997; Furuta *et al.*, 1998). Finally, the inventors' data indicate that the human endocrine cell mass that developed in NOD/*scid* mice is able to perfectly regulate the glycemia of NOD/*scid* mice deficient in endogenous beta cells, and hence is functional. These human endocrine cells remain functional and can regulate the glycemia of the mice for at least 43 days, the longest period tested before removing the graft.

The inventors demonstrate here that newly differentiated human beta cells that are able to regulate the glycemia of the host deprived of its own

beta cells can be produced from human early embryonic pancreas. Human embryonic pancreas does thus represent an alternative source of tissue useful to generate functional human beta cells for transplantation. Moreover, the model of mice grafted with human embryonic pancreas can now be used to progress in the study of the development of the human pancreas, a type of study that were difficult to perform due to the lack of human embryonic pancreases and of proper experimental systems.

2.6 Development of rat embryonic pancreases engrafted in Scid mice and search for conditions to transduce insulin-expressing cells using recombinant lentiviruses

The inventor first studied the capacity of pancreatic tissue to grow and develop when engrafted to *scid* mice. E16,5 pancreases were dissected and next transplanted under the kidney capsule of *scid* mice. Seven days after grafting, the mice were killed, and the grafts were removed. As previously shown (Rall et al., 1973), at E16, before grafting, the vast majority of the endocrine cells in the pancreas are glucagon-expressing cells, very rare insulin-expressing cells being present at this stage. Such insulin-expressing cells were found dispersed (Fig. 9A). On the other hand, when E16 pancreases were grafted for 7 days, insulin-expressing cells developed and were now found associated forming islets of Langerhans (Fig. 9B). The inventor next used an HIV-1 based vector, containing the green fluorescent protein (GFP) gene as reporter. GFP was driven by the

rat insulin promoter (RIP), to restrict the expression of GFP to beta cells. E16 pancreases were infected and grafted during 7 days. As show in Fig. 9C, no GFP-expressing cells were detected under such conditions.

5 The inventor next repeated the experiments but dissociated the tissue before infection and graft. As shown in Fig. 9D, under such conditions, the endocrine tissue developed properly. Moreover, a large number of cells expressed GFP when the tissue was dissociated

10 with Dispase before infection (Fig. 9E). GFP was never detected in non infected tissues (data not shown).

2.7 Long term expression of GFP in insulin-expressing cells.

15 The next step was to define the cell types expressing GFP. Double immunohistochemistry was performed using anti-insulin, -glucagon and -cytokeratin, to determine whether the GFP-expressing cells were beta cells, alpha cells or duct cells respectively. As shown in Fig. 10,

20 GFP was clearly detected in insulin-expressing cells. On the other hand, GFP was never detected in glucagon-expressing cells or cytokeratin-expressing cells.

Finally as shown in Fig. 11, sustained expression of GFP during more than one month (the longest time point

25 analyzed) could be achieved when GFP was driven by the rat insulin promoter.

2.8 Beta cells expressing GFP derive from infected progenitors.

Two sets of arguments indicate that beta cells expressing GFP derive from progenitors that were first
5 infected with RIP-GFP and next differentiated into beta cells and not by infection of the few beta cells present at E16 that would next proliferate. First, in the model of development of E16 pancreas grafted under the kidney capsule of Scid mice, beta cell
10 differentiation occurs. Indeed, as shown in Fig. 12A, during a 1 week grafting period, insulin content per pancreas measured by radio immunoassay was multiplied by more than 250 fold. As shown in Fig 12B, while at this stage, as expected, proliferation of pancreatic
15 cells is high, insulin-expressing cells do not proliferate. To further demonstrate that insulin/GFP cells derive from infected progenitor cells and not from preexisting mature beta cells, infection was repeated using dissociated pancreases at E13. As
20 previously shown, at this stage, very few insulin-expressing cells are present that are fully immature (Jackerott et al., 1996; Miralles et al., 1998; Pictet and Rutter, 1972). It is now established that such immature insulin-expressing cells will not give rise
25 later to mature beta cells (Jensen et al., 2000). As shown in Fig. 12C, when dissociated E13 pancreases were infected with RIP-GFP and transplanted under the kidney capsule of Scid mice, beta cells expressing GFP could be detected one week later, further indicating that

Insulin/GFP double positive cells derived from
endocrine progenitors that were infected at E13.

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SEQUENCE LISTING

<110> UNIVERSITE PARIS 7
CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (CNRS)

<120> METHOD OF PRODUCING HUMAN BETA CELL LINES

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C L A I M S

1. A method of regenerating pancreas function in an individual, the method comprising:

- 5 (a) introducing an effective amount of animal embryonic pancreatic cells not older than 10 weeks of development, into the kidney capsule of non-obese diabetic/severe combined immunodeficiency (NOD/*scid*) animal, excepted
10 human, wherein said NOD/ *scid* is of a different species than said animal from which are obtained said embryonic pancreatic cells; and
(b) allowing the animal embryonic pancreatic cells to develop, to differentiate and to regenerate
15 at least a pancreatic function ; and
(c) transplantation of an effective amount of the animal functional pancreatic cells obtained at step (b), into said individual.

20 2. The method of claim 1, wherein the individual is a mammal.

3. The method of claim 2, wherein the mammal is a human.

25

4. The method of claim 1, wherein said animal embryonic pancreatic cells are human embryonic pancreatic cells.

5. The method of claim 4, wherein said human embryonic pancreatic cells is of 6 to 9 weeks of development.

5 6. The method of claim 1, wherein the non-obese diabetic / severe combined immunodeficiency animal is a mouse.

7. The method of claim 1, wherein the effective
10 amount of the animal pancreatic cells transplanted at step (c) is comprised between about 10^3 to about 10^{12} animal pancreatic cells.

8. The method of claim 1, wherein the animal
15 pancreatic cells transplanted at step (c) are introduced into the pancreas of said individual.

9. The method of claim 1, wherein said pancreatic function is the regulation of glycemia.

20

10. The method of claim 1, wherein said individual is an insulin-dependant diabetic.

11. A method of treatment of diabetes in a human
25 patient in need of such treatment, the method comprising the steps of:

(a) introducing an effective amount of human embryonic pancreatic cells not older than 10 weeks of development, into the kidney capsule
30 of non-obese diabetic/severe combined

- immunodeficiency (NOD/scid) animal, excepted human; and
- (b) allowing the embryonic pancreatic cells to develop, to differentiate and to regenerate at least the pancreatic function ; and
- (c) transplantation of an effective amount of the human functional pancreatic cells obtained at step (b), into said patient,
- (d) and treating diabetics, wherein said treatment is effected by the regeneration of said pancreatic function of regulation of glycemia.

12. The method of claim 11, wherein the non-obese diabetic / severe combined immunodeficiency animal is a mouse.

13. A method of producing functional animal pancreatic cell wherein said method comprises the steps of:
- (a) introducing an effective amount of animal embryonic pancreatic cells not older than 10 weeks of development, into the kidney capsule of non-obese diabetic/severe combined immunodeficiency (NOD/scid) animal, excepted human, wherein said NOD/scid is of a different species than said animal from which are obtained said embryonic pancreatic cells; and
- (b) allowing the animal embryonic pancreatic cells to develop, to differentiate and to regenerate at least a pancreatic function ; and

(c) collecting animal pancreatic cells obtained at step (b), and

(d) optionally *in vitro* culturing the cells obtained at step (c).

5

14. The method of claim 13, wherein said pancreatic function is the regulation of glycemia.

15. The method of claim 13 further comprising the
10 step of immortalizing the cells obtained at step (d).

16. The method of claim 13 comprising the preliminary step of immortalizing said animal embryonic pancreatic cells not older than 10 weeks of
15 development.

17. The method of claims 15 and 16 wherein the cell is immortalized with a compound selected in the group comprising a natural virus, a recombinant virus,
20 or a fragment thereof, a virus based vector, said virus being selected among lentivirus, simian virus SV40, Epstein-Bahr virus, Moloney leukemia virus.

18. The method of claim 17 wherein the compound is
25 a lentivirus based vector, preferably a HIV-1 based vector.

19. The method of claim 17 wherein the virus based vector comprises an expression cassette that comprises

a gene of interest operatively linked to the rat insulin gene promoter.

20. The method of claim 19 wherein the gene of
5 interest is a reporter gene, preferably a gene coding for the green fluorescence protein (GFP).

21. The method of claim 19 wherein the gene of interest is an oncogene.

10

22. The method according to claim 13 further comprising the step of genetically modifying the cells obtained at step (d).

15 23. The method according to claim 13 further comprising the preliminary step of genetically modifying said animal pancreatic cells not older than 10 weeks of development.

20 24. Functional animal pancreatic cell obtained by the method according to claim 13 wherein said cell is a pancreatic beta cell.

25 25. Pancreatic beta cell of claim 24 wherein said cell express insulin in response to glucose.

