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미세 피막화 이종장기 췌도를 위한 형질전환돼지 개발

Development of transgenic pigs for microencapsulated xeno-islet

2013 년 2 월

서울대학교 대학원
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This thesis is submitted by the undersigned for examination for the degree of Doctor of Philosophy to the Seoul National University. This thesis has not been submitted for the purposes of obtaining any other degree or qualification from any other academic institution.

I hereby declare that the composition and experiment of this thesis and the work presented in it are entirely my own.

Sol Ji Park

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Development of transgenic pigs for microencapsulated xeno-islet

by Sol Ji Park

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OF DOCTOR OF PHILOSOPHY
in
Theriogenology Department of Veterinary Medicine, Graduate School
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We accept this thesis as confirming
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Development of transgenic pigs for microencapsulated xeno-islet

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ABSTRACT

Islet transplantation has been considered as a safer alternative than whole organ transplantation and a potentially alternative treatment to conventional exogenous insulin therapy in type 1 diabetes (T1D). The discrepancy between the number of donors and recipients requires a new alternative and xenotransplantation of pig islet is currently the most advanced approach with respect to a possible clinical application. Immune rejection remains a serious limitation of this therapeutic regimen to a broad usage. Transgenic (TG) pigs and encapsulation for bio-artificial pancreas make this technique a clinically feasible treatment option. Somatic cell nuclear transfer (SCNT) is known to be suitable for production of TG pigs because of accurate gene targeting, however, low efficiency restricts its clinical application. Also, incomplete immunoprotection of

the microencapsulation lead a gradual decrease in islet function. Accordingly, the objective of this thesis is the improvement of porcine cloning efficacy and encapsulation for bioartificial pancreas.

In order to improve pig cloning efficiency, investigation of optimum activation condition for porcine SCNT and epigenetic reprogramming were performed. Activation with short-term treatment *vs* long-term treatment of 6-DMAP, demecolcine and with electrical based activation *vs* thimerosal/ dithiothreitol (Thi/DTT) based activation were compared by evaluation of *in vitro* development. Then oxamflatin, a histone deacetylase inhibitor, was evaluated whether it can reprogram as to mimic the events that occur during the ordinary fertilization and support *in vivo* development to term. With improved activation and reprogramming protocol transgenic pigs were produced. After that, improvement of microencapsulation was investigated to widen the application of encapsulation. Exendin-4, GLP-1 analogue, was integrated into the capsule to release from the capsule and the protective effects were assessed. Then, genetically modified (GM) islets encapsulated in exendin-4 were analyzed in view of viability and insulin release capacity.

As results, 0.2 mM Thi for 10 min followed by 8 mM DTT for 30 min with 2 mM 6-DMAP and 0.4 μg/ml demecolcine was most effective to activate embryo. 1 μM oxamflatin enhanced developmental potential of SCNT embryos by alteration of epigenetic status and increase of pluripotency-releated and antiapoptotic genes expression resulting in significantly enhanced cloning efficiency from 0.9 to 3.2 %. Also, double-transgenic pigs for shTNFRI-Fc and HA-tagged-human heme oxygenerase-1 (HA HO-1) using 2A self-cleavage peptide were produced. Subsequently, the multilayered alginate microcapsules

with an outer alginate layer were generated to deliver exendin-4. Exendin-4 released from the capsule reduced proinflammatory cytokine-induced apoptosis in islets. The function of GM islets encapsulated in exendin-4 releasing capsule was assessed *in vitro*. As a result, insulin release respond to glucose stimulation and the viability increased after TNFα and CHX treatment (2.8±0.06 and 53.3±2.2, respectively).

In summary, SCNT and encapsulation technique were established for bioartificial pancreas. SCNT procedure was improved by chemical activation condition and HDACi treatment. CAG-shTNFR1 Fc-HA hHO1 pigs were born by these optimized SCNT protocols. Also encapsulation technique was enhanced by exendin-4 releasing microencapsulation. GM islets encapsulated in exendin-4 were successfully generated and its function as a bio-artificial pancreas were confirmed

……..…….
Key Words: pig, transgenic, somatic cell nuclear transfer, encapsulation, xenotransplantation, bio-artificial pancreas

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LIST OF ABBREVIATIONS

6-DMAP	6-dimethylaminopurine
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
COC	Cumulus-Oocyte Complex
DMEM	Dulbecco's Modified Eagle's Medium
DPBS	Dulbecco's Phosphate Buffered Saline
DTT	Dithiothreitol
EGF	Epidermal Growth factor
ET	Embryo Transfer
FBS	Fetal Bovine Serum
FSH	Follicle Stimulating Hormone
HBSS	Hank's balanced salt solution
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HO1	Heme oxygenerase 1
IBMX	3-isobutyl-1-methylxanthine
IVC	*In Vitro* Culture
IVM	*In Vitro* Maturation
LH	Luteinizing Hormone
NPCC	Neonatal Pancreatic Cell Cluster
PCR	Polymerase Chain Reaction
PFF	Porcine follicular Fluid
PVA	Polyvinyl Alcohol
PZM-3	Porcine Zygote Medium-3

RT	Reverse Transcript
SCNT	Somatic Cell Nuclear Transfer
TCM	Tissue Culture Medium

PART I.

GENERAL INTRODUCTION AND LITERATURE REVIEW

1. Introduction

1.1 Type 1 dibetes is emerging pandemic.

Type 1 diabetes (T1D) is a presumed autoimmune disease resulting in the destruction of insulin producing β cells in pancrease, which lead uncontrolled blood glucose level [1]. Prolonged hyperglycemia and ketoacidosis explains the typical complications of atherosclerosis, heart attack, heart failure, stroke, renal failure and neuropathy. This affects around 240 million people in the world and it has been estimated that the costs of its complication account for 10% of the total healthcare expenditure around the world. Moreover, according to World Hearth Organization the number of patients are rocketing, 300 million people are expeted to develop by the year 2025 [2].

1.2 Disadvantages of conventional treatment of T1D

The general treatment is insulin therapy administered by subcutaneous injection or via an insulin pump. Although insulin therapy has decreased mortality, it cannot maintain blood glucose level with the physiological rage as endogenous insulin does. Intensive glucose control causes microvascular complication or repetitive hypoglycemic episodes which can be fatal. The inability of exogenous insulin administration to achieve complete blood glucose level and drawbacks require cell therapy like islet transplantation. However, unfortunately, the demand for organ transplantation already surpasses by far the number of available donors [3].

1.3 Xenotransplantation with porcine islets as an alternative

The xenotransplantation can be the answer to this limited donor availability. The pig is known similar to humans with regard to genetics, physiology and anatomy and evoke less moral disgust to people as pig has been raised for food sources. Porcine islet transplantation currently represents a very promising solution to the limited donor supply [4]. Especially, porcine insulin differs from human insulin by only one amino acid, pig islets have lower sensitivity to destruction by recurrent T1D autoimmunity than human islets [5], they do not accumulate amyloid [6], besides pig can be genetically modified to improve immunomodulation and cytoprotection.

1.4 The outcome of pig islet cell xenografts

Nine *in vivo* studies reported pig islet graft sulrvival of two months despite the use of strong immune suppressive regimens [7-8]. In 2006, a number of studies [9-10] demonstrated that the use of a cocktail of immunosuppressive regimen allowed neonatal and adult pig islets to survive in primates for longer than a half of year. However, such an intensive immunosuppressive regimen has severe side effets and is not appropriate for application in humans. Although the field of islet transplantation has progressed dramatically, the life-long immunosuppressive drugs severly restricts the wide application of this therapy as an alternative to exogenous insulin. Therefore, strategies aimed at overcoming life-long immunosuppression using encpaulation technology.

1.5 Research objective

Transgenic pigs have been generated to control immune rejection and promising results with TG pigs suggest that protection from complement activation is beneficial to the survival after the graft [11-12]. On the other hand, still there are rooms for improvement with regard to the best encapsulation device and material and factors support islet survival. Therefore, combination of encapsulation and transgenic pigs may demonstrate synergic effects. In this study the GM islets with exendin-4 releasing encapsulation were generated and *in vitro* functions were tested.

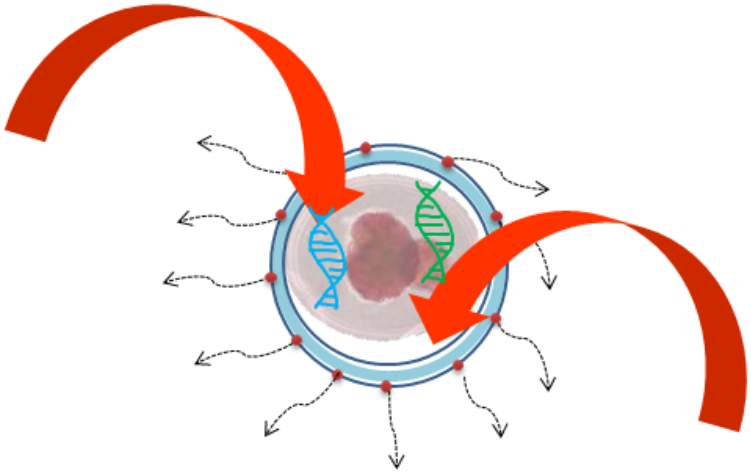

Figure 1. Purpose of the study is to development of transgenic pig and encapsulation for Bio-artificial pancreas.

1.6 Outline of studies

In part I, as a general introduction, it was explained why I have designed and performed this study. Through part II to IV, detailed experimental results were

described. In part II, the cloning efficiency of porcine somatic cell nuclear transfer (SCNT) was improved. Then, transgenic pigs were generated via SCNT. In part III, improvement of encapsulation of porcine neonatal pancreatic cell clusters (NPCC) was performed. Finally, GM islets were encapsulated in exendin-4 relealsing microencpaule for bio-artificial pancreas and *in vitro* fuction was tested in part IV. In part V, summary and prospectives were described.

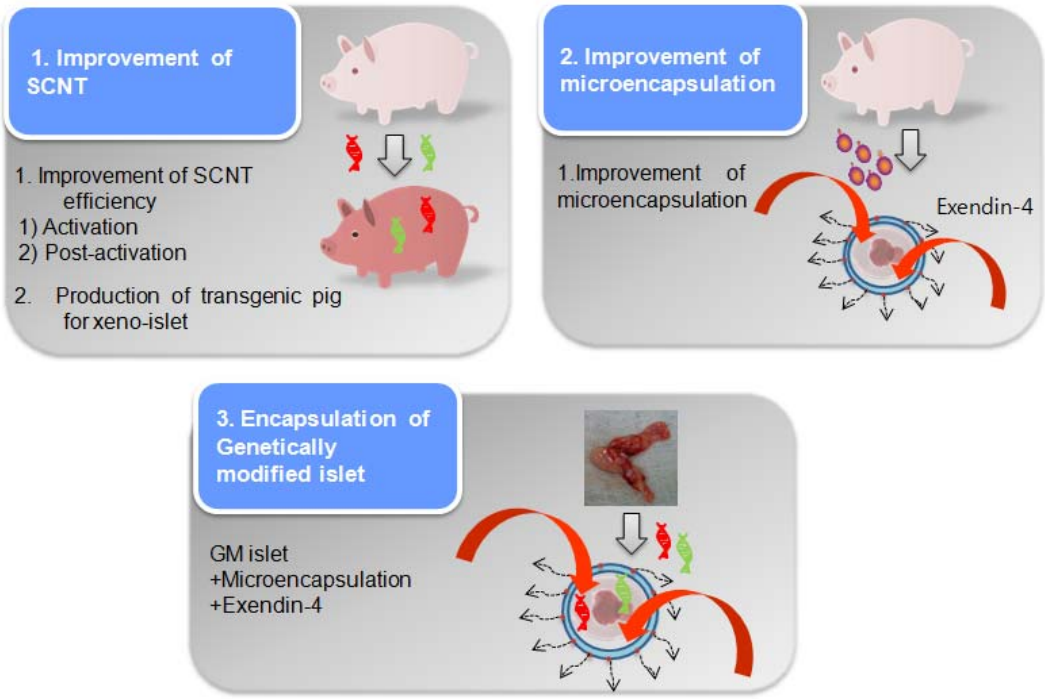

Figure 2. Outline of the study.

2. Literature review

2.1 Method to produce transgenic pigs

Technical improvements in generation of GM pigs have facilitated the usage of minipigs for pig-to-human transplantation [13]. To date the available GM pigs are generated by microinjection (MI) of transgenes in plasmid constructs or lentiviral vehicles into zygotes, sperm-mediated gene transfer (SMGT) and SCNT. Before the development of generating pigs by cloning, genetically modified pigs were generated by MI and SMGT. The first transgenic pig was produced by MI in 1985 [14] and many transgenic pigs have been generated in MI. However, the large-scale application of MI is hampered by its relatively low efficiency with low number of transgenic offspring and transgenic mosaicism [15-16]. SMGT is a direct method that has been used for GM pigs and shows higher gene integration efficiency than that of MI [17]. Only a few GM pigs are produced using SMGT [18] Insertion is still random and transgene can be reaaranged, thus affecting the expression level [19]. The long-term expression of the transgene remains controversial [20]. SCNT overcomes many limitations of previous procedures, making precise gene targeting to pig genome and providing a less expensive way to perform genetic engineering [19].

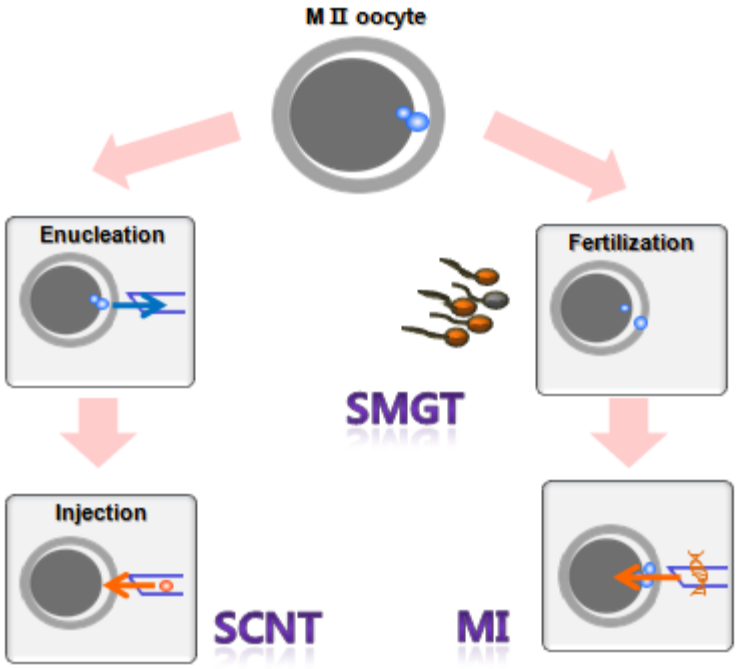

Figure 3. Current techniques for the genetic modification of pigs include DNA microinjection into the nuclei of fertilized oocytes (MI), spermmediated gene transfer (SMGT) and somatic cell nuclear transfer (SCNT) using genetically modified nuclear donor cells.

2.2 Low cloning efficiency of SCNT

The efficiency of SCNT is affected by many factors including the quality of recipient oocytes [21], stage of donor cell cycle [22], duration of exposure of the donor nucleus into inactivated oocytes [23], activation method [24] and epigenetic and genetic status of the donor cell genome [25]. In particular, the mode of activation [26].and epigenetic reprogramming [27] are regarded as critical factors directly affecting the developmental competence of SCNT embryos.

2.2.1 *In vitro* maturation of oocyte

The purpose of IVM is to lead the oocytes which are obtained from ovaries collected at the local abattoir to metaphase-II (M II) stage and make them ready for SCNT, because oocytes are immature both nuclear and cytoplasmic respects.

One critical point of the maturation culture is selection of oocytes, because immature oocytes are generally recovered from ovaries at different growth phases. Marchal *et al.* [28] showed that oocytes from large follicles (more than 5 mm in diameter) have more ability to develop into embryos than oocytes from small follicles (<3 mm in diameter). However, in terms of efficiency, the diameter of follicles is difficult to control. Furthermore, after aspiration of the follicles, the oocytes are commonly selected using various criteria such as their morphology, the numbers of cumulus cell layers and evaluation of the granulation of the cytoplasm. Those morphological evaluations are subjective, and categorization standards vary among investigators. As an alternative, exposure of the oocytes to brilliant cresyl blue (BCB) has been suggested to select developmentally competent oocytes, according to colour [29-30]. The BCB test permits assessment of the intracellular activity of glucose-6-phosphate dehydrogenase, an enzyme synthesized in growing oocytes, but with decreased activity in oocytes that have finished their growth phase.

For the onset of nuclear maturation, a various hormones and growth factors are reqired in IVM medium. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [31] transforming growth factor and androstenedione [32], pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) [33-34], insulin like growth factor-I (IGF-I) [35] and estradiol-17 [36]. To

synchronize the progress of maturation, protein synthesis inhibitors including cycloheximide [37] and dibutyryl cyclic adenosine monophosphate (dbcAMP) [38] have been added into IVM media. These were expected to prevent aging caused by premature meiotic resumption and accumulate developmental factors resulting in improvement of development to blastocyst [38].

Although nuclear maturation seems to be completely established during IVM, the maturation of the cytoplasm is still inappropriate [39]. Cytoplasmic maturation can be improved by reduction of oxidative stress caused by reactive oxygen species (ROS) production from stressed cumulus-oocyte complexs (COCs) due to improper *in vitro* culture environment [40] using anti-oxidant like melatonin [41-42]. GSH level can be increased by supplementation of thiol compounds such as cysteine [43], cysteamine, glutamine and β-mercaptoethanol and/or follicular fluid to the IVM medium [44]. Besides, GSH also increases amino acid transport, and stimulates DNA and protein synthesis [45].

Various basic culture medium types including North Carolina State University 23 (NCSU23) [46], modified tissue culture medium have been used for maturation of porcine oocytes. Most of laboratories supplement the maturation medium with porcine follicular fluid (pFF) or fetal calf serum (FCS). The beneficial effect of the addition of pFF and FCS as the only protein supplements have been demonstrated in several studies [47]. Recently, Takemoto e*t al.*[48] found that pFF showed anti-apoptotic effect. However, the exact role of pFF in the maturation medium remains unclear. Besides they contain many unknown factors and chemically-defined media should be used to standardize the investigations. Recently, successful piglet production has been reported using

oocytes matured in a chemically defined system [49-50], even by making it gonadotropin-free [51].

2.2.2 Nuclear reprogramming

Nuclear remodeling is an architectural change of the chromatin resulting in a reprogramming of the pattern of gene expression in the cloned embryos. Such a modification of chromatin structure is thought to result in a recapitulation of the expression of many genes. There have been many studies that evaluated gene expression in cloned mammalian embryos and tissues, each set of donor cells appears to be different, as some can readily be reprogrammed and others not [52-55]. While the majority of genes appear to be correctly reprogrammed, there is a different subset of genes in many different donor cells that are not reprogrammed and are expressed at inappropriate times. Thus, a consistent pattern of aberrant gene expression has not been identified. This suggests that there are numerous defects in the genomic architecture after SCNT. For example, there is mounting evidence of more variation in DNA methylation within clones than between non-clonal controls [56] and this is also inconsistent with phenotypic variation [57]. Recently, it has been argued that therapeutic cloning, in spite of this aberrant gene expression, could move forward because the developmental defects are most likely to show up in the placenta [58]. If remodeling is not sufficient during the first cell cycle, then all descendants would be affected. Just because the placenta has a stricter requirement for maintaining the fidelity of gene expression does not mean that the cells contributing to the inner cell mass (ICM) are normal, and thus they might be suspect for therapeutic uses.

2.2.3 Oocyte Activation

After the nucleus is transferred to the cytoplasm of the oocyte, the oocyte must be activated to reduce maturation promotion factor (MPF) activity so that the cell cycle may resume and the developing embryo can proceed to the first interphase. In most mammalian species, oocytes are ovulated at the MⅡ phase of meiosis and remain arrested until fertilized by sperm. A series of long-lasting oscillations of intracellular Ca^{2+} is triggerd after the sperm binds to the oocyte resulting in the release of meiotic arrest and subsequent development. Increases of intracellular Ca^{2+} can be mimicked by applying a high voltage DC electrical field pulse [59] to the oocytes in a activation chamber in Ca^{2+} containing medium. After the electrical pulse, pores are open in the plasma membrane allowing oocyte activation caused by the influx of Ca^{2+} [60]. There are several procedures to activate the oocyte after SCNT including electrical activation as discussed above or chemical activation. Initial attempts were made to use the chemical (calcium ionophore and 6-dimethylaminopurine: 6-DMAP) activation. Zgotes are treated with compounds such as the broad spectrum protein synthesis inhibitor, cyclyheximide, or protein kinase inhibitor 6-DMAP, thus blocking cyclin B from functioning and reducing the level of MPF that is maintaining meiotic arrest [61-62]. Commonly, reconstructed bovine zygotes are fused electrically followed by activation with calcium ionophore or ionomycin to elevate intracellular Ca^{2+} levels. The presumptive zygotes are activated by treatment with Thi/DTT. Thi, a sulfhydryl-oxidizing compound, will induce Ca^{2+} transients in metaphase II arrested oocytes [63]. Subsequent treatment with DTT will then reduce those disulfide bonds and the oocyte will continue to develop as if it were fertilized with sperm. In bovine and sheep, SCNT embryos activated and fused simultaneously

resulted in poorer development [64] than delayed activation, thus leading to a more complicated activation protocol. One study found that delaying activation in reconstructed bovine zygotes for 4 h after fusion resulted in a much higher blastocyst rate (26% *vs*. 5%) further suggesting delaying meiotic resumption allows factors within the oocyte to exchange with the metaphase chromosomes and better remodel the donor nuclei [65].

2.2.4 *In Vitro* Embryo Culture (IVC)

In Vitro embryo culture is an important procedure for improving the developmental competence of in vitro embryos produced by SCNT. Furthermore, Yamanaka *et al*. [66] have demonstrated that pig SCNT embryos are more easily affected by culture environment compared with *In vitro* fertilization (IVF) embryos. Therefore, the developmental competence of SCNT embryos would be further enhanced by developing more appropriate culture conditions [66]. For IVC of pigs, several media have been developed and used. The most often used IVC media was the NCSU-23 and NCSU-37 reported by Petters and Wells [46], Nevertheless, these media are still not entirely appropriate for two reasons: first, because they are supplemented with BSA throughout the whole culture period and secondly, because they are still suboptimal for embryonic development. Porcine embryos cultured *in vitro* have lesser developmental competence than embryos cultured *in vivo*. Kikuchi *et al.* [67] demonstrated that IVM–IVF oocytes have higher potential for developing to the blastocyst stage, equal to that of *in vivo* matured oocytes, when they are cultured with oviductal epithelial cells, than when they are cultured *in vitro* (mean cell number 181.5 and 38.4 per blastocyst when cultured *in vivo* and *in vitro*, respectively). Culture in modified NCSU 23 or NCSU 37 without glucose, but supplemented with low levels of lactate and

pyruvate for the first 48–72 h and followed by culture in IVC medium with glucose, improved blastocyst development and total cell number compared with those cultured for the whole period in the presence of glucose [67-68]. It seems that *in vitro*–derived 2–4 cell embryos utilized less glucose than morulae or blastocysts [69], where it plays a major role during the compaction and blastocyst formation. Recently, Yoshioka *et al.* [49] developed a chemically defined medium (porcine zygote medium: PZM5) for *in vitro* culture of zygotes based on the composition of pig oviduct fluid. For the culture with PZM medium, embryo development was optimized in an atmosphere of 5% CO_2, 5% O_2, 90% N_2 compared to 5% CO_2 in air [49,70]. Reducing oxygen tension during in vitro culture from 20% to 5–7% could be also an important factor for in vitro development to blastocysts. However, controversial reports have been published. Some authors suggest that low (5–7%) O_2 concentration is helpful to in vitro development of embryos [71-73], others, for instance Machaty *et al.* [74], reported that developmental rates and total cell numbers of blastocyst were higher in embryos cultured in an atmosphere of 5% CO_2 in air than under 5% CO_2, 5% O_2, 90% N_2. Booth *et al*. [75] suggested that the effect of oxygen tension on embryonic development is dependent on embryo type. Similarly, Abeydeera *et al*. [68] suggested the use of a sequential culture environment: 20% O_2 up to the morula stage and 5% O_2 for later stages. Further studies are required to better understand the exact role of oxygen tension during *in vitro* culture on the development of porcine oocytes.

2.3Transgenic pigs for xenotransplantation

Although fetal and neonatal pig islets express galactose-α-1,3-galactose, the expression become lost with age and adult pig islets have no or negligible

expression [76]. Transplantation of wild-type adult or neonatal pig islets into non-human primates demonstrated clinically inapplicable despite of immunosuppressive drugs [9-10]. The immunological obstacles in xenotransplantation are inevitable and significant. A way to reduce the immune response after transplantation of porcine islet is the use of transgenic pigs. Transgenic pigs expressing hCD46 maintained normoglyceamia for more than 1 year in a diabetic cynomolgus monkeys suggesting inhibition of complement activation [12]. Recently, Klymiul *et al*. [11] reported that neonatal islets from transgenic pigs expressing LEA29Y, a high-affinity variant of the T-cell costimulation inhibitor CTLA-4Ig, under the control of the porcine insulin gene promoter were transplanted into streptozotocin-induced hyperglycemic NOD-scid IL-2R null mice and normalized glucose homeostasis.

2.4 Encapsulation technology

Cell encapsulation strategies have been suggested as a solution to immunosuppression. These technologies are based on the principle that foreign cells are protected against the host immune systems, including antibodies and cytotoxic cells, by sequestering them within a biocompatible artificial membrane. The membrane has pores which allow the passage of small molecules such as insulin and glucose (6 kDa) however, prevent the entry of immune cells (7 μm) or antibodies (150-900 kDa) [77]. Immunoprotection provided by encapsulation enables xenotransplantation without immunosuppressive drugs. Since 1980s, researchers have studied a diverse type for a bioartificial pancreas to replace the endocrine function of the pancreas without graft rejection caused by immune response.

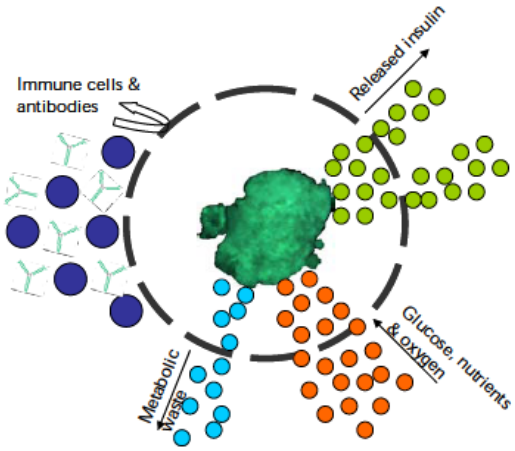

Figure 4. Encapsulation principle. The semi-permeable membrane allows the diffusion of glucose, insulin, nutrients and oxygen whereas it prevents the immune cells and antibodies (Rev Diabet Stud (2011) 8: 51-67)

2.4.1 Microencapsulation

2.4.1.1 Polymers for microcapsule formation

Generally, microcapsules are produced using polymers which form hydrogels derived either naturally or through synthetic routes. In islet transplantation, the naturally obtained alginate hydrogels are widely used as they can be produced under a certain physiological condition without the use of toxic chemicals and without influencing islet function [78]. The commercially available alginates are obtained from seaweed (brown algae) and are polysaccharides composed of β-D-mannuronic acid and α-L-guluronic acid linked together. There are both high guluronic acid (G) and high mannuronic acid (M) containing alginate and alginates vary in composition and ratio of G: M depending on the source. Alginate when dissolved to a highly viscous solution hence it can be used

to create small droplets. Then the droplets are stabilized to become microcapsules by immersion in a solution of polycations such as Ca^{2+} or Ba^{2+} which forms strong cross-links with the monomers guluronic and mannuronic acids. Microcapsules with high G alginate are more rigid and stable compared to the high M microcapsules. The latter are associated with increased swelling indicating inadequately encapsulated islets. Apart from alginate, many other polymers such as agarose, chitosan, methacrylic acid, methyl methacrylate, polyethylene glycol and 2-hydroxyethyl methacrylate have been used for islet encapsulation studies resulting in limited success [79]. A large number of strategies are currently under investigation to create an immunoprivileged environment at islet transplantation site to minimize immunosuppressives. These include pretransplant islet culture in the presence of mitomycin C [80], surface heparinization, pegylation [81] of islets, and transfection with immunorepellant stromal cell-derived factor.

2.4.1.2 Factors influencing microencapsulated islet graft

Biocompatibility

Survival rates of encapsulated islets of either allograft or xenograft are similar suggesting that immune rejection is not the only cause of graft failure [82]. Biocompatibility is defined as the ability of a biomaterial to perform with an appropriate host response in a 'specific application' [83]. Prevention of pericapsular fibrotic overgrowth of microcapsules is considered to be a crucial factor in biocompatibility of microcapsules. The fibrosis, comprised mostly of macrophages and fibroblasts, occurs in the immediate post-transplantation period affecting only a small percentage of microcapsules [82]. Lack of biocompatibility may be attributed to the purity or shape of the encapsulation. Highly purified alginate which is removed impurities such as polyphenols, proteins and

endotoxins greatly enhanced the biocompatibility of alginate microcapsules [84], improved the biocompatibility and graft outcomes [85]. Commercially-available alginates contain many contaminants which are immunogenic and affect the biofunctional properties of microcapsules compromising biocompatibility [85]. Unfortunately even after the removal of endogenous contaminants, purified alginates still contain residual protein contaminants which are immunogenic. Irregularities in microcapsule shape also lead pericapsular fibrotic overgrowth and islet necrosis, although this accounts for fewer than 5% of the capsules. The physical irregularities are due to inadequate encapsulation leading protuberance of the islets, thereby initiating an immune response which causes fibrotic overgrowth [86].

Immunoprotection

Immunoprotection refers to protection of the encapsulated islets from both the usual rejection and recurring autoimmune destruction. Although a proper encapsulation system can protect against immune cells and large antibodies, the islets are still vulnerable to small molecules such as chemokines, cytokines and nitric oxide (NO) [87]. Insufficient immunoprotection results from permeability of the microcapsules to these small molecules. These small molecules are produced by the islets even themselves, which can leak through the pores of the microcapsule and attract macrophages [88]. This chemotaxis, activate macrophages, thereby releasing pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and small molecules including NO. These small molecules (2-30 kDa) can easily pass through the microcapsule membrane. Although reduced microcapsular permeability may prevent the passage of these small molecules, unfortunately

reduced pore size will also interfere with the diffusion of nutrients and insulin. Thus, achieving a optimal pore size which provides immunoprotection without compromising islet function is crucial [77]. Several strategies, co-encapsulation with various types of cells such as erythrocytes and sertoli cells [89-90] releasing immunosuppressive factors have been studied to protect the encapsulated islets and improve islet function. Cross-linking hemoglobin on the microcapsular surface has shown to protect islets from the NO induced cellular damage, thus improving islet function [91]. Recent studies involving encapsulated islets in a modified microcapsule such as sulphated glucomannan-barium-alginate, resulted in decreased secretion of proinflammatory cytokines and improved functional capacity of rat islets due to its heparin-like properties in absorbing and filtering cytokines [76]. Also, genetic engineering of the islets to secrete anti-inflammatory molecules such as inhibitor of TNF-α and IL-1 receptor antagonist leads to reduced β cell apoptosis from the deleterious small molecules secreted by activated macrophages [92-93].

