EVALUATION OF PIG SKIN FOR WOUND THERAPY IN TAIWAN

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ABSTRACT

The primary function of the skin is to serve as a protective barrier against the environment. Loss of the integrity of large portions of the skin as a result of injury or illness may lead to major disability or even death. The many similarities between man and pig would lead one to believe that the pig should make an excellent animal model for human wound healing. Many pig breeds were adopted in wound study, the disadvantages of large body size and weight in using breeds such as Landrance and Yorkshire had been specified. Recently, the Lanyu pig, a Taiwan domestic minipig, draws a lot of attention because of its small body size and weight. However, the skin wound healing profile of this breed is still unknown. The present study was performed to characterize Lanyu pig skin with different age and establish standardized procedure on creating a suitable wound size and wound thickness for investigating the progress of wound healing without treatment and establish wound array for potential drug treatment in these pigs.

Further, Skin regeneration is an important field of tissue engineering. Especially in larger burns and chronic wounds, present treatments are insufficient in preventing scar formation and promoting healing. The remarkable potential of embryonic stem cells is their ability to develop into many different cell types. The technology combine somatic cell nuclear transfer（SCNT）and stem cell technology to acquire multiple different types of cells for repair or replace damaged and diseased cells, known as therapeutic cloning. The in vitro differentiation of ES cells may provide an excellent model for studying the cellular and molecular mechanisms of early epidermal development and eventually the generation of donor cells for transplantation therapies of wounds. Logically, before application of stem cell therapies into a human medicine, it is necessary to verify the efficiency and safety of these methods in an acceptable animal model.

Key words: Lanyu pig, Skin, Wound healing

FULL-THICKNESS WOUND MODEL IN PIGS

The purpose of this study is to build an incision wound healing model in pigs and using this model to evaluate multiple full-thickness excisional wounds (3cm × 3cm、 5cm × 5cm、 10cm × 10cm) without any further treatment. First of all, wounds were created on the dorsum of 2-month-old Landrace pig (n = 4). The wound were approximately 8 mm deep. These wounded pigs dressed with Tegaderm, a polyurethane occlusive dressing and protected cloth. At each week after surgery four of the pigs right flank appearance of the wounds was photography, evaluate time for complete reepithelialization, scar area measurement and wound contraction determination. At the end of observation ,transverse sections, approximately 3 mm thick, were cut through the central part of the wounds, fixed in buffered 4 % formaldehyde. Sections (5 μm) were stained with hematoxylin and eosin and examined using light microscorpy. The results indicate that from small to large wound size can heal spontaneously. The fully reepithelialization was shown to complete on day 14 (3cm × 3cm), 28 (5cm × 5cm) and 56 (10cm × 10cm). From week one to week four, area of wounds will decrease to 25 % of original size. On week five, wound contraction slow down and maintain the same wound area. Later, wound area no more change till the end of observation. Sections to compare with health skin show that epidermis is much thicker and flattened. There are more collagen bundles with irregular orientation in dermis. Burning wound not only with large area but depth damage to dermis may not heal well and need longer wound care time. In most research, small wounds (3cm × 3cm) were adopted to evaluate how well the treatment will be by combining clinical skin products. In our opinion, information and method using in small wound may not fulfill the demand for treatment in large wound. Therefore, the interaction, application and evaluate methods when combining clinical skin products or not in large wound treatment may need further study in the future.

LARGER WOUND IN PORCINE FULL-THICKNESS WOUND HEALING

The purpose of this study was to establish standardized procedure on wound creation and understand re-epithelialization, contraction rate and scar formation in case of larger full-thickness wounds in swine. Multiple larger full-thickness excision wounds (10 × 10 cm) were created on the dorsum of 7-week-old (n = 5) Landrace pigs. The extents of wound closure area and wound contraction were measured using clear plastic sheets from weeks 0 to 10. These transparent sheets were scanned and then ImageJ software was used to measure the area of each wound, and a contraction rate was calculated for each wound on each week using week 0 as “0 % contracted.” Based on the gross appearance and biopsies taken from the center of the wound, we estimated the time to completed re-epithelialization for wound was 6 ± 0.5 weeks (mean ± SD). The mean wound closure area in each week was 8.0 ± 2.2, 5.7 ±