26. Pancreatic cell according to claims 24 to 25 wherein said cell is a human cell.

27. Pancreatic cell according to claims 24 and 25 wherein said cell is a rat cell.

28. Pancreatic cell according to claims 24 and 25
5 as a medicament.

29. Use of a pancreatic cell according to claims 24 to 26 for preparing a medicament to treat diabetics.

10 30. Use of a pancreatic cell according to claims 24 to 26 for cell therapy.

31. Use of a pancreatic cell according to claims 24 to 27 for studying the physiopathological development
15 of diabetics.

32. A method of producing animal pancreatic cell at different stages of development wherein said method comprises the steps of:

- 20 (a) introducing an effective amount of animal embryonic pancreatic cells not older than 10 weeks of development, into the kidney capsule of non-obese diabetic/severe combined immunodeficiency (NOD/scid) animal, excepted
25 human, wherein said NOD/scid is of a different species than said animal from which are obtained said embryonic pancreatic cells; and
(b) allowing the animal embryonic pancreatic cells to develop, optionally to differentiate and

optionally to regenerate at least a pancreatic function; and

(c) collecting animal pancreatic cells obtained at step (b) at different periods of time, and

5 (d) optionally *in vitro* culturing the cells obtained at step (c).

33. Use of pancreatic cells obtained by the method of claim 32 for studying pancreas development.

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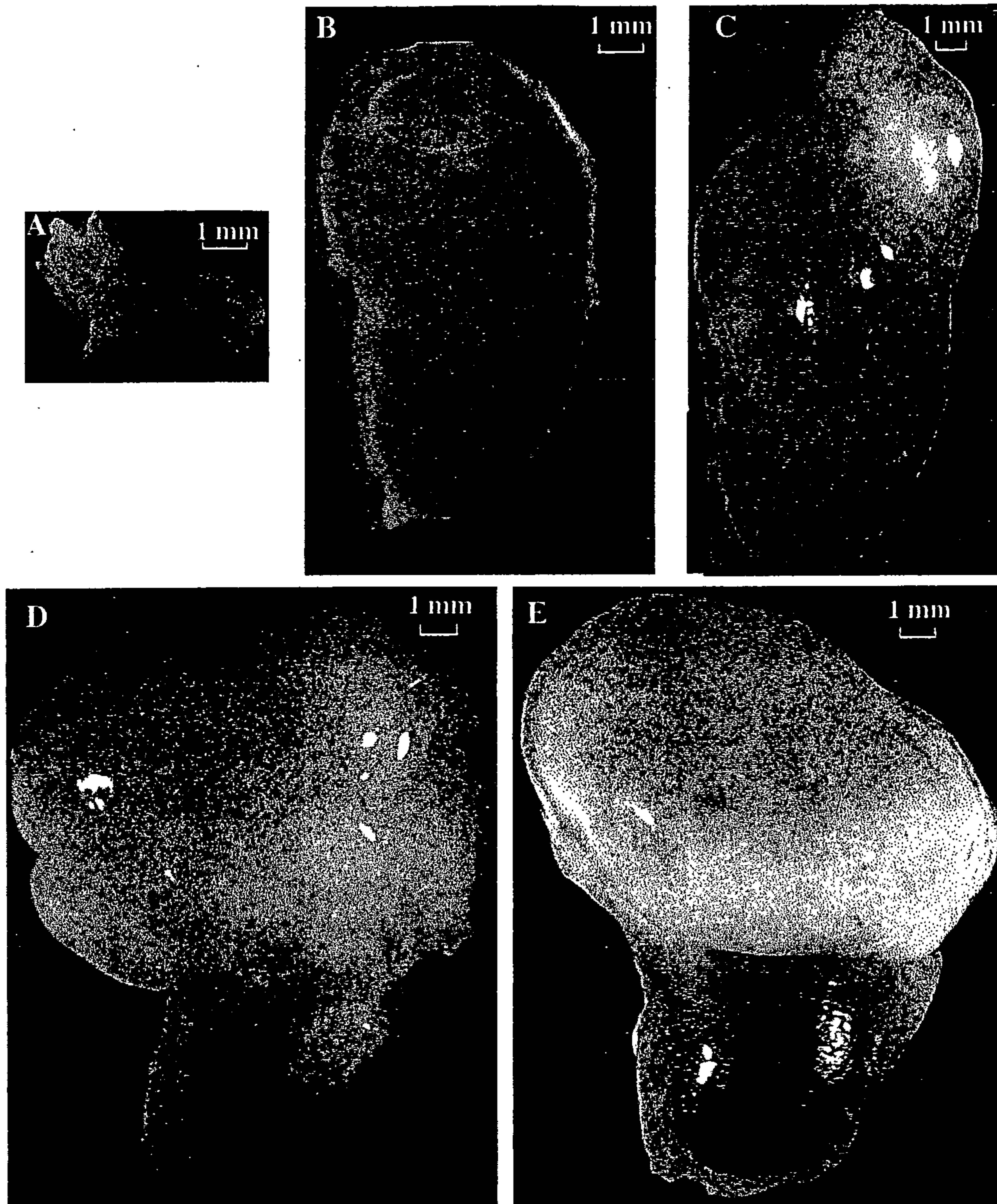


FIGURE 1

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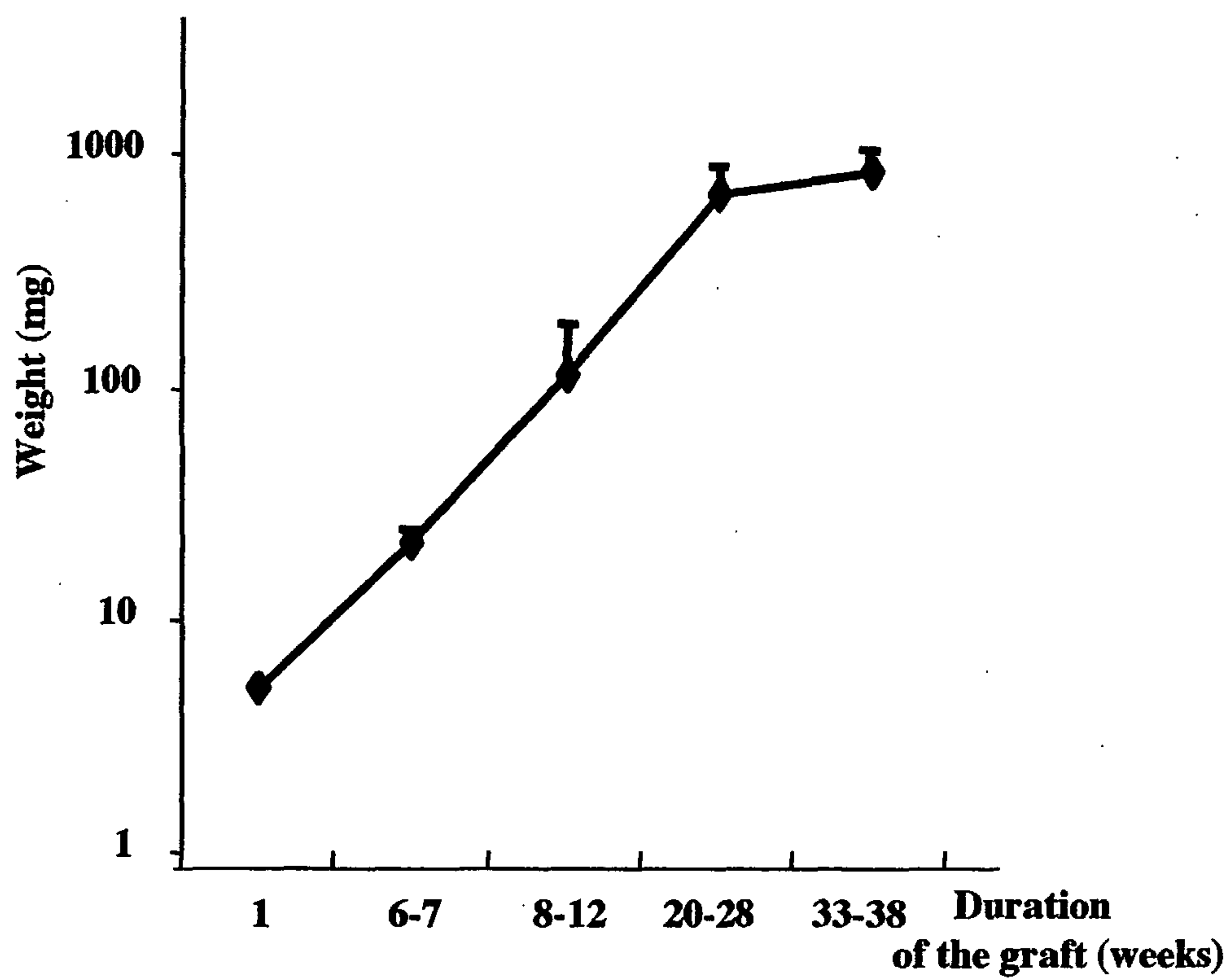
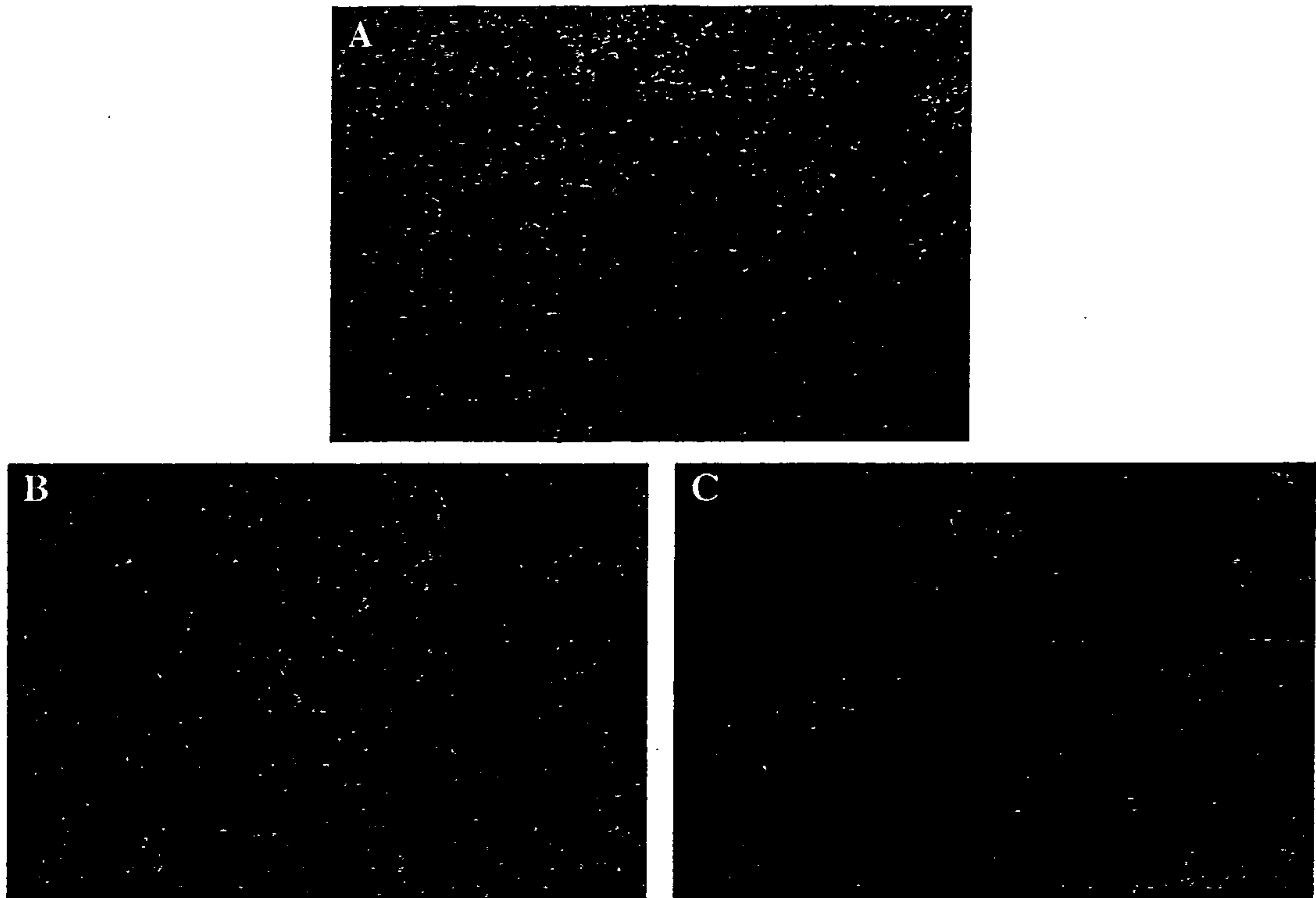