Hypoxia

Another issue which contributes to islet graft failure is hypoxia. Ordinary islets are supplied abundant oxygen and nutrients from a dense network of capillaries [94]. However, the capillary networks are destroyed during the isolation thus the islets suffer from post-isolation hypoxic stress. Furthermore, transplanted islets are exposed to hypoxia before revascularization resulting in reperfusion injury and apoptosis. Microcapsules also prevent the revascularization process, subjecting the islets to further severe hypoxic stress. Limited oxygen supply also hampers the ability of the microencapsulated islets to release insulin and function properly thereby requiring large numbers of encapsulated islets to

reverse diabetes compared to non-encapsulated islets [95]. Several strategies such as prevascularization [96-97], heat shock [98], ischemic reconditioning [99], and stimulation of Bcl-2/Bcl-xl complex [100] have been investigated to minimize hypoxic stress, and prevent apoptosis. Aforementioned strategies are effective only in the immediate post-transplantation period but not in the long-term. Revascularization towards the microencapsulated islets would enhance oxygen supply and prevent long-term hypoxic damage. This may be achieved by incorporation of growth factors [101-103], vascular precursor cells, or even microvessel fragments into biomaterials [104]. Encapsulation of islets in a rough surface employing hydroxymethyl polysulfones resulted in enhanced vascularization and improved islet function [105]. Direct oxygen supply by co-encapsulation of algae which have photosynthetic capacity with islets in alginate microbeads induced greater insulin release in response to glucose under light when perfused with oxygen-free medium [106].

Enhancing islet function

Protection of islets in microcapsules can be achieved either through pharmacological strategies or bioengineering approaches. Inducing expression of superoxide dismutase, catalase or glutathione peroxidase in islets protects them from free radical toxicity and the adverse effect of NO [107]. It was demonstrated the beneficial effects of superoxide dismutase and catalase by entrapping them within a polymer matrix to release gradually [108]. Other drugs such as nicotinamide and 15-deoxyspergualin protect islets and promote long-term graft survival and function [109-110]. It could also be achieved by modifying the microcapsules or coating the microcapsules with anti-inflammatory agents. Recently, it has been shown that cross-linking the anti-inflammatory peptide IL-1

receptor inhibitory peptide to methoxy-polyethylene glycol (PEG) hydrogels protected the encapsulated insulin-producing cells from pro-inflammatory cytokines and T lymphocytes [111].

Large volumes of islets are required for efficacy, which generally restricts the transplantation site. Attempts to reduce this number might be achieved by increasing the functional efficiency of the islets, rather enhancing insulin secretion. Capitalizing on this, Hwang *et al.* and Park *et al.* [112-113] prepared a polymeric conjugate of sulfonylureas and demonstrated increased insulin secretion by coencapsulating the conjugate with islets and insulin-producing cell lines respectively. However, the increased insulin secretion was seen only at basal levels and did not increase at elevated glucose concentrations. Co-encapsulation the islets with polymeric conjugate of glucagon like peptide-1 were tried to overcome this drawback [114].

2.4.2 Macroscale encapsulation

Macroencapsulation devices generally classified in two categories according to implantation strategies: intravascular or extravascular. Intravascular device is a synthetic hollow fiber, semipermeable membrane that passes through a compartment seeded with islets [115-117] . The device is directly connected to the host systemic circulation by vascular anastomoses creating an intravascular shunt. The major advantage of these intravascular devices is their close proximity with the recipient's blood stream ensures sufficient oxygen and nutrient supply, hence enhancing islet survival and rapid glucose-stimulated insulin release. Despite few successful animal studies [118], thrombus formation within the lumen of the device, membrane collapse and limitations in transport properties have been suggested to be major obstacles [115,119]. Extravascular devices refer to macoencapsulated cells that are implanted out of the vessel. Although these devices, such as hollow fibers, diffusion chamber and polymeric sheets yielded encouraging [120-125], their large size and exclusive reliance on diffusive transport resulted in islet dysfunction and graft failure. Mathematical modeling predicts inadequate transport profiles, scalability will be problematic after islet density optimization, thereby rendering such implants bulky or requiring multiple devices [126-127].

2.4.3 Nanoscale Encapsulation

As covalent attachment of PEG to exogenous proteins increased half-life and reduced immunogenicity without affecting function [128-129], researchers attempted to generate a biocompatible nanometer-scale isolating a biocompatible

steric barrier to the cell surface by cross linking the cell surface proteins with PEG. Several groups have carried out PEGylation on the surface of islets through varying approaches, which include covalent surface attachment of polyethylene glycol (PEG), known as 'PEGylation' [130] , linking islet surface amine groups with isocyanate and *N*-hydroxysuccinimide functionalized PEG polymers [81,130-132] or inserting lipid moieties linked to a PEG chain within islet cell membranes [133]. Not only did PEGylation have no adverse effects on islet viability or function but it was also found to reduce islet recognition and activation of immune cells *in vitro* [132,134], prolong survival of the allograft in the absence of immunosuppression [135], and reverse diabetes when combined with mild immunosuppression in rodent models [136]. Covalent modification of amine groups on islet surface proteins presents a problem due to periodic turnover of membrane components [131] and possible interference with cell surface protein activity. Wilson and colleagues [137] have used a noncovalent approach of coating islets via electrostatic interactions with modified PLL. Exposure to PLL alone, along with other polycations, results in high levels of cytotoxicity, however, if modified to the appropriate degree with PEG, the PLL can interact with the islet surface without inducing apoptosis [138]. In addition, the chemoselective reactive groups like hydrazide, azide, and biotin were introduced by functionalization of the PEG macromers prior to PLL modification [137,139]. a recent study using conformal coating to encapsulate islets via layer-by-layer deposition of poly(L-lysine)-*g*-PEG-(biotin) and streptavidin failed to show any improvement in the survival and function of the implanted allo-islets [137]. Effective immunoprotection using nanocoating thus remains a challenge in islet transplantation.

PART II.

IMPROVEMENT OF SOMATIC CELL NUCLEAR TRANSFER

Chapter I. Short-term treatment with 6-DMAP and demecolcine improves developmental competence of electrically- or Thi/DTT-activated porcine parthenogenetic embryos

1. Introduction

The development of somatic cell nuclear transfer (SCNT) technology to produce cloned animals opened a new era in reproductive biology and biomedical science [140]. In the porcine research field, numerous experiments have been conducted in attempts to realise this potential, ever since the first successful cloning of pigs by SCNT [141]. However, the overall developmental ability of porcine SCNT embryos to term is still low. The efficiency of SCNT is affected by many factors including the quality of recipient oocytes [21], stage of donor cell cycle [22], duration of exposure of the donor nucleus into inactivated oocytes [23], activation method [24] and epigenetic and genetic status of the donor cell genome [25]. In particular, the mode of activation is regarded as one of the most critical factors directly affecting the developmental competence of SCNT embryos [26].

Since the first cloned piglets have been produced by electrical activation [141], electrical activation of oocytes has been frequently employed for the activation of porcine SCNT, and this approach resulted in a number of cloned piglets. Further research is needed to improve the oocyte activation step in porcine SCNT because electrical activation induces only a single transient rise in intracellular Ca^{2+} concentrations [142-143], rather than the multiple rises called 'calcium oscillations' that is observed during fertilization. A single transient rise in

intracellular Ca^{2+} is not enough to support high rates of development beyond resumption of the second meiotic division. Thus, several chemicals such as 6-dimethylaminopurine (6-DMAP) [144] or cytochalasin B [145] have been studied for supplementing successful electrical activation of porcine oocytes, and chemical treatments were found to improve developmental competence in combination with electrical pulses [146]. However, data were obtained only using long-term (3 to 6 hours) chemical treatment. In previous reports, treatment with cytochalasin B [145], 6-DMAP [147], demecolcine (DE) [148] and cyclohexamide [149] need two, three, four and six hours, respectively, for activation. It is still an open question as to whether such long-term treatment to cause sufficient activation is really necessary considering possible adverse effect on embryo quality [150]. If short-term treatment triggers efficient activation, it would improve the technology of oocyte activation with practical advantages.

On the other hand, it was suggested that combined treatment with thimerosal/dithiothreitol (Thi/DTT) treatment induced efficient activation in porcine embryos [63] without electrical pulses. In a previous study of porcine oocyte activation, short-term treatment (40 min) with Thi/DTT increased not only the developmental competence of SCNT embryos but also the mean cell number with an increased ratio of inner cell mass (ICM) to trophectoderm (TE) [151]. Thimerosal triggers a series of Ca^{2+} spikes in the oocytes, moreover, if followed by incubation with dithiothreitol, it can stimulate pronucleus formation. The combined Thi/DTT treatment also causes cortical granule exocytosis, subsequent zona pellucida hardening and development of the activated oocytes to the blastocyst stage [63]. As there have been many successful efforts to improve developmental competence using a combination of electric pulses and different chemicals, it was expected that the combination of Thi/DTT with other chemicals

would enhance the developmental competence of parthenogenetic porcine embryos.

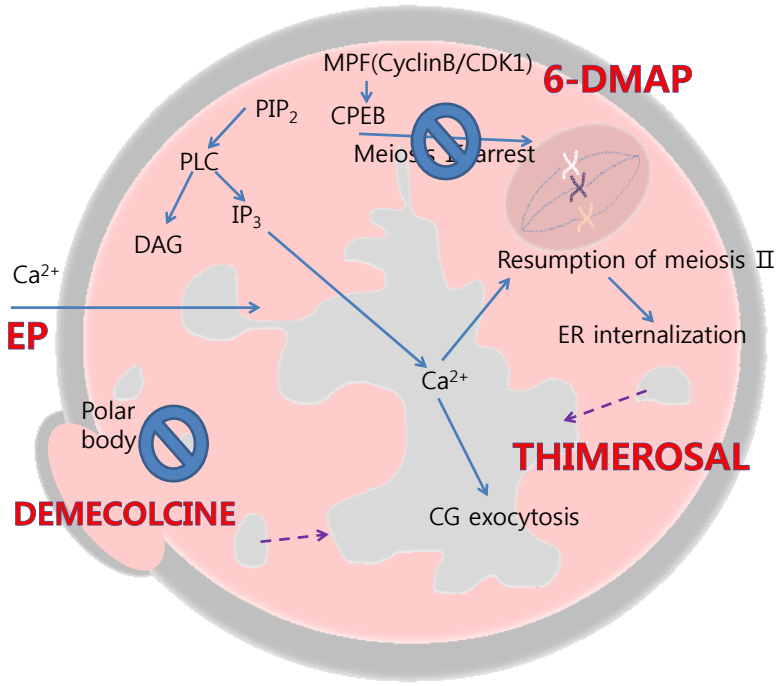

Figure 5. Mechanism of electric pulse (EP), 6-dimethylaminopurine (6-DMAP), demecolcine and thimerosal.

Accordingly, as to determine the optimal activation protocol of pathenogenetic porcine embyo we examined whether short-term treatment with 6-DMAP and DE improves the developmental competence of electrically- or Thi/DTT-activated porcine parthenogenetic embryos, then investigated the optimal activation protocol for activation of porcine embryos.

2. Materials and methods

2.1 Chemicals and reagents

All chemicals and reagents used for oocyte maturation, activation and embryo culture in this study were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), unless otherwise stated.

2.2 Collection of oocytes and *In Vitro* Maturation

Ovaries were collected at a local abattoir and stored in sterile physiological saline at 30-35°C during transportation. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles (3-6 mm) with 18-gauge needle connected to a 10 mL disposable syringe. COCs with several layers of cumulus cells and uniform cytoplasm were chosen and cultured in tissue culture medium (TCM)-199 (Invitrogen, Carlsbad, USA) supplemented with 10 ng/mL EGF, 0.57 mM cysteine, 0.91 mM sodium pyruvate, 5 μg/mL insulin, 1% (v/v) Pen-Strep (Invitrogen), 1 mM dibutyryl adenosine 3',5'-cyclic monophosphate (dbcAMP), 0.5 μg/mL follicle stimulating hormone, 0.5 μg/mL luteinizing hormone and 10% porcine follicular fluid at 39°C in a humidified atmosphere of 5% CO_2, first, with GnRH and dbcAMP for 22 h and then without them for a further 22 h. COCs were washed at each steps. After a total of 44 h maturation culture, oocytes were denuded by pipetting with 0.1% hyaluronidase in Dulbecco's PBS (DPBS) (Invitrogen) supplemented with 0.1% polyvinyl alcohol. Denuded oocytes with evenly-granulated and homogeneous cytoplasm were selected and then assigned randomly to different activation groups.

2.3 Electrical and Thi/DTT activation of porcine oocytes

After denuding, COCs were divided into four groups for electrical and/or Thi/DTT activation.

For electrical activation, the first group was electric pulse (EP) control. COCs were equilibrated in pulsing medium then transferred to a chamber containing two electrodes overlaid with the pulsing medium. The pulsing medium was 0.26 M mannitol solution containing 0.5 mM Hepes, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$. Oocytes were activated with a single DC pulse of 1.5 kV/cm for 60 μsec utilizing BTX electro-cell Manipulator 2001 (BTX, Inc., San Diego, USA). The other three groups were exposed to 2 mM 6-DMAP (EP+6-DMAP), 0.4 μg/mL demecolcine (EP+DE) or both together (EP+6-DMAP+DE) for 40 min at 39°C in a humidified atmosphere of 5% CO_2 after EP treatment.

For Thi/DTT activation, the first group was thimerosal/ dithiothreitol (Thi/DTT) control. Oocytes were treated with 0.2 mM Thi (Sigma, T8784) for 10 min, followed by 8 mM DTT for 30 min. The other three groups were Thi/DTT+6-DMAP, Thi/DTT+DE and Thi/DTT+6-DMAP+DE. i) 6-DMAP, ii) DE, iii) both 6-DMAP and DE were added to Thi/DTT respectively.

2.4 *In vitro* culture

Embryos were washed and transferred into 500 μl of porcine zygote medium-3 (PZM-3) covered with mineral oil. The culture medium was 39°C, 5% CO_2, 5% O_2 and 90% N_2. Embryos were evaluated for cleavage on Day 2. On Day 4, 10% fetal bovine serum was added to the IVC medium. Blastocyst formation and the

number of nuclei were determined in Day 7.

2.5 Total blastocyst cell counts

Briefly, blastocysts were fixed in absolute alcohol then nuclei were stained with 5 μg/mL bisbenzamide (Hoechst 33258) overnight at 4°C. Fixed and stained blastocysts were mounted on a glass slide in a drop of glycerol, gently flattened with a cover glass and visualized for cell counting with a fluorescence microscope using a 460 nm excitation filter.

2.6 Experimental design

2.6.1 Effect of short-term treatment with DE on inhibition of polar body extrusion

As DE maintains diploid DNA content by inhibiting second polar body (PB2) extrusion and thereby indirectly affects embryo development ability, it is needed to confirm whether short-term treatment with DE could sufficiently disturb PB2 extrusion. Hoechst 33258 staining was performed to evaluate polar body extrusion in parthenogenetic embryos. At 6 hr after activation, embryos were fixed for 24 hr 4°C in 4% formaldehyde in D-PBS. The fixed embryos were stained with 5 μg/mL Hoechst 33258 and then mounted on slides. Extrusion of PB2 was examined under UV light using a fluorescence microscope with a 460 nm excitation filter.

2.6.2 Effects of short- term treatment with 6-DMAP and/or DE on either electrical or Thi/DTT activation of oocytes.

This experiment was to find out whether short-term treatment with 6-DMAP and DE improved developmental competence of electrically- or Thi/DTT-activated parthenogenetic porcine embryos. As a control, EP was compared to other groups with or without 6-DMAP and DE (EP vs. EP+6-DMAP, EP+DE and EP+6-DMAP+DE). Evaluation of development competence, cleavage rate, rate of blastocyst formation and total blastocyst cell number were checked on Days 2 and 7. Then, results with Thi/DTT were compared to other groups with or without 6-DMAP and DE (Thi/DTT vs. Thi/DTT+6-DMAP, Thi/DTT+DE and Thi/DTT+6-DMAP+DE).

2.6.3 Comparison of short-term and long-term treatment with 6-DMAP and DE on electrical or Thi/DTT activation of oocytes

Short-term treatments of 6-DMAP and De improved embryo development ability with electrical or Thi/DTT activation. Therefore, I compared these combinations of treatments on both electrical and Thi/DTT activation to validate the effect of short-term treatment with 6-DMAP and DE.

2.6.4 Optimization of short-term treatment with 6-DMAP and DE on electrical and Thi/DTT activation of oocytes

Finally, to optimise the activation protocol, the best conditions in electrically- and Thi/DTT-activated groups were compared.

2.7 Statistical analysis

All data were subjected to one-way ANOVA followed by Tukey's test using Prism version 4.0 (Graphpad Software, San Diego, USA) to determine differences among experimental groups. Statistical significance was determined when P value was less than 0.05.

3. Results

3.1 Effect of short-term treatment of DE on inhibition of polar body extrusion

Inhibition of PB2 extrusion after oocyte activation would be consistent with maintenance of diploid DNA content in parthenogenetic embryos. The number of embryos showing only 1PB was higher in both EP and Thi/DTT groups treated with demecolcine 6 hr after activation (39.0±4.5, 46.8±4.5 vs. 20.5±3.8, 24.1±4.0%, respectively, $P<0.05$) (Table 1).

Table 1. Effect of short-term treatment with DE on inhibition of polar body extrusion in porcine oocytes

Activation	No. of oocytes	1PB (%)	2PB (%)
EP	112	23(20.5±3.8)[a]	89(79.5±3.8)[a]
EP+DE	118	46(39.0±4.5)[b]	72(61.0±4.5)[b]
Thi/DTT	116	28(24.1±4.0)[a]	88(75.9±4.0)[a]
Thi/DTT+DE	124	58(46.8±4.5)[b]	66(53.2±4.5)[b]

EP: electric pulse; DE:demecolcine; Thi/DTT: thimerosal/dithiothreitol; PB:polar body

[a, b] Values for different superscripts in the same column are significantly different ($P<0.05$)

Experiments were repeated at least four times.

3.2 Effects of short-term treatment with 6-DMAP and DE on either electrical activation or Thi/DTT activation of oocytes

Cleavage rates and blastocyst formation rate of EP+6-DMAPand EP+6-DMAP+DE were significantly different among treatments (69.1±3.2, 75.6±3.0% and 31.4±3.2, 33.8±3.2%, respectively, $P<0.05$) (Table 2). Blastocysts activated with 6-DMAP and DE showed significantly more cell number compared to others (48.2±1.0). Similar to short-term electrical activation treatment, embryos Thi/DTT activated with 6-DMAP and DE showed the highest cleavage rate (73.9 ±3.1%, $P<0.05$), blastocyst formation (32.9±3.3%, $P<0.05$) and total cell number (47.9±3.0, $P<0.05$) (Table 3).

Table 2. Effects of short- term treatment with 6-DMAP and DE on electrical activation of porcine oocytes

Activation	No. of oocytes	Cleavage (%)	BL (%)	No. of cells in BL
EP	210	142(67.6±3.2)[a]	32(15.2±2.5)[a]	31.1±1.1[a]
EP+6-DMAP	210	145(69.1±3.2)[b]	66(31.4±3.2)[b]	41.7±1.1[b]
EP+DE	210	134(63.8±3.3)[a]	42(20.0±2.8)[a]	42.8±0.9[b]
EP+6-DMAP+DE	213	161(75.6±3.0)[a, b]	72(33.8±3.2)[a, b]	48.2±1.0[c]

EP: electric pulse; 6-DMAP: 6-dimethylaminopurine; DE: demecolcine

[a-c] Values for different superscripts in the same column are significantly different ($P<0.05$). Experiments were repeated at least six times.

Table 3. Effects of short- term treatment with 6-DMAP and DE on Thi/DTT activation of porcine oocytes

Activation	No. of oocytes	Cleavage (%)	BL (%)	No. of cells in BL
Thi/DTT	206	130(63.1±3.4)[a]	25(12.1±2.3)[a]	33.7±1.5[a]
Thi/DTT+6-DMAP	208	147(70.7±3.2)[a]	47(22.6±2.9)[b]	43.9±2.0[b]
Thi/DTT+DE	205	127(62.0±3.4)[a]	19(9.3±2.0)[a]	38.3±3.2[a, b]
Thi/DTT+6-DMAP+DE	207	153(73.9±3.1)[a, b]	68(32.9±3.3)[c]	47.9±3.0[b]

Thi/DTT: thimerosal + dithiothreitol; 6-DMAP: 6-dimethylaminopurine; DE: demecolcine [a-c] Values for different superscripts in the same column are significantly different ($P<0.05$). Experiments were repeated at least six times.

3.3 Comparison of short-term and long-term treatment with 6-DMAP and DE on electrical or Thi/DTT activation of oocytes

Together with short and long-term with 6-DMAP and DE on electrical activation, cleavage or blastocyst formation rates and total cell number were not significantly different (Table 4, 67.4±3.5 vs. 72.7±3.3, 32.7±3.3 vs. 30.0±3.4% and 41.9±1.2 vs. 40.3±2.4, respectively). Same as electrical activation, cleavage rate, blastocyst formation rate and total cell number of short and long- term treatment with 6-DMAP and DE on Thi/DTT activation were not considerably different (76.0±3.2 vs. 82.8±2.8, 36.1±3.6 vs. 38.3±3.6% and 47.2±1.7 vs. 42.2±3.3, respectively).

Table 4. Comparison of short-term and long-term treatment with 6-DMAP and DE on electrical or Thi/DTT activation of porcine oocytes

Activation		No. of oocytes	Cleavage (%)	BL (%)	No. of cells in BL
EP*	Short	181	122(67.4±3.5)	47(32.7±3.3)	41.9±1.2
	Long	183	133(72.7±3.3)	55(30.0±3.4)	40.3±2.4
Thi/DTT*	Short	183	139(76.0±3.2)	66(36.1±3.6)	47.2±1.7
	Long	180	149(82.8±2.8)	69(38.3±3.6)	42.2±3.3

*EP: electric pulse; 6-DMAP: 6-dimethylaminopurine; DE: demecolcine; Thi/DTT: thimerosal/dithiothreitol

There were no statistically significant differences among the groups (P>0.05).

Experiments were repeated at least three times.

3.4 Optimization of short-term treatment of 6-DMAP and DE together with electrical activation and Thi/DTT activation

Thi/DTT combined with 6-DMAP and DE induced more effective activation of oocytes than both EP+6-DMAP+DE and EP+Thi/DTT+6-DMAP+DE did: blastocyst formation rate was significantly higher (36.1±3.5 vs. 23.3±3.0, 17.2±2.7%, $P<0.05$) and blastocyst cell numbers increased (46.9±1.0 vs. 39.0±2.2, 36.7±1.5, $P<0.05$) (Table 5).

Table 5. Optimization of short-term treatment with 6-DMAP and DE on electrical and Thi/DTT activation of porcine oocytes

Activation	No. of oocytes	Cleavage (%)	BL (%)	No. of cells in BL
EP+6-DMAP+DE	206	147(71.4±3.2)	48(23.3±3.0)[a]	39.0±2.2[a]
Thi/DTT+6-DMAP+DE	191	145(75.9±3.1)	69(36.1±3.5)[b]	46.9±1.0[b]
EP+Thi/DTT+6-DMAP+DE	198	139(70.2±3.3)	34(17.2±2.7)[a]	36.7±1.5[a]

EP: electric pulse; Thi/DTT: thimerosal/dithiothreitol; 6-DMAP: 6-dimethylaminopurine; DE: demecolcine

[a, b] Means with different superscripts in same column were significantly difference ($P<0.05$).

Experiments were repeated at least six times.

4. Discussion

Oocyte activation is one of the important factors affecting SCNT efficiency and a prerequisite for partheonogenesis. There have been many papers in the past years reporting minor variations to the activation protocols. Sequential artificial activation induced similar results [152-153] and did not cause sufficient improvement. In the present study, I examined the effects of DC pulse or Thi/DTT combined with 6-DMAP and DE on the embryonic development of *in vitro* matured porcine oocytes. Each stimulus simutaneously activates oocytes in different mechanisms and exposure time was decreased different from previous studies. This study demonstrated that short-term treatment with 6-DMAP and DE on electrical and Thi/DTT activation of porcine embryos (EP+6-DMAP+DE, Thi/DTT+6-DMAP+DE) substantially improved their developmental competence. In addition, comparison of EP+6-DMAP+DE, Thi/DTT+6-DMAP+DE and EP+Thi/DTT+6-DMAP+DE reveals that Thi/DTT+6-DMAP+DE is the optimal protocol for activation of porcine embryo.

Recently, studies have focused on the improvement of oocyte activation protocols by combining electrical stimulation with administration of chemicals. Additional administration of 6-DMAP is one of the most widely used activation protocols for reconstructed oocytes [154-156]. Such a strategy was applied successfully to produce cloned piglets [157]. Consistent with previous work, the present results show that treatment with 6-DMAP remarkably accelerates embryo development compared to EP or Thi/DTT only. Interestingly, oocytes exposed to 6-DMAP for a short time showed similar developmental ability compared to long-term treatment with respect to cleavage, blastocyst formation rate and total cell numbers (Table 6). It is ascertained that short vs. long exposure to 6-DMAP did

not produce significantly different results.

6-DMAP, a serine protease (phosphorylation) inhibitor, initiates pronuclear formation via complex mechanisms which involve blocking activity of key cell cycle regulatory proteins such as mitogen activated protein kinase (MAPK), myosin light chain kinase and the cyclin dependent $p34^{cdc2}$. In oocytes that were treated with two electrical pulses (1.2 KV/cm for 60 μsec), MAPK activity dropped markedly after 1 hr and reached its lowest value 3 hr after electroporation [158]. However, by 4 hr the activity increased again and remained at elevated levels until the time of pronuclear formation. This seems to explain ordinary treatment time with 6-DMAP for 3-4 hr. 6-DMAP may suppress the MAPK activity faster and maintain the low level longer, by complementing the effect of EP on MAPK.

Our experiments suggest that 40 min exposure to 6-DMAP sufficiently dephosphorylates MAPK or serine/threonine kinase $p90^{RSK}$ within the MAPK pathway. More potent electrical stimuli (1.5 kV/cm for 60 μsec) and Thi may downregulate MPF activity faster, which prevents maternal c-mos translation. This reinforces the effect of 6-DMAP on MAPK inhibition. Thereby, responses to short-term treatment with 6-DMAP on electrically or Thi/DTT activated porcine embryos were equivalent to established long-term treatment. Besides, there have been several reports that long-term treatment with 6-DMAP can do harm on embryo. The first report claimed that 6-DMAP may cause alteration in the cell's DNA content owing to an abnormal pattern of karyokinesis [159] without cytokinesis [150], although longer exposure to 6-DMAP increased pronuclear formation, cleavage rate and blastocyst formation [160]. 6-DMAP is also a

mutagenic agent that may influence the genetic background [161]. Therefore, it is necessary to minimise adverse effects and maximise the efficiency of activation. If short-term treatment with 6-DMAP is sufficient to activate oocytes, there is no reason to administer it for a long time and risk severe side effects on embryo development.

On the other hand, synergistic effect was observed in this study with 6-DMAP and DE together, while there was no significant improvement in embryo development using DE alone. DE was generally used for 0.4 μg/mL after EP from 1.5-2.5 hr and 1.5-3.5 hr, because the second polar body is extruded in the majority of oocytes 4 hr after activation [162]. Retention of the diploid DNA complement has influences on the developmental competence of SCNT embryos receiving donor cells in the G0/G1 phase [163], because the DNA contents affect the subsequent development of SCNT embryos during the first cell cycle [164]. Unless SCNT embryos injected with donor nuclei in the G0/G1 phase are treated with cytoskeletal inhibitors such as DE or cytochalasin, they exclude some chromosomes as a pseudo-polar body after artificial activation, resulting in aneuploidy [165]. DE impairs second polar body extrusion completely by the change of microtubules [166]. However, DE assisted enucleation during the SCNT, which changes microtubule and facilitates removal of chromatin, only take 30-45 min to impair microtubule [167-168]. Thus we would expect that even brief exposure to DE perturbs microtubules. Not surprisingly, it was observed that DE effectively disturbs second polar body extrusion and thus maintains diploid DNA content (Table 1). DE administration itself did not support efficient development, but the combined treatment of DE together with 6-DMAP did improve developmental competence of activated oocytes (Table 2, 3). This indicates that DE advances development ability indirectly via inhibition of 2nd polar body

extrusion.

To further investigate the optimal activation protocol, I compared effects of short-term treatment with several combinations of 6-DMAP and DE on electrical activation and Thi/DTT activation. Development potential induced by Thi/DTT activation surpassed that of electrical activation (Table 5). Peculiar mechanisms of action of Thi/DTT can support this result. Thi, a sulfhydryl reagent, induces repetitive Ca^{2+} spikes in oocytes by oxidizing critical sulfhydryl groups on intracellular Ca^{2+} release proteins which are suspected to be either inositol triphosphate 1,4,5 receptors [169] or ryanodine receptors [170]. It also oxidizes the tubulin sulfhydryl groups and thus causes disassembly of the meiotic spindle which is required for transition from the M phase to interphase. However, this latter effect can be reversed by the reducing agent DTT to complete the subsequent series of mitotic divisions [63]. The Ca^{2+} oscillation profile generated by thimerosal exceeds the single Ca^{2+} spike induced by an electric pulse. Contrary to expectations that electrical and Thi/DTT activation combined would cause optimal consequences, it was noted that EP+Thi/DTT+6-DMAP+DE treatment did not result in better development than the other treatments (EP+6-DMAP+DE and Thi/DTT+6-DMAP+DE). It might be deduced that excessively increased Ca^{2+} levels deteriorate development [171], hence developmental ability would be impaired.

Embryos activated by Thi/DTT+6-DMAP+DE were transferred embryos which were to 30 surrogates. Among them, three became pregnant and 16 foetuses were collected as samples for another experiment and 1 was born. Now another sow is pregnant. Even though this result did not show the comparison between Thi/DTT+6-DMAP+DE and other conditions, the present study demonstrated that

Thi/DTT+6-DMAP+DE is compatible with the establishment of pregnancy and offspring production after SCNT.

