

Transgenic expression of human cytotoxic T-lymphocyte associated antigen4-Immunoglobulin (hCTLA4Ig) by porcine skin for xenogeneic skin grafting

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Abstract Porcine skin is frequently used as a substitute of human skin to cover large wounds in clinic practice of wound care. In our previous work, we found that transgenic expression of human cytotoxic T-lymphocyte associated antigen4-immunoglobulin (hCTLA4Ig) in murine skin graft remarkably prolonged its survival in xenogeneic wounds without extensive immunosuppression in recipients, suggesting that transgenic hCTLA4Ig expression in skin graft

may be an effective and safe method to prolong xenogeneic skin graft survival. In this work, using a transgene construct containing hCTLA4Ig coding sequence under the drive of human Keratine 14 (k14) promoter, hCTLA4Ig transgenic pigs were generated by somatic nuclear transfer. The derived transgenic pigs were healthy and exhibited no signs of susceptibility to infection. The hCTLA4Ig transgene was stably transmitted through germline over generations, and thereby a transgenic pig colony was established. In the derived transgenic pigs, hCTLA4Ig expression in skin was shown to be genetically stable over generations, and detected in heart, kidney and corneal as well as in skin. Transgenic hCTLA4Ig protein in pigs exhibited expected biological activity as it suppressed human lymphocyte proliferation in human mixed lymphocyte culture to extents comparable to those of commercially purchased purified hCTLA4Ig protein. In skin grafting from pigs to rats, transgenic porcine skin grafts exhibited remarkably prolonged survival compared to the wild-type skin grafts derived from the same pig strain (13.33 ± 3.64 vs. 6.25 ± 2.49 days, $P < 0.01$), further indicating that the transgenic hCTLA4Ig protein was biologically active and capable of extending porcine skin graft survival in xenogeneic wounds. The transgenic pigs generated in this work can be used as a reproducible resource to provide porcine skin grafts with extended survival for wound coverage, and also as donors to investigate the impacts of hCTLA4Ig on xenotransplantation of other organs (heart, kidney and

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corneal) due to the ectopic transgenic hCTLA4Ig expression.

Keywords hCTLA4Ig · Transgenic pigs · Skin grafting · Xenotransplantation

Introduction

Skin grafting following early excision of necrotic burned tissues is often required for the treatment of severe large burn wounds (Orgill 2009; Desai et al. 1990; Wang et al. 2007). Although human autologous or allogeneic skins are preferred materials to cover burn wounds in clinic practice, their disadvantages include limited or unstable supply, easier disease transmission and ethic considerations. For these reasons, xenogeneic skins, especially porcine skins, are frequently used as substitutes of human skins for large burn wound coverage in clinic burn wound care (Weiner et al. 2010). However, the severe immune rejection after transplantation limits its application (Luo et al. 2005).

Skin is avascular tissue and T cells play critical roles in the rapid immune rejection following skin grafting. Cytotoxic T-lymphocyte associated antigen4-immunoglobulin (CTLA4Ig), a soluble chimeric molecule consisting of the extracellular domain of CTLA4 on the N-terminus and the constant and hinge regions of IgG on the C terminus, is a well established molecule capable of down-regulating T cell activation by blocking co-stimulatory pathway and inducing T cell energy, and thereby prolonging graft survival in different experimental models (Linsly et al. 1992; Lenschow et al. 1992; Turka et al. 1992; Jin and Xie 2003; Wang et al. 2004; Stell et al. 2003; Guo et al. 2003). Due to the confirmed functionality of CTLA4Ig, human CTLA4Ig (hCTLA4Ig) protein has been developed as a drug (Abatacept) for the treatment of autoimmunity-related diseases, such as rheumatoid arthritis (RA), juvenile idiopathic arthritis, systemic lupus erythematosus and psoriasis vulgaris (Larsen et al. 2005; Kremer et al. 2005; Genovese et al. 2005; Weisman et al. 2006; Ruperto et al. 2008; Abrams et al. 1999). In our previous work, biologically active hCTLA4Ig protein was transgenically expressed in mouse skins and the hCTLA4Ig transgenic mouse skin grafts exhibited remarkably prolonged survival in

xenogeneic rat wounds, suggesting that transgenic expression of hCTLA4Ig in skin graft may be a potential method to prolong clinic xenogeneic skin graft survival (Jiang et al. 2012; Wang et al. 2008).