2.7, 4.2 ± 2.3, 4.1 ± 2.2, 4.0 ± 2.2, 3.6 ± 1.9, 3.8 ± 1.8, 3.8 ± 1.8, 3.9 ± 1.8 and 3.9 ± 1.8cm^2**.** The mean wound contraction rate was about 35 ± 5, 67± 8, 77 ± 5, 83 ± 5, 84 ± 5, 87 ± 4, 85 ± 3, 85 ± 3, 85 ± 3 and 85 ± 3%. Although wound contraction measurements from weeks 4 to 10 showed no statistical differences among the pigs, the wound contraction of full-thickness wounds was faster from the first week to third week *($P < 0.05$)*. These results suggest that this larger wound model can be used to test the effects of therapeutic approaches such as dermal and epidermal substitutes, efficacy, fine-tuning of required cell densities and safety aspects relating to immunoreactivity and biocompatibility intended to treat larger full-thickness wound.

SKIN GROWTH IN DIFFERENT PIG BREEDS

The purpose of this study was to investigate the profile and to determine the breed-specific constant of skin growth among different pig breeds. This skin growth constant may be used to calibrate and verify the real wound closer rate after wound creation. The pigs were clipped and their skin were scrubbed by brush clean with soap and then rinsed with tap water. On each flank of the pig, the margins of three squares of different sizes were tattooed with the aid of templates (3cm × 3cm, 5cm × 5cm and 10cm × 10cm). The length of outer margins of each square were measured and recorded weekly until puberty of the pig for determination of skin growth constant. The results shows that the growth rate of in Lanyu and Landrance were 1.34 and 2.97 kg/week, with the rate of skin growth of 1.55 and 1.22 cm^2/week, respectively. That could be explained as each kg of weight gain equates to the growth of 1.16 cm^2 and 0.41 $cm^2$of skin area in the Lanyu pig and the Landrance, respectively. It is concluded that the Lanyu pig has a higher skin growth rate than that of the Landrance. Furthermore, the skin growth constant of Lanyu pigs and Landrance were calculated as 1.1181 and 1.0733. Based on these result, it is important to verify with the skin growth constant for determination of the real wound closer rate after wound creation.

AGE EFFECTS ON WOUND CLOSURE AND REEPITHELIALIZATION OF FULL-THICKNESS WOUND HEALING IN LANYU MINIPIGS

The aim of this study was to investigate the ages of Lanyu pigs on the profile of re-epithelialization, contraction rate and scar formation in case of full-thickness skin wounds. Multiple full-thickness excision wounds such as small (3 × 3 cm), middle (5 × 5 cm) and large (10 × 10cm) were created on the dorsum of 3.5-month-old (n = 1) and 9-month-old (n = 2) Lanyu pigs. The photography of the wounds of the pigs was taken at each week after surgery.

The evaluation of the period for complete re-epithelialization, scar area, and wound contraction were also determined weekly. The results indicates that the period for complete re-epithelialization in adult pigs were 1 week faster than young pigs with small and middle wounds. In large wounds, the fully re-epithelialization was shown to be no difference between young and adult pigs. Furthermore, the mean wound closure area and contraction rate of three wound sizes in young pigs were larger and stronger than adult pigs. These results suggested that Lanyu minipig wound model could be used to test the effects of therapeutic approaches such as dermal and epidermal substitutes, efficacy, fine-tuning of required cell densities and safety aspects relating to immunoreactivity and biocompatibility intended to treat full-thickness wound.