Taking our results together, I verified that short-term treatment with 6-DMAP and DE can induce efficient activation of oocytes and concluded that the optimal activation protocol is Thi/DTT+6-DMAP+DE. This combined activation method was highly effective in supporting blastocyst formation (Table 5) and increasing total blastocyst cell number, and thus may be part of a successful oocyte-activating scheme during porcine SCNT procedures. In addition, embryo transfer to sow after short-term activation can reduce the damage of embryo quality and save both time and labour.

Chapter II. Oxamflatin improves developmental competence of porcine somatic cell nuclear transfer embryos

1. Introduction

Since the first success of animal cloning [172], somatic cell nuclear transfer (SCNT) gives a promise to application in both agriculture and regenerative medicine. Although cloning animals has been developed successfully for several decades, there are significant obstacles to their applications, especially the low cloning efficiency [173] and a high incidence of abnormalities in SCNT clones including placental deficiency, increased or decreased growth and oversized organs (large offspring syndrome), obesity, short life span, prolonged gestation, dystocia, and perinatal death [58,174-175]. It is generally believed that aberrant nuclear reprogramming of cloned embryos attributes low cloning efficiency. Accumulating evidence supports that defective epigenetic reprogramming, mainly involving DNA methylation and histone modification, may result in abnormal epigenetic modifications [176-178] and gene expression [179-182] in cloned embryos. Because the epigenetic pattern of donor nuclei must be reversed into embryonic state within a limited and short time after the SCNT, the relaxation of chromatic structure by histone acetylation, which corresponds to a transcriptionally permissive state, might contribute to a successful cloning [27].

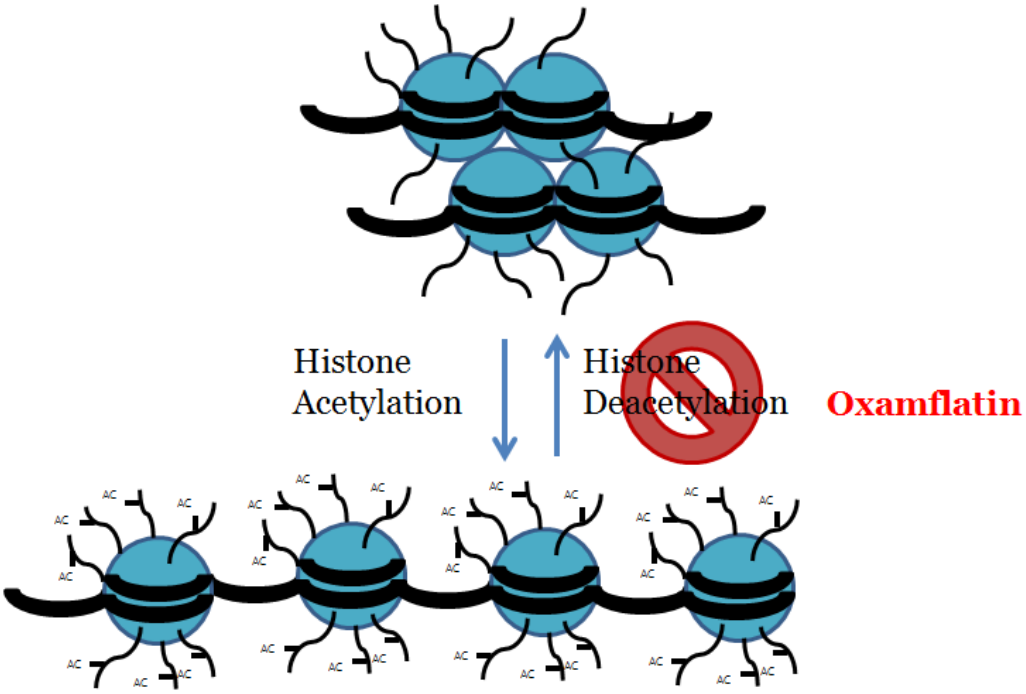

Figure 6. Mechanism of oxamflatin.

Several epigenetic remodeling drugs such as histone deaceylase inhibitors (HDACi) Trichostatin A (TSA) [183-186], valporic acid [187-188], sodium butyrate [189], suberoylanilide hydoxamic acid (SAHA) [190] or DNA methyltransferase inhibitor 5-azacytidine [186,191] have been studied in attempts to improve the developmental potential of SCNT embryos. Notably, several groups have been reported that scriptaid, another HDACi enhances development, especially in porcine embryos [27,192], however still low efficiency and cellular toxicity requires further studies. Recently, Wakayama et al. found that oxamflatin, which can inhibit classes I and IIa/b HDACs [193-194] is improves full-term development of cloned mice and significantly increases establishment of nuclear transfer-generated embryonic stem cell lines without obvious abnormalities [190].

Hence, I explored the effects of oxamflatin and scriptaid on *in vitro* and *in vivo* development of porcine embryos. The objectives of this study were to investigate, optimize and compare effects of these compounds on the reprogramming of somatic nuclei after SCNT and on *in vitro* and *in vivo* embryo development.

2. Materials and Methods

2.1 Chemicals and reagents

All chemicals and reagents used for oocyte maturation, activation and embryo culture in this study were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), unless otherwise stated.

2.2 Collection of oocytes and *in vitro* maturation

Ovaries were collected at a local abattoir and stored in sterile physiological saline at 30-35°C during transportation. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles (3-6 mm) with an 18-gauge needle attached to a 10 mL disposable syringe. COCs with several layers of cumulus cells and uniform cytoplasm were chosen and cultured in tissue culture medium (TCM)-199 (Invitrogen, Carlsbad, USA) supplemented with 10 ng/mL EGF, 0.57 mM cysteine, 0.91 mM sodium pyruvate, 5 μg/mL insulin, 1% (v/v) Pen-Strep (Invitrogen), 0.5 μg/mL follicle stimulating hormone, 0.5 μg/ mL luteinizing hormone and 10% porcine follicular fluid at 39°C in a humidified atmosphere of 5% CO_2 in air, first, with GnRH for 22 h and then without it for a further 22 h. The COCs were washed after each step. After a total of 44 h maturation culture, oocytes were denuded by pipetting with 0.1% hyaluronidase in Dulbecco's PBS (DPBS) (Invitrogen) supplemented with 0.1% polyvinyl alcohol (PVA). Denuded oocytes with evenly-granulated and homogeneous cytoplasm were selected and then utilized for parthenogenesis and SCNT.

2.3 Parthenogenesis

Selected oocytes were equilibrated in pulsing medium then transferred to a chamber containing two electrodes overlaid with the pulsing medium. The pulsing medium was 0.26 M mannitol solution containing 0.5 mM Hepes, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$. Oocytes were activated with a single DC pulse of 1.5 kV/ cm for 60 μsec utilizing a BTX electro-cell Manipulator 2001 (BTX, Inc., San Diego, USA).

2.4 Somatic cell nuclear transfer

For SCNT, a micromanipulator (NT-88-V3, Nikon-Narishige, Tokyo, Japan) attached to an inverted microscope (TE2000, Nikon Instrument, Tokyo, Japan) was used. A cumulus-free oocyte was held with a holding micropipette and the zona pellucida was partially dissected with a fine glass needle to make a slit near the adjacent cytoplasm, presumably containing the metaphase-II chromosomes, were extruded by aspiration with the same needle. Enucleation was confirmed by staining the cytoplasm with 5 μg/mL bisbenzimide (Hoechst 33342) during manipulation. Two white Yucatan miniature transgenic cell lines (SNU1 and SNU2) are used for donor cell. A single fibroblast cell with a smooth surface was selected under a microscope and transferred into the perivitelline space of the enucleated oocyte. Membrane fusion was performed as described by Cho and Koo *et al* [26]. Briefly, cell-oocyte complexes were placed in a 280 mM mannitol solution (pH 7.2) containing 0.15 mM $MgSO_4$, 0.01% (w/v) PVA and 0.5 mM HEPES and held between two electrode needles. Membrane fusion was induced with an electro cell fusion generator (LF101, Nepagene, Ichikawa, Japan) by applying a single direct current pulse (200 V/mm, 20 μsec) and a pre- and post-

pulse alterating current field of 5 V, 1 MHz, for 5 sec, respectively. The reconstructed embryos were cultured in porcine zygote medium-5 (PZM-5) (IFP0410P, Funakoshi, Tokyo, Japan) for 1 to 1.5 h and then subjected to electrical activation. Electrical activation was performed in the same way as for parthenogenetic embryos.

2.5 Post activation treatment and embryo culture

Electrically activated embryos were washed and chemically activated using 2 mM 6-DMAP and 7.5 μg/mL cytochalasin B supplemented with various concentrations of oxamflatin (0, 0.1, 1, 10 μM) or scriptaid (500 nM) for 3 h in PZM-5 (IFP0410P, Funakoshi, Tokyo, Japan). After chemical activation Embryos were then cultured in PZM-5 at the same concentrations of oxamflatin or with 500 nM scriptaid as above for a further 6 or 11-13 h, respectively. After three washes in PZM-5, embryos were cultured in 450 μl of PZM-5 covered with mineral oil. The embryos were cultured in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C for 7 days. Cleavage rates were evaluated on Day 2. Blastocyst formation and the number of nuclei were determined on Day 7. Briefly, blastocysts were fixed in absolute alcohol and then nuclei were stained with 5 μg/mL bisbenzamide (Hoechst 33342) overnight at 4°C. Fixed and stained blastocysts were mounted on a glass slide in a drop of glycerol, gently flattened with a cover glass and visualized for cell counting with a fluorescence microscope using a 346 nm excitation filter.

2.6 Total RNA extraction, RT-PCR and Real Time PCR

Total RNA was isolated from fresh or previously frozen (-80°C) blastocysts

using the easy-spin™ (DNA free) Total RNA Extraction Kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions, quantified by a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and immediately stored at -80°C until used for RT-PCR and qRT-PCR. cDNA was produced from 1 µg of total RNA from blastocyst samples, using a SuperScript™ III First-Strand cDNA Synthesis Kit (Invitrogen) primed with oligonucleotide-dT (18mer) and followed by RNase H digestion of RNA, in a total volume of 20 µl as per the manufacturer's instructions. Quantitative real time PCR was performed in a 7300 Real Time PCR System (Applied Biosystems, Foster, USA) using the SYBR premix ExTaq perfect Real Time (TAKARA Bio Inc., Shiga, Japan) with little modification. In brief, all the primers were standardized with a standard curve. The PCR plate (MicroAmp optical 96-well reaction plate, Singapore) was made by adding 2 µl cDNA, 1µl (10 pM) forward primer, 1 µl (10 pM) reverse primer, 10 µl SYBR Premix Ex Tag (Takara Bio Inc), 0.4 µl ROX Reference Dye (Takara Bio Inc.) and 5.6 µl of Nuclease-free water (Ambion Inc., Austin, USA). For each sample, four replications were performed. Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 6 containing the specific primer to amplify POU5F1, NANOG, HDAC1, HDAC2, BCL2L and BAX.

Table 6. Sequence-specific primers for quantitative reverse transcription-polymerase chain reaction

Gene name	Primer sequences (5'-3')	Length (bp)	Gene ID
POU5F1	F: TTTGGGAAGGTGTTCAGCCAAACG	198	NM_001113060
	R: TCGGTTCTCGATACTTGTCCGCTT		
NANOG	F: GTACCTCAGCCTCCAGCA	161	AJ877915
	R: CTGAGCCCTTCTGAATCAC		
CDX2	F: TGTGCGAGTGGATGCGGAAG R: CCGAATGGTGATGTAGCGACTG	149	GI 262070767
REX01	F: TTTCTGAGTACGTGCCAGGCAA	201	TC206866
	R: TCTGAGAAAGCATCTCTCCGTTC		
HDAC1	F: GTGAGGACTGTCCGGTGTTT	227	NM_008228
	R: CGCAGTAGCTGAAACCACAA		
HDAC2	F: TGGAGTACTGACTGCCTGGA	236	AY555
	R: CCTGAACCTTTGTGGTGCTT		
BCL2L1	F: TGGTGGTTGACTTTCTCTCC	139	AF216205
	R: ATTGATGGCACTAGGGGTTT		
BAX	F: GCCGAAATGTTTGCTGACGG	152	AJ606301
	R: CGAAGGAAGTCCAGCGTCCA		
ACTB	F: GTGGACATCAGGAAGGACCTCTA	137	U07786
	R: ATGATCTTGATCTTCATGGTGCT		

2.7 Embryo transfer and pregnancy diagnosis

Embryo transfer was followed as in the previous report [195]. Briefly, 70 to 130 SCNT embryos cultured for 1 or 2 days were loaded into a sterilized 0.25 mL straw (Minitüb, Tiefenbach, Germany) and kept in a portable incubator (Minitüb) during transportation to the embryo transfer facility. An oestus-synchronized recipient was anaesthetized by a combination of ketamine (1.13 mg/mL; Yuhan ketamine®, Yuhan crop., Seoul , Korea) and xylazine (0.3 mg/kg; Celactal®, Bayer Animal Health corp., Tokyo, Japan) through IV for induction and 3% of isoflurane (Ifran®, Hana Pharm Co., Ltd, Hwasung, Korea) for maintenance. One oviduct was exposed by laparotomy. The straw containing the embryos was inserted directly into the oviduct of the recipient and embryos were expelled from the straw using 1 mL syringe (Becton Dickinson). Embryos were transferred to eleven recipients: when the SNU1 cell line was used as the nuclear donor, oxamflatin-treated embryos were transferred to four surrogates and scriptaid-treated embryos were transferred to three. When the SNU2 cell line was used, the oxamflatin treatment embryos were transferred to two and the scriptaid groups were transferred to two. Recipients were checked for pregnancy by transabdominal ultrasound examination at day 30 after embryo transfer.

2.8 Statistical analysis

All data were subjected to one-way ANOVA followed by Tukey's test using Prism version 4.0 (Graphpad Software, San Diego, USA) to determine differences among experimental groups. Statistical significance was determined when P value was less than 0.05.

3. Results

3.1 Effect of different concentration of oxamflatin on development of porcine parthenogenetic embryos *in vitro*

Developmental competence was assessed in terms of cleavage rate, blastocyst rate and blastocyst total cell numbers. No significant difference was observed in these parameters among parthenogenetic embryos treated with 0.1 or 1 μM oxamflatin. However, cleavage rate, blastocyst rate and blastocyst total cell numbers in the 10 μM oxamflatin-treated group were all significantly lower ($P<0.05$) than those of the controls and of the 0.1 and 1 μM oxamflatin treatment groups (Table 7)

Table 7. Effect of different concentration of oxamflatin on development of porcine parthenogenetic embryos *in vitro*

	Control	0.1 μM	1 μM	10 μM
No. of embryos cultured	77	76	78	77
Cleavage	54(70.1±5.3^{a})	60(79.0±4.7^{a})	61(76.9±4.8^{a})	45(58.4±5.7^{b})
Blastocyst	8(14.8±4.9^{a})	9(15.0±4.6^{a})	12(19.7±5.1^{a})	2(4.4±3.1^{b})
Total cell number in blastocyst	64.7±5.0 ^{a}	75.0±5.8 ^{a}	76.2±4.6 ^{a}	50.5±3.5 ^{b}

Values are mean ± SEM. Values with different superscripts in the same column differ significantly ($P<0.05$). Experiments were repeated at least five times.

3.2 Effect of different concentration of oxamflatin on development of porcine SCNT embryos *in vitro*

After treatment of SCNT embryos with 0.1 or 1 μM oxamflatin, the cleavage rate was similar to that of the control group, however, blastocyst development rate and blastocyst total cell numbers in the 1 μM oxamflatin treated group (35.7±6.5 % and 72.3±2.3, respectively) were increased significantly compared to the control or 0.1 μM groups (13.2±4.7% and 50.6±3.5, 15.4±5.1 % and 57.6±3.8, respectively) (Table 8).

Table 8. Effect of different concentration of oxamflatin on development of porcine SCNT embryos *in vitro*

	Control	0.1 μM	1 μM
No. of embryos cultured	75	74	73
Cleavage	53(70.7±5.3[a])	52(70.3±5.4[a])	56(76.7±5.0[a])
Blastocyst	7(13.2±4.7[a])	8(15.4±5.1[a])	20(35.7±6.5[b])
Total cell number in blastocyst	50.6±3.5[a]	57.6±3.8[a]	72.3±2.3[b]

Values are mean ± SEM. Values with different superscripts in the same column differ significantly ($P<0.05$). Experiments were repeated at least five times.

3.3 Comparison of effect of oxamflatin and scriptaid on development of porcine SCNT embryos *in vitro*

Oxamflatin at 1 μM significantly increased blastocyst development rate and blastocyst total cell numbers (33.3±6.0 % and 73.1±1.6, respectively) compared with control embryos (10.3±3.7 % and 54.1±3.5), respectively or with the scriptaid group (16.4±4.6 % and 64.4±2.1, respectively) (Table 9)

Table 9. Comparison of the effect between oxamflatin and scriptaid on development of porcine SCNT embryos *in vitro*

	Control	Oxamflatin	Scriptaid
No. of embryos cultured	89	88	88
Cleavage	68(76.4±4.5^{a})	63(71.6±4.8^{a})	67(76.1±4.6^{a})
Blastocyst	7(10.3±3.7^{a})	21(33.3±6.0^{b})	12(16.4±4.6^{a})
Total cell number in blastocyst	54.1±3.5^{a}	73.1±1.6^{b}	64.4±2.1^{c}

Values are mean ± SEM. Values with different superscripts in the same column differ significantly ($P<0.05$). Experiments were repeated at least five times.

3.4 Gene expression profiles among non-treated, oxamflatin or scriptaid treated SCNT embryos during *in vitro* development

To test whether the effects of oxamflatin on gene expression during the preimplantation development the expression levels of pluripotency-related genes (POU5F1, NANOG, CDX2, REX01), histone deacetylase genes (HDAC1, HDAC2) and apoptosis-related genes (BCL2L1, BAX) were studied in control, oxamflatin or scriptaid treated blastocysts using real-time PCR. The expression levels of POU5F1, NANOG, REX01 and BCL2L1 were significantly higher in oxamflatin treated group than control or scriptiad treated groups. At the 2 cell stages, the oxamflatin or scriptaid treated embryos showed significantly higher expression levels of HDAC1 and HDAC2 than control embryos. However, expression pattern of HDAC1 and HDAC2 in oxamflatin treated group was significantly decreased compared to those of the control or scriptaid treated groups at the 4 cell stages. HDAC1 expression in scriptaid treated blastocysts was significantly higher than those in control or oxamflatin treated blastocysts, whereas both scriptaid and oxamflatin treated blastocysts demonstrated significantly higher expression than control. Oxamflatin-treated blastocysts showed higher level in BCL2L1 and lower level in BAX than counterparts ($P< 0.05$) (Figure 7).

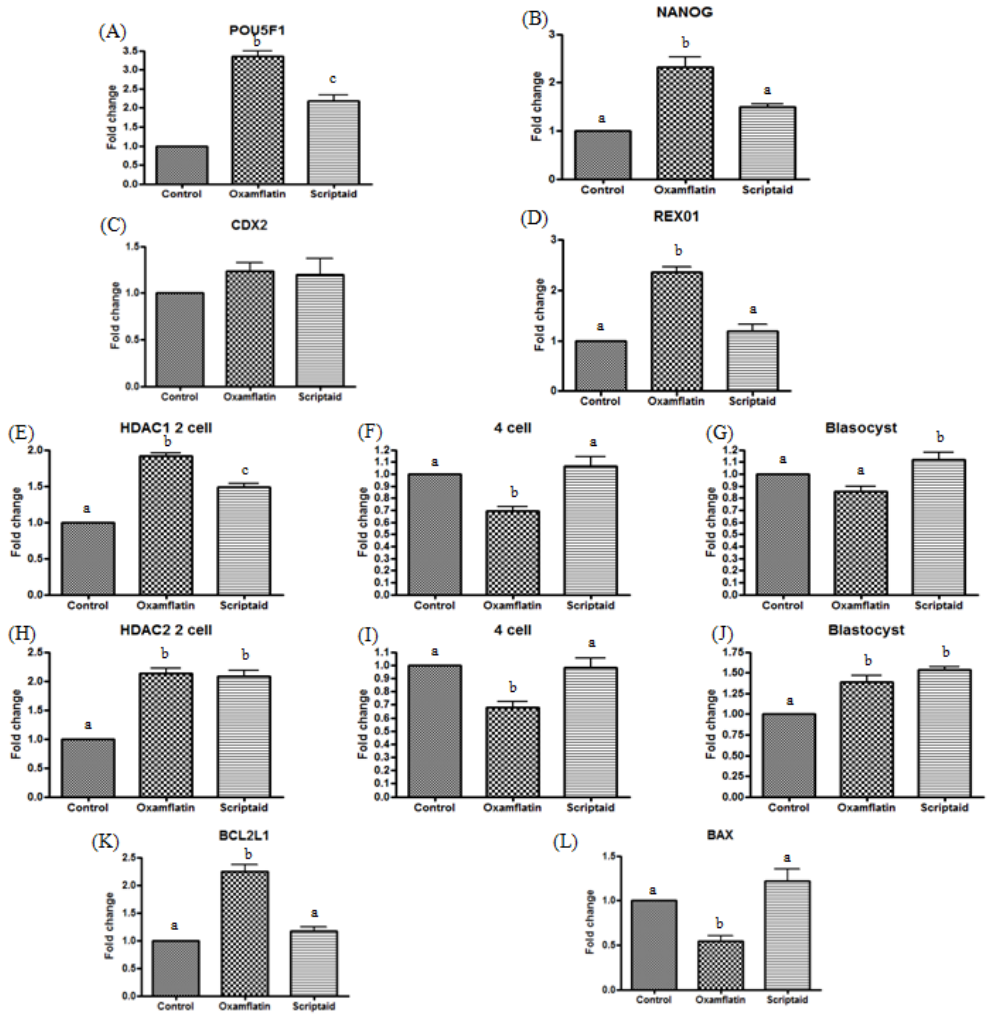

Figure 7. Expression profiles of POU5F1, NANOG, CDX2, REX01, HDAC1, HDAC2, BCL2L1 and BAX on SCNT embryos during *In vitro* development. Relative expression patterns of the (A) POU5F1in blastocysts, (B) NANOG in blastocysts, (C) CDX2 in blastocysts, (D) REX01 in blastocysts (E) HDAC1 in 2 cell, (F) HDAC1in 4 cell, (G) HDAC1 in blastocysts, (H) HDAC2 in 2 cell, (I) HDAC2 in 4 cell, (J) HDAC2 in blastocysts, (K) BCL2L1 in blastocysts (L) BAX in blastocysts during *in vitro* development of control, oxamflatin, scriptaid-treated SCNT embryos. Experiments were repeated at least four times.

3.5 Full term development of SCNT embryos following oxamflatin or scriptaid treatment

The SCNT embryos which were reconstructed with two white Yucatan miniature transgenic cell lines (SNU1 and SNU2) were transferred to eleven surrogates to investigate the effect of oxamflatin treatment on full term development. Oxamflatin treatment resulted in a higher percentage of full term births compared to the scriptaid-treated group. When the SNU1 cell line was used as the nuclear donor, all three surrogates became pregnant in the oxamflatin treatment group and one went to term with cloning efficiency of 0.9% (Table 10); however, while two of four recipients became pregnant in the scriptaid treatment group, no live piglet was obtained. When SNU2 was the donor cell, one of two surrogates became pregnant and three piglets were born in the oxamflatin treated group with a cloning efficiency of 3.2%, whereas two piglets were obtained from one litter in the scriptaid treatment group with a cloning efficiency of 1.8%.

Table 10. Full term development of SCNT embryos following oxamflatin or scriptaid

Sex	Cell type	No. of trasferred embryos	Treatment	Pregnancy	Piglet	Cloning efficiency
Male	SNU1	96	Scriptaid	+	/	0[a]
Male	SNU1	98	Scriptaid	+	/	
Male	SNU1	115	Scriptaid	-	/	
Male	SNU1	116	Scriptaid	-	/	
Male	SNU1	80	Oxamflatin	+	/	
Male	SNU1	80	Oxamflatin	+	/	
Male	SNU1	117	Oxamflatin	+	1 died after birth + 1 Mummy	0.9[b]
Female	SNU2	113	Scriptaid	+	1 + 1 died after birth	1.8[c]
Female	SNU2	125	Scriptaid	-	/	
Female	SNU2	94	Oxamflatin	+	2 + 1 died after birth	3.2[d]
Female	SNU2	74	Oxamflatin	-	/	

Values with different superscripts in the same column differ significantly ($P<0.05$).

+ : Pregnant

- : Not pregnant

Cloning efficiency: No. of piglets/ No. of embryos transferred.

4. Discussion

Reprogramming following the transfer of somatic nuclei into oocyte cytoplasm occurs at the epigenetic level [196]. Considering the previous reports, insufficient and abnormal epigenetic modifications including DNA methylation and histone acetylation occur [185,197] during SCNT and these errors seem to be associated with the low success rate of cloning [198].

Increased histone acetylation by scriptaid could dramatically enhance the developmental competence of SCNT embryos in pig [27,192,199], further studies are required because of the still low efficiency and cellular toxicity when this compound is used at its effective concentration [200]. A recent study in mice and cow showed that oxamflatin treatment (1 μM oxamflatin for 9 h after nuclear transfer) significantly improved the *in vitro* culture and full-term development of cloned mice [190,201]. In the present study, I investigated the effect of oxamflatin on *in vitro* and *in vivo* development of porcine SCNT embryos and compared it with a scriptaid-treated group. I found that oxamflatin treatment was superior to scriptaid and enhanced the development of reconstructed embryos both *in vitro* and *in vivo*.

It is found that 1 μM of oxamflatin was effective while 10 μM of oxamflatin was toxic to embryos, thus 1 μM concentration was used for subsequent experiments. Compared to non-treated SCNT embryos, both scriptaid and oxamflatin treated embryos exhibited increased total cell numbers in blastocysts, although there was no difference in embryo quality as judged by blastocyst formation rate between the non-treated and scriptaid-treated groups. HDAC

inhibitors including scriptaid and oxamflatin can induce hyperacetylation of the core histone, indicating structural alleviation in chromatin to the transcriptionally permissive state, as well as DNA methylation of the donor cell-derived genome [202-203]. Frequently, histone methylation is reduced and thus genes are activated which are crucial for embryo development [204].

Oxamflatin significantly increased both blastocyst formation rate and total cell numbers compared to the scriptaid treated group, while both HDACi treated groups showed improvement of *in vitro* development compared with the non-treated group. The mechanism underlying how oxamflatin improves *in vitro* development and cloning efficiency remains unclear.

To elucidate this mechanism, I measured the relative expression levels of the pluripotency-related genes (POU5F, NANOG) the histone deacetylase genes (HDAC1, HDAC2) and apoptosis-related genes (Bcl, Bax) in the non-treated, oxamflatin-treated and scriptaid-treated SCNT blastocysts using real-time PCR. POU5F1, the homeodomain containing transcription factor, is a key regulator of pluripotency and cell differentiation [205]. It seems that POU5F1 plays an important role in determining early steps in embryogenesis and differentiation [206]. POU5F1 deficient embryos lose their developmental pluripotency and low Oct-4 levels resulted in differentiation into only trophectoderm [207]. Lower expression of POU5F1 was found in porcine SCNT blastocyst than in IVF counterparts [208] and could be associated with low developmental competence of SCNT embryos. However, oxamflatin-treated SCNT blastocysts had higher POU5F1 expression levels than both non-treated and scriptaid-treated SCNT blastocysts in this study. NANOG is also an important transcription factor for maintaining the pluripotent cells of the ICM [209] and is known to be regulated

by POU5F1 [210]. Oxamflatin-treated SCNT blastocysts showed significantly higher expression levels of NANOG than the control group and this observation coincided with NANOG expression under POU5F1 regulation. From our results, I can conclude that oxamflatin treatment can correct abnormally reprogrammed porcine SCNT embryos by increasing POU5F1 and NANOG.

It is known that histone acetylation regulates transcription mediated throughout activities of HDACs and HATs. Dependence on embryonic genome expression cannot be detected until the mid 2-cell stage in the mouse, the 4-cell stage in the pig, and the 8-cell stage in sheep [211]. Embryonic gene activation (EGA) is considered to be important evidence of nuclear reprogramming, which transforms the genome from transcriptional quiescence at fertilization into robust transcriptional activity. The same events are likely involved when the oocyte initiates the reprogramming process after SCNT. Reprogramming involves a series of molecular events that control gene expression and has a profound effect on nuclear architecture [212]. HDAC1 and HDAC2 are implicated as candidate genes involved in genome activation [213]. Mcgaw ***et al.*** looked at the expression patterns of bovine HDAC1 from immature oocytes to blastocysts. The lowest level of mRNA of HDAC1 is at the 8-cell stage when EGA starts, however, HDAC1 is strongly expressed at the blastocyst stage [214] . Repression could be associated with structural changes in the chromatin and the discovery that histone deacetylation can be associated with chromatin offers enlightenment regarding how histone deacetylation relates to repression of gene expression [215]. In a previous experiment, HDAC2 showed that a low level of expression at the 4-cell stage with a dramatic decrease from the 2-cell stage and then an increase reaching a maximum at the blastocyst stage in IVF and *in vivo* produced porcine embryos, whereas immaterial changes were observed in NT embryos [208]. In addition

there are reports that embryonic activated genes are suppressed [216-217] and nucleolar re-activation is delayed in SCNT-generated embryos [218]. It was observed that oxamflatin treatment enhances *in vitro* developmental competence and cloning efficiency along with decreased HDAC1 and HDAC2 expression at the 4-cell stage. Taken together, these findings suggest that HDACi treatment promotes the onset of EGA to relieve the incompetence of enucleated oocytes to induce early embryonic stage-specific gene expression. Therefore, oxamflatin promoted normal EGA to overcome the delayed activation of nucleolar function in SCNT-generated embryos. I believe that oxamflatin treatment affected EGA in SCNT-generated cloned embryos to support more normal regulation of embryonic development.

The proapoptotic gene, Bax regulates apoptosis while the anti-apoptotic gene Bcl protects against apoptosis during embryo development. There was lower expression of Bax and higher expression of Bcl in oxamflatin-treated blastocysts compared with those in scriptaid or non-treated embryos. Oxamflatin also suppresses apoptosis in bovine SCNT embryos [201] indicating reduced apoptosis of cells in oxamflatin treated blastocysts.

Embryo transfer results showed that oxamflatin treatment can produce viable cloned piglets and the development of SCNT embryos to the piglets after treatment with oxamflatin was twice than that of scriptaid treated group.

In conclusion, the expression of development-related genes and modification of histone acetylation subsequently improves nuclear reprogramming, culminating in increased development of porcine SCNT embryos both *in vitro* and *in vivo*.