FIGURE 2

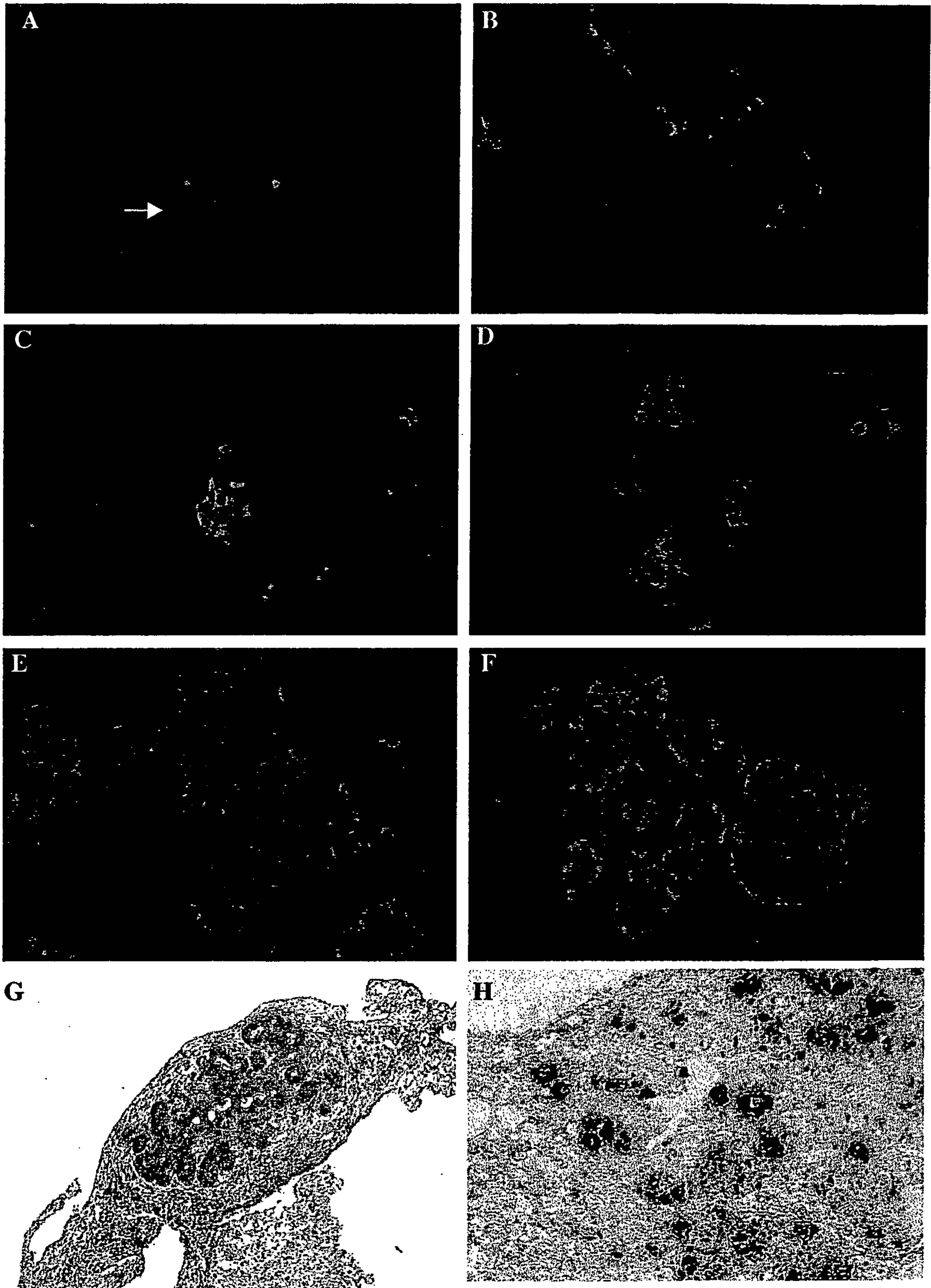
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Vimentin / pan cytokeratin

FIGURE 3

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Insulin/Glucagon**Proinsulin mRNA****FIGURE 4**