MOLECULAR AND CELLULAR BIOLOGY OF SKIN WOUND HEALING IN LANYU PIG

The purpose of this study was to define the pattern of mRNA and cellular change at various time post-wounding. The DNA and RNA were isolated from normal skin and samples at various time post-wounding .The mRNA levels for relevant molecules were assessed by semiquantitative RT-PCR using porcine specific primer sets. Analysis of cellular change was assessed by DNA quantification and histology of tissue sections. The results demonstrated that the changes in the pattern of RNA and DNA content of the scar tissue were consistent with the observed increasing cellularity. The mRNA levels for Growth factor (CTGF, bFGF, KGF, IGF-1, IGF-2, TGF-β) , Growth factor receptor and others (GR, IGF-1R, PAFR, PDGFR) ,Cytokine (IL-1,IL-6,TNF-α), Proteoglycan (Biglycan, Decorin, Fibromodulin, Versican), Matrix metalloproteinases (MMP-1,MMP-2,MMP-9), Matrix metalloproteinases inhibitors (TIMP-1,TIMP-2,TIMP-4), Type I collagen, bone morphogenetic protein-1 and heat shock protein 47 were significantly elevated during healing; the mRNA levels for TIMP-3 were depressed. These findings suggest that skin wound healing is a series of complex matrix-cell interactions that involve cellular migration and inflammation, followed by proliferation of fibroblasts with new collagen synthesis, and lastly tissue remodeling of the scar. Further definition of this model should identify unique points in the healing processs, and such information could lead to development of therapeutic intervenetions to improve skin wound healing.

PIG AS ANIMAL MODEL FOR THERAPEUTIC CLONING

The technology combine somatic cell nuclear transfer（SCNT）and stem cell technology to acquire multiple different types of cells for repair or replace damaged and diseased cells,

known as therapeutic cloning. Logically, before application of stem cell therapies into a human medicine, it is necessary to verify the efficiency and safety of these methods in an acceptable animal model. Therefore the purpose of this study was conducted to take healthy cells from a pig's ear and create cloned cells that can be used as a source of perfectly matched cells to replace or repair damaged or diseased tissues by using domestic mininature Lan-yu pigs. Here, we established the cell line of ear skin fibroblasts derived from Lan-yu boar (206-01) and gilt (206-02). These cell lines also transfected with fluorescent gene (pZsgreen-N1) for further ex vivo tracing. Recipient gilt oocytes were derived from slaughter house and in vitro cultured for 42hr for maturation. After SCNT, electrofusion of donor cell and recipient oocyte membrane were conducted with a single pulse of 3.0 kV/cm for 20 μs. Four h after fusion, reconstructed embryos were activated for 5 min with Ca^{2+} ionophore and 6-DMAP at 4 h separately. Activated reconstructed embryos were cultured in NCSU-23 medium, 38.5℃, 5 % CO_2, R.H. 100% incubator. SCNT-derived blastocysts at day 7-9 were culture as a whole or used for isolation of ICMs on murine embryonic fibroblasts（MEF）or mouse STO feeder layers in porcine embryonic stem cell（pESC）medium for 3 days. All blastocysts or ICMs were monitored daily for the attachment to the feeder layer and colony formation. Among 1536 reconstructed embryos 81 qualified blastocysts had generated. Totally, 29 of blastocysts or ICMs had been transferred to MEF feeder layer and other 52 had been transferred to STO feeder layer. There are more primary colonies formed from the culture of ICMs than the intact blastocysts. Some of the primary colonies that inoculated did not survive the first passage and the other were all consequently degenerated or lost by passage 2. In conclusion, the attempt for deriving porcine ntES cell lines is still unsuccessful and required further study.

IN VITRO DIFFERENTIATION OF TRANSPLANTABLE KERATINOCYTE PRECURSORS FROM PIG EMBRYONIC STEM

Porcine embryonic stem (pES) cells had been isolated and characterized in our previous study. The remarkable potential of embryonic stem cells is their ability to develop into many different cell types. The in vitro differentiation of ES cells may provide an excellent model for studying the cellular and molecular mechanisms of early epidermal development and eventually the generation of donor cells for transplantation therapies. The objective of this study is to use porcine ES cells as a reproducible cellular model to test the potential of ES cells to differentiate into epidermal cells without formation of EB. The results indicated that the derivation of epidermal cells from porcine ES cells has been successfully induced by exposing the cells to precisely controlled instructive conditions including extracellular matrix (ECM) and the morphogen BMP-4. This in vitro approach will be an invaluable tool for

recapitulating the cellular events of the differentiation from uncommitted ES cells to functional epidermal cells, including the characterization of putative epidermal cells, the delineation of cell fate selection, as well as cell commitment and specialization in vitro.

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