Chapter III. Production and characterization of human shTNFRI-Fc and hHO-1 double transgenic pigs using 2A peptide

1. Introduction

The continuously increasing shortage of human donor organs has prompted the research of pig-to-human xenotransplantation. The first immunological barrier by hyperacute rejection can be overcome by pigs homozygous for α 1,3-galactosyltransferase knockout (α 1,3-GalT KO) [219-220] or transgenic pigs expressing human complement regulatory proteins [221]. While deletion of the α 1,3-GalT gene represented a significant advance in the field of xenotransplantation, it did not provided a complete solution to the problem. The unresolved problem is acute humoral xenograft rejection (AHXR) caused by the activation of the porcine endothelium [222-223]. AHXR is characterized by anti-non-Gal antibodies, up-regulated expression of adhesion molecules, platelet adhesion, and aggregation, followed by fibrin and thrombin deposition, and ultimately thrombosis and disseminated intravascular coagulation (DIC) which eventually induce inflammation and coagulopathy through activation of porcine endothelial cell.

Heme oxygenase-1 (HO-1) is a 32 kDa ubiquitously expressed enzyme encoded by the HMOX1 gene [224]. HO-1 induced by various stimuli including oxidative stress, inflammation, hypoxia degrade heme into three end-products, carbon monoxide, free iron and biliverdin. All three end-products have anti-oxidative, anti-inflammatory and anti-apoptotic effect underlying the role of HO-1

[225]. Besides, the expression of HO-1 on endothelial cells and cardiac myocytes has been associated with the accommodation and prolonged xenograft survival of rodent cardiac and lung xenografts [226-227]. These findings renders HO-1 a fundamental molecule for genetically modified pigs in xenotransplantation.

On the other hand, human TNF-α (hTNF-α) can induce xenograft rejection by direct stimulation of porcine endothelial cell resulting in up-regulation of SLA class I, class II, B-7-1, B-7-2, VCAM-1, E-selectin [220,228] and ICAM-1 expression [229]. A resistance of HO-1 to apoptotic damage after hTNF- α exposure was reported [230-231], however, HO-1 did not suppress TNF-α induced apoptosis fully and another strategy to express sTNFRI or sTNFRII was developed [232-234]. Previously, Cho and Koo *et al*. produced transgenic pig secreting soluble human tumor necrosis factor receptor I-Fc (shTNFRI-Fc) and confirmed that shTNFRI-Fc neutralizes hTNF-α [235]. It was hypothesized that expression of shTNFR1-Fc in combination with hHO1 could provide more effective protection to the transplanted pig tissues.

Until now, most multi-transgenic pigs have been produced by breeding programs that utilize different single transgenic pigs [236-237]. This method requires different single transgenic pigs to breed, which is time-consuming and costly. Also it could not guarantee the production of multi-transgenic pigs if the founders are heterozygous. A possible method to generate multi-transgenic pigs is transfection of multiple plasmids simultaneously to porcine fibroblasts, which are then used for somatic cell nuclear transfer (SCNT). However, the efficiency is low in both cell transfection and expression [238].

The viral 2A peptide has recently become an alternative for mediating

polycistronic expression in gene therapy and somatic cells reprogramming [239]. The viral 2A peptide originated from picornaviruses is approximately 19 amino acids long. Briefly, a normal peptide bond formation between the glycine and the proline is unparied at the 2A site causing the ribosomal to skip and begin to translate from the second codon resulting in the expression of two independent proteins from a single transcription event [224].

In this study, multi-cistronic vector system using 2A peptide enables HA hHO-1 and shTNFRI-Fc double transgenic pigs. Transgenic pigs expressing shTNFRI-Fc and HA hHO-1 are produced, characterized and investigated the protective effects of HA hHO-1 and shTNFRI-Fc against hypoxic condtion or inflammatory stimuli in an *in vitro* system.

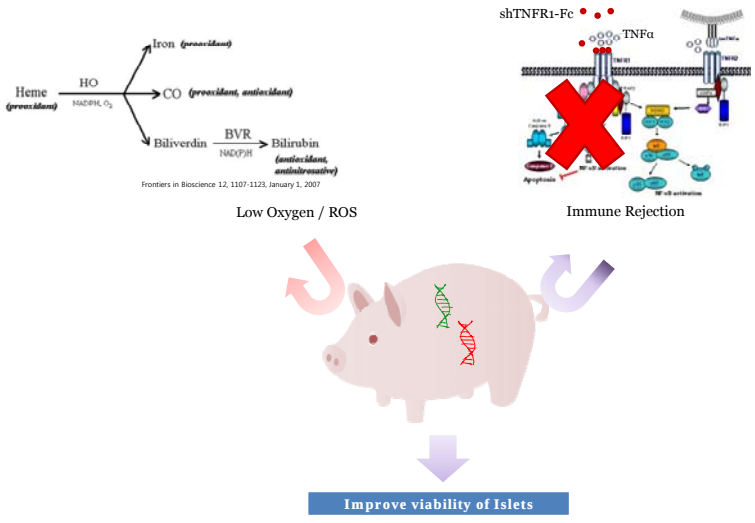

Figure 8. Heme oxygenerase and soluble human tumor necrosis factor recptor 1-Fc (shTNFR1 Fc) protect islet from apoptosis and immune rejection.

2. Material and methods

2.1 Chemicals and reagents

All chemicals and reagents used for oocyte maturation, activation and embryo culture in this study were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), unless otherwise stated.

2.2 Construction of expression vector containing shTNFRI-Fc-2A- HA hHO-1gene

shTNFRI-Fc fusion gene was constructed by previous study [235]. Hemagglutinin tagged human heme oxygenase-1 (HA hHO-1) gene was constructed. shTNFRI-Fc and HA hHO-1gene was linked with nucleotide sequences encoding 20 amino acids length F2A peptide (shTNFRIFc-2A- HA hHO-1). The shTNFRI-Fc-2A- HA hHO-1 gene was inserted into expression vector containing CAG promoter.

2.3Generation of adenovirus expressing single and double genes containing 2A peptide

The generation of adenoviruses used in this study previously described [240]. In brief, target genes (shTNFRI-Fc, HA hHO-1, and shTNFRI-Fc-2A- HA hHO-1) were inserted into the shuttle vector (pAdTrack-CMV) and linearized with *Pme*I restriction enzyme. The linearized shuttle vector was mixed adenoviral plasmid, pAdEasy-1, for homologous recombination. The viral plasmid was linearized with *Pac*I restriction enzyme and transfected into HEK293 cells. After 9 days,

transfected cells were lysed by three cycles of freezing and thawing. GFP expressing adenovirus was also generated as a control.

2.4 Confirmation of shTNFRI-Fc-2A- HA hHO-1 expression and function

In order to confirm the expression of protein and function of both genes, isolated NPCCs were infected with adenovirus expressing shTNFRI-Fc-2A- HA hHO-1 (50 MOI). After 48 hr of infection, the culture media and lysate of NPCCs was collected. Enzyme-linked immunosorbent assay (ELISA) was carried out using shTNFRI specific ELISA kit (R&D system, MN, USA) for shTNFRI-Fc expression and western blot was performed for HA hHO-1 expression using HO-1 antibody (Rabbit monoclonal, 1:2000, Abcam, , USA). In order to protection effects of both genes, adenovirus infected NPCCs were treated TNF-α (20 ng/mL, eBioscience, CA, USA) and Cycloheximide (10 μg/mL) for 24 hr or H_2O_2 (400 uM) for 1 hr. After treatment, cell viability was estimated by CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan)

2.5 Establishment of donor cells for SCNT

The 35-day-old male White Yucatan miniature pig fetuses were used as source of fetal fibroblasts. Isolation of fetal pig fibroblasts was described previous studies [241]. The shTNFRI-Fc-2A-HA hHO-1 expression vector was introduced into the isolated fetal fibroblasts by electroporation.

2.6 Generation of shTNFRI-Fc-2A- HA hHO-1double transgenic pig using SCNT

Somatic cell nuclear transfer was performed as described in previous studies [241] with modifications. Briefly, oocytes were enucleated by removal of the first polar body along with the adjacent cytoplasm, presumably containing the metaphase-II chromosomes. Single transgenic fibroblast cell from one of the positive cell clones was transferred into the perivitelline space of enucleated oocytes. Transgenic cell-oocyte complexes were placed in a 280 mM mannitol solution (pH 7.2) containing 0.15 mM $MgSO_4$, 0.01% (w/v) PVA and 0.5 mM HEPES and held between two electrode needles. Membrane fusion was induced with an electro cell fusion generator (LF101, Nepagene, Ichikawa, Japan) by applying a single direct current pulse (200 V/mm, 20 μsec) and a pre- and post-pulse alterating current field of 5 V, 1 MHz, for 5 sec, respectively. The reconstructed embryos were cultured in porcine zygote medium-5 (PZM-5) (Funakoshi, IFP0410P) for 1 to 1.5 hr and then subjected to chemical activation with 0.2 mM Thi+2 mM 6-DMAP+0.4 μg/mL demecolcine for 10 min, followed by 8 mM DTT+2 mM 6-DMAP+0.4 μg/mL demecolcine for 30 min. Embryos were then cultured in PZM-5 (IFP0410P, Funakoshi, Tokyo, Japan) at the same concentrations of 1 μM oxamflantin for 9 hr. Reconstructed transgenic embryos were cultured *in vitro* for 1-2 days and then ninety to 120 embryos were transferred to an estrus-synchronized surrogate pig (Landrace x Yorkshire) by laparatomy. Pregnancy was monitored by ultrasonography, and the transgenic pigs were delivered by caesarian section.

2.7 Analysis of transgene expression in shTNFRI-Fc-2A- HA hHO-1transgenic pigs.

Genomic DNA isolated from tails of TG piglets and cDNA synthesized from whole blood of transgenic piglets were used for confirmation of transgenes expression by polymerase chain reaction (PCR) with specific primer sets. For the confirmation of the protein expression of transgenes, western blot and immunochemistry (IHC) was performed. Western blot was performed with various organ samples from transgenic piglets using HRP-conjugated human IgG (1:2000, The binding Site, Birmingham , UK), HO-1 (1:2000, Abcam, MA, USA), and HA (1:4000, Abcam, MA, USA) antibodies. Immunohistochemistry (IHC) was also carried out in various organs using HO-1 (1:50) and HA (1:100) antibodies.

3. Results

3.1 Generation of shTNFRI-Fc and HA hHO-1 double transgenic pigs

The shTNFRI-Fc-2A- HA hHO-1transgene was constructed under the control of CAG promoter (Figure 9A). The constructed transgene was introduced into fetal fibroblast cells originated from White yucatan pig. After G418 selection for two weeks, shTNFRI-Fc and HA hHO-1 expressed cell line was established. The established cell line was used as donor cells for nuclear transfer. On average, 120 reconstructed embryos were transferred into each of eight estrus-synchronized surrogate pigs. Three of them became pregnant and one farrowed four live male piglets (Table 11 and Figure 9B). The one (#012) piglet of the four piglets was died in few hours after birth. Genomic DNA PCR showed that all cloned transgenic pigs had integrated the shTNFRI-Fc and HA hHO-1construct. RT-PCR and western blot analysis revealed that all transgenic pigs successfully expressed shTNFRI-Fc and HA hHO-1 mRNAs and proteins (Figure 9C). In addition, shTNFRI-Fc which is secretory protein exist in blood serum at high concentration (Figure 9D) in the all transgenic piglets except of #012 transgenic pig. Because #012 transgenic pig was died suddenly, blood was not harvested.

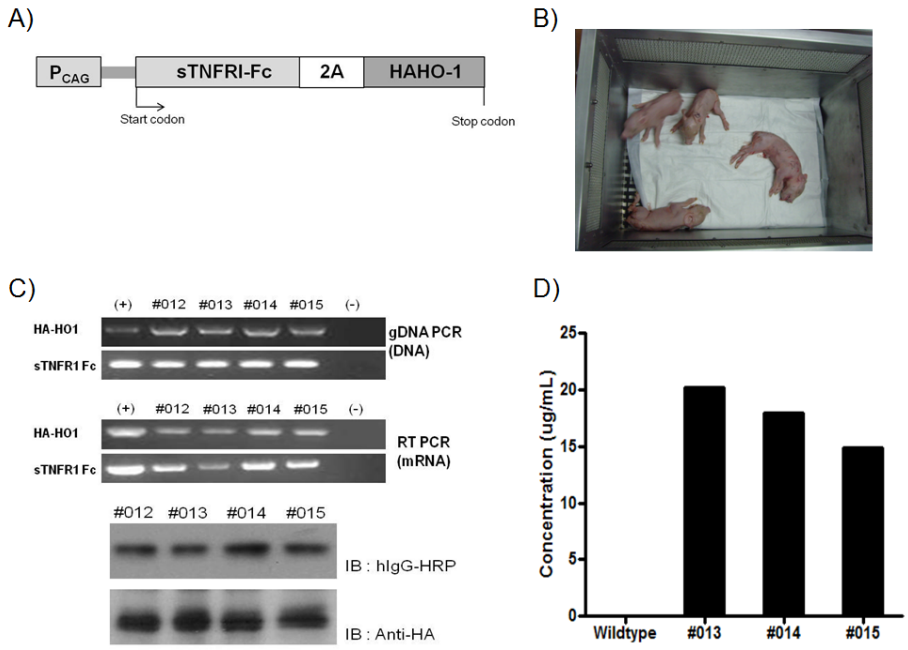

Figure 9. Generation and characterization of shTNFRI-Fc-2A-HA hHO1 transgenic pig.

Table 11. Production of cloned shTNFRI-Fc-2A-HA hHO-1 transgenic pigs

ID	Number of transferred embryo	Pregnancy	Born piglets	Current Status
1	128	Yes	4	2 Alive
2	118	No	-	-
3	114	No	-	-
4	129	No	-	-
5	130	No	-	-
6	96	Yes	0	Abortion
7	127	No	-	-
8	116	Yes	0	Abortion

3.2The expression and the tissue distribution of transgenes in transgenic pigs

The organs were harvested from the two piglets (#012, #014) of the four transgenic piglets. The one (#012) was died in few hours after birth, the other (#014) was sacrificed at 2 days after birth. In order to check the tissue distribution of transgenes (shTNFRI-Fc and HA hHO-1), western blot and was performed in each transgenic piglet's organ (Heart, Liver, Lung, Kidney, Pancreas, and Spleen). Unfortunately, the adrenal gland was harvested in #014 TG pig instead of pancreas. Although protein expression level is different depending on the tissue, shTNFRI-Fc protein was expressed in most organs of two piglets except of pancreas tissue of #012 transgenic pig. In order to check the expression of HA hHO-1 protein, anti-HO-1 antibody andante-HA antibody was used separately. The HA hHO-1 protein was expressed in heart, lung, kidney in the #012 transgenic pig. In the #014 transgenic pig, HA hHO-1 protein was showed higher expression than the HA hHO-1 expression of #012 transgenic pig in all tissue. In liver tissues of two piglets, HA hHO-1 was not expressed (Figure 10).

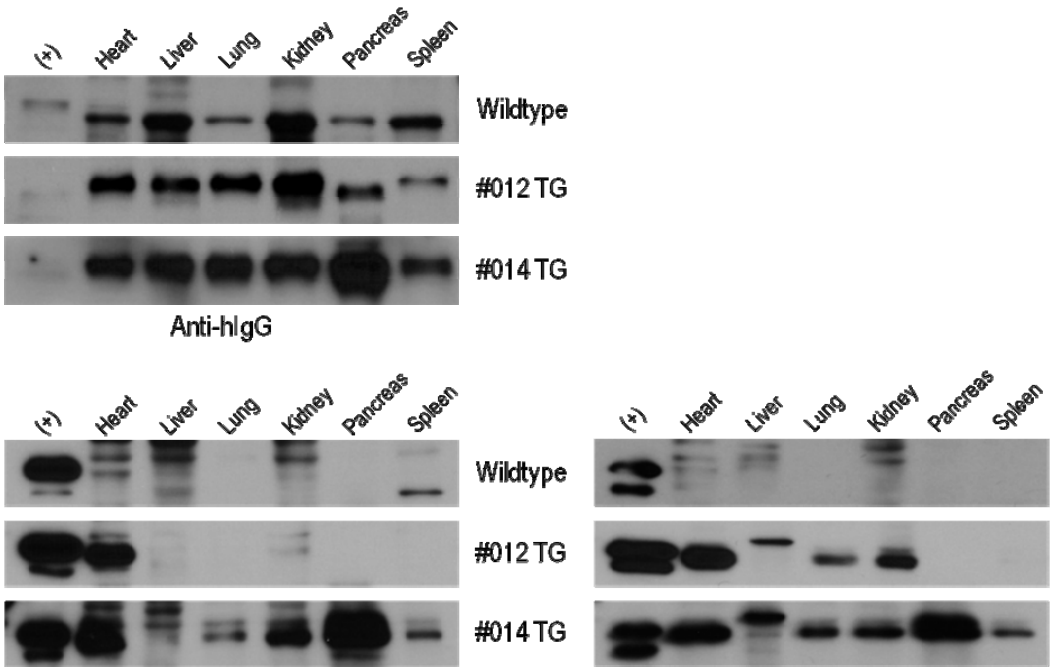

Figure 10. Tissue distribution of transgenes in shTNFRI-Fc-2A-HA hHO-1 transgenic pig.

4. Discussion

The potential use of pigs as alternative organ donors for xenotransplantation is considered an obvious solution for the shortage of human donor organs worldwide due to its physiological, anatomical, metabolic, genetic and pathologic similarity with humans [242-243]. However, immunological challenges and other barriers associated with xenotransplantation are required to be overcome. Transplantation of organs from genetically modified pigs into non-human primates is now not substantially limited by hyperacute, but other issues have become more prominent, such as induction of inflammation and coagulopathy through activation of porcine endothelial cell (PEC) in the recipient [244] and ischemia/reperfusion injury (IRI) [245]. As infiltrated host immune cells produce TNFα and amplify further inflammation [246], acitivated PECs play a major role in the initial recruitment of host immune cells [247].

IRI is caused by cytotoxic mediators, such as reactive oxygen species (ROS) during organ procurement, which also induce the expression of chemokines and adhesion molecules and the infiltration of innate inflammatory cells [248]. To address these problems, the development of genetically modified pigs for xenotransplantation goes towards multi-transgenic pig models encompassing transgenes against thromboagulopathy, inflammation and IRI.

Recently, Cho and Koo *et al.* reported that sera from transgenic pig secreting shTNFR1-Fc inhibited induction of chemokines, and E-selectin in PECs stimulated with Human TNF-α [235]. Transgenic expression of hHO-1 in pigs could provide significant protection against post-transplant vasculopathy and ischemia reperfusion injury in a xenotransplantation setting [231]. In addition,

Yeom and Koo *et al*. studied that HA tagged HO-1 transgenic pig fibroblasts have the resistances against apoptosis, inflammation, and hypoxic condition [249]. Therefore, hHO1 and shTNFR1-Fc transgenic pigs may exhibit promising inhibition of TNFα mediated activation in the PEC and protection against IRI.

Many transgenic pigs have been generated by breeding [236-237], however, Zhou *et al*. reported that triple transgenic pig co-expressing human CD59, human DAF and human MCP pig by breeding showed different expression patterns of hD59 between PBMCs and endothelial cells[237]. Moreover, different single transgenic pigs should first be generated then sexual maturity and gestation period are additionally needed, which is time-consuming and costly. Sperm-mediated gene transfer (SMGT) has also been reported to produce multiple-transgenic pigs [250]. Although, SMGT shorten time to generate multiple-piglets and reduced cost , the gene integration efficiency is reportedly from 57% to 80% [251] and the gene insertion is random which indicates different expression level for different genes in the piglets [252] Also, the transgenes introduced by SMGT have been reported to be transiently transmitted to the offspring and be likely lost [20].

A possible method to generate multi-transgenic pigs is transfection of multiple plasmids simultaneously to porcine fibroblasts, which are then used for somatic cell nuclear transfer (SCNT). However, the efficiency is low in both cell transfection and expression. To date, the most common way to express more than one protein in a single vector is through the use of internal ribosomal entry sites (IRESs) [238],however, downstream genes are often expressed at substantially lower level than the upstream gene [253]. Another problem with the use of IRESs is their relatively large size, in the range of 500 nucleotides which can be an important consideration when using viral vectors with limited cloning capacity

such as adeno-associated virus vectors [254-255]. Even though, efforts have been tried to address the first of the above issues [256], the other problems are harder to address [257].

The use of tandem cDNAs are separated by 18-22 amino acid viral sequences, referred to as 2A peptide in multicistronic constructs has emerged as an attractive alternative to the IRES [239] . Compared with the aforementioned method, the 2A system has the following advantages: (1) Small size [257], (2) Abundant number of sequences [239], (3) High degree of coordinate expression [258], (4)The use of 2A peptides allows for stiochimetric expression of multiple proteins [238]. (5) It only requires a single round of nuclear transfer and saves significant time and money [259].

The 2A peptide efficiently mediated the co-translational cleavage of artificial polypeptides by inserting it between the coding sequences of two reporter genes [260]. Subsequently, 2A system has demonstrated its function in cells in a wide variety of eukaryotes, ranging from yeast to plants, insects and mammals [261]. The 2A peptide was first shown to work *in vivo* in the rat brain with recombinant adeno-associated viruses containing 2A dependent vectors [258]. To date, 2A technology has been known to be robust in generation of long-term, stable expression in both plant and animal system [262]. Recently, Deng *et al*. [259] reported that the strategy using 2A peptide successfully poroduced the multi-transgenic pigs and confirmed uniformly co-expression.

In this study, shTNFRI-Fc and HA hHO-1 expressing double transgenic pigs were generated by SCNT using 2A system. The expression of shTNFRI-Fc and

HA hHO-1 was confirmed on tail biopsies in all transgenic piglets expressed in their tail tissue and blood serum. The major tissues including the heart, liver, lung, spleen and kidney and pancreas were collected from two transgenic piglets for expression analysis of the shTNFRI-Fc and HA hHO-1 proteins. Although two transgenes were well expressed in most organs, the expression levels were varied. Expression levels in heart, lung and the kidney were higher than in other tissues. Due to the high activity of the β-actin promoter-based CAG promoter in heart, which are rich in actin filaments. These observations are consistent with previous studies heart [263].

In conclusion, multi-transgenic pigs co-expressing shTNFR1- Fc and HA hHO-1 were successfully produced using 2A peptide and this study will pave the way for multi-transgenic pigs generated with a single nuclear transfer for xenotransplantation.

PART III.

SYNTHESIS OF MULTILAYERED ALGINATE MICROCAPSULES FOR RELEASE OF EXENDIN-4 TO TREAT TYPE1 DIABTES

Synthesis of multilayered alginate microcapsules for release of exendin-4 to treat type 1 diabetes mellitus

1. Introduction

In recent years, advances have been made in the use of islet transplantation as a treatment for T1D owing to immunosuppressive drugs [264-265]. Even though, clinical islet transplantation has shown promise, routine application is hindered because patients must be treated with diverse immune-suppressants and large volumes of islets are required for efficacy [266-267]. The apply immune-suppression by semi-permeable membranes to protect donor against antibodies and cytotoxic cells in the host immune system suggests an attractive means to overcome the requirement for immune-suppression [83]. Microencapsulation of cells in alginate-based capsules by Lim and Sun [268] is the most commonly applied procedure for immune-isolation.

Although the use of alginate-based capsule provides certain immune-isolation to the encapsulated islets, the encapsulated islet transplantation is still challenged under the sophisticated host immune response and fails to function normally in the long term. The failure of exclusion barriers is mainly caused by reactive oxygen species (ROS), hypoxia and small cytotoxic cytokines. Therefore, recent studies are focused on the synthesis of bioactive encapsulation rather than unmodified alginate, which can protect encapsulated islets from the damage by hypoxia [101,103,106] and cytotoxic cytokines [111].

Several therapeutic agents have been proposed to increase the insulinotropic

effect of pancreatic β cells [269], to increase β cell mass [270-271] and to actively protect them from apoptosis [272]. Among these therapeutic agents, GLP-1 has been extensively studied both on its glucose-dependent insulinotropic and antiapoptotic eggects to islet cells [273-275] The incretin hormone glucagon-like peptide-1(GLP-1) is a 30 amino acid peptide that is sec L cells in response to nutrient ingestion [276]. This peptide and its long-acting receptor agonist, exendin-4 promote multiple regulatory actions both on pancreatic islet and in peripheral tissues which contribute to metabolic adaptation to meal ingestion and overall glucose homeostasis [277]. In addition, GLP-1 has been shown to enhance β cells in rodents, though inhibition of β cell apoptosis and stimulation of β cell proliferation and islet neogenesis [273,278-279].

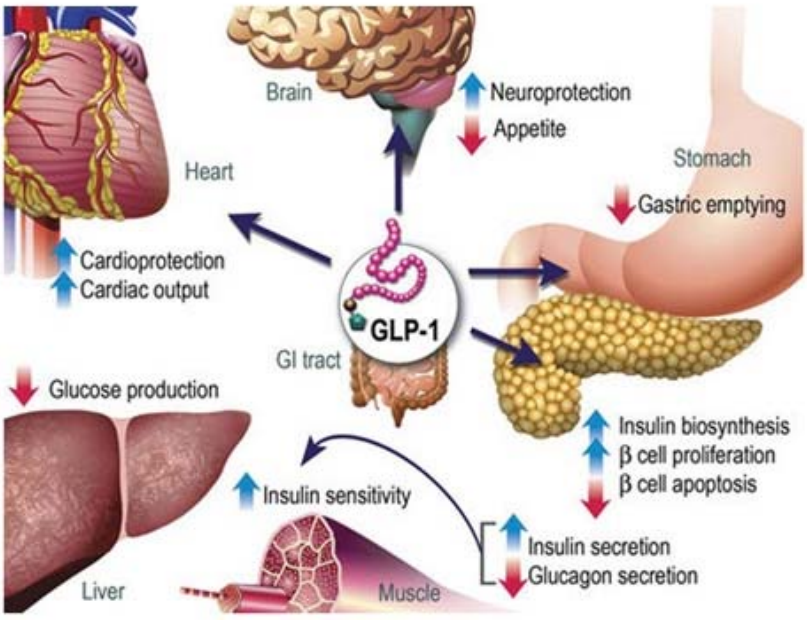

Figure 11. Action on GLP-1 receptors in different organs and tissues (Rev Diabet Stud 2008;5:73-94).

GLP-1 has a bioactive site located at the N-terminus of peptide (His-Ala) and its inactivation is caused by the removal of the peptide's first two bioactive residues by dipeptidyl peptidase [276,280]. GLP-1 has an extremely short half-life less than 2 min *in vivo* and not suitable for clinical application [281]. Exendin-4, long-acting and potent agonist's gloucose-dependent insulinotropic and antiapoptotic effects to islet cell has been extensively studied [282-284].

Here, I aimed to fabricate exdenin-4 functionalized alginate and exendin-4 release from PLL promote the survival and functions of the encapsulated islets *in vitro*.

2. Materials and Methods

2.1 Materials

All chemicals and reagents used in this study for isolation, culture and encapsulation of neonatal pancreatic cell clusters (NPCCs) in this study were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), unless otherwise stated.

2.2 NPCCs isolation

One to three-day-old neonatal pigs were used as pancreatic donors. NPCCs were isolated according to previously established methods [285]. Briefly, the piglets were intramuscularly administered with 0.1 mg/kg azaperon (stresnil, Janssen, Bruxelles, Belgium), 125 mg/kg tiletamine hydrochloride and zolazepam hydrochloride (zoletil, Virbac, Carros, France), sequentially. The piglets underwent laparotomy to expose the pancreas by midline incision. The pancreas was then carefully dissected from its surrounding pylorus, duodenum and arteries. Particular care was administered to avoid bacterial contamination which is especially induced by bowel nicking. The pancreas was cut into small pieces (2 mm^3) and washed in Hank's balanced salt solution (HBSS). The tissue was then digested using 2.5 mg/mL collagenase V (Roche Diagnostics, S.p.A., Italy) for 7 to 10 min in a 37°C distilled water (DW) bath until its color appeared milk-like. The digested pellets were centrifuged at 200 g for 2 min and the pellets subsequently washed twice in HBSS supplemented with 100 U/mL penicillin, 0.1 mg streptomycin (Invitrogen, Carlsbad, USA), 0.25% bovine serum albumin fraction V (BSA) and 12.5 mM HEPES. Finally the tissue was resuspended in Hams-F10 supplemented with 0.5% BSA fraction V, 50 mM 3-isobutyl-1-methylxanthine (IBMX), 10 mM nicotinamide, 2 mM L-glutamine, 100 U/mL

penicillin and 0.1 mg/mL streptomycin and was plated in 100 × 15 mm Petri dishes. The culture medium was changed every 48 h.

2.3 Multilayer microcapsule synthesis

The NPCCs were cultured *in vitro* and then harvested by centrifugation and resuspended in a sodium alginate solution, resulting in a final concentration of 2% (w/v) in saline. The alginate solution containing NPCC was gently pipetted several times until the islets were homogeneously dispersed, and the air bubbles inside the solution were removed by centrifugation. Droplets were hardened via crosslinking in 110 mM $CaCl_2$ solution for 5 min and washed twice with saline. The poly-L-lysine (PLL) layer was generated by transferring the microcapsules to a 0.1% (w/v) solution of PLL in normal saline and gently mixed, in resulting in the formation of a PLL coating on the microcapsules. Microcapsules were washed three times with 22 mM $CaCl_2$ and 0.9% NaCl solution. The PLL-coated microcapsules were transferred into alginate solutions of varying concentration and viscosity. The composition of the outer layer was varied from 1, 1.5 to 2% for low viscosity alginate and high viscosity alginate. 5 U/mL heparin and exendin-4 were incorporated into the outer layer by adding to the outer alginate solution prior to addition of microcapsules.

2.4 Exendin-4 release

For release studies, the microcapsules were incubated in 2 mL medium (Hams-F10) in 6-well plates on a rotational shaker at 37 °C for 7 d. Half the medium was replaced daily and the amount of released exendin-4 into the medium was determined by an Exendin-4 EIA kit (EK-070-94, Phoenix pharmaceuticals,

CA, USA) according to the manufacturer's instructions.

2.5 *In vitro* function test

Encapsulated NPCCs were cultured *in vitro* and examined for insulin release. Briefly, the encapsulated NPCCs were tested at day 7 for their insulin secretion in response to glucose stimulation. Selected NPCCs were sequentially incubated with 2.8 and 20 mM glucose for 2 h, respectively. The supernatants were retrieved at the end of each incubation period and stored at -20°C. The insulin content in the supernatants was measured using an electrochemiluminescence immunoassay (Elecsys insulin reagents kit, Roche Diagnostics, Mannheim, Germany) with an automated Roche Modular Analytics E170 (Roche Diagnostics). The stimulation index (SI) represents the capacity for insulin release and was calculated as the ratio of stimulated insulin to basal insulin (response to 20 mM glucose/response to 2.8 mM glucose).