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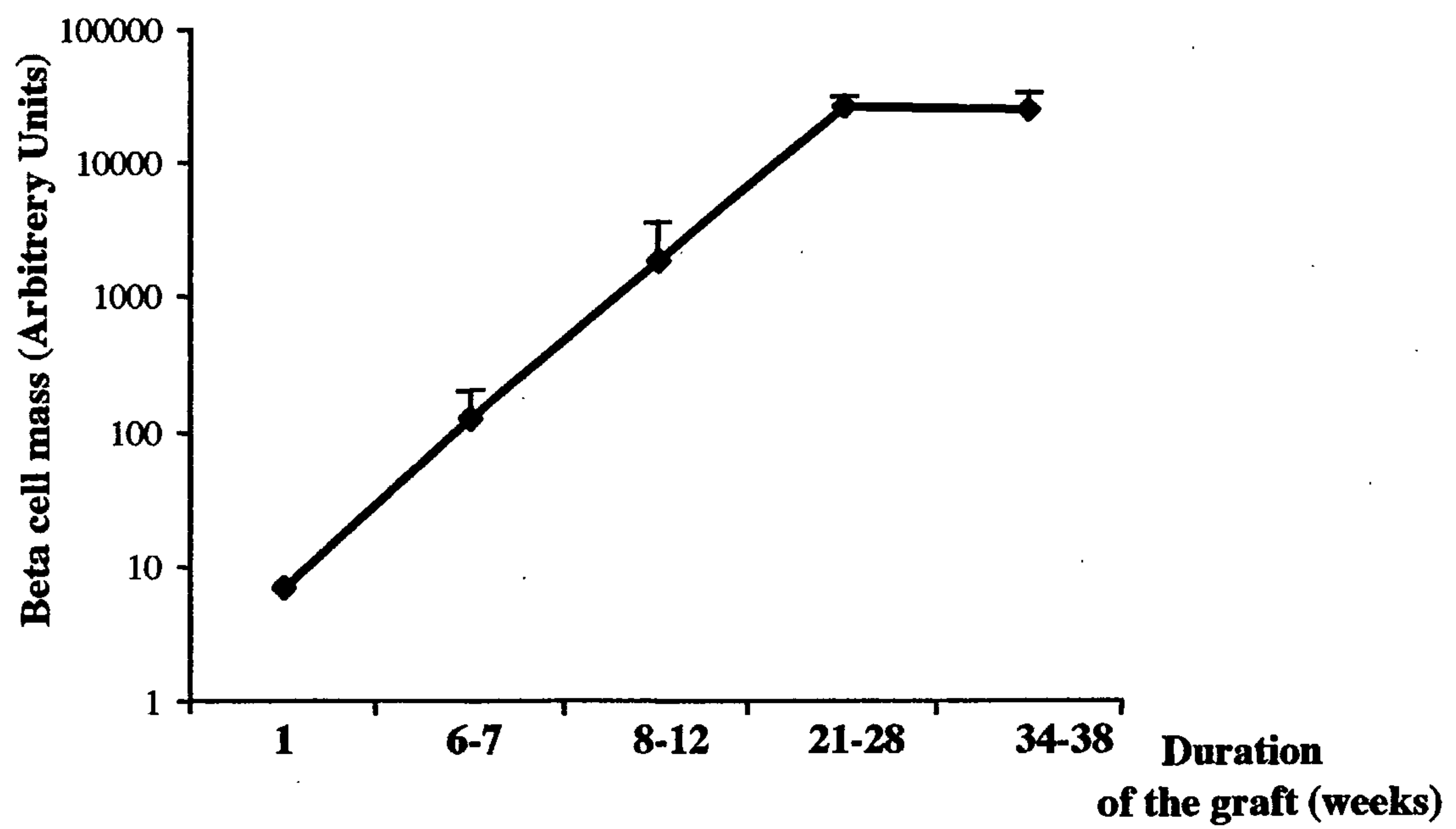


FIGURE 5

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Pan cytokeratin /BrdU



Insulin / BrdU

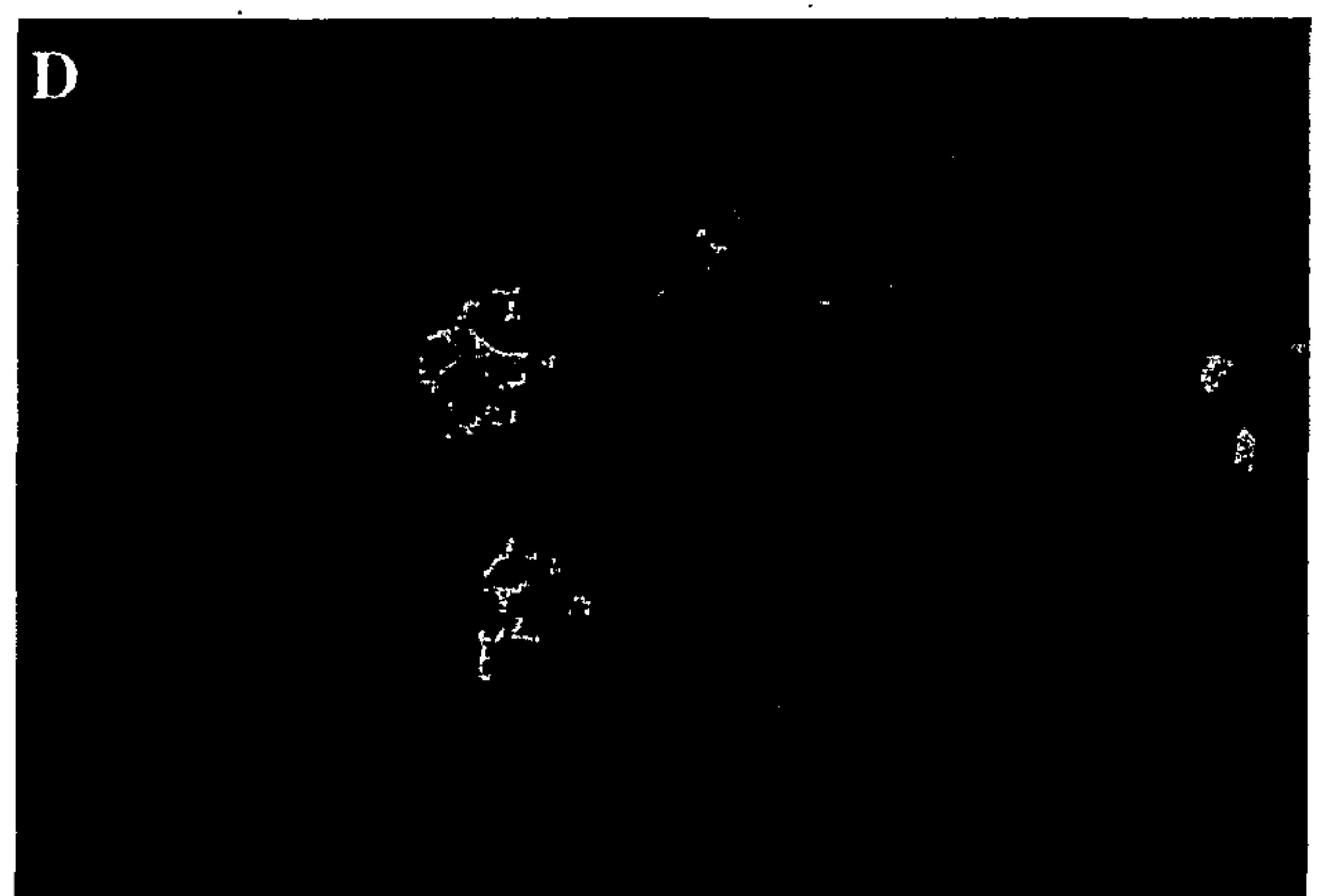
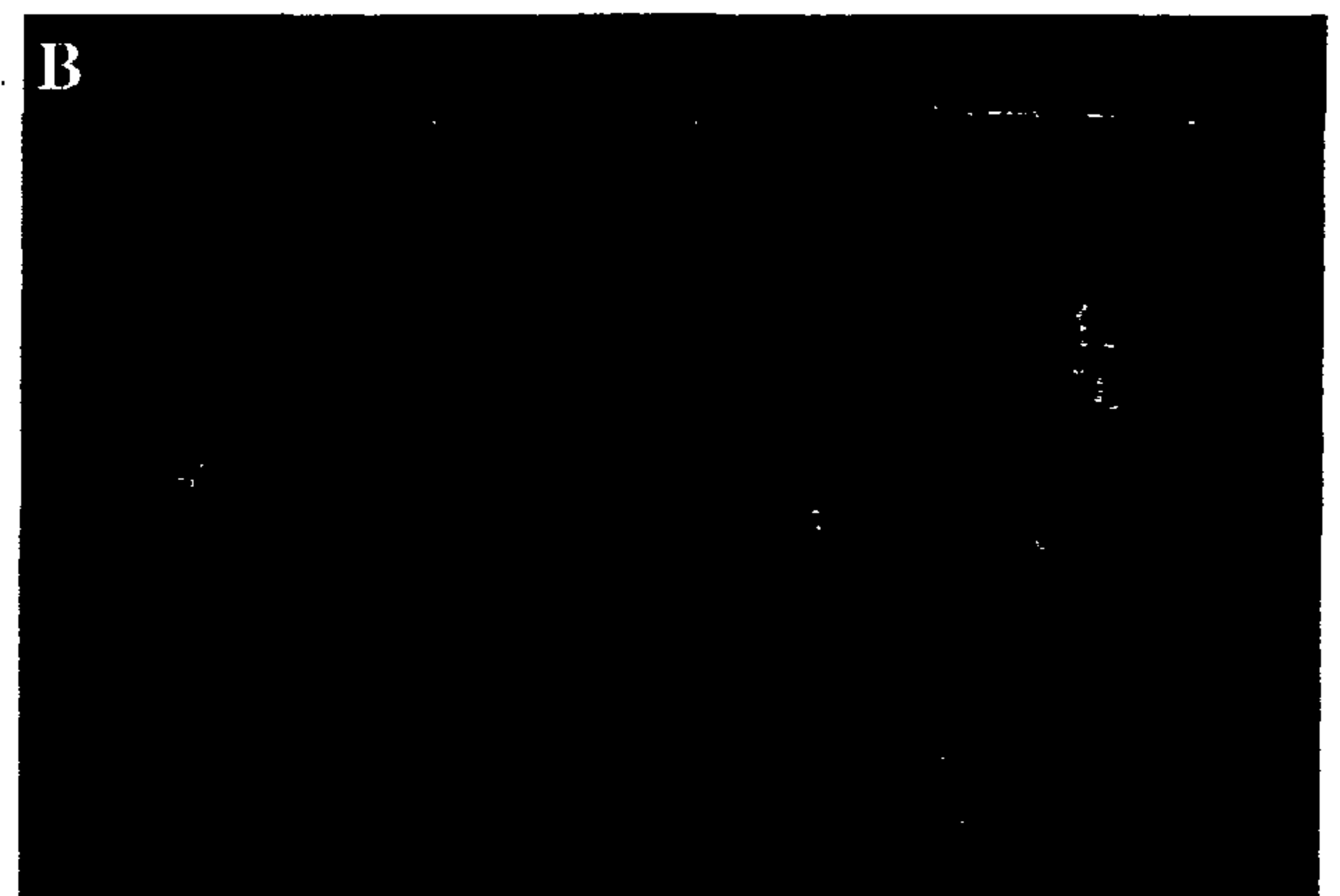