2.6 Inflammatory stimulation and apoptosis assay

Encapsulated NPCCs were cultured for another 7 days. The resistance of NPCCs against apoptotic damage after challenging with 20 ng/mL recombinant human TNFα and 20 μg/mL cyclohexamide for 30 min were determined with Alexa fluor 488 annexin V/Dead cell apoptosis Kit (Invitrogen V13245, CA, USA) following the manufacturer's instruction. Briefly, 5 μl of PE–annexin V conjugate and 5 μl of 7-AAD (BD Bioscience, CA, USA) or 5μl of PI was added to each sample tube and incubated for 15 min at room temperature in the dark. After that, 400 μl of binding buffer was added to each sample tube and the cells were analyzed by flow cytometry (FACS Calibur; BD Biosciences, CA, USA).

2.7 Realtime PCR

After 7 days culture, total RNA was isolated from NPCCs using the easy-spin™ (DNA free) Total RNA Extraction Kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions. cDNA was produced from 1 μg of total RNA using a SuperScript™ III First-Strand cDNA Synthesis Kit (Invitrogen) primed with oligonucleotide-dT (18mer) and followed by RNase H digestion of RNA, in a total volume of 20 μl as per the manufacturer's instructions. Quantitative real time PCR was performed in a 7300 Real Time PCR System (Applied Biosystems, Foster, USA) using the SYBR premix ExTaq perfect Real Time (TAKARA Bio Inc., Shiga, Japan) with little modification. For each sample, four replications were performed. Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 12.

Table 12. Sequence specific primers for quantitive reverse transcription polymerase chain reaction

Gene name	Primer sequences (5'-3')	Length (bp)	Gene ID
PDX1	F: AGTTTACGGGGAAAGTGGTT	102	213983078
	R: CAGACCCTGAAACAAGCAAT		
GLUT2	F: TTCTCTTTGCTGGAGTGGTC	118	148236168
	R: GCTGAGCCACTCTTCTTTTG		
BCL2L1	F: TGGTGGTTGACTTTCTCTCC	139	AF216205
	R: ATTGATGGCACTAGGGGTTT		
BAX	F: GCCGAAATGTTTGCTGACGG	152	AJ606301
	R: CGAAGGAAGTCCAGCGTCCA		
ACTB	F: GTGGACATCAGGAAGGACCTCTA	137	U07786
	R: ATGATCTTGATCTTCATGGTGCT		

2.8 Statistical analyses

All data were subjected to one-way ANOVA, followed by Tukey's test using Prism version 4.0 (Graphpad Software, San Diego, USA) to determine the experimental group differences. The statistical significance was determined when the P value was <0.05.

3. Results

3.1 Release of exendin-4 from the outer alginate layer

Varied concentration and viscosity of alginate microcapsules loaded with exendin-4 were fabricated in order to evaluate whether there is effect on exendin-4 release upon viscosity and concentration of alginate. Figure 1 depicts the cumulative release profiles of exendin-4 from the capsules, either high viscosity or high concentration. The data shows a first-order kinetics curve and a high burst release was observed during first 2 h, followed by a much lower rate of exelndin-4 release. Collectively the results show high concentration and high viscosity of alginate deliver high level of exendin-4 (Figure 12).

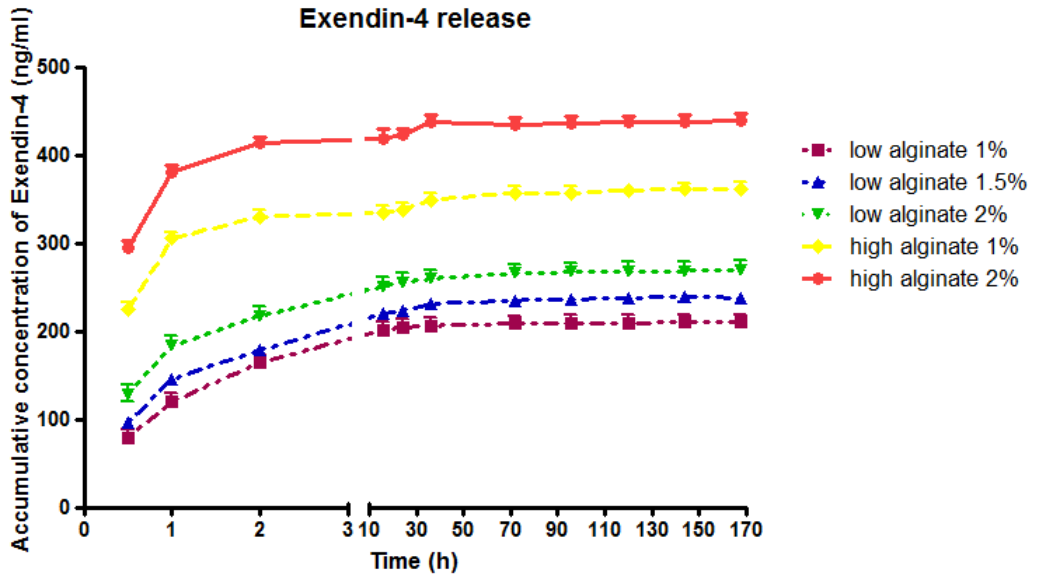

Figure 12. Release of exendin-4 from the outer alginate layer varied based on the concentration and viscosity of alginate. The values for the different superscripts are significantly different ($P<0.05$).

3.2 Insulin release respond to glucose stimulation

The insulin secretion of the islets in microencapsule loaded with exendin-4 or without it was investigated by measuring the glucose-stimulated insulin secretion with 2.8 and 20 mM glucose (Fig. 7). In terms of insulin secretion, the encapsulated islets secreted insulin in response to glucose in static culture medium. The NPCCs in microencapsules loaded with exendin-4 (1.47±0.06) showed a significantly higher SI than that of the NPCCs in microencapsules (1.30±0.03) in response to glucose stimulation at day 14 after encapsulation (Figure 13).

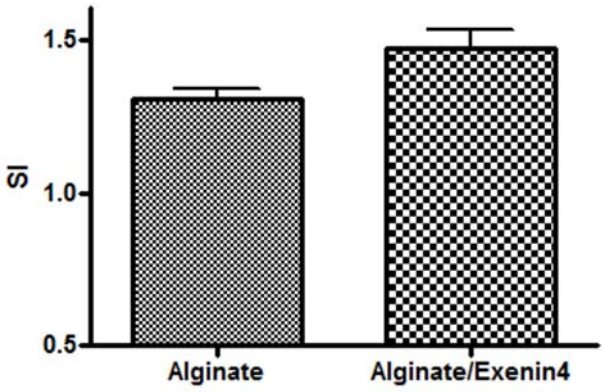

Figure 13. Insulin release respond to glucose stimulation in alginate capsules and exendin-4 releasing capsules.

The insulin amounts that were secreted from the NPCCs in response to glucose concentration changes ranging from 2.8 to 20 mM were determined by electrochemiluminescence immunoassay analyses. The assays were replicated six times. The values for the different superscripts are significantly different ($P<0.05$).

3.3 Effects of exendin-4 release against anti-inflammatory cytokine induced apoptosis

To define the anti-inflammatory effects against inflammatory cytokines-induced cell death, islets were exposed to 20 ng/mL recombinant human TNFα and 20 μg/mL cyclohexamide for 30 min. An Annexin V-FITC/PI quantification demonstrated that TNFα and CHX induced islet cell death mediated by apoptosis and that exendin-4 released from capsule protected islet cells from TNFα and CHX-induced apoptosis (Figure 14).

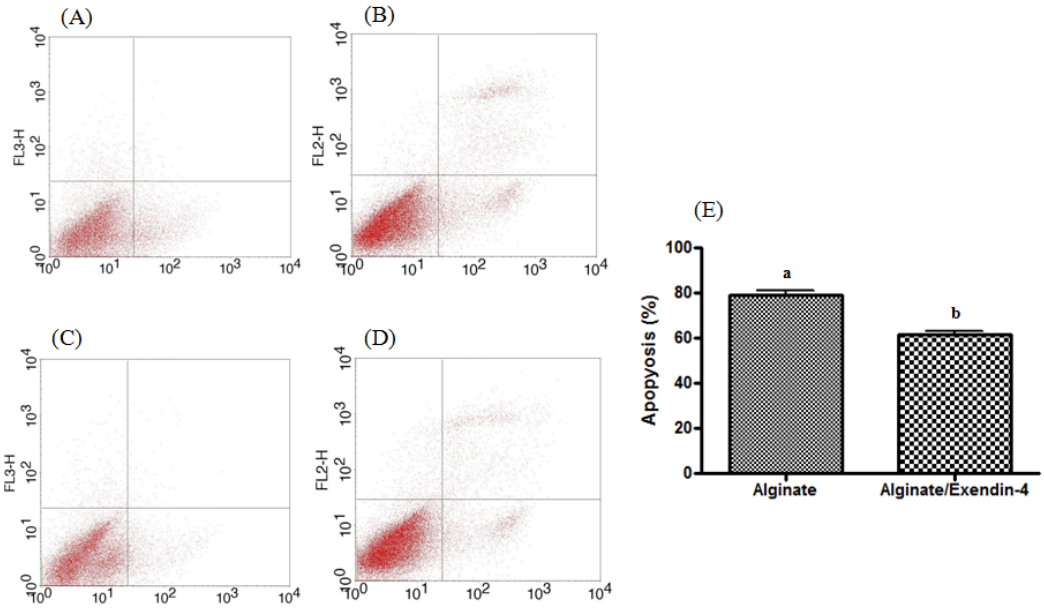

Figure 14. Effects of exendin-4 release from alginate capsule on anti-inflammatory cytokine induced apoptosis in NPCC. (A) Encapsulated islet alone (B) encapsulated islet+TNFα+CHX (C) Exendin-4 releasing encapsulated islet alone (D) Exendin-4 releasing encapsulated islet +TNFα+CHX (E) Quantification of apoptotic cells. The values for the different superscripts are significantly different ($P<0.05$).

3.4 Gene expression profile of NPCC in exendin-4 releasing alginate capsule.

To test whether the effects of exendin-4 release from microcapsule on gene expression during the *in vitro* culture of NPCC, the expression levels of key regulator of insulin gene expression (PDX1), glucose transporter genes (GLUT2) and apoptosis-related genes (BCL2L1, BAX) were studied in NPCCs in alginate microcapsule and NPCCs in alginate microcapsule loaded with exendin-4 using real-time PCR. The expression levels of PDX1, GLUT2 and BCL2L1 were significantly higher in NPCCs in alginate microcapsule loaded with exendin-4 than NPCCs in alginate microcapsule (Figure 15).

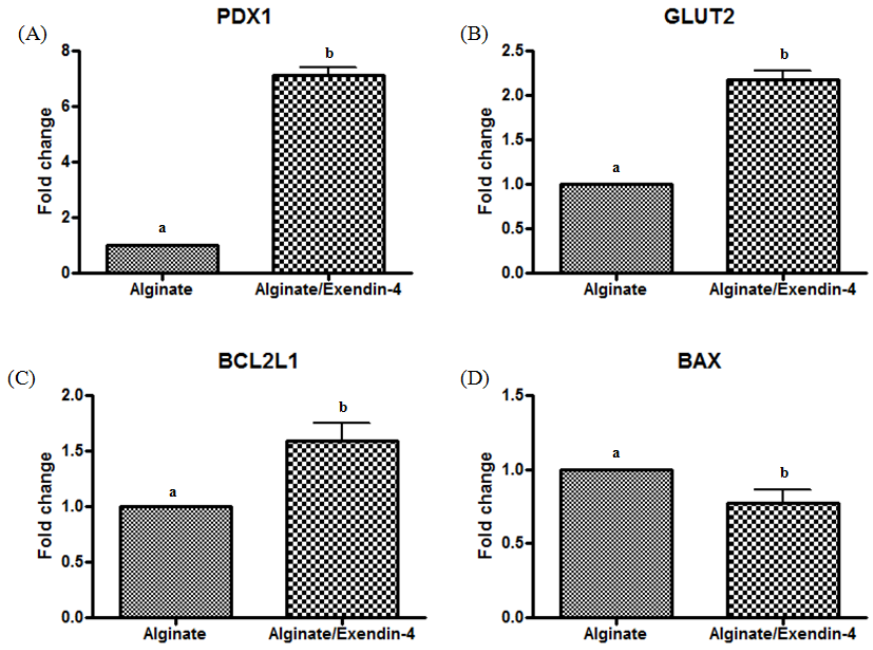

Figure 15. Gene expression profile of NPCC in exendin-4 releasing alginate capsule. Relative expression pattern of the (A) PDX1 (B) GLUT2 (C) BCL2L1 (D) BAX in encapsulated islet and Exendin-4 releasing. The values for the different superscripts are significantly different ($P<0.05$).

4. Discussion

Since the 1980s, researchers have tested a variety of designs for a bioactificial pancreas capable of replacing the endocrine function of the pancreas while preventing graft rejection due to immune response [138]. In principle, a stable biocompatible semipermeable barrier made from a variety of natural and synthetic materials should separate the tissue graft from the host's immuno effectors both cellular and humoral, whereas allowing for proper diffusion of nutrients and metabolic waste and therapeutic cell products [286].

Even though encapsulation could prevent immune reaction by the direct antigen presentation pathway, antigen shedding from the transplanted cells and subsequent indirect pathway activation is difficult to be evaded due to permeability requirements that must be satisfied to allow nutrient influx and insulin outflux. Furthermore, proinflammatory cytokines freely diffuse through capsule, instigateing graft cellular apoptosis [287].

To address these problems, polymer functionalization seeks to confer additional immune-protective effects *in vivo* via immune-modulation of the host environment. Su *et al*. described PEG hydrogel confunctionalized with an interleukin (IL)-1β antagonist peptide and enhanced viability and function as measured by glucose-stimulated insulin secretion in MIN-6 after exposure of cytokines [111]. Lin *et al*. reported PEG-diacrylate hydrogel with an RGD adhesive peptide and tumor necrosis factor (TNF) α and murine islets decreased caspase 3/7 activity [288]. Therefore, modification to encapsulation materials how promise in enhancing islet function and prolonging graft survival once implanted in the recipient.

Recently, Lin showed a PEG-diacrylate-derived hydrogel cofunctionalized with the laminin adhesive sequence IKVAV and a glucagon-like peptide-1(GLP-1) analog modified with a carboxyl terminal cysteine group. GLP-1 has been previously described to protect islets from cytokine-induced apoptosis and enhance insulin secretion [289]

The permeselectivity of PLL and alginate seems to suggest that islet cells would be protected from molecules that contribute to rejection upon transplantation. It is observed that exendin-4 released from outlayer of capsule protect islet cells from sTNFα and IFNγ treatment in our study. The bioconjugation of exendin-4 with alginate protected from enzymatic proteolysis likely due to the physical masking of the proteins provided by the polymer [290]. The formation of the alginate hydrogel may create a local reservoir, thus providing an additional barrier for protein diffusion and release. However, the release of the exendin-4 was limited to only short-term which were not seen after 1 week.

There are several reports that exendin-4 protects insulin-secreting cells from TNFα-induced apoptosis by inhibiting JNK signaling and the consequent proapoptotic signal [291-292]. It is observed that exendin-4 released from capsule significantly reduced the percentage of cells that underwent apoptosis when NPCCs were exposed to TNFα and IFN γ. There were lower expression of BAX and higher expression of BCL2L1 in NPCCs in microencapsules loaded with exendin-4 compared with those in microencapsules. Taken together, these findings suggest that exendin-4 released from capsule protects NPCCs in capsule from inflammatory cytokines. The delivery of exendin-4 from capsule in the transplanted kidney increased insulin-positive islet cells and high expression of

PDX1. These results agree with previous studies showing the positive effect of exendin-4 on islet regeneration and proliferation in islet cell culture [293]. Pdx1 plays a key regulator of insulin gene expression, but also it is an essential for normal development of the pancreas, most probably by determining maturation and differentiation of common pancreatic precursor cells in the development [294-296].

Besides improving beta cell survival, GLP-1 is also known to improve pancreatic beta cell function [297]. It is observed that exendin-4 from the capsule was able to ameliorate insulin secretion. The observed improved glucose-induced insulin secretion when exeindin-4 was released from the capsule would be related to increased sensitivity to glucose. This interpretation is in agreement with higher expression of GLUT2 and one of the mechanisms through which GLP-1 increases glucose stimulated insulin secretion involves up-regulation of glucose-sensing elements [298].

Results herein show that the bioactive exendin-4 immobilized in alginate capsule enhances the survival of pancreatic β cell and increases insulin release from the β cells. However, further studies may be needed to extend the functionality and survivability of transplanted NPCCs in microcapsules loaded with exendin-4 in *in vivo* model.

PART IV.

ENCAPSULATION OF GENETICALLY-MODIFIED ISLET

Encapsulation of genetically-modified islet

1. Introduction

Current estimate for the prevalence of diabetes mellitus (DM) is approximately 285 billion people indicationg that 6.4% of the global population have diabetes [299]. Approximately 4 million people die each year from DM and DM causes serious complication like blindness, renal failure, ischemic heart disease. Type 1 diabetes mellitus (T1D) is a disorder characterized by targeted autoimmune destruction of a patient's β cells within the pancreatic islets of Langerhans usually diagnosed in children and young adults [300]. This affects around 240 million people in the world and it has been estimated that the costs of its complication account for 10% of the total healthcare expenditure around the world [2]. The current standard treatment for T1D is daily injections of exogenous insulin to control blood glucose level where manual insulin delivery is dictated by periodic monitoring of blood glucose levels. Given this lack of precise control, T1D patients face earlier mortality and a higher risk of angiopathic lesions, often resulting in neutropathy, nephropathy, and retinopathy [301].

Islet replacement through cellular transplantation has the promise of providing a long-term cure for T1D [138]. To date, these trials found strong improvements in metabolic control, with 57% of patients' insulin independent and 70% with measureable C-peptide levels at five years [302-303]. The immunological obstacles to xenotransplantation of islets are significant. A way to reduce the immune response after implantation of pig islet cells in the recipient is the use of transgenic animals. Promising results with TG pigs suggest that

protection from complement activation is beneficial to the survival after the graft [12]. Though clinical trial is promising, there is a need to investigate supporting devices to develop means to minimize the powerful inflammatory and immunological responses. Tissue engineering approaches which combine biomaterials and cells to fabricate have the potential to improve clinical islet transplantation outcomes by providing scaffold for enhancing islet survival and engraftment.

My strategy is the use of devices to enhance islet function, protect islet from inflammatory cytokines. Additionally, promoted islet survival was explored the transgenic islet and I explore the synergy effects of these strategies *in vitro* function of islet.

2. Materials and method

2.1 Materials

All chemicals and reagents used in this study for isolation, culture and encapsulation of neonatal pancreatic cell clusters (NPCCs) in this study were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), unless otherwise stated.

2.2 NPCCs isolation

One to three-day-old neonatal pigs were used as pancreatic donors. NPCCs were isolated according to previously established methods [285]. Briefly, the piglets were intramuscularly administered with 0.1 mg/kg azaperon (stresnil, Janssen, Bruxelles, Belgium), 125 mg/kg tiletamine hydrochloride and zolazepam hydrochloride (zoletil, Virbac, Carros, France), sequentially. The piglets underwent laparotomy to expose the pancreas by midline incision. The pancreas was then carefully dissected from its surrounding pylorus, duodenum and arteries. Particular care was administered to avoid bacterial contamination which is especially induced by bowel nicking. The pancreas was cut into small pieces (2 mm^3) and washed in Hank's balanced salt solution (HBSS). The tissue was then digested using 2.5 mg/mL collagenase V (Roche Diagnostics, S.p.A., Italy) for 7 to 10 min in a 37°C distilled water (DW) bath until its color appeared milk-like. The digested pellets were centrifuged at 200 g for 2 min and the pellets subsequently washed twice in HBSS supplemented with 100 U/mL penicillin, 0.1 mg streptomycin (Invitrogen, Carlsbad, USA), 0.25% bovine serum albumin fraction V (BSA) and 12.5 mM HEPES. Finally the tissue was resuspended in Hams-F10 supplemented with 0.5% BSA fraction V, 50 mM 3-isobutyl-1-

methylxanthine (IBMX), 10 mM nicotinamide, 2 mM L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin and was plated in 100 x 15 mm Petri dishes. The culture medium was changed every 48 h.

2.3 Generation of adenovirus containing shTNFRI-Fc, HA hHO1 and GFP

The generation of adenoviruses used in this study previously described [240]. In brief, target genes (shTNFRI-Fc-2A-HA hHO1-GFP) were inserted into the shuttle vector (pAdTrack-CMV) and linearized with *Pme*I restriction enzyme. The linearized shuttle vector was mixed adenoviral plasmid, pAdEasy-1, for homologous recombination. Isolated NPCCs were transfected with adenovirus expressing shTNFRI-Fc-2A-HA hHO1--GFP (50 MOI).

2.4 Multilayer microcapsule synthesis

The NPCCs were cultured *in vitro* and then harvested by centrifugation and resuspended in a sodium alginate solution, resulting in a final concentration of 2% (w/v) in saline. The alginate solution containing NPCC was gently pipetted several times until the islets were homogeneously dispersed, and the air bubbles inside the solution were removed by centrifugation. Droplets were hardened via crosslinking in 110 mM $CaCl_2$ solution for 5 min and washed twice with saline. The poly-L-lysine (PLL) layer was generated by transferring the microcapsules to a 0.1% (w/v) solution of PLL in normal saline and gently mixed, in resulting in the formation of a PLL coating on the microcapsules. Three times washed with 22 mM $CaCl_2$ and 0.9% NaCl solution. The PLL-coated microcapsules were transferred into alginate solutions of varying concentration and viscosity. The composition of the outer layer was varied from 1, 1.5 to 2% for low viscosity

alginate and high viscosity alginate. 5 U/ml heparin and exendin-4 were incorporated into the outer layer by adding to the outer alginate solution prior to addition of microcapsules.

2.5 *In vitro* function test

Encapsulated NPCCs were cultured *in vitro* and examined for insulin release. Briefly, the encapsulated NPCCs were tested at day 7 for their insulin secretion in response to glucose stimulation. Selected NPCCs were sequentially incubated with 2.8 and 20 mM glucose for 2 h, respectively. The supernatants were retrieved at the end of each incubation period and stored at -20°C. The insulin content in the supernatants was measured using an electrochemiluminescence immunoassay (Elecsys insulin reagents kit, Roche Diagnostics, Mannheim, Germany) with an automated Roche Modular Analytics E170 (Roche Diagnostics). The stimulation index (SI) represents the capacity for insulin release and was calculated as the ratio of stimulated insulin to basal insulin (response to 20 mM glucose/response to 2.8 mM glucose).

2.6 Inflammatory stimulation and apoptosis assay

Encapsulated NPCCs were cultured for another 7 days. The viabilith of NPCCs after challenging with 20 ng/ml recombinant human TNFα and 20 μg/mL cyclohexamide for 30 min were evaluated by trypan blue exclusion method using an automated cell counter (Invitrogen) [304].

2.7 Statistical analyses

All data were subjected to one-way ANOVA, followed by Tukey's test using Prism version 4.0 (Graphpad Software, San Diego, USA) to determine the experimental group differences. The statistical significance was determined when the P value was <0.05.

3. Results

3.1 Viability of islet cells: naïve NPCCs, encapsulated NPCCs in alginate capsule, encapsulated NPCCs in exendin-4 releasing alginate capsule and genetically modified NPCCs in exendin-4 releasing alginate capsule

Viability of all groups except naïve group did not show significant difference 7 days after encapsulation. However, the viability demonstrated significant difference among groups after inflammatory cytokine treatment. To define the anti-inflammatory effects against inflammatory cytokines-induced cell death, islets were exposed to 20 ng/mL recombinant human TNFα and 20 μg/mL cyclohexamide for 30 min. An Annexin V-FITC/PI quantification demonstrated that TNFα and CHX induced islet cell death mediated by apoptosis and that exendin-4 released from capsule protected islet cells from TNFα and CHX-induced apoptosis. Transgenic islet encapsulated in exendin-releasing capsule showed 53.3±2.2 where as the viability of naïve islet and encapsulated islet were 20.3±0.9, 21.3±1.9 (Figure 16).

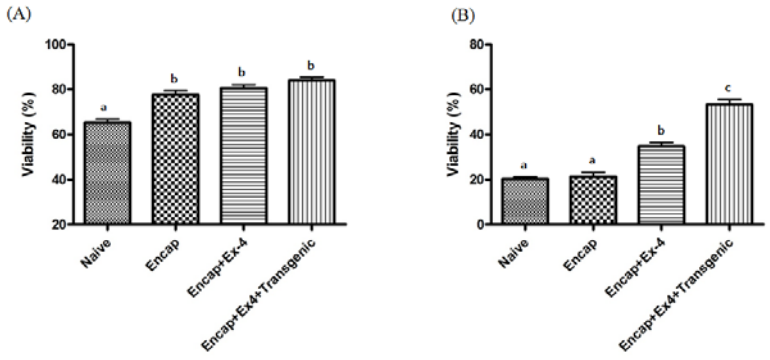

Figure 16. Viability of islet cells: naïve NPCCs, encapsulated NPCCs in alginate capsule, encapsulated NPCCs in exendin-4 releasing alginate capsule and genetically modified NPCCs in exendin-4 releasing alginate capsule. (A) viability 7 days after encapsulation (B) viability after TNFα and CHX treatment. The viability values are displayed as the average from six performed trials ($p<0.05$).

3.2 Insulin release respond to glucose stimulation

The insulin secretion of the islets in microencapsule loaded with exendin-4 or without it was investigated by measuring the glucose-stimulated insulin secretion with 2.8 and 20 mM glucose (Fig. 16). In terms of insulin secretion, the encapsulated islets secreted insulin in response to glucose in static culture medium. The GM NPCCs in microencapsules loaded with exendin-4 (2.8±0.1) showed a significantly higher SI than that of the naïve NPCCs (1.7±0.1) in response to glucose stimulation at day 14 after encapsulation (Figure 17).

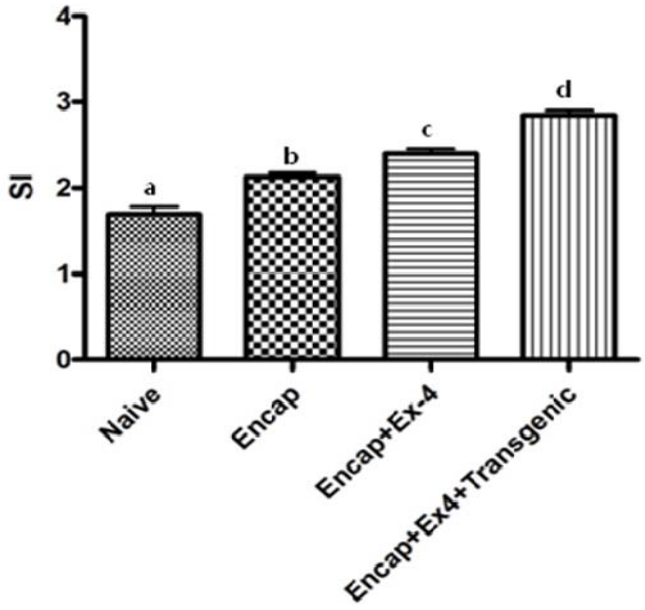

Figure 17. Insulin release respond to glucose stimulation: naïve NPCCs, encapsulated NPCCs in alginate capsule, encapsulated NPCCs in exendin-4 releasing alginate capsule and genetically modified NPCCs in exendin-4 releasing alginate capsule.The insulin amounts that were secreted from the NPCCs in response to glucose concentration changes ranging from 2.8 to 20 mM were determined by electrochemiluminescence immunoassay analyses. The values for the different superscripts are significantly different ($P<0.05$).

4. Discussion

Islet transplantation has been considered as a safer alternative than whole organ transplantation and a potentially alternative treatment to conventional exogenous-insulin therapy [305]. While significant progress has been made in the islets transplantation field, many obstacles that currently preclude its widespread application remain. The most important limitations are low tension of O_2 where the islets are implanted [2] and insufficient immunoprotection [88].

More than half of transplanted islets may be lost in the first few days and this is thought to be due to hypoxic death before vascularization develops [306]. I enclosed islets in semi-permeable membranes, which are more favorable for substance and O_2 exchange [307]. This strategy focuses at decreasing capsule size so that it may increase surface area to volume ration and in turn oxygen diffusion, which improve islet viability *in vitro*. However, implanted islets should be adequate oxygenation and real-time access to blood glucose levels by intimate vascular supply. Islets make up 2% of the pancreatic tissue yet they need over 10% of the pancreatic blood flow [308]. Vascularization develops take 7-10 days and more especially when there are already ischemic damages [103,309]. This delayed and insufficient revascularization deprives these islets of oxygen, resulting in cell death and graft failure [310]. I suggest genetic engineering to improve resistance against hypoxic damage. Heme oxygenase-1 (HO-1) is a 32 kDa ubiquitously expressed enzyme encoded by the HMOX1 gene [224]. HO-1 induced by various stimuli including oxidative stress, inflammation, hypoxia degrade heme into three end-products, carbon monoxide, free iron and biliverdin. All three end-products have anti-oxidative, anti-inflammatory and anti-apoptotic effect underlying the

role of HO-1 [225].

Despite of protection against immune cells and large antibodies in proper encapsulation system, the islets are still vulnerable to small molecules (2-30 kDa) such as chemokines/cytokines and nitric oxide (NO) [79]. It has been shown that pancreatic islets secret chemokines/cytokines upon stress [311], which can leak through the pores of the microcapsule and attract macrophages [88]. This chemotaxis can cause graft failure. Chemoattractants can activate macrophages, thereby releasing pro-inflammatory cytikines, interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) and NO [77]. An alternative to these are co-encapsulation with various agents such as erythrocytes [89] and sertoli cell [90], which release immunosuppressive factors. Another option is the use of genetic engineering of the islets to secrete anti-inflammatory molecules such as inhibitor of TNF-α [92] and IL-1 receptor antagonist [93]. In this study, I suggest genetic engineering and modified microcapsules. Soluble forms of TNFR (sTNFR) can bind to TNF receptors and inhibit TNF-induced cell activation [233]. Among two types of TNF receptors, TNF receptor I has a higher affinity to TNF-α than TNF receptor II [312] and binds to both membrane-bound and soluble forms of TNF-α. Therefore, it is generated genetic engineered islets to secret sTNFR1 Fc to neutralize TNF-α. Furthermore, it is expected that exendin-4 released from microcapsule counteracts TNF-α -mediated apoptosis [313] and interleukin-1 beta-induced apoptosis by interfering with the c-Jun NH2-terminal kinase pathway [314] , resulting in promotion of cell survival.

Significant progress has been made in islets transplantation, many problems remain to be overcome. A variety of strategies such as genetic engineering, improvement in oxygen supply and incorporating factor into the encapsulation

material improved the islet performance. This may give a future perspective to the application of immunoprotective capsules and viability in clinical practice.