FIGURE 6

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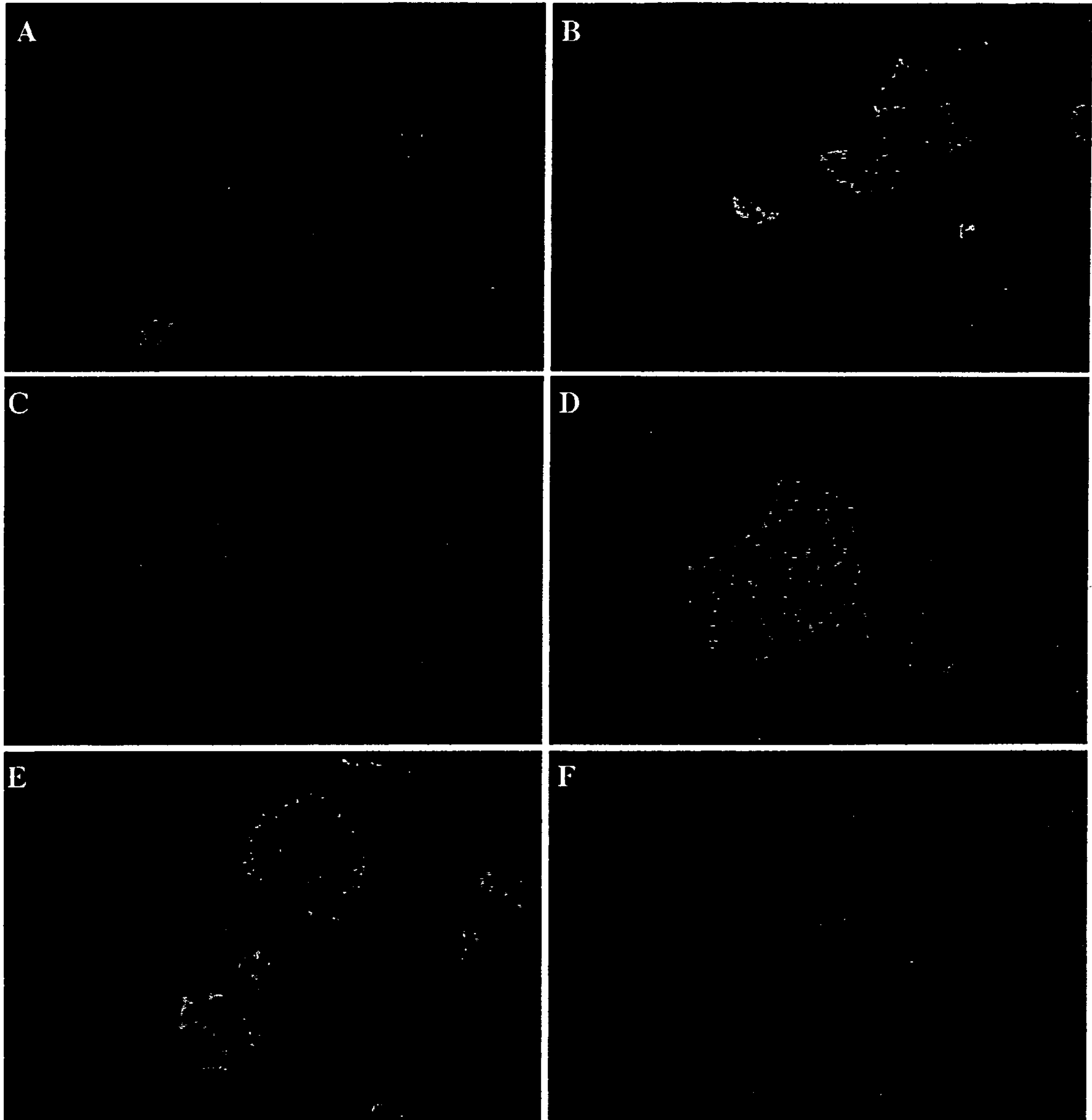
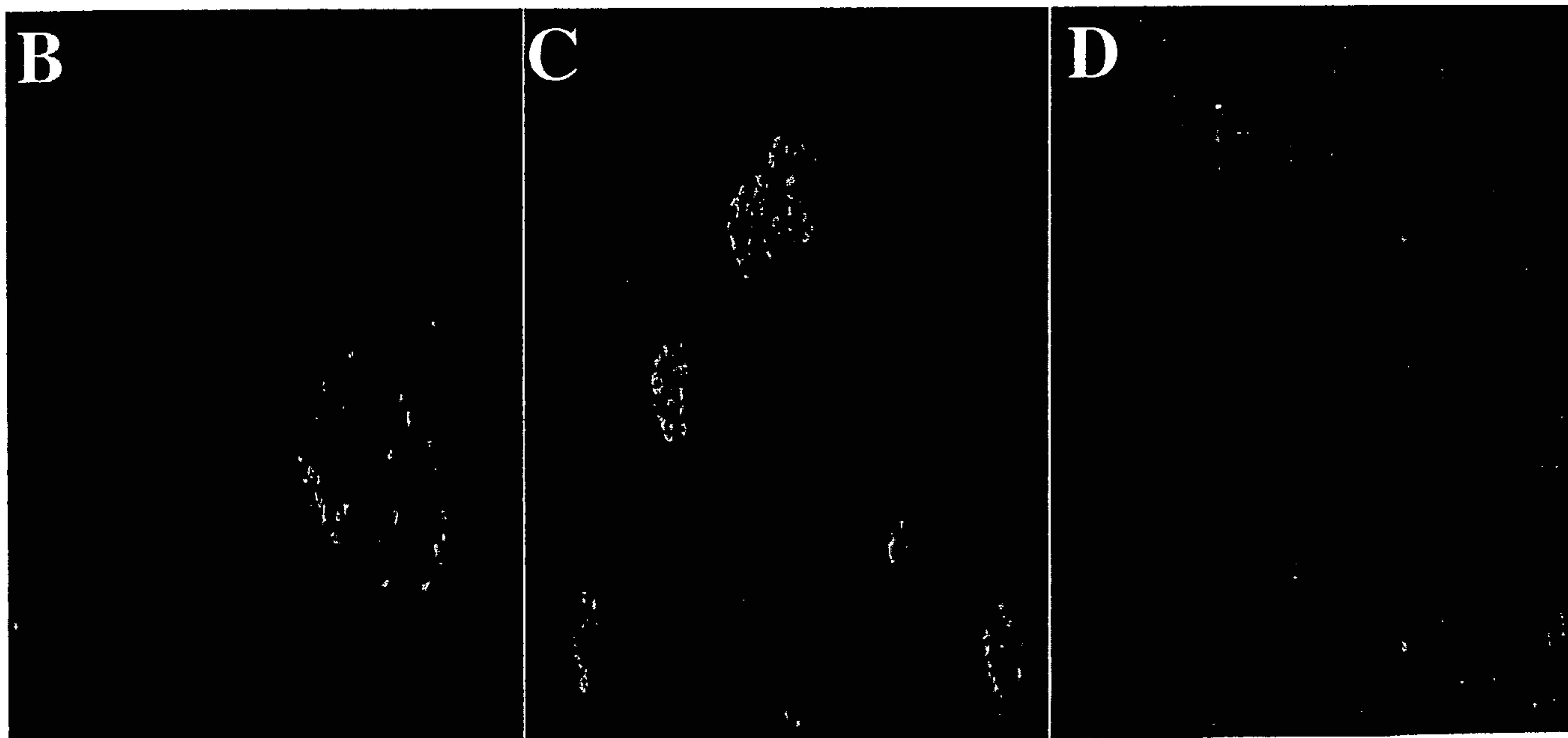
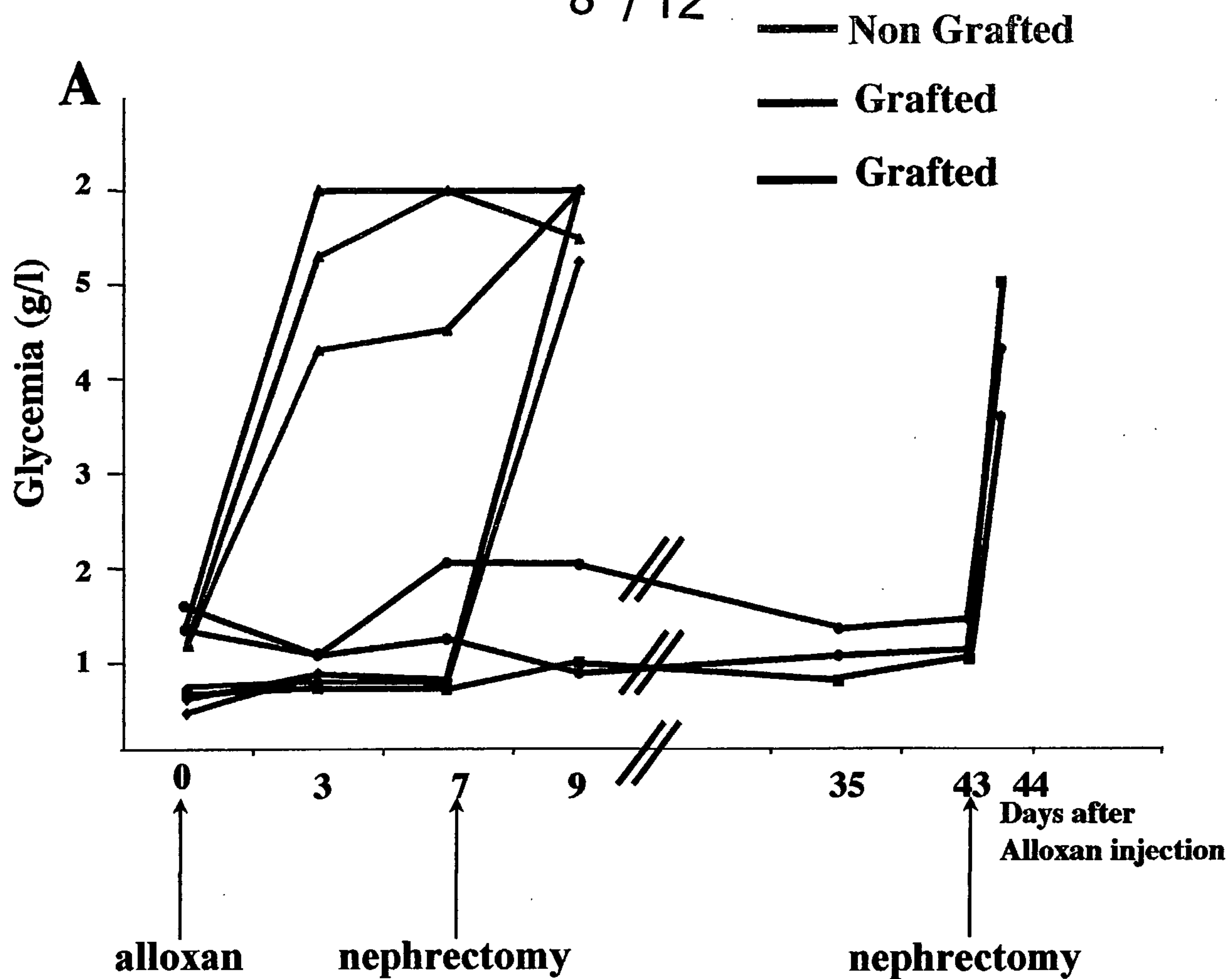


FIGURE 7

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Insulin/Glucagon

FIGURE 8

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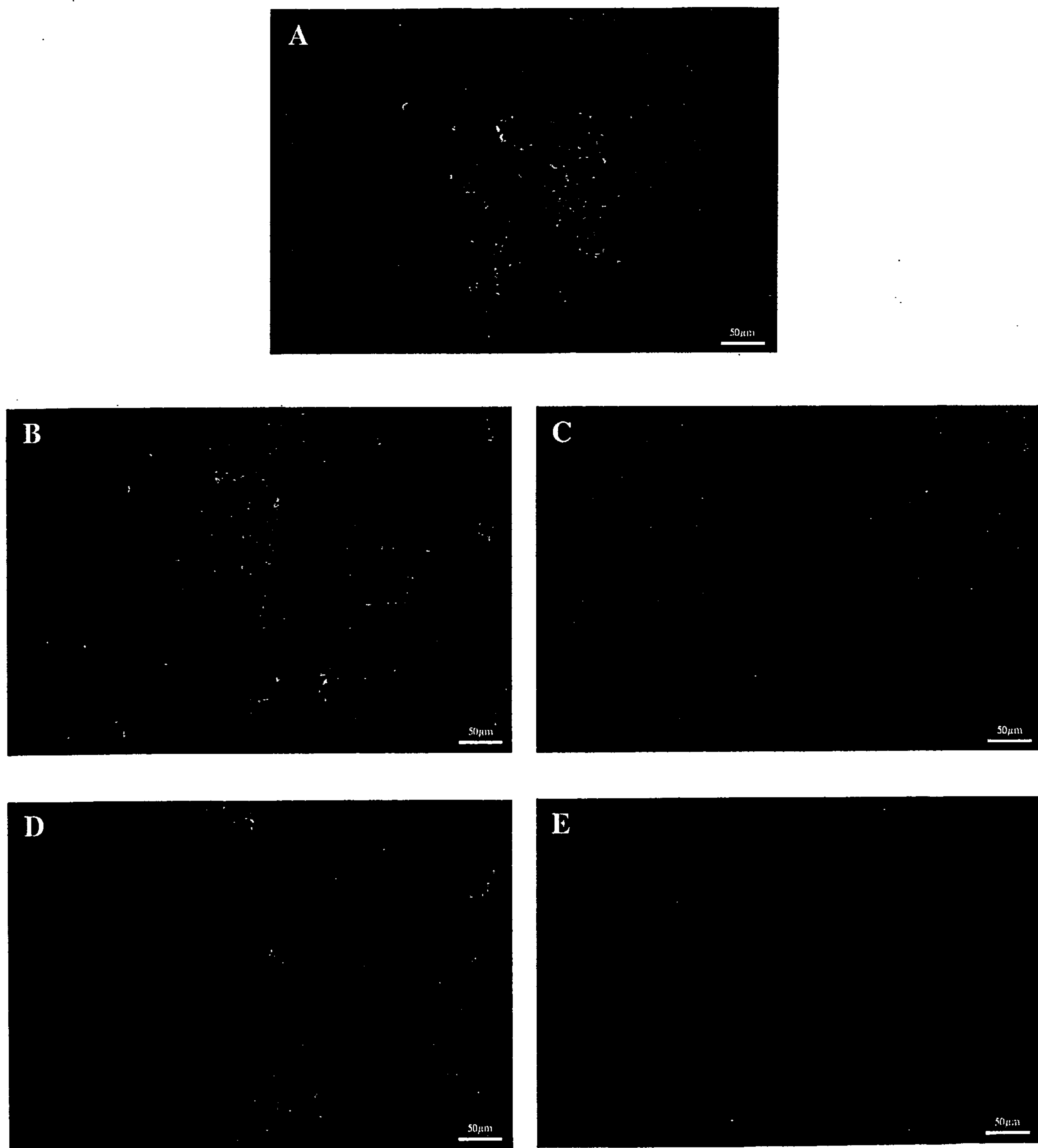