PART V.

SUMMARY AND PERSPECTIVES

1. Summary

The purpose of this study was to improve porcine somatic cell nuclear transfer and microencapsulation for bio-artificial pancreas. SCNT enables more accurate gene targeting in pig genome compared to previous technology for TG pig production. Refinement of SCNT protocol may give the answer to low cloning efficiency which restricts its application. Another strategy for bio-artificial pancreas, microencapsulation has the potential to improve islet transplantation, although further improvements are needed. Extended functionality of microencapsulation would support TG islet in bio-artificial pancreas.

The present study was performed to establish and improve a procedure of SCNT technique and microencapsulation for bio-artificial pancreas. First of all, SCNT technique was improved with 1) short-term treatment of 6-DMAP and demecolcine with Thi/DTT 2) epigenetic reprogramming with oxamflatin both *in vitro* (36.7%) and *in vivo* (3.2%). With optimized SCNT technique transgenic pigs expressing hsTNFR1 Fc and HA hHO1 were produced as bio-artificial pancreas source. Islet cells in microcapsules were protected from immune cells' attack and allow O_2 supply or insulin release response to glucose with increased viability via conformal microencapsulation. Furthermore, exendin-4 leached from microcapsule 1) stimulates β cell proliferation 2) protects β cell from inflammatory cytokine-mediated apoptosis 3) increases glucose-dependent insulin secretion. Collectively, improvement of SCNT and microencapsulation would attribute to bio-artificial pancreas. However, further studies may be needed to extend the functionality and survivability of transplanted GM islets in microcapsules loaded with exendin-4 in *in vivo* model.

2. Perspectives

Despite the progress in the *in vitro* production (IVP) of pig embryos, the methodologies involved in IVP need to be improved to enhance oocyte maturation and embryo development. The increase in overall efficiency of these grounding technologies combined with advances in SCNT and freezing procedures will further allow studies on pig cloning and the establishment of a gene bank of genetically modified pigs.

Pig to human xenotransplantation faces the problem of strong rejection. Promising results using pigs expressing hCD46 [12] or LEA29Y [11] suggest that inhibition of complement activation and the T cell–mediated immune response are effective. Although biological effects have been shown for individual genetic modifications, clinical xenotransplantation will require multi-transgenic pigs transmitting effective genetic modifications in a non-segregating manner.

Alginate-based microcapsules, either alginate-poly-L-lysine-alginate (APA) cross-linked with calcium or barium are the most widely studied encapsulation systems for the generation of a bioartificial pancreas. Microencapsulated insulin producing cells in alginate is safe and optimization of encapsulation protocols may prove to be a successful approach, as promising results indicate [315-316]. However, further modifications of the microcapsules are required to prevent fibrosis completely and to become encapsulated cell-based therapies a viable option as therapy for T1D in humans. A large number of islet product-directed strategies are currently under investigation to allow minimal immunosuppressive treatment. Integrating controlled release of immunoregulatory, cytoprotective, and proangiogenic factors together with the use of bioscaffolds, microspheres and

nanoparticles [315,317] could be a key to success. Another major interest of study is the tracking of encapsulated cells in a non-invasive way to determine when microcapsules become surrounded by fibrous tissue in a certain transplant situations. Scaling-up of the manufacturing process to produce encapsulated islet and preservation until the transplantation are essential if encapsulation strategy is to entered and create therapeutic impact on people with diabetes.

The simultaneous development of suitable sources of genetically modified pig islets and more immunoprivileged environment provided by encpasulation, along with the establishment of clinically applicable immunosuppressive regimens, could be translated into tangible benefits for patients with T1D in the very near future.

국문초록

미세 피막화 이종장기 췌도를 위한 형질전환돼지 개발

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췌도 이식은 제 1형 당뇨를 치료하기 위한 기존의 인슐린 요법과 달리 저혈당 발생없이 정상혈당을 유지할 수 있다는 점에서 그리고 췌장 이식보다 시술과 관련된 합병증 및 위험성이 매우 낮다는 점에서 안전하고 획기적인 치료 방법이다. 그러나 기증자와 대기자 수의 차이로 이종장기이식이 새로운 차선책으로 제안되었다. 면역 거부 반응으로 이종장기이식의 임상적 적용에 한계가 있어 형질전환돼지나 피막화기술을 통한 바이오 인공 췌장이 임상적 적용이 가능하도록 연구되고 있다. 체세포핵이식기술은 다른 형질전환돼지 생산기술과 비교하여 보다 정확한 유전자 적중이 가능하다는 장점이 있으나 아직은 효율이 낮아 개선할 필요가 있다. 또한 피막화가 면역 세포는 막을 수 있으나 염증성 사이토카인이나 NO와 같은 작은 크기의 물질은 그대로 통과하여 면역거부

반응차단이 불완전하므로 이식 후 췌도 세포의 기능상실을 야기시키므로 이를 개선할 필요가 있다.

형질전환돼지 생산 효율을 향상시키기 위하여 체세포 핵이식 기술의 난자 활성화와 리프로그래밍 과정의 개선을 위한 연구가 진행되었다. 6-DMAP과 demecolcine의 단기 및 장기 처리의 효과를 비교하였고 전기 및 thimerosal/dithiothreitol 기반의 난자 활성화의 효과를 난자 발달율을 통해 비교하였다. 그 후 HDACi인 oxamflatin을 이용하여 정상적인 수정에서와 같이 형질전환된 체세포가 리프로그래밍되어 하나의 완전한 개체로 태어날 수 있는지 그리고 배아의 체외성숙율을 비교하였다. 이렇게 향상된 난자활성화와 리프로그래밍 방법을 이용하여 췌도 이식을 목적으로 하는 형질전환돼지를 생산하였다. 그 후 피막화 기술의 불완전한 면역거부반응 차단을 개선하기 위하여 GLP-1 유사체의 일종인 exendin-4를 캡슐에서 서서히 분비되어 캡슐 안의 췌도 세포가 염증성 사이토카인의 공격으로부터 보호받을 수 있도록 제작하였고 보호 기능을 평가하였다. 또한 형질전환된 췌도를 앞서 개발한 exendin-4가 분비되는 피막화하여 생존능과 포도당에 반응하여 인슐린을 분비하는 기능이 향상되는지 평가하였다.

그 결과 2 mM 6-DMAP과 0.4 μg/mL demecolcine을 0.2 mM의 thimerosal과 함께 10분간 처리 후 8 mM dithiothreitol에 30분간 처리한 그룹에서 배아의 체외발달율이 유의적으로 증가하였다. 또한 1 μM의 oxamflatin 처리가 epigenetic status를 교정하고 pluripotency 및 항 세포사 관련 유전자의 발현 증가를 통하여 배아의 체외 발달율이

향상하였고 형질전환복제돼지 생산율도 유의적으로 증가한 것을 확인하였다. 또한 위 방법을 이용하여 췌도 이식을 목적으로 한 shTNFR1 Fc와 hHO1가 발현되는 형질전환복제돼지를 생산하는데 성공하였다. 한편 exendin-4가 피막에서 분비되어 피막화된 췌도를 염증성 사이토카인의 공격으로부터 보호하는 것을 확인하였다. shTNFR1 Fc와 hHO1이 발현하는 췌도 세포를 exendin-4가 피막에서 분비되록 피막화하였더니 생존율과 인슐린 분비능이 유의적으로 증가하는 것을 확인하였다.

결론적으로 바이오 인공췌장을 만들기 위한 체세포 핵이식 기술과 피막화 기술이 확립되었고 이전보다 효율 및 기능이 향상되었다.

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주요어: 돼지, 형질전환, 체세포 핵이식, 피막화, 이종장기이식, 인공췌장
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PUBLICATION LISTS

PUBLICATION PAPERS

1. **SJ Park**, OJ Koo, DK Kwon, Gomez MN, JT Kang, Atikuzzaman M, SJ Kim, G Jang, BC Lee, Short-term treatment with 6-DMAP and demecolcine improves developmental competence of electrically or Thi/DTT-activated porcine parthenogenetic embryos, Zygote. 2011; 19(1):1-8.
2. **SJ Park**[1], S Shin[1], OJ Koo, JH Moon, G Jang, C Ahn, BC Lee, YJ Yoo, Functional improvement of porcine neonatal pancreatic cell clusters via conformal encapsulation using an air-driven encapsulator, Exp Mol Med. 2012; 44(1):20-5 ([1]Both authors are contributed equally)
3. **SJ Park**, HJ Park, OJ Koo, WJ Choi, JH Moon, DK Kwon, JT Kang, SJ Kim, JY Choi, G Jang, BC Lee, Oxamflatin improves developmental competence of porcine somatic cell nuclear transfer embryos, Cellular reprogramming, 2012; 14: 398-406
4. **SJ Park**, B Cho[1], JT Kang, JH Moon, SJ Kim, JY Choi, HJ Yeom, JI Hwang, EM Lee, SH Hur, JH Hong, J Yang, BC Lee, C Ahn, Production and characterization of human shTNFRI-Fc and hHO-1 double transgenic pigs using 2A peptide, (manuscript prepared/[1]Both authors are contributed equally)
5. **SJ Park**, S Shin, OJ Koo, JY Choi, J Yang, G Jang, YJ Yoo, C Ahn, BC Lee, Release of exendin-4 from multilayered alginate microcapsules protect islet cells (manuscript prepared)
6. DK Kwon, JT Kang, **SJ Park**, Gomez MN, SJ Kim, Atikuzzaman M, OJ Koo, G Jang, BC Lee, Blastocysts derived from adult fibroblasts of a rhesus monkey (Macaca mulatta) using interspecies somatic cell nuclear transfer, Zygote. 2011; 19(3):199-204.
7. M Atikuzzaman, OJ Koo, JT Kang, DK Kwon, **SJ Park**, SJ Kim, M N Gomez, HJ Oh, SG Hong, G Jang, BC Lee, The 9-cis retinoic acid signaling pathway and its regulation of prostaglandin-endoperoxide synthase 2 during *in vitro* maturation of pig cumulus cell-oocyte

complexes and effects on parthenogenetic embryo production, Biol Reprod. 2011; 84(6):1272-81

8. HJ Yeom[1], R Han[1], **SJ Park**, JH Hong, B Cho, H Kim, SJ Kim, JI Hwang, BC Lee, C Ahn, J Yang, Adenovirus-mediated heme oxygenase-1 gene transfer to neonatal porcine islet-like cluster cells: the effects on gene expression and protection from cell stress, BioChip J, 2012; 6(1): 56-64, ([1]Both authors are contributed equally)
9. JH Moon, SJ Kim, HJ Park, JT Kang, **SJ Park**, OJ Koo, da Torre BR, Saadeldin IM, BC Lee, G Jang, Production of porcine cloned embryos derived from cells conditionally expressing an exogenous gene using Cre-loxP, Zygote. 2012; 25:1-3 (In press).
10. JT Kang , Atikuzzaman M, DK Kwon, **SJ Park**, SJ Kim, JH Moon, OJ OJ Koo, G Jang, BC Lee, Developmental competence of porcine oocytes after *in vitro* maturation and *in vitro* culture under different oxygen concentrations, Zygote. 2012; 20(1):1-8
11 MN Gomez, JT Kang, OJ Koo, SJ Kim, DK Kwon, **SJ Park**, Atikuzzaman M, SG Hong, G Jang, BC Lee., Effect of oocyte-secreted factors on porcine *in vitro* maturation, cumulus expansion and developmental competence of parthenotes, Zygote. 2012; 20(2): 135-45.
12. I M Saadeldin, OJ Koo, JT Kang, DK Kwon, **SJ Park**, SJ Kim, JH Moon, HJ Oh, G Jang, BC Lee, Paradoxical effects of kisspeptin: it enhances oocyte *in vitro* maturation but has an adverse impact on hatched blastocysts during *in vitro* culture, Reprod Fertil Dev. 2012; 24(5): 656-68
13. HJ Yeom[1], OJ Koo[1], J Yang, B Cho, JI Hwang, **SJ Park**, S Hurh, H Kim, EM Lee, H Ro, JK Won, SJ Kim, H Kim, C D Surh, BC Lee, C Ahn, Generation and Characterization of Human Heme Oxygenase-1 Transgenic Pigs, Plosone,2012; 7: e46646
14. SJ Kim, JH Moon, B R da Torre, JT Kang**, SJ Park**, JY Choi, BC Lee, G Jang, DsRed gene expression by doxycycline in porcine fibroblasts and cloned embryo using transposon, Zygote, 2012, submission
15. JY Choi, JT Kang, **SJ Park**, SJ Kim, JH Moon, I M. Saadeldin, G Jang, BC Lee, Effect of 7,8-Dihydroxyflavone as an antioxidants on *In Vitro* Maturation of Oocytes and Development of Parthenogenetic Embryos in Pigs, Reproduction, Fertility and Development, 2012, submission

ABSTRACTS and PRESENTATIONS

1. **SJ Park**, OJ Koo, Dae-Kee Kwon, Hee-Jung Park and Byeong-Chun Lee, Short term electrical-chemical parthenogenetic activation for *in vitro* matured porcine oocytes, Conference of the Korean Society of Veterinary Science,2008
2. **SJ Park**, OJ Koo, DK. Kwon, HJ. Park, SS. Lee, YH Nam, BC Lee, Short term treatment of 6-DMAP and demecolcine improve developmental competence of porcine parthenogenetic embryos, Annual Meeting of International Embryo Transfer Society, 2009
3. **SJ Park**, OJ Koo, DK Kwon, M. N Gomez. , JT Kang, M Atikuzzaman, SJ Kim, G Jang, BC Lee, Optimization of artificial activation in porcine embryos using short-term treatment of 6-DMAP and demecolcine, Annual Conference of society for the study of Reproduction, 2009
4. **SJ Park,** OJ Koo, DK Kwon, M. N Gomez, JT Kang, M Atikuzzaman, SJ Kim, Goo Jang and Byeong Chun Lee, Optimization of producing transgenic animals for pancreatic islet xenotransplantation, International Congress of the Immunology of Diabetes Society, 2010
5. **SJ Park**, HJ Park, OJ Koo, DK Kwon, JT Kang, SJ Kim, JH Moon, JY Choi, G Jang, BC Lee, Oxamflatin improves in developmental competence of somatic cell nuclear transfer porcine embryos, Swine in Biomedical Research conference, 2011
6. **SJ Park**, S Shin, OJ Koo, JY Choi, JH Moon, YJ Yoo , G Jang ,C Ahn, BC Lee, Synthesis of multilayered alginate microcapsules for release of exendin-4 to treat type 1 diabetes mellitus, CTS-IXA, 2011
7. **SJ Park**, S Shin, OJ Koo, JY Choi, JH Moon, YJ Yoo , G Jang ,C Ahn, BC Lee, Release of exendin-4 from multilayered alginate microcapsules protect islet cells after transplantation, TERMIS, 2012
8. JT Kang, OJ Koo, DK Kwon, **SJ Park**, M. Atikuzzaman, M N Gomez, G Jang , BC Lee, Effects of melatonin on preimplantation development of porcine parthonogenetic embyos, International Embryo Transfer Society, 2010
9. DK Kwon, JT Kang, **SJ Park**, M N.Gomez, SJ Kim, M. Atikuzzaman, OJ Koo, G Jang, BC Lee, Blastocysts derived from adult fibroblasts of rhesus monkey(MACACA MULATTA) using interspecies somatic cell

nuclear transfer, International Embryo Transfer Society, 2010

10. SJ Kim, OJ Koo, **SJ Park**, JH Moon, DK Kwon, JT Kang, M N Gomez, M Atikuzzaman, BC Lee, G. Jang, Inducible red fluorescent protein (RFP) expression in porcine fibroblasts and transgenic cloned embryos using piggyback transposition, International Embryo Transfer Society, 2010
11. OJ Koo,JT Kang, DK Kwon, **SJ Park**, SJ Kim, G Jang, JI Hwang, J Yang, C Ahn, BC Lee, Production of multiple gene transgenic pig for xenotransplantation of pancreatic islet, International Congress of the Immunology of Diabetes Society, 2010
12. JT Kang, M Atikuzzaman, DK Kwon, **SJ Park**, SJ Kim, JH Moon, M N Gomez, OJ Koo, G Jang, BC Lee, Developmental competence of porcine oocytes after *in vitro* maturation and *in vitro* culture under comparative oxygen tension, Conference of the Korean Society of Veterinary Science, 2010
13. OJ Koo, DW Lee, JT Kang, DK Kwon, HJ Park, **SJ Park**, SJ Kim, G Jang, BC Lee, Selection of early cleaved embryos and optimal recipients to optimize pig cloning, The 10th international symposium on developmental biotechnology, 2010
14. SJ Kim, OJ Koo, DK Kwon, JT Kang, HJ Park, **SJ Park**, M Na Gomez, M Atikuzzaman, G Jang, BC Lee, The 10th international symposium on developmental biotechnology, 2010
15. JT Kang, DK Kwon, **SJ Park**, SJ Kim, JH Moon, BC Lee, Quercetin improges *in vitro* development of porcine oocytes by acting as an antioxidant, Swine in biomedical research, 2011
16. I M Saadeldin, OJ Koo, JT Kang, DK Kwon, **SJ Park**, SJ Kim, JH Moon, OJ Oh, BC Lee, Kisspeptin enhances porcine oocyte *in vitro* maturation but may adversely affect early embryonic ,44tn annual meeting of the Society for the Study of Reproduction, 2011
17. JT Kang, DK Kwon, **SJ Park**, SJ Kim, JH Moon, I M. Saadeldin, OJ Koo, BC Lee ,Quercetin improves *in vitro* development of porcine oocytes by decreasing reactive oxygen species levels, 44tn annual meeting of the Society for the Study of Reproduction, 2011
18. I M. Saadeldin, JT Kang, **SJ Park**, SJ Kim, JH Moon, BC Lee ,Using of porcine granulosa cells as feeders for porcine and bovine trophectoderm

cell culture, International Embryo Transfer Society, 2011

19. JY Choi, JT Kang, **SJ Park**, SJ Kim, JH Moon, I M. Saadeldin, TW Kim, G Jang, BC Lee, 7,8-Dihydroxyflavone improves *in vitro* development of porcine oocytes/embryos by decreasing reactive oxygen species levels, 44tn annual meeting of the Society for the Study of Reproduction, 2012
20. B Cho, **SJ Park**, H Kim, JT Kang, EM Lee, JH Moon, EW Lee, SJ Kim, JY Choi, J Yang, K Bang, BC Lee, C Ahn, Generation of soluble human TNFRI-Fc/hHO-1 double transgenic pigs using 2A self-cleavage peptide and their characterization, TTS, 2012
21. JY Choi, JT Kang, **SJ Park**, SJ Kim, JH Moon, I M. Saadeldin, G Jang, BC Lee, 7,8-Dihydroxyflavone enhances development of oocytes and parthenogenetic embryos in pigs, Conference of the Korean Society of Veterinary Science, 2012

REFERENCES

[1] Gan MJ, Albanese-O'Neill A, Haller MJ. Type 1 diabetes: current concepts in epidemiology, pathophysiology, clinical care, and research. Curr Probl Pediatr Adolesc Health Care 2012;42:269-91.
[2] Souza YE, Chaib E, Lacerda PG, Crescenzi A, Bernal-Filho A, D'Albuquerque LA. Islet transplantation in rodents. Do encapsulated islets really work? Arq Gastroenterol 2011;48:146-52.
[3] Klymiuk N, Aigner B, Brem G, Wolf E. Genetic modification of pigs as organ donors for xenotransplantation. Mol Reprod Dev 2010;77:209-21.
[4] Prabhakaran S, Hering BJ. What strain of pig should be used? Xenotransplantation 2008;15:83-6.
[5] Koulmanda M, Qipo A, Smith RN, Auchincloss H, Jr. Pig islet xenografts are resistant to autoimmune destruction by non-obese diabetic recipients after anti-CD4 treatment. Xenotransplantation 2003;10:178-84.
[6] Potter KJ, Abedini A, Marek P, Klimek AM, Butterworth S, Driscoll M, Baker R, Nilsson MR, Warnock GL, Oberholzer J, Bertera S, Trucco M, Korbutt GS, Fraser PE, Raleigh DP, Verchere CB. Islet amyloid deposition limits the viability of human islet grafts but not porcine islet grafts. Proc Natl Acad Sci U S A 2010;107:4305-10.
[7] Kirchhof N, Shibata S, Wijkstrom M, Kulick DM, Salerno CT, Clemmings SM, Heremans Y, Galili U, Sutherland DE, Dalmasso AP, Hering BJ. Reversal of diabetes in non-immunosuppressed rhesus macaques by intraportal porcine islet xenografts precedes acute cellular rejection. Xenotransplantation 2004;11:396-407.
[8] Cantarovich D, Blancho G, Potiron N, Jugeau N, Fiche M, Chagneau C, Letessier E, Boeffard F, Loth P, Karam G, Soulillou JP, Le Mauff B. Rapid failure of pig islet transplantation in non human primates. Xenotransplantation 2002;9:25-35.
[9] Hering BJ, Wijkstrom M, Graham ML, Hardstedt M, Aasheim TC, Jie T, Ansite JD, Nakano M, Cheng J, Li W, Moran K, Christians U, Finnegan C, Mills CD, Sutherland DE, Bansal-Pakala P, Murtaugh MP, Kirchhof N, Schuurman HJ. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. Nat Med 2006;12:301-3.
[10] Cardona K, Korbutt GS, Milas Z, Lyon J, Cano J, Jiang W, Bello-Laborn H, Hacquoil B, Strobert E, Gangappa S, Weber CJ, Pearson TC, Rajotte RV, Larsen CP. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. Nat Med 2006;12:304-6.
[11] Klymiuk N, van Buerck L, Bahr A, Offers M, Kessler B, Wuensch A, Kurome M, Thormann M, Lochner K, Nagashima H, Herbach N, Wanke R, Seissler J, Wolf E. Xenografted islet cell clusters from INSLEA29Y transgenic pigs rescue diabetes and prevent immune rejection in humanized mice. Diabetes 2012;61:1527-32.
[12] van der Windt DJ, Bottino R, Casu A, Campanile N, Smetanka C, He J, Murase N, Hara H, Ball S, Loveland BE, Ayares D, Lakkis FG, Cooper DK, Trucco M. Long-term controlled normoglycemia in diabetic non-human primates after transplantation with hCD46 transgenic porcine islets. Am J Transplant 2009;9:2716-26.
[13] Aigner B, Renner S, Kessler B, Klymiuk N, Kurome M, Wunsch A, Wolf E. Transgenic pigs as models for translational biomedical research. J Mol Med (Berl) 2010;88:653-64.

[14] Hammer RE, Pursel VG, Rexroad CE, Jr., Wall RJ, Bolt DJ, Ebert KM, Palmiter RD, Brinster RL. Production of transgenic rabbits, sheep and pigs by microinjection. Nature 1985;315:680-3.
[15] Bleck GT, White BR, Miller DJ, Wheeler MB. Production of bovine alpha-lactalbumin in the milk of transgenic pigs. J Anim Sci 1998;76:3072-8.
[16] Wall RJ. Pronuclear microinjection. Cloning Stem Cells 2001;3:209-20.
[17] Lavitrano M, Busnelli M, Cerrito MG, Giovannoni R, Manzini S, Vargiolu A. Sperm-mediated gene transfer. Reprod Fertil Dev 2006;18:19-23.
[18] Luo Y, Lin L, Bolund L, Jensen TG, Sorensen CB. Genetically modified pigs for biomedical research. J Inherit Metab Dis 2012;35:695-713.
[19] Galli C, Perota A, Brunetti D, Lagutina I, Lazzari G, Lucchini F. Genetic engineering including superseding microinjection: new ways to make GM pigs. Xenotransplantation 2010;17:397-410.
[20] Wu Z, Li Z, Yang J. Transient transgene transmission to piglets by intrauterine insemination of spermatozoa incubated with DNA fragments. Mol Reprod Dev 2008;75:26-32.
[21] Wakayama T, Yanagimachi R. Mouse cloning with nucleus donor cells of different age and type. Mol Reprod Dev 2001;58:376-83.
[22] De Sousa PA, Dobrinsky JR, Zhu J, Archibald AL, Ainslie A, Bosma W, Bowering J, Bracken J, Ferrier PM, Fletcher J, Gasparrini B, Harkness L, Johnston P, Ritchie M, Ritchie WA, Travers A, Albertini D, Dinnyes A, King TJ, Wilmut I. Somatic cell nuclear transfer in the pig: control of pronuclear formation and integration with improved methods for activation and maintenance of pregnancy. Biol Reprod 2002;66:642-50.
[23] Akagi S, Adachi N, Matsukawa K, Kubo M, Takahashi S. Developmental potential of bovine nuclear transfer embryos and postnatal survival rate of cloned calves produced by two different timings of fusion and activation. Mol Reprod Dev 2003;66:264-72.
[24] Yang XY, Zhao JG, Li HW, Li H, Liu HF, Huang SZ, Zeng YT. Improving in vitro development of cloned bovine embryos with hybrid (Holstein-Chinese Yellow) recipient oocytes recovered by ovum pick up. Theriogenology 2005;64:1263-72.
[25] Inoue K, Noda S, Ogonuki N, Miki H, Inoue S, Katayama K, Mekada K, Miyoshi H, Ogura A. Differential developmental ability of embryos cloned from tissue-specific stem cells. Stem Cells 2007;25:1279-85.
[26] Solter D. Mammalian cloning: advances and limitations. Nat Rev Genet 2000;1:199-207.
[27] Zhao J, Hao Y, Ross JW, Spate LD, Walters EM, Samuel MS, Rieke A, Murphy CN, Prather RS. Histone deacetylase inhibitors improve in vitro and in vivo developmental competence of somatic cell nuclear transfer porcine embryos. Cell Reprogram 2010;12:75-83.
[28] Marchal R, Vigneron C, Perreau C, Bali-Papp A, Mermillod P. Effect of follicular size on meiotic and developmental competence of porcine oocytes. Theriogenology 2002;57:1523-32.
[29] Wongsrikeao P, Otoi T, Yamasaki H, Agung B, Taniguchi M, Naoi H, Shimizu R,

Nagai T. Effects of single and double exposure to brilliant cresyl blue on the selection of porcine oocytes for in vitro production of embryos. Theriogenology 2006;66:366-72.
[30] Ishizaki C, Watanabe H, Bhuiyan MM, Fukui Y. Developmental competence of porcine oocytes selected by brilliant cresyl blue and matured individually in a chemically defined culture medium. Theriogenology 2009;72:72-80.
[31] Mattioli M, Bacci ML, Galeati G, Seren E. Effects of LH and FSH on the maturation of pig oocytes in vitro. Theriogenology 1991;36:95-105.
[32] Singh B, Barbe GJ, Armstrong DT. Factors influencing resumption of meiotic maturation and cumulus expansion of porcine oocyte-cumulus cell complexes in vitro. Mol Reprod Dev 1993;36:113-9.
[33] Funahashi H, Day BN. Effects of the duration of exposure to hormone supplements on cytoplasmic maturation of pig oocytes in vitro. J Reprod Fertil 1993;98:179-85.
[34] Funahashi H, Cantley T, Day BN. Different hormonal requirements of pig oocyte-cumulus complexes during maturation in vitro. J Reprod Fertil 1994;101:159-65.
[35] Illera MJ, Lorenzo PL, Illera JC, Petters RM. Developmental competence of immature pig oocytes under the influence of EGF, IGF-I, follicular fluid and gonadotropins during IVM-IVF processes. Int J Dev Biol 1998;42:1169-72.
[36] Bing YZ, Naga T, Rodriguez-Martinez H. Effects of cysteamine, fsh and estradiol-17beta on in vitro maturation of porcine oocytes. Theriogenology 2001;55:867-76.
[37] Ye J, Campbell KH, Craigon J, Luck MR. Dynamic changes in meiotic progression and improvement of developmental competence of pig oocytes in vitro by follicle-stimulating hormone and cycloheximide. Biol Reprod 2005;72:399-406.
[38] Funahashi H, Cantley TC, Day BN. Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following in vitro fertilization. Biol Reprod 1997;57:49-53.
[39] Schoevers EJ, Bevers MM, Roelen BA, Colenbrander B. Nuclear and cytoplasmic maturation of sow oocytes are not synchronized by specific meiotic inhibition with roscovitine during in vitro maturation. Theriogenology 2005;63:1111-30.
[40] Tatemoto H, Ootaki K, Shigeta K, Muto N. Enhancement of developmental competence after in vitro fertilization of porcine oocytes by treatment with ascorbic acid 2-O-alpha-glucoside during in vitro maturation. Biol Reprod 2001;65:1800-6.
[41] Tamura H, Takasaki A, Miwa I, Taniguchi K, Maekawa R, Asada H, Taketani T, Matsuoka A, Yamagata Y, Shimamura K, Morioka H, Ishikawa H, Reiter RJ, Sugino N. Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. J Pineal Res 2008;44:280-7.
[42] Kang JT, Koo OJ, Kwon DK, Park HJ, Jang G, Kang SK, Lee BC. Effects of melatonin on in vitro maturation of porcine oocyte and expression of melatonin receptor RNA in cumulus and granulosa cells. J Pineal Res 2009;46:22-8.
[43] Yoshida M, Ishigaki K, Nagai T, Chikyu M, Pursel VG. Glutathione

concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. Biol Reprod 1993;49:89-94.
[44] Jeong BS, Yang X. Cysteine, glutathione, and Percoll treatments improve porcine oocyte maturation and fertilization in vitro. Mol Reprod Dev 2001;59:330-5.
[45] Lafleur MV, Hoorweg JJ, Joenje H, Westmijze EJ, Retel J. The ambivalent role of glutathione in the protection of DNA against singlet oxygen. Free Radic Res 1994;21:9-17.
[46] Petters RM, Wells KD. Culture of pig embryos. J Reprod Fertil Suppl 1993;48:61-73.
[47] Algriany O, Bevers M, Schoevers E, Colenbrander B, Dieleman S. Follicle size-dependent effects of sow follicular fluid on in vitro cumulus expansion, nuclear maturation and blastocyst formation of sow cumulus oocytes complexes. Theriogenology 2004;62:1483-97.
[48] Tatemoto H, Muto N, Sunagawa I, Shinjo A, Nakada T. Protection of porcine oocytes against cell damage caused by oxidative stress during in vitro maturation: role of superoxide dismutase activity in porcine follicular fluid. Biol Reprod 2004;71:1150-7.
[49] Yoshioka K, Suzuki C, Onishi A. Defined system for in vitro production of porcine embryos using a single basic medium. J Reprod Dev 2008;54:208-13.
[50] Mito T, Yoshioka K, Nagano M, Suzuki C, Yamashita S, Hoshi H. Transforming growth factor-alpha in a defined medium during in vitro maturation of porcine oocytes improves their developmental competence and intracellular ultrastructure. Theriogenology 2009;72:841-50.
[51] Akaki Y, Yoshioka K, Noguchi M, Hoshi H, Funahashi H. Successful piglet production in a chemically defined system for in-vitro production of porcine embryos: dibutyryl cyclic amp and epidermal growth factor-family peptides support in-vitro maturation of oocytes in the absence of gonadotropins. J Reprod Dev 2009;55:446-53.
[52] Inoue K, Ogonuki N, Miki H, Hirose M, Noda S, Kim JM, Aoki F, Miyoshi H, Ogura A. Inefficient reprogramming of the hematopoietic stem cell genome following nuclear transfer. J Cell Sci 2006;119:1985-91.
[53] Somers J, Smith C, Donnison M, Wells DN, Henderson H, McLeay L, Pfeffer PL. Gene expression profiling of individual bovine nuclear transfer blastocysts. Reproduction 2006;131:1073-84.
[54] Jouneau A, Zhou Q, Camus A, Brochard V, Maulny L, Collignon J, Renard JP. Developmental abnormalities of NT mouse embryos appear early after implantation. Development 2006;133:1597-607.
[55] Aston KI, Li GP, Hicks BA, Sessions BR, Davis AP, Rickords LF, Stevens JR, White KL. Abnormal levels of transcript abundance of developmentally important genes in various stages of preimplantation bovine somatic cell nuclear transfer embryos. Cell Reprogram 2010;12:23-32.
[56] de Montera B, El Zeihery D, Muller S, Jammes H, Brem G, Reichenbach HD, Scheipl F, Chavatte-Palmer P, Zakhartchenko V, Schmitz OJ, Wolf E, Renard JP, Hiendleder S. Quantification of leukocyte genomic 5-methylcytosine levels reveals epigenetic plasticity in healthy adult cloned cattle. Cell Reprogram 2010;12:175-81.