FIGURE 9

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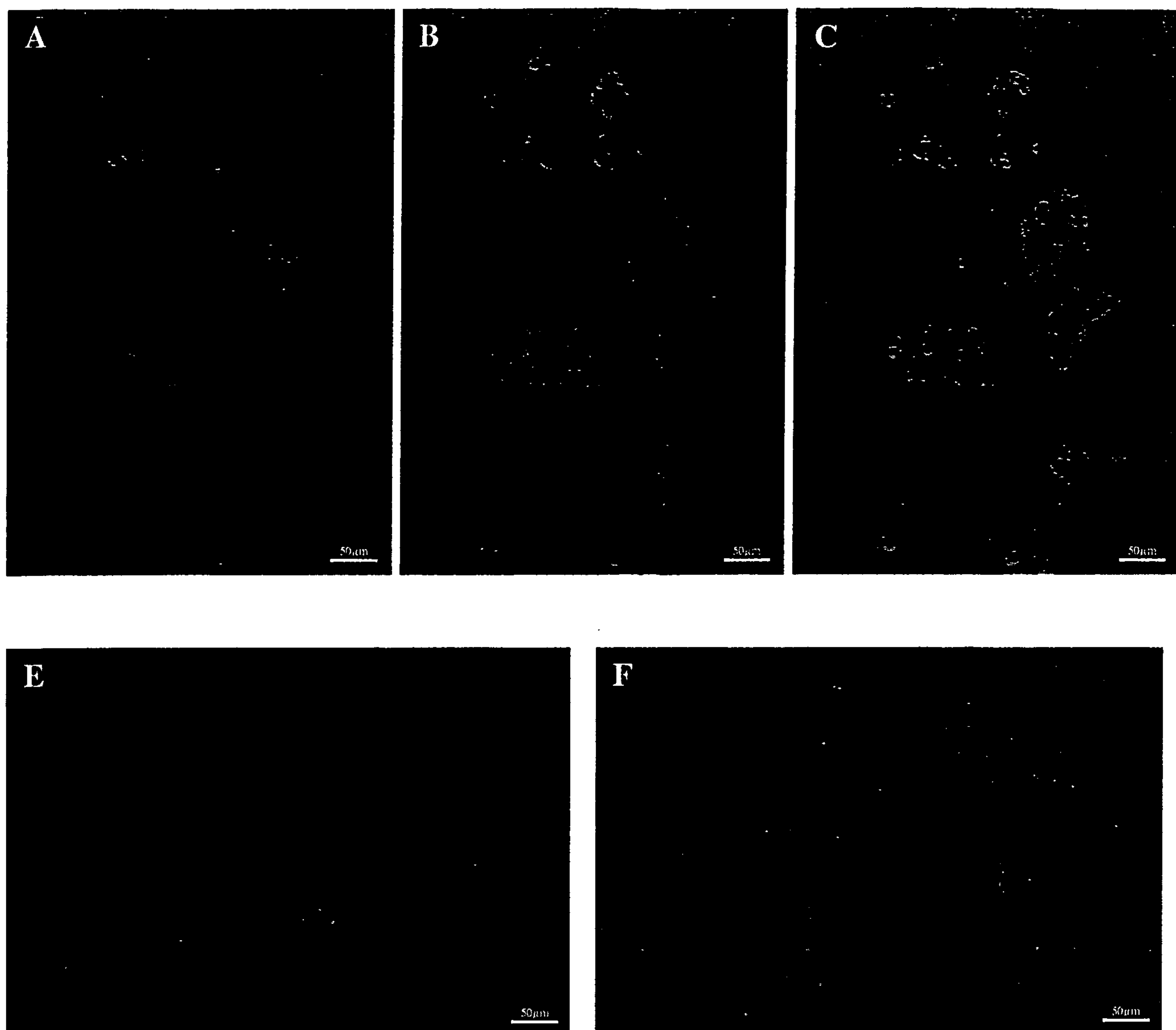


FIGURE 10

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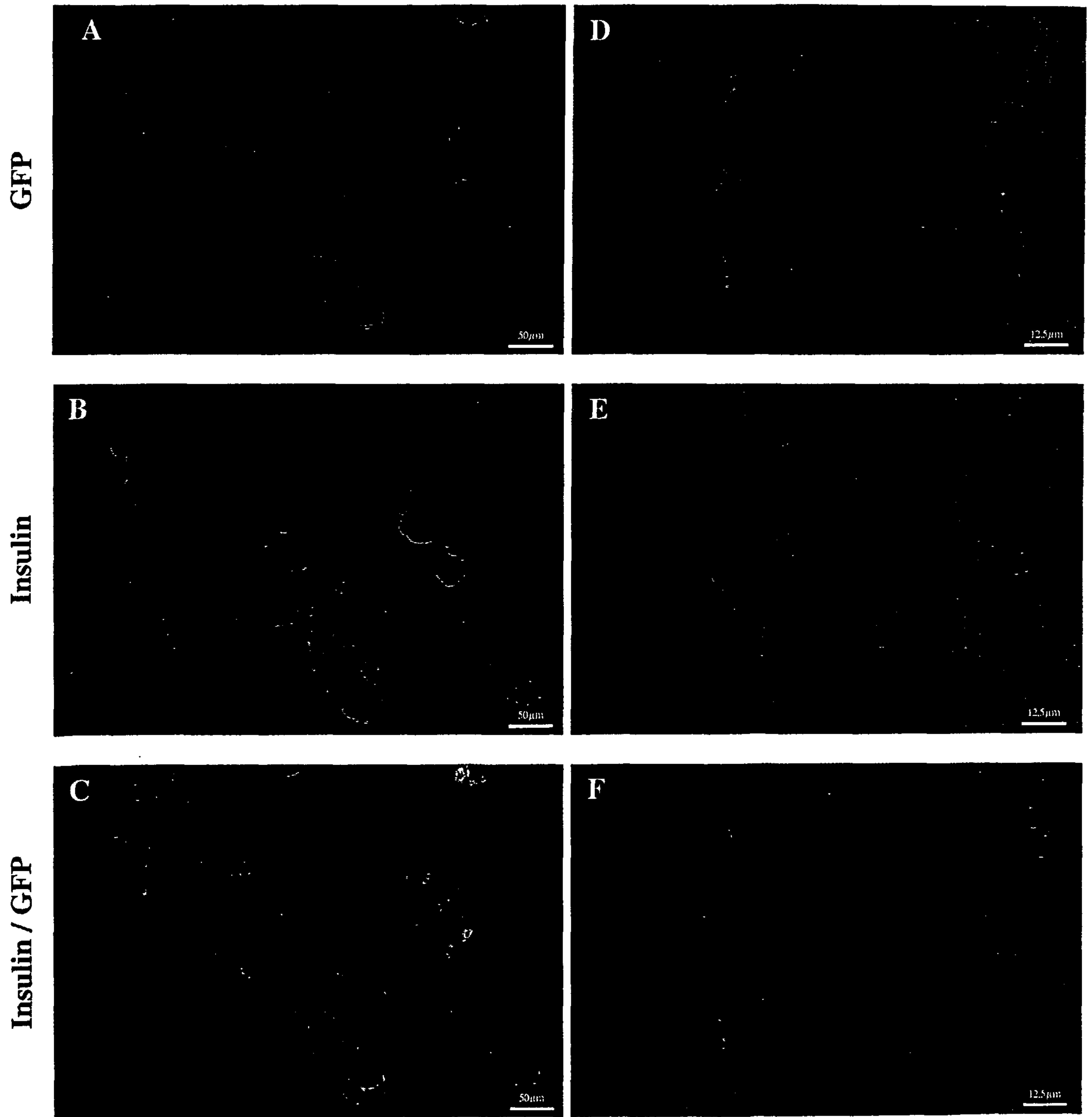
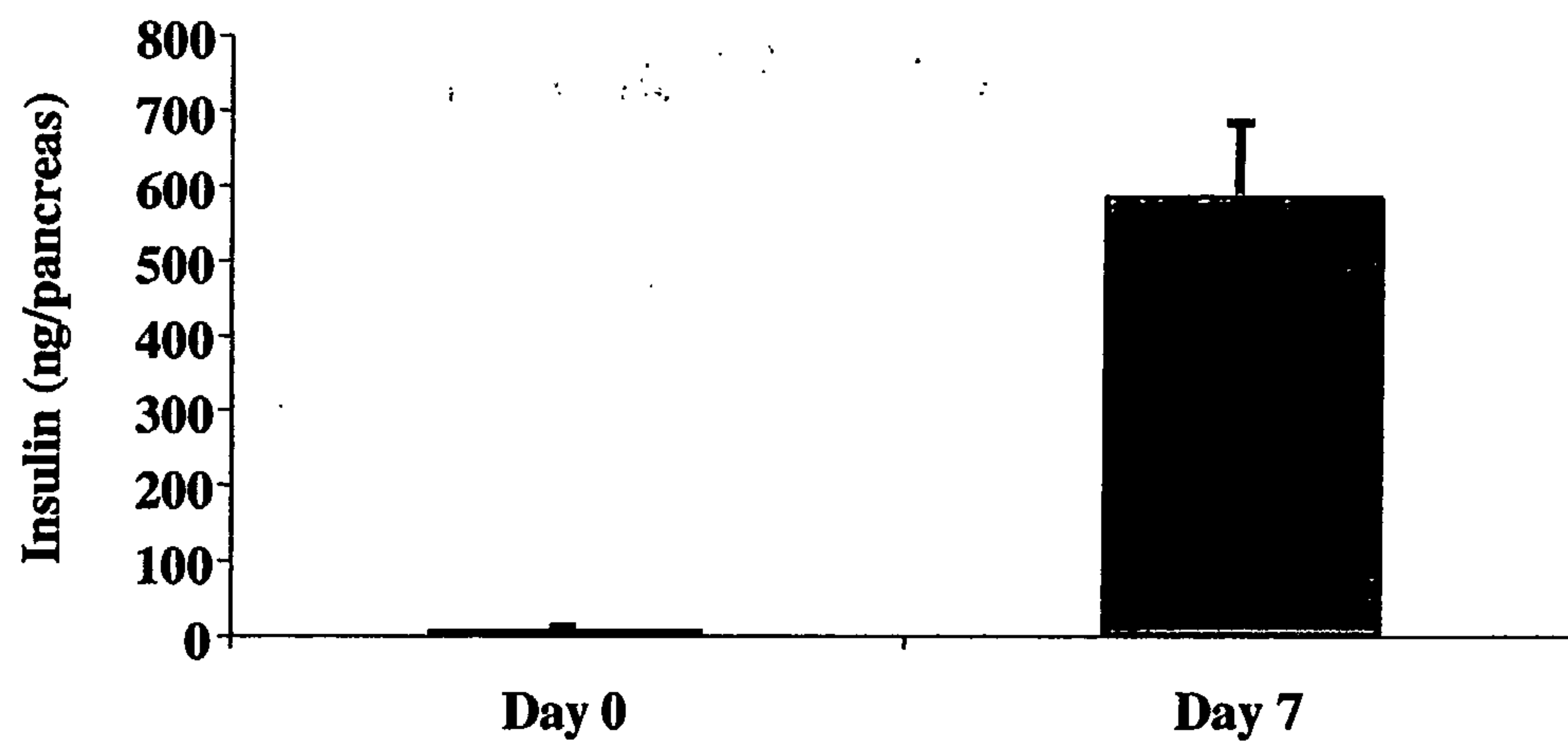
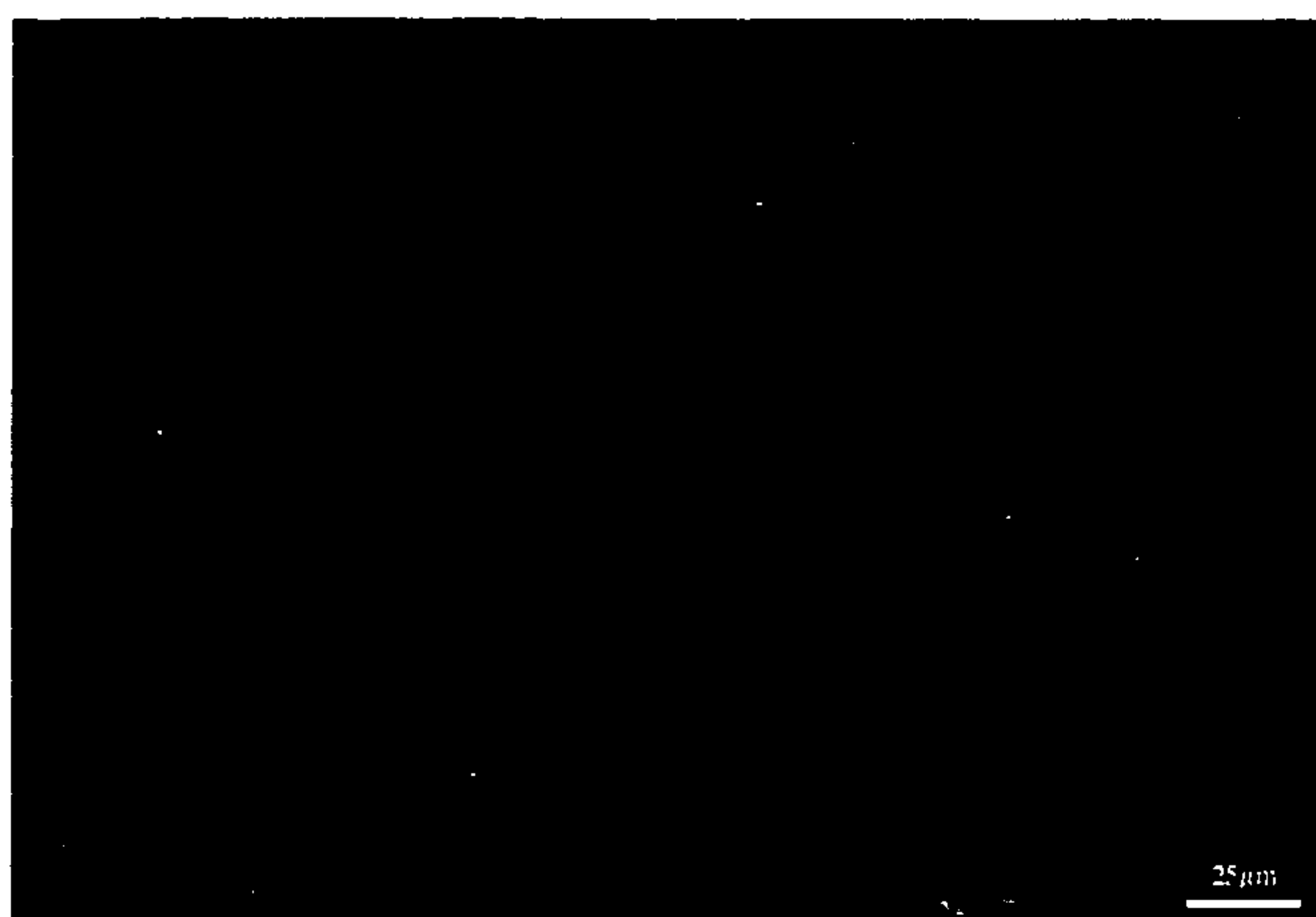