[57] Lee RS, Peterson AJ, Donnison MJ, Ravelich S, Ledgard AM, Li N, Oliver JE, Miller AL, Tucker FC, Breier B, Wells DN. Cloned cattle fetuses with the same nuclear genetics are more variable than contemporary half-siblings resulting from artificial insemination and exhibit fetal and placental growth deregulation even in the first trimester. Biol Reprod 2004;70:1-11.
[58] Yang X, Smith SL, Tian XC, Lewin HA, Renard JP, Wakayama T. Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. Nat Genet 2007;39:295-302.
[59] Zimmermann U, Vienken J. Electric field-induced cell-to-cell fusion. J Membr Biol 1982;67:165-82.
[60] Machaty Z, Rickords LF, Prather RS. Parthenogenetic activation of porcine oocytes after nuclear transfer. Cloning 1999;1:101-9.
[61] Liu L, Ju JC, Yang X. Differential inactivation of maturation-promoting factor and mitogen-activated protein kinase following parthenogenetic activation of bovine oocytes. Biol Reprod 1998;59:537-45.
[62] Liu L, Ju JC, Yang X. Parthenogenetic development and protein patterns of newly matured bovine oocytes after chemical activation. Mol Reprod Dev 1998;49:298-307.
[63] Machaty Z, Wang WH, Day BN, Prather RS. Complete activation of porcine oocytes induced by the sulfhydryl reagent, thimerosal. Biol Reprod 1997;57:1123-7.
[64] Campbell KH, McWhir J, Ritchie WA, Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. Nature 1996;380:64-6.
[65] Shen PC, Lee SN, Liu BT, Chu FH, Wang CH, Wu JS, Lin HH, Cheng WT. The effect of activation treatments on the development of reconstructed bovine oocytes. Anim Reprod Sci 2008;106:1-12.
[66] Yamanaka K, Sugimura S, Wakai T, Kawahara M, Sato E. Difference in sensitivity to culture condition between in vitro fertilized and somatic cell nuclear transfer embryos in pigs. J Reprod Dev 2009;55:299-304.
[67] Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H, Akita T, Nagai T. Successful piglet production after transfer of blastocysts produced by a modified in vitro system. Biol Reprod 2002;66:1033-41.
[68] Abeydeera LR. In vitro production of embryos in swine. Theriogenology 2002;57:256-73.
[69] Gandhi AP, Lane M, Gardner DK, Krisher RL. Substrate utilization in porcine embryos cultured in NCSU23 and G1.2/G2.2 sequential culture media. Mol Reprod Dev 2001;58:269-75.
[70] Yoshioka K, Suzuki C, Tanaka A, Anas IM, Iwamura S. Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol Reprod 2002;66:112-9.
[71] Karja NW, Wongsrikeao P, Murakami M, Agung B, Fahrudin M, Nagai T, Otoi T. Effects of oxygen tension on the development and quality of porcine in vitro fertilized embryos. Theriogenology 2004;62:1585-95.
[72] Kitagawa Y, Suzuki K, Yoneda A, Watanabe T. Effects of oxygen concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen

species (ROS), and DNA fragmentation in porcine embryos. Theriogenology 2004;62:1186-97.
[73] Kang JT, Atikuzzaman M, Kwon DK, Park SJ, Kim SJ, Moon JH, Koo OJ, Jang G, Lee BC. Developmental competence of porcine oocytes after in vitro maturation and in vitro culture under different oxygen concentrations. Zygote 2012;20:1-8.
[74] Machaty Z, Day BN, Prather RS. Development of early porcine embryos in vitro and in vivo. Biol Reprod 1998;59:451-5.
[75] Booth PJ, Holm P, Callesen H. The effect of oxygen tension on porcine embryonic development is dependent on embryo type. Theriogenology 2005;63:2040-52.
[76] McKenzie IF, Koulmanda M, Mandel TE, Sandrin MS. Pig islet xenografts are susceptible to "anti-pig" but not Gal alpha(1,3)Gal antibody plus complement in Gal o/o mice. J Immunol 1998;161:5116-9.
[77] Vaithilingam V, Tuch BE. Islet transplantation and encapsulation: an update on recent developments. Rev Diabet Stud 2011;8:51-67.
[78] de Vos P, Bucko M, Gemeiner P, Navratil M, Svitel J, Faas M, Strand BL, Skjak-Braek G, Morch YA, Vikartovska A, Lacik I, Kollarikova G, Orive G, Poncelet D, Pedraz JL, Ansorge-Schumacher MB. Multiscale requirements for bioencapsulation in medicine and biotechnology. Biomaterials 2009;30:2559-70.
[79] de Vos P, Spasojevic M, Faas MM. Treatment of diabetes with encapsulated islets. Adv Exp Med Biol 2010;670:38-53.
[80] Gunji T, Saito T, Sato Y, Matsuyama S, Ise K, Kimura T, Anazawa T, Gotoh M. Mitomycin-C treatment followed by culture produces long-term survival of islet xenografts in a rat-to mouse model. Cell Transplant 2008;17:619-29.
[81] Yun Lee D, Hee Nam J, Byun Y. Functional and histological evaluation of transplanted pancreatic islets immunoprotected by PEGylation and cyclosporine for 1 year. Biomaterials 2007;28:1957-66.
[82] De Vos P, Van Straaten JF, Nieuwenhuizen AG, de Groot M, Ploeg RJ, De Haan BJ, Van Schilfgaarde R. Why do microencapsulated islet grafts fail in the absence of fibrotic overgrowth? Diabetes 1999;48:1381-8.
[83] de Vos P, Faas MM, Strand B, Calafiore R. Alginate-based microcapsules for immunoisolation of pancreatic islets. Biomaterials 2006;27:5603-17.
[84] Langlois G, Dusseault J, Bilodeau S, Tam SK, Magassouba D, Halle JP. Direct effect of alginate purification on the survival of islets immobilized in alginate-based microcapsules. Acta Biomater 2009;5:3433-40.
[85] Tam SK, Dusseault J, Polizu S, Menard M, Halle JP, Yahia L. Impact of residual contamination on the biofunctional properties of purified alginates used for cell encapsulation. Biomaterials 2006;27:1296-305.
[86] Hobbs HA, Kendall WF, Jr., Darrabie M, Opara EC. Prevention of morphological changes in alginate microcapsules for islet xenotransplantation. J Investig Med 2001;49:572-5.
[87] Narang AS, Mahato RI. Biological and biomaterial approaches for improved islet transplantation. Pharmacol Rev 2006;58:194-243.
[88] Siebers U, Horcher A, Brandhorst H, Brandhorst D, Hering B, Federlin K,

Bretzel RG, Zekorn T. Analysis of the cellular reaction towards microencapsulated xenogeneic islets after intraperitoneal transplantation. J Mol Med (Berl) 1999;77:215-8.
[89] Wiegand F, Kroncke KD, Kolb-Bachofen V. Macrophage-generated nitric oxide as cytotoxic factor in destruction of alginate-encapsulated islets. Protection by arginine analogs and/or coencapsulated erythrocytes. Transplantation 1993;56:1206-12.
[90] Yang H, Wright JR, Jr. Co-encapsulation of Sertoli enriched testicular cell fractions further prolongs fish-to-mouse islet xenograft survival. Transplantation 1999;67:815-20.
[91] Chae SY, Lee M, Kim SW, Bae YH. Protection of insulin secreting cells from nitric oxide induced cellular damage by crosslinked hemoglobin. Biomaterials 2004;25:843-50.
[92] Dobson T, Fraga D, Saba C, Bryer-Ash M, Gaber AO, Gerling IC. Human pancreatic islets transfected to produce an inhibitor of TNF are protected against destruction by human leukocytes. Cell Transplant 2000;9:857-65.
[93] Giannoukakis N, Rudert WA, Ghivizzani SC, Gambotto A, Ricordi C, Trucco M, Robbins PD. Adenoviral gene transfer of the interleukin-1 receptor antagonist protein to human islets prevents IL-1beta-induced beta-cell impairment and activation of islet cell apoptosis in vitro. Diabetes 1999;48:1730-6.
[94] Carlsson PO, Palm F, Andersson A, Liss P. Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. Diabetes 2001;50:489-95.
[95] Siebers U, Horcher A, Bretzel RG, Klock G, Zimmermann U, Federlin K, Zekorn T. Transplantation of free and microencapsulated islets in rats: evidence for the requirement of an increased islet mass for transplantation into the peritoneal site. Int J Artif Organs 1993;16:96-9.
[96] Wang W, Gu Y, Tabata Y, Miyamoto M, Hori H, Nagata N, Touma M, Balamurugan AN, Kawakami Y, Nozawa M, Inoue K. Reversal of diabetes in mice by xenotransplantation of a bioartificial pancreas in a prevascularized subcutaneous site. Transplantation 2002;73:122-9.
[97] Sorenby AK, Kumagai-Braesch M, Sharma A, Hultenby KR, Wernerson AM, Tibell AB. Preimplantation of an immunoprotective device can lower the curative dose of islets to that of free islet transplantation: studies in a rodent model. Transplantation 2008;86:364-6.
[98] Bellmann K, Wenz A, Radons J, Burkart V, Kleemann R, Kolb H. Heat shock induces resistance in rat pancreatic islet cells against nitric oxide, oxygen radicals and streptozotocin toxicity in vitro. J Clin Invest 1995;95:2840-5.
[99] Hagerkvist R, Sandler S, Mokhtari D, Welsh N. Amelioration of diabetes by imatinib mesylate (Gleevec): role of beta-cell NF-kappaB activation and anti-apoptotic preconditioning. FASEB J 2007;21:618-28.
[100] Klein D, Ribeiro MM, Mendoza V, Jayaraman S, Kenyon NS, Pileggi A, Molano RD, Inverardi L, Ricordi C, Pastori RL. Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets. Biochem Biophys Res Commun 2004;323:473-8.

[101] Sigrist S, Mechine-Neuville A, Mandes K, Calenda V, Braun S, Legeay G, Bellocq JP, Pinget M, Kessler L. Influence of VEGF on the viability of encapsulated pancreatic rat islets after transplantation in diabetic mice. Cell Transplant 2003;12:627-35.
[102] Lee KW, Yoon JJ, Lee JH, Kim SY, Jung HJ, Kim SJ, Joh JW, Lee HH, Lee DS, Lee SK. Sustained release of vascular endothelial growth factor from calcium-induced alginate hydrogels reinforced by heparin and chitosan. Transplant Proc 2004;36:2464-5.
[103] Moya ML, Garfinkel MR, Liu X, Lucas S, Opara EC, Greisler HP, Brey EM. Fibroblast growth factor-1 (FGF-1) loaded microbeads enhance local capillary neovascularization. J Surg Res 2010;160:208-12.
[104] Dziubla TD, Lowman AM. Vascularization of PEG-grafted macroporous hydrogel sponges: a three-dimensional in vitro angiogenesis model using human microvascular endothelial cells. J Biomed Mater Res A 2004;68:603-14.
[105]Lembert N, Wesche J, Petersen P, Doser M, Zschocke P, Becker HD, Ammon HP. Encapsulation of islets in rough surface, hydroxymethylated polysulfone capillaries stimulates VEGF release and promotes vascularization after transplantation. Cell Transplant 2005;14:97-108.
[106] Bloch K, Papismedov E, Yavriyants K, Vorobeychik M, Beer S, Vardi P. Immobilized microalgal cells as an oxygen supply system for encapsulated pancreatic islets: a feasibility study. Artif Organs 2006;30:715-8.
[107] Tiedge M, Lortz S, Munday R, Lenzen S. Protection against the co-operative toxicity of nitric oxide and oxygen free radicals by overexpression of antioxidant enzymes in bioengineered insulin-producing RINm5F cells. Diabetologia 1999;42:849-55.
[108] Giovagnoli S, Luca G, Casaburi I, Blasi P, Macchiarulo G, Ricci M, Calvitti M, Basta G, Calafiore R, Rossi C. Long-term delivery of superoxide dismutase and catalase entrapped in poly(lactide-co-glycolide) microspheres: in vitro effects on isolated neonatal porcine pancreatic cell clusters. J Control Release 2005;107:65-77.
[109] Melo SS, Arantes MR, Meirelles MS, Jordao AA, Jr., Vannucchi H. Lipid peroxidation in nicotinamide-deficient and nicotinamide-supplemented rats with streptozotocin-induced diabetes. Acta Diabetol 2000;37:33-9.
[110] Kenmochi T, Miyamoto M, Mullen Y. Protection of mouse islet isografts from nonspecific inflammatory damage by recipient treatment with nicotinamide and 15-deoxyspergualin. Cell Transplant 1996;5:41-7.
[111] Su J, Hu BH, Lowe WL, Jr., Kaufman DB, Messersmith PB. Anti-inflammatory peptide-functionalized hydrogels for insulin-secreting cell encapsulation. Biomaterials 2010;31:308-14.
[112] Hwang JS, Chae SY, Lee MK, Bae YH. Synthesis of sulfonylurea conjugated copolymer via PEO spacer and its in vitro short-term bioactivity in insulin secretion from islets of Langerhans. Biomaterials 1998;19:1189-95.
[113] Park KH, Kim SW, Bae YH. Interaction of sulfonylurea-conjugated polymer with insulinoma cell line of MIN6 and its effect on insulin secretion. J Biomed Mater Res 2001;55:72-8.

[114] Kim S, Wan Kim S, Bae YH. Synthesis, bioactivity and specificity of glucagon-like peptide-1 (7-37)/polymer conjugate to isolated rat islets. Biomaterials 2005;26:3597-606.
[115] Reach G, Jaffrin MY. Kinetic modelling as a tool for the design of a vascular bioartificial pancreas: feedback between modelling and experimental validation. Comput Methods Programs Biomed 1990;32:277-85.
[116] Silva AI, Mateus M. Development of a polysulfone hollow fiber vascular bio-artificial pancreas device for in vitro studies. J Biotechnol 2009;139:236-49.
[117] Petruzzo P, Pibiri L, De Giudici MA, Basta G, Calafiore R, Falorni A, Brunetti P, Brotzu G. Xenotransplantation of microencapsulated pancreatic islets contained in a vascular prosthesis: preliminary results. Transpl Int 1991;4:200-4.
[118] Maki T, Lodge JP, Carretta M, Ohzato H, Borland KM, Sullivan SJ, Staruk J, Muller TE, Solomon BA, Chick WL, et al. Treatment of severe diabetes mellitus for more than one year using a vascularized hybrid artificial pancreas. Transplantation 1993;55:713-7; discussion 7-8.
[119] Maki T, Ubhi CS, Sanchez-Farpon H, Sullivan SJ, Borland K, Muller TE, Solomon BA, Chick WL, Monaco AP. The biohybrid artificial pancreas for treatment of diabetes in totally pancreatectomized dogs. Transplant Proc 1991;23:754-5.
[120] Olivares E, Piranda S, Malaisse WJ. Long-term correction of hyperglycaemia in streptozotocin-induced diabetic rats transplanted with islets placed in an implantation device. Horm Metab Res 2001;33:687-8.
[121] Young TH, Chuang WY, Hsieh MY, Chen LW, Hsu JP. Assessment and modeling of poly(vinyl alcohol) bioartificial pancreas in vivo. Biomaterials 2002;23:3495-501.
[122] Qi M, Gu Y, Sakata N, Kim D, Shirouzu Y, Yamamoto C, Hiura A, Sumi S, Inoue K. PVA hydrogel sheet macroencapsulation for the bioartificial pancreas. Biomaterials 2004;25:5885-92.
[123] Yang KC, Wu CC, Sumi S, Tseng CL, Wu YH, Kuo TF, Lin FH. Calcium phosphate cement chamber as an immunoisolative device for bioartificial pancreas: in vitro and preliminary in vivo study. Pancreas 2010;39:444-51.
[124] Edamura K, Itakura S, Nasu K, Iwami Y, Ogawa H, Sasaki N, Ohgawara H. Xenotransplantation of porcine pancreatic endocrine cells to total pancreatectomized dogs. J Vet Med Sci 2003;65:549-56.
[125] Grundfest-Broniatowski SF, Tellioglu G, Rosenthal KS, Kang J, Erdodi G, Yalcin B, Cakmak M, Drazba J, Bennett A, Lu L, Kennedy JP. A new bioartificial pancreas utilizing amphiphilic membranes for the immunoisolation of porcine islets: a pilot study in the canine. ASAIO J 2009;55:400-5.
[126] Dulong JL, Legallais C. What are the relevant parameters for the geometrical optimization of an implantable bioartificial pancreas? J Biomech Eng 2005;127:1054-61.
[127] Dulong JL, Legallais C. A theoretical study of oxygen transfer including cell necrosis for the design of a bioartificial pancreas. Biotechnol Bioeng 2007;96:990-8.
[128] Abuchowski A, van Es T, Palczuk NC, Davis FF. Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. J

Biol Chem 1977;252:3578-81.
[129] Abuchowski A, McCoy JR, Palczuk NC, van Es T, Davis FF. Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. J Biol Chem 1977;252:3582-6.
[130] Lee DY, Yang K, Lee S, Chae SY, Kim KW, Lee MK, Han DJ, Byun Y. Optimization of monomethoxy-polyethylene glycol grafting on the pancreatic islet capsules. J Biomed Mater Res 2002;62:372-7.
[131] Panza JL, Wagner WR, Rilo HL, Rao RH, Beckman EJ, Russell AJ. Treatment of rat pancreatic islets with reactive PEG. Biomaterials 2000;21:1155-64.
[132] Jang JY, Lee DY, Park SJ, Byun Y. Immune reactions of lymphocytes and macrophages against PEG-grafted pancreatic islets. Biomaterials 2004;25:3663-9.
[133] Teramura Y, Iwata H. Surface modification of islets with PEG-lipid for improvement of graft survival in intraportal transplantation. Transplantation 2009;88:624-30.
[134] Lee DY, Nam JH, Byun Y. Effect of polyethylene glycol grafted onto islet capsules on prevention of splenocyte and cytokine attacks. J Biomater Sci Polym Ed 2004;15:753-66.
[135] Teramura Y, Iwata H. Islets surface modification prevents blood-mediated inflammatory responses. Bioconjug Chem 2008;19:1389-95.
[136] Lee DY, Park SJ, Lee S, Nam JH, Byun Y. Highly poly(ethylene) glycolylated islets improve long-term islet allograft survival without immunosuppressive medication. Tissue Eng 2007;13:2133-41.
[137] Wilson JT, Cui W, Chaikof EL. Layer-by-layer assembly of a conformal nanothin PEG coating for intraportal islet transplantation. Nano Lett 2008;8:1940-8.
[138] Giraldo JA, Weaver JD, Stabler CL. Tissue engineering approaches to enhancing clinical islet transplantation through tissue engineering strategies. J Diabetes Sci Technol 2010;4:1238-47.
[139] Stabler CL, Sun XL, Cui W, Wilson JT, Haller CA, Chaikof EL. Surface re-engineering of pancreatic islets with recombinant azido-thrombomodulin. Bioconjug Chem 2007;18:1713-5.
[140] Tian XC, Kubota C, Enright B, Yang X. Cloning animals by somatic cell nuclear transfer--biological factors. Reprod Biol Endocrinol 2003;1:98.
[141] Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KH. Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 2000;407:86-90.
[142] Swann K, Ozil JP. Dynamics of the calcium signal that triggers mammalian egg activation. Int Rev Cytol 1994;152:183-222.
[143] Wang WH, Abeydeera LR, Prather RS, Day BN. Functional analysis of activation of porcine oocytes by spermatozoa, calcium ionophore, and electrical pulse. Mol Reprod Dev 1998;51:346-53.
[144] Betthauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J, Forsythe T, Golueke P, Jurgella G, Koppang R, Lesmeister T, Mallon K, Mell G, Misica P, Pace M, Pfister-Genskow M, Strelchenko N, Voelker G, Watt S, Thompson S, Bishop M. Production of cloned pigs from in vitro systems. Nat Biotechnol 2000;18:1055-9.

[145] Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H, Perry AC. Pig cloning by microinjection of fetal fibroblast nuclei. Science 2000;289:1188-90.
[146] Lee SH, Kim DY, Nam DH, Hyun SH, Lee GS, Kim HS, Lee CK, Kang SK, Lee BC, Hwang WS. Role of messenger RNA expression of platelet activating factor and its receptor in porcine in vitro-fertilized and cloned embryo development. Biol Reprod 2004;71:919-25.
[147] Loi P, Ledda S, Fulka J, Jr., Cappai P, Moor RM. Development of parthenogenetic and cloned ovine embryos: effect of activation protocols. Biol Reprod 1998;58:1177-87.
[148] Russell DF, Ibanez E, Albertini DF, Overstrom EW. Activated bovine cytoplasts prepared by demecolcine-induced enucleation support development of nuclear transfer embryos in vitro. Mol Reprod Dev 2005;72:161-70.
[149] Varga E, Pataki R, Lorincz Z, Koltai J, Papp AB. Parthenogenetic development of in vitro matured porcine oocytes treated with chemical agents. Anim Reprod Sci 2008;105:226-33.
[150] Campbell KH, Fisher P, Chen WC, Choi I, Kelly RD, Lee JH, Xhu J. Somatic cell nuclear transfer: Past, present and future perspectives. Theriogenology 2007;68 Suppl 1:S214-31.
[151] Im GS, Seo JS, Hwang IS, Kim DH, Kim SW, Yang BC, Yang BS, Lai L, Prather RS. Development and apoptosis of pre-implantation porcine nuclear transfer embryos activated with different combination of chemicals. Mol Reprod Dev 2006;73:1094-101.
[152] Cheng WM, Sun XL, An L, Zhu SE, Li XH, Li Y, Tian JH. Effect of different parthenogenetic activation methods on the developmental competence of in vitro matured porcine oocytes. Anim Biotechnol 2007;18:131-41.
[153] Yi YJ, Park CS. Parthenogenetic development of porcine oocytes treated by ethanol, cycloheximide, cytochalasin B and 6-dimethylaminopurine. Anim Reprod Sci 2005;86:297-304.
[154] Jiang JY, Mizuno S, Mizutani E, Sasada H, Sato E. Parthenogenetic activation and subsequent development of rat oocytes in vitro. Mol Reprod Dev 2002;61:120-5.
[155] Meo SC, Yamazaki W, Ferreira CR, Perecin F, Saraiva NZ, Leal CL, Garcia JM. Parthenogenetic activation of bovine oocytes using single and combined strontium, ionomycin and 6-dimethylaminopurine treatments. Zygote 2007;15:295-306.
[156] Alexander B, Coppola G, Di Berardino D, Rho GJ, St John E, Betts DH, King WA. The effect of 6-dimethylaminopurine (6-DMAP) and cycloheximide (CHX) on the development and chromosomal complement of sheep parthenogenetic and nuclear transfer embryos. Mol Reprod Dev 2006;73:20-30.
[157] Holker M, Petersen B, Hassel P, Kues WA, Lemme E, Lucas-Hahn A, Niemann H. Duration of in vitro maturation of recipient oocytes affects blastocyst development of cloned porcine embryos. Cloning Stem Cells 2005;7:35-44.
[158] Nanassy L, Lee K, Javor A, Machaty Z. Changes in MPF and MAPK activities in porcine oocytes activated by different methods. Theriogenology 2007;68:146-52.
[159] De La Fuente R, King WA. Developmental consequences of karyokinesis

without cytokinesis during the first mitotic cell cycle of bovine parthenotes. Biol Reprod 1998;58:952-62.
[160] Szollosi MS, Kubiak JZ, Debey P, de Pennart H, Szollosi D, Maro B. Inhibition of protein kinases by 6-dimethylaminopurine accelerates the transition to interphase in activated mouse oocytes. J Cell Sci 1993;104 (Pt 3):861-72.
[161] Katoh M, Araki A, Ogura T, Valdivia RP. 6-Dimethylaminopurine (6-DMAP), which is used to produce most cloned animals, is mutagenic in Salmonella typhimurium TA1535. Mutat Res 2004;560:199-201.
[162] Tian JH, Wu ZH, Liu L, Cai Y, Zeng SM, Zhu SE, Liu GS, Li Y, Wu CX. Effects of oocyte activation and sperm preparation on the development of porcine embryos derived from in vitro-matured oocytes and intracytoplasmic sperm injection. Theriogenology 2006;66:439-48.
[163] Sugimura S, Kawahara M, Wakai T, Yamanaka K, Sasada H, Sato E. Effect of cytochalasins B and D on the developmental competence of somatic cell nuclear transfer embryos in miniature pigs. Zygote 2008;16:153-9.
[164] Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 1998;394:369-74.
[165] Lai L, Tao T, Machaty Z, Kuhholzer B, Sun QY, Park KW, Day BN, Prather RS. Feasibility of producing porcine nuclear transfer embryos by using G2/M-stage fetal fibroblasts as donors. Biol Reprod 2001;65:1558-64.
[166] Ibanez E, Albertini DF, Overstrom EW. Demecolcine-induced oocyte enucleation for somatic cell cloning: coordination between cell-cycle egress, kinetics of cortical cytoskeletal interactions, and second polar body extrusion. Biol Reprod 2003;68:1249-58.
[167] Saraiva NZ, Perecin F, Meo SC, Ferreira CR, Tetzner TA, Garcia JM. Demecolcine effects on microtubule kinetics and on chemically assisted enucleation of bovine oocytes. Cloning Stem Cells 2009;11:141-52.
[168] Li J, Villemoes K, Zhang Y, Du Y, Kragh PM, Purup S, Xue Q, Pedersen AM, Jorgensen AL, Jakobsen JE, Bolund L, Yang H, Vajta G. Efficiency of two enucleation methods connected to handmade cloning to produce transgenic porcine embryos. Reprod Domest Anim 2009;44:122-7.
[169] Miyazaki S, Shirakawa H, Nakada K, Honda Y, Yuzaki M, Nakade S, Mikoshiba K. Antibody to the inositol trisphosphate receptor blocks thimerosal-enhanced Ca(2+)-induced Ca2+ release and Ca2+ oscillations in hamster eggs. FEBS Lett 1992;309:180-4.
[170] Swann K. Different triggers for calcium oscillations in mouse eggs involve a ryanodine-sensitive calcium store. Biochem J 1992;287 (Pt 1):79-84.
[171] Collas P, Fissore R, Robl JM, Sullivan EJ, Barnes FL. Electrically induced calcium elevation, activation, and parthenogenetic development of bovine oocytes. Mol Reprod Dev 1993;34:212-23.
[172] Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Cloning Stem Cells 2007;9:3-7.
[173] Tamada H, Kikyo N. Nuclear reprogramming in mammalian somatic cell

nuclear cloning. Cytogenet Genome Res 2004;105:285-91.
[174] Young LE, Sinclair KD, Wilmut I. Large offspring syndrome in cattle and sheep. Rev Reprod 1998;3:155-63.
[175] Farin PW, Piedrahita JA, Farin CE. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. Theriogenology 2006;65:178-91.
[176] Dean W, Santos F, Stojkovic M, Zakhartchenko V, Walter J, Wolf E, Reik W. Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. Proc Natl Acad Sci U S A 2001;98:13734-8.
[177] Santos F, Zakhartchenko V, Stojkovic M, Peters A, Jenuwein T, Wolf E, Reik W, Dean W. Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. Curr Biol 2003;13:1116-21.
[178] Ohgane J, Wakayama T, Senda S, Yamazaki Y, Inoue K, Ogura A, Marh J, Tanaka S, Yanagimachi R, Shiota K. The Sall3 locus is an epigenetic hotspot of aberrant DNA methylation associated with placentomegaly of cloned mice. Genes Cells 2004;9:253-60.
[179] Humpherys D, Eggan K, Akutsu H, Friedman A, Hochedlinger K, Yanagimachi R, Lander ES, Golub TR, Jaenisch R. Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. Proc Natl Acad Sci U S A 2002;99:12889-94.
[180] Inoue K, Kohda T, Lee J, Ogonuki N, Mochida K, Noguchi Y, Tanemura K, Kaneko-Ishino T, Ishino F, Ogura A. Faithful expression of imprinted genes in cloned mice. Science 2002;295:297.
[181] Suemizu H, Aiba K, Yoshikawa T, Sharov AA, Shimozawa N, Tamaoki N, Ko MS. Expression profiling of placentomegaly associated with nuclear transplantation of mouse ES cells. Dev Biol 2003;253:36-53.
[182] Jiang L, Lai L, Samuel M, Prather RS, Yang X, Tian XC. Expression of X-linked genes in deceased neonates and surviving cloned female piglets. Mol Reprod Dev 2008;75:265-73.
[183]Li J, Svarcova O, Villemoes K, Kragh PM, Schmidt M, Bogh IB, Zhang Y, Du Y, Lin L, Purup S, Xue Q, Bolund L, Yang H, Maddox-Hyttel P, Vajta G. High in vitro development after somatic cell nuclear transfer and trichostatin A treatment of reconstructed porcine embryos. Theriogenology 2008;70:800-8.
[184] Beebe LF, McIlfatrick SJ, Nottle MB. Cytochalasin B and trichostatin a treatment postactivation improves in vitro development of porcine somatic cell nuclear transfer embryos. Cloning Stem Cells 2009;11:477-82.
[185] Kishigami S, Mizutani E, Ohta H, Hikichi T, Thuan NV, Wakayama S, Bui HT, Wakayama T. Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. Biochem Biophys Res Commun 2006;340:183-9.
[186] Ding X, Wang Y, Zhang D, Guo Z, Zhang Y. Increased pre-implantation development of cloned bovine embryos treated with 5-aza-2'-deoxycytidine and trichostatin A. Theriogenology 2008;70:622-30.
[187] Miyoshi K, Mori H, Mizobe Y, Akasaka E, Ozawa A, Yoshida M, Sato M. Valproic acid enhances in vitro development and Oct-3/4 expression of miniature pig

somatic cell nuclear transfer embryos. Cell Reprogram 2010;12:67-74.
[188] Costa-Borges N, Santalo J, Ibanez E. Comparison between the effects of valproic acid and trichostatin A on the in vitro development, blastocyst quality, and full-term development of mouse somatic cell nuclear transfer embryos. Cell Reprogram 2010;12:437-46.
[189] Das ZC, Gupta MK, Uhm SJ, Lee HT. Increasing histone acetylation of cloned embryos, but not donor cells, by sodium butyrate improves their in vitro development in pigs. Cell Reprogram 2010;12:95-104.
[190] Ono T, Li C, Mizutani E, Terashita Y, Yamagata K, Wakayama T. Inhibition of class IIb histone deacetylase significantly improves cloning efficiency in mice. Biol Reprod 2010;83:929-37.
[191] Enright BP, Kubota C, Yang X, Tian XC. Epigenetic characteristics and development of embryos cloned from donor cells treated by trichostatin A or 5-aza-2'-deoxycytidine. Biol Reprod 2003;69:896-901.
[192] Zhao J, Ross JW, Hao Y, Spate LD, Walters EM, Samuel MS, Rieke A, Murphy CN, Prather RS. Significant improvement in cloning efficiency of an inbred miniature pig by histone deacetylase inhibitor treatment after somatic cell nuclear transfer. Biol Reprod 2009;81:525-30.
[193] Blackwell L, Norris J, Suto CM, Janzen WP. The use of diversity profiling to characterize chemical modulators of the histone deacetylases. Life Sci 2008;82:1050-8.
[194] Chuang DM, Leng Y, Marinova Z, Kim HJ, Chiu CT. Multiple roles of HDAC inhibition in neurodegenerative conditions. Trends Neurosci 2009;32:591-601.
[195] Koo OJ, Park HJ, Kwon DK, Kang JT, Jang G, Lee BC. Effect of recipient breed on delivery rate of cloned miniature pig. Zygote 2009;17:203-7.
[196] Rybouchkin A, Kato Y, Tsunoda Y. Role of histone acetylation in reprogramming of somatic nuclei following nuclear transfer. Biol Reprod 2006;74:1083-9.
[197] Kang YK, Koo DB, Park JS, Choi YH, Chung AS, Lee KK, Han YM. Aberrant methylation of donor genome in cloned bovine embryos. Nat Genet 2001;28:173-7.
[198] Kishigami S, Bui HT, Wakayama S, Tokunaga K, Van Thuan N, Hikichi T, Mizutani E, Ohta H, Suetsugu R, Sata T, Wakayama T. Successful mouse cloning of an outbred strain by trichostatin A treatment after somatic nuclear transfer. J Reprod Dev 2007;53:165-70.
[199] Whitworth KM, Zhao J, Spate LD, Li R, Prather RS. Scriptaid corrects gene expression of a few aberrantly reprogrammed transcripts in nuclear transfer pig blastocyst stage embryos. Cell Reprogram 2011;13:191-204.
[200] Su GH, Sohn TA, Ryu B, Kern SE. A novel histone deacetylase inhibitor identified by high-throughput transcriptional screening of a compound library. Cancer Res 2000;60:3137-42.
[201] Su J, Wang Y, Li Y, Li R, Li Q, Wu Y, Quan F, Liu J, Guo Z, Zhang Y. Oxamflatin Significantly Improves Nuclear Reprogramming, Blastocyst Quality, and In Vitro Development of Bovine SCNT Embryos. PLoS One 2011;6:e23805.
[202] Armstrong L, Lako M, Dean W, Stojkovic M. Epigenetic modification is central to genome reprogramming in somatic cell nuclear transfer. Stem Cells 2006;24:805-14.
[203] Simonsson S, Gurdon J. DNA demethylation is necessary for the epigenetic

reprogramming of somatic cell nuclei. Nat Cell Biol 2004;6:984-90.
[204] Cervoni N, Szyf M. Demethylase activity is directed by histone acetylation. J Biol Chem 2001;276:40778-87.
[205] Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H, Smith A. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 1998;95:379-91.
[206] Thompson JR, Gudas LJ. Retinoic acid induces parietal endoderm but not primitive endoderm and visceral endoderm differentiation in F9 teratocarcinoma stem cells with a targeted deletion of the Rex-1 (Zfp-42) gene. Mol Cell Endocrinol 2002;195:119-33.
[207] Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 2000;24:372-6.
[208] Kumar BM, Jin HF, Kim JG, Ock SA, Hong Y, Balasubramanian S, Choe SY, Rho GJ. Differential gene expression patterns in porcine nuclear transfer embryos reconstructed with fetal fibroblasts and mesenchymal stem cells. Dev Dyn 2007;236:435-46.
[209] Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 2003;113:643-55.
[210] Hatano SY, Tada M, Kimura H, Yamaguchi S, Kono T, Nakano T, Suemori H, Nakatsuji N, Tada T. Pluripotential competence of cells associated with Nanog activity. Mech Dev 2005;122:67-79.
[211] Braude P, Bolton V, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature 1988;332:459-61.
[212] Misteli T. A nucleolar disappearing act in somatic cloning. Nat Cell Biol 2003;5:183-4.
[213] Zeng F, Schultz RM. RNA transcript profiling during zygotic gene activation in the preimplantation mouse embryo. Dev Biol 2005;283:40-57.
[214] McGraw S, Robert C, Massicotte L, Sirard MA. Quantification of histone acetyltransferase and histone deacetylase transcripts during early bovine embryo development. Biol Reprod 2003;68:383-9.
[215] Schultz RM, Davis W, Jr., Stein P, Svoboda P. Reprogramming of gene expression during preimplantation development. J Exp Zool 1999;285:276-82.
[216] Vassena R, Han Z, Gao S, Latham KE. Deficiency in recapitulation of stage-specific embryonic gene transcription in two-cell stage cloned mouse embryos. Mol Reprod Dev 2007;74:1548-56.
[217] Suzuki T, Minami N, Kono T, Imai H. Zygotically activated genes are suppressed in mouse nuclear transferred embryos. Cloning Stem Cells 2006;8:295-304.
[218] Svarcova O, Dinnyes A, Polgar Z, Bodo S, Adorjan M, Meng Q, Maddox-Hyttel P. Nucleolar re-activation is delayed in mouse embryos cloned from two different cell lines. Mol Reprod Dev 2009;76:132-41.
[219] Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, Ball S, Specht SM, Polejaeva IA, Monahan JA, Jobst PM, Sharma SB, Lamborn AE, Garst AS, Moore M, Demetris AJ, Rudert WA, Bottino R, Bertera S, Trucco M, Starzl TE, Dai Y,

Ayares DL. Production of alpha 1,3-galactosyltransferase-deficient pigs. Science 2003;299:411-4.
[220] Kolber-Simonds D, Lai L, Watt SR, Denaro M, Arn S, Augenstein ML, Betthauser J, Carter DB, Greenstein JL, Hao Y, Im GS, Liu Z, Mell GD, Murphy CN, Park KW, Rieke A, Ryan DJ, Sachs DH, Forsberg EJ, Prather RS, Hawley RJ. Production of alpha-1,3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations. Proc Natl Acad Sci U S A 2004;101:7335-40.
[221] Takahagi Y, Fujimura T, Miyagawa S, Nagashima H, Shigehisa T, Shirakura R, Murakami H. Production of alpha 1,3-galactosyltransferase gene knockout pigs expressing both human decay-accelerating factor and N-acetylglucosaminyltransferase III. Mol Reprod Dev 2005;71:331-8.
[222] Robson SC, Cooper DK, d'Apice AJ. Disordered regulation of coagulation and platelet activation in xenotransplantation. Xenotransplantation 2000;7:166-76.
[223] Chen G, Qian H, Starzl T, Sun H, Garcia B, Wang X, Wise Y, Liu Y, Xiang Y, Copeman L, Liu W, Jevnikar A, Wall W, Cooper DK, Murase N, Dai Y, Wang W, Xiong Y, White DJ, Zhong R. Acute rejection is associated with antibodies to non-Gal antigens in baboons using Gal-knockout pig kidneys. Nat Med 2005;11:1295-8.
[224] Abraham NG, Kappas A. Pharmacological and clinical aspects of heme oxygenase. Pharmacol Rev 2008;60:79-127.
[225] Maines MD. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. FASEB J 1988;2:2557-68.
[226] Tabata T, de Perrot M, Keshavjee S, Liu M, Downey GP, Waddell TK. Accommodation after lung xenografting from hamster to rat. Transplantation 2003;75:607-12.
[227] Sato K, Balla J, Otterbein L, Smith RN, Brouard S, Lin Y, Csizmadia E, Sevigny J, Robson SC, Vercellotti G, Choi AM, Bach FH, Soares MP. Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants. Journal of immunology 2001;166:4185-94.
[228] Shen J, SS TT, Schrieber L, King NJ. Early E-selectin, VCAM-1, ICAM-1, and late major histocompatibility complex antigen induction on human endothelial cells by flavivirus and comodulation of adhesion molecule expression by immune cytokines. J Virol 1997;71:9323-32.
[229] Stocker CJ, Sugars KL, Yarwood H, Delikouras A, Lechler RI, Dorling A, Landis RC, Morley BJ, Haskard DO. Cloning of porcine intercellular adhesion molecule-1 and characterization of its induction on endothelial cells by cytokines. Transplantation 2000;70:579-86.
[230] Soares MP, Lin Y, Anrather J, Csizmadia E, Takigami K, Sato K, Grey ST, Colvin RB, Choi AM, Poss KD, Bach FH. Expression of heme oxygenase-1 can determine cardiac xenograft survival. Nat Med 1998;4:1073-7.
[231] Petersen B, Ramackers W, Lucas-Hahn A, Lemme E, Hassel P, Queisser AL, Herrmann D, Barg-Kues B, Carnwath JW, Klose J, Tiede A, Friedrich L, Baars W, Schwinzer R, Winkler M, Niemann H. Transgenic expression of human heme oxygenase-1 in pigs confers resistance against xenograft rejection during ex vivo

perfusion of porcine kidneys. Xenotransplantation 2011;18:355-68.
[232] Becher B, Blain M, Giacomini PS, Antel JP. Inhibition of Th1 polarization by soluble TNF receptor is dependent on antigen-presenting cell-derived IL-12. J Immunol 1999;162:684-8.
[233] Croxford JL, Triantaphyllopoulos KA, Neve RM, Feldmann M, Chernajovsky Y, Baker D. Gene therapy for chronic relapsing experimental allergic encephalomyelitis using cells expressing a novel soluble p75 dimeric TNF receptor. J Immunol 2000;164:2776-81.
[234] Costa C, Bell NK, Stabel TJ, Fodor WL. Use of porcine tumor necrosis factor receptor 1-Ig fusion protein to prolong xenograft survival. Xenotransplantation 2004;11:491-502.
[235] Cho B, Koo OJ, Hwang JI, Kim H, Lee EM, Hurh S, Park SJ, Ro H, Yang J, Surh CD, D'Apice AJ, Lee BC, Ahn C. Generation of soluble human tumor necrosis factor-alpha receptor 1-Fc transgenic pig. Transplantation 2011;92:139-47.
[236] Zhou CY, McInnes E, Parsons N, Langford G, Lancaster R, Richards A, Pino-Chavez G, Dos Santos Cruz G, Copeman L, Carrington C, Thompson S. Production and characterization of a pig line transgenic for human membrane cofactor protein. Xenotransplantation 2002;9:183-90.
[237] Zhou CY, McInnes E, Copeman L, Langford G, Parsons N, Lancaster R, Richards A, Carrington C, Thompson S. Transgenic pigs expressing human CD59, in combination with human membrane cofactor protein and human decay-accelerating factor. Xenotransplantation 2005;12:142-8.
[238] Szymczak AL, Vignali DA. Development of 2A peptide-based strategies in the design of multicistronic vectors. Expert Opin Biol Ther 2005;5:627-38.
[239] Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S, Vanin EF, Vignali DA. Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. Nat Biotechnol 2004;22:589-94.
[240] Hwang JI, Oh YS, Shin KJ, Kim H, Ryu SH, Suh PG. Molecular cloning and characterization of a novel phospholipase C, PLC-eta. Biochem J 2005;389:181-6.
[241] Park SJ, Park HJ, Koo OJ, Choi WJ, Moon JH, Kwon DK, Kang JT, Kim S, Choi JY, Jang G, Lee BC. Oxamflatin improves developmental competence of porcine somatic cell nuclear transfer embryos. Cell Reprogram 2012;14:398-406.
[242] Kues WA, Niemann H. Advances in farm animal transgenesis. Prev Vet Med 2011;102:146-56.
[243] Whyte JJ, Prather RS. Genetic modifications of pigs for medicine and agriculture. Mol Reprod Dev 2011;78:879-91.
[244] Yang YG, Sykes M. Xenotransplantation: current status and a perspective on the future. Nat Rev Immunol 2007;7:519-31.
[245] Charniot JC, Bonnefont-Rousselot D, Albertini JP, Zerhouni K, Dever S, Richard I, Nataf P, Pavie A, Monsuez JJ, Delattre J, Artigou JY. Oxidative stress implication in a new ex-vivo cardiac concordant xenotransplantation model. Free Radic Res 2007;41:911-8.
[246] Lee EM, Park JO, Kim D, Kim JY, Oh KH, Park CG, Oh BH, Kim S, Ahn C. Early up-regulation of CXC-chemokine expression is associated with strong cellular

immune responses to murine skin xenografts. Xenotransplantation 2006;13:328-36.
[247] Millan MT, Geczy C, Stuhlmeier KM, Goodman DJ, Ferran C, Bach FH. Human monocytes activate porcine endothelial cells, resulting in increased E-selectin, interleukin-8, monocyte chemotactic protein-1, and plasminogen activator inhibitor-type-1 expression. Transplantation 1997;63:421-9.
[248] Li L, Okusa MD. Macrophages, dendritic cells, and kidney ischemia-reperfusion injury. Semin Nephrol 2010;30:268-77.
[249] Yeom HJ, Koo OJ, Yang J, Cho B, Hwang JI, Park SJ, Hurh S, Kim H, Lee EM, Ro H, Kang JT, Kim SJ, Won JK, O'Connell PJ, Surh CD, Lee BC, Ahn C. Generation and characterization of human heme oxygenase-1 transgenic pigs. PLoS One 2012;7:e46646.
[250] Webster NL, Forni M, Bacci ML, Giovannoni R, Razzini R, Fantinati P, Zannoni A, Fusetti L, Dalpra L, Bianco MR, Papa M, Seren E, Sandrin MS, Mc Kenzie IF, Lavitrano M. Multi-transgenic pigs expressing three fluorescent proteins produced with high efficiency by sperm mediated gene transfer. Mol Reprod Dev 2005;72:68-76.
[251] Lavitrano M, Bacci ML, Forni M, Lazzereschi D, Di Stefano C, Fioretti D, Giancotti P, Marfe G, Pucci L, Renzi L, Wang H, Stoppacciaro A, Stassi G, Sargiacomo M, Sinibaldi P, Turchi V, Giovannoni R, Della Casa G, Seren E, Rossi G. Efficient production by sperm-mediated gene transfer of human decay accelerating factor (hDAF) transgenic pigs for xenotransplantation. Proc Natl Acad Sci U S A 2002;99:14230-5.
[252] Sachs DH, Galli C. Genetic manipulation in pigs. Curr Opin Organ Transplant 2009;14:148-53.
[253] Mizuguchi H, Xu Z, Ishii-Watabe A, Uchida E, Hayakawa T. IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector. Mol Ther 2000;1:376-82.
[254] Walther W, Stein U. Viral vectors for gene transfer: a review of their use in the treatment of human diseases. Drugs 2000;60:249-71.
[255] Kay MA, Glorioso JC, Naldini L. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. Nat Med 2001;7:33-40.
[256] Martin P, Albagli O, Poggi MC, Boulukos KE, Pognonec P. Development of a new bicistronic retroviral vector with strong IRES activity. BMC Biotechnol 2006;6:4.
[257] Trichas G, Begbie J, Srinivas S. Use of the viral 2A peptide for bicistronic expression in transgenic mice. BMC Biol 2008;6:40.
[258] Furler S, Paterna JC, Weibel M, Bueler H. Recombinant AAV vectors containing the foot and mouth disease virus 2A sequence confer efficient bicistronic gene expression in cultured cells and rat substantia nigra neurons. Gene Ther 2001;8:864-73.
[259] Deng W, Yang D, Zhao B, Ouyang Z, Song J, Fan N, Liu Z, Zhao Y, Wu Q, Nashun B, Tang J, Wu Z, Gu W, Lai L. Use of the 2A peptide for generation of multi-transgenic pigs through a single round of nuclear transfer. PLoS One 2011;6:e19986.
[260] Ryan MD, Drew J. Foot-and-mouth disease virus 2A oligopeptide mediated cleavage of an artificial polyprotein. EMBO J 1994;13:928-33.
[261] de Felipe P, Ryan MD. Targeting of proteins derived from self-processing polyproteins containing multiple signal sequences. Traffic 2004;5:616-26.

[262] de Felipe P, Luke GA, Hughes LE, Gani D, Halpin C, Ryan MD. E unum pluribus: multiple proteins from a self-processing polyprotein. Trends Biotechnol 2006;24:68-75.
[263] Araki K, Araki M, Miyazaki J, Vassalli P. Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. Proc Natl Acad Sci U S A 1995;92:160-4.
[264] Omer A, Duvivier-Kali VF, Trivedi N, Wilmot K, Bonner-Weir S, Weir GC. Survival and maturation of microencapsulated porcine neonatal pancreatic cell clusters transplanted into immunocompetent diabetic mice. Diabetes 2003;52:69-75.
[265] Zimmermann H, Zimmermann D, Reuss R, Feilen PJ, Manz B, Katsen A, Weber M, Ihmig FR, Ehrhart F, Gessner P, Behringer M, Steinbach A, Wegner LH, Sukhorukov VL, Vasquez JA, Schneider S, Weber MM, Volke F, Wolf R, Zimmermann U. Towards a medically approved technology for alginate-based microcapsules allowing long-term immunoisolated transplantation. J Mater Sci Mater Med 2005;16:491-501.
[266] Opara EC, Kendall WF, Jr. Immunoisolation techniques for islet cell transplantation. Expert Opin Biol Ther 2002;2:503-11.
[267] Beck J, Angus R, Madsen B, Britt D, Vernon B, Nguyen KT. Islet encapsulation: strategies to enhance islet cell functions. Tissue Eng 2007;13:589-99.
[268] Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science 1980;210:908-10.
[269] Ropero AB, Alonso-Magdalena P, Soriano S, Juan-Pico P, Roepke TA, Kelly MJ, Nadal A. Insulinotropic effect of the non-steroidal compound STX in pancreatic beta-cells. PLoS One 2012;7:e34650.
[270] Gahr S, Merger M, Bollheimer LC, Hammerschmied CG, Scholmerich J, Hugl SR. Hepatocyte growth factor stimulates proliferation of pancreatic beta-cells particularly in the presence of subphysiological glucose concentrations. J Mol Endocrinol 2002;28:99-110.
[271] Garcia-Ocana A, Vasavada RC, Takane KK, Cebrian A, Lopez-Talavera JC, Stewart AF. Using beta-cell growth factors to enhance human pancreatic Islet transplantation. J Clin Endocrinol Metab 2001;86:984-8.
[272] Edlund H. Factors controlling pancreatic cell differentiation and function. Diabetologia 2001;44:1071-9.
[273] Brubaker PL, Drucker DJ. Minireview: Glucagon-like peptides regulate cell proliferation and apoptosis in the pancreas, gut, and central nervous system. Endocrinology 2004;145:2653-9.
[274] Drucker DJ. Development of glucagon-like peptide-1-based pharmaceuticals as therapeutic agents for the treatment of diabetes. Curr Pharm Des 2001;7:1399-412.
[275] Drucker DJ. Minireview: the glucagon-like peptides. Endocrinology 2001;142:521-7.
[276] Drucker DJ. Biological actions and therapeutic potential of the glucagon-like peptides. Gastroenterology 2002;122:531-44.
[277] Giorgino F, Natalicchio A, Leonardini A, Laviola L. Exploiting the pleiotropic actions of GLP-1 for the management of type 2 diabetes mellitus and its complications.

Diabetes Research and Clinical Practice 2007;78:S59-S67.
[278] Dai YT, Chen Y, Yao LS, Yang R, Sun ZY, Wen DG. [Expression of nerve growth factor in cavernous tissue and its effects on the treatment of rats with diabetic erectile dysfunction]. Zhonghua Nan Ke Xue 2005;11:748-51, 54.
[279] Li Y, Hansotia T, Yusta B, Ris F, Halban PA, Drucker DJ. Glucagon-like peptide-1 receptor signaling modulates beta cell apoptosis. J Biol Chem 2003;278:471-8.
[280] Holz GG, Chepurny OG. Glucagon-like peptide-1 synthetic analogs: new therapeutic agents for use in the treatment of diabetes mellitus. Curr Med Chem 2003;10:2471-83.
[281] Lee SH, Lee S, Youn YS, Na DH, Chae SY, Byun Y, Lee KC. Synthesis, characterization, and pharmacokinetic studies of PEGylated glucagon-like peptide-1. Bioconjug Chem 2005;16:377-82.
[282] Buss JL, Rajab A, Essig ED, Bergdall VK, Wang J, Osei K. Exenatide pretreatment improved graft function in nonhuman primate islet recipients compared to treatment after transplant only. J Transplant 2012;2012:382518.
[283] Chen DL, Xiang JN, Yang LY. Role of ERp46 in beta-cell lipoapoptosis through endoplasmic reticulum stress pathway as well as the protective effect of exendin-4. Biochem Biophys Res Commun 2012;426:324-9.
[284] Derosa G, Maffioli P. GLP-1 agonists exenatide and liraglutide: a review about their safety and efficacy. Curr Clin Pharmacol 2012;7:214-28.
[285] Mazzitelli S, Tosi A, Balestra C, Nastruzzi C, Luca G, Mancuso F, Calafiore R, Calvitti M. Production and characterization of alginate microcapsules produced by a vibrational encapsulation device. J Biomater Appl 2008;23:123-45.
[286] Korsgren O, Nilsson B. Improving islet transplantation: a road map for a widespread application for the cure of persons with type I diabetes. Curr Opin Organ Transplant 2009;14:683-7.
[287] Safley SA, Cui H, Cauffiel S, Tucker-Burden C, Weber CJ. Biocompatibility and immune acceptance of adult porcine islets transplanted intraperitoneally in diabetic NOD mice in calcium alginate poly-L-lysine microcapsules versus barium alginate microcapsules without poly-L-lysine. J Diabetes Sci Technol 2008;2:760-7.
[288] Lin CW, Chen LJ, Lee PL, Lee CI, Lin JC, Chiu JJ. The inhibition of TNF-alpha-induced E-selectin expression in endothelial cells via the JNK/NF-kappaB pathways by highly N-acetylated chitooligosaccharides. Biomaterials 2007;28:1355-66.
[289] Lin CC, Anseth KS. Glucagon-like peptide-1 functionalized PEG hydrogels promote survival and function of encapsulated pancreatic beta-cells. Biomacromolecules 2009;10:2460-7.
[290] Ruvinov E, Leor J, Cohen S. The effects of controlled HGF delivery from an affinity-binding alginate biomaterial on angiogenesis and blood perfusion in a hindlimb ischemia model. Biomaterials 2010;31:4573-82.
[291] Wang LX, Wang YP, Chen Z, Liu XY, Liu XH, Liu L, Chen WJ, Liu LB. Exendin-4 protects murine pancreatic beta-cells from dexamethasone-induced apoptosis through PKA and PI-3K signaling. Diabetes Res Clin Pract 2010;90:297-304.

[292] Liu L, Wang Y, Wang L, Lin Y, Liu X. Exendin-4 Protects Murine Pancreatic beta-Cells from Free Fatty Acid-Induced Apoptosis through PI-3K Signaling. Endocr Res 2012.
[293] Egan JM, Bulotta A, Hui H, Perfetti R. GLP-1 receptor agonists are growth and differentiation factors for pancreatic islet beta cells. Diabetes Metab Res Rev 2003;19:115-23.
[294] Kim SK, Hebrok M. Intercellular signals regulating pancreas development and function. Genes Dev 2001;15:111-27.
[295] Butler PC, Meier JJ, Butler AE, Bhushan A. The replication of beta cells in normal physiology, in disease and for therapy. Nat Clin Pract Endocrinol Metab 2007;3:758-68.
[296] Jonsson J, Carlsson L, Edlund T, Edlund H. Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 1994;371:606-9.
[297] Farilla L, Bulotta A, Hirshberg B, Li Calzi S, Khoury N, Noushmehr H, Bertolotto C, Di Mario U, Harlan DM, Perfetti R. Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets. Endocrinology 2003;144:5149-58.
[298] Wang Y, Egan JM, Raygada M, Nadiv O, Roth J, Montrose-Rafizadeh C. Glucagon-like peptide-1 affects gene transcription and messenger ribonucleic acid stability of components of the insulin secretory system in RIN 1046-38 cells. Endocrinology 1995;136:4910-7.
[299] Zhang P, Zhang X, Brown J, Vistisen D, Sicree R, Shaw J, Nichols G. Global healthcare expenditure on diabetes for 2010 and 2030. Diabetes Res Clin Pract 2010;87:293-301.
[300] Mathis D, Vence L, Benoist C. beta-Cell death during progression to diabetes. Nature 2001;414:792-8.
[301] Silva AI, de Matos AN, Brons IG, Mateus M. An overview on the development of a bio-artificial pancreas as a treatment of insulin-dependent diabetes mellitus. Med Res Rev 2006;26:181-222.
[302] Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, Secchi A, Brendel MD, Berney T, Brennan DC, Cagliero E, Alejandro R, Ryan EA, DiMercurio B, Morel P, Polonsky KS, Reems JA, Bretzel RG, Bertuzzi F, Froud T, Kandaswamy R, Sutherland DE, Eisenbarth G, Segal M, Preiksaitis J, Korbutt GS, Barton FB, Viviano L, Seyfert-Margolis V, Bluestone J, Lakey JR. International trial of the Edmonton protocol for islet transplantation. N Engl J Med 2006;355:1318-30.
[303]Faradji RN, Froud T, Messinger S, Monroy K, Pileggi A, Mineo D, Tharavanij T, Mendez AJ, Ricordi C, Alejandro R. Long-term metabolic and hormonal effects of exenatide on islet transplant recipients with allograft dysfunction. Cell Transplant 2009;18:1247-59.
[304] Gregoriades N, Luzardo M, Lucquet B, Ryll T. Heat inactivation of mammalian cell cultures for biowaste kill system design. Biotechnol Prog 2003;19:14-20.
[305] Hara Y, Fujino M, Nakada K, Kimura K, Adachi K, Li XK. Influence of the numbers of islets on the models of rat syngeneic-islet and allogeneic-islet transplantations. Transplant Proc 2006;38:2726-8.

[306] O'Sullivan ES, Johnson AS, Omer A, Hollister-Lock J, Bonner-Weir S, Colton CK, Weir GC. Rat islet cell aggregates are superior to islets for transplantation in microcapsules. Diabetologia 2010;53:937-45.
[307] Park SJ, Shin S, Koo OJ, Moon JH, Jang G, Ahn C, Lee BC, Yoo YJ. Functional improvement of porcine neonatal pancreatic cell clusters via conformal encapsulation using an air-driven encapsulator. Exp Mol Med 2012;44:20-5.
[308] Jansson L, Hellerstrom C. Stimulation by glucose of the blood flow to the pancreatic islets of the rat. Diabetologia 1983;25:45-50.
[309] Johansson A, Olerud J, Johansson M, Carlsson PO. Angiostatic factors normally restrict islet endothelial cell proliferation and migration: implications for islet transplantation. Transpl Int 2009;22:1182-8.
[310] Figliuzzi M, Cornolti R, Perico N, Rota C, Morigi M, Remuzzi G, Remuzzi A, Benigni A. Bone marrow-derived mesenchymal stem cells improve islet graft function in diabetic rats. Transplant Proc 2009;41:1797-800.
[311] Cardozo AK, Proost P, Gysemans C, Chen MC, Mathieu C, Eizirik DL. IL-1beta and IFN-gamma induce the expression of diverse chemokines and IL-15 in human and rat pancreatic islet cells, and in islets from pre-diabetic NOD mice. Diabetologia 2003;46:255-66.
[312] Yang L, Lindholm K, Konishi Y, Li R, Shen Y. Target depletion of distinct tumor necrosis factor receptor subtypes reveals hippocampal neuron death and survival through different signal transduction pathways. J Neurosci 2002;22:3025-32.
[313] Natalicchio A, De Stefano F, Orlando MR, Melchiorre M, Leonardini A, Cignarelli A, Labarbuta R, Marchetti P, Perrini S, Laviola L, Giorgino F. Exendin-4 prevents c-Jun N-terminal protein kinase activation by tumor necrosis factor-alpha (TNFalpha) and inhibits TNFalpha-induced apoptosis in insulin-secreting cells. Endocrinology 2010;151:2019-29.
[314] Ferdaoussi M, Abdelli S, Yang JY, Cornu M, Niederhauser G, Favre D, Widmann C, Regazzi R, Thorens B, Waeber G, Abderrahmani A. Exendin-4 protects beta-cells from interleukin-1 beta-induced apoptosis by interfering with the c-Jun NH2-terminal kinase pathway. Diabetes 2008;57:1205-15.
[315] Ludwig B, Zimerman B, Steffen A, Yavriants K, Azarov D, Reichel A, Vardi P, German T, Shabtay N, Rotem A, Evron Y, Neufeld T, Mimon S, Ludwig S, Brendel MD, Bornstein SR, Barkai U. A novel device for islet transplantation providing immune protection and oxygen supply. Horm Metab Res 2010;42:918-22.
[316] Dufrane D, Goebbels RM, Gianello P. Alginate macroencapsulation of pig islets allows correction of streptozotocin-induced diabetes in primates up to 6 months without immunosuppression. Transplantation 2010;90:1054-62.
[317] Bloch K, Bloch O, Tarasenko I, Lazard D, Rapoport M, Vardi P. A strategy for the engineering of insulin producing cells with a broad spectrum of defense properties. Biomaterials 2011;32:1816-25.