FIGURE 11

A

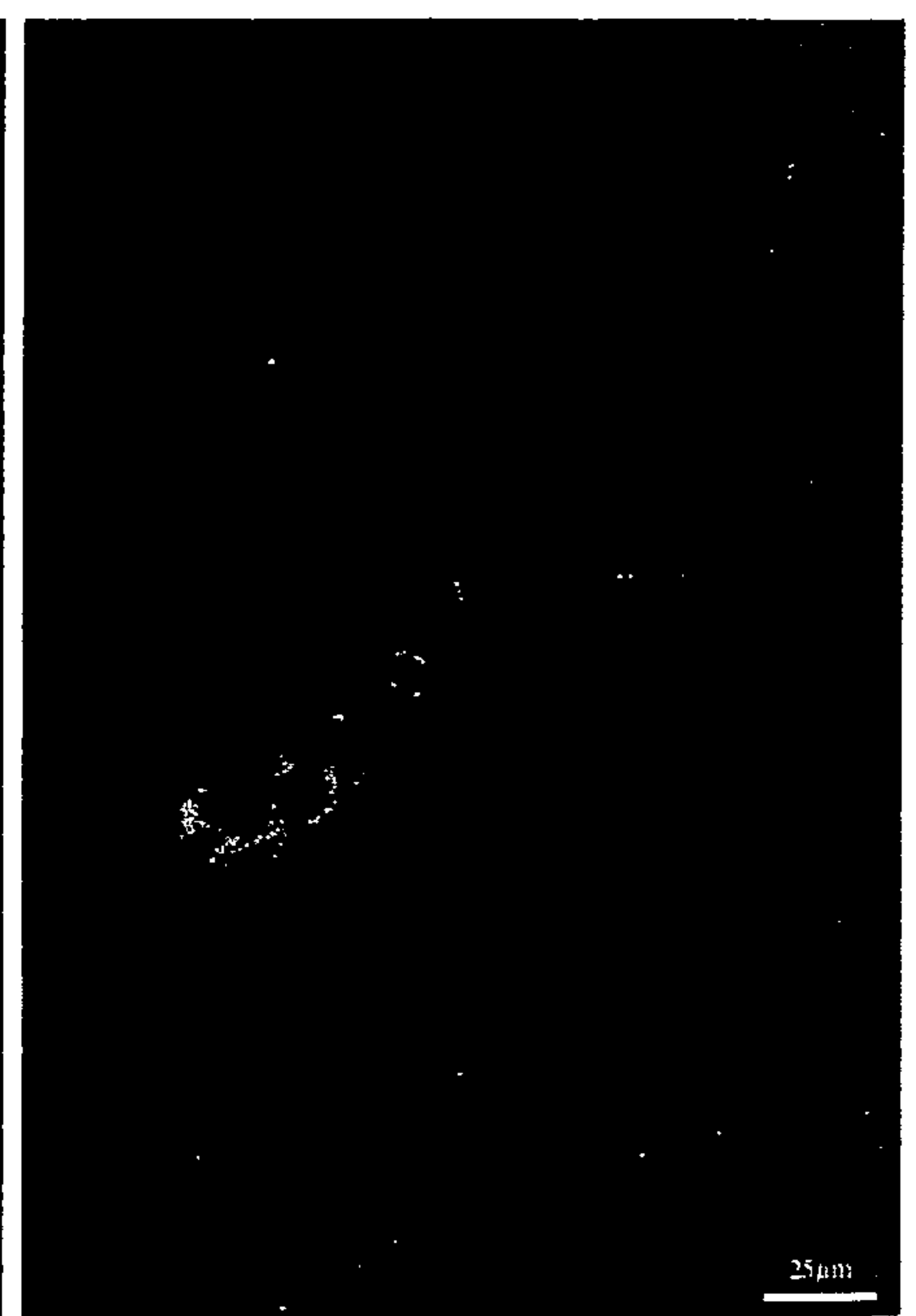
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**B**

BrdU/insulin



BrdU/insulin/glucagon

C**FIGURE 12**