Porcine skin is the most frequently used xenogeneic skin in clinic practice as a substitute of human skin, therefore transgenic expression of biologically active hCTLA4Ig in porcine skin is of important clinic implication. In this work, using a transgene construct containing hCTLA4Ig coding sequence (CDS) under the drive of human keratin14 gene (K14) promoter, transgenic pigs expressing hCTLA4Ig protein in skin were generated by somatic cell nuclear transfer (SCNT). The transgenic hCTLA4Ig protein exhibited expected bio-activity as it suppressed human lymphocyte proliferation in mixed lymphocyte culture (MLC), and the survival of hCTLA4Ig transgenic porcine skin grafts in xenogeneic (rat) wounds was remarkably prolonged compared to that of the wild-type skins. These results indicated that transgenic expression of hCTLA4Ig in skin is a potential method to prolong the survival of porcine skin grafts in xenogeneic wounds, and the hCTLA4Ig transgenic pigs can be used as a reproducible resource providing transgenic porcine skin grafts with extended survival.

Materials and methods

Transgene construct

The transgene construct used for the generation of transgenic pigs is as previously described (Wang et al. 2005, 2006). This transgene construct, called K14-hCTLA4Ig in this article, contained a complete hCTLA4Ig CDS which is downstream human K14 promoter and followed by human K14 poly (A) signal sequence. Besides, to stabilize transcripts and improve transgene expression, an intronic sequence from rabbit globulin gene was inserted between K14 promoter and hCTLA4Ig CDS. To add a selection marker gene to the K14-hCTLA4Ig construct, the eGFP expression cassette in pEGFP-C1 vector which contains a selection marker neomycine resistance (Neo^r) gene, was replaced with the DNA fragment containing the K14-hCTLA4Ig construct by blunt end ligation. As a result, a recombinant vector, designated as pK14-hCTLA4Ig-C1, was

constructed and used for the generation of transgenic pigs.

Preparation of transgenic nuclei donor cells

Porcine embryonic fibroblasts (PEFs) were derived from fetus at 32–35 days of gestation as previously described (Betthausen et al. 2000). For transfection of PEFs with the pK14-hCTLA4Ig-C1 vector, the PEFs cultured to subconfluence in DMEM media containing 15 % fetal bovine serum (FBS) were digested using DMEM media containing 0.25 % trypsin, and then centrifuged (250 g, 5 min) and resuspended in 500 μ L phosphate buffered saline. The concentrations of suspended cells were adjusted to be 5×10^6 cells/mL, and the cells were mixed with purified pK14-hCTLA4Ig-C1 vector (50 ng/ μ L) which was linearized by digestion with the restrictive enzyme ApaLI. The PEFs were then subjected to electroporation in cuvettes (Bio-rad) with 4 mm gap using electroporation system Gene Pulser MXcell (Bio-rad) at the following condition: voltage (V): 230; pulse length(ms): 5.0; number of pulse: 1. The treated cells were equally divided into ten culture plates with 10 cm diameter, and cultured in DMEM media containing 15 % FBS at 38.5 °C in humidified air containing 5 % CO₂. After 24–48 h of culture, the neomycin analog G418 were added into the culture at the final concentration of 800 μ g/mL. Thereafter, the culture media was changed every 3 d, and the selection culture continued until cell colonies emerged. The G418-resistant cell colonies were harvested, cultured and expanded. The presence of transgene in the transfected cells were detected by PCR using the following primer set which covered the junction region between the artificially added Oncostain M secretory signal and the native hCTLA4Ig CDS: hCTLA4Ig-F1 (forward): 5'-GACGCTGCTCAGTCTGGT-3'; hCTLA4Ig-R1 (reverse): 5'-TGTAGAGTCCCGTGTCCAT-3', and the size of PCR product was 326 bp.

SCNT and embryo transfer

The transgenic PEFs were cultured to confluence, and then digested, washed and resuspended in fresh DMEM media containing 15 % FBS. The suspended transgenic PEFs were used as donor cells for SCNT. SCNT was performed as previously described (Lai

et al. 2002; Betthausen et al. 2000). Briefly, cumulus-oocyte complexes (COCs) were aspirated from follicles with 2–8 mm diameter on the surfaces of ovaries collected from slaughter house. The COCs were cultured for maturation in TCM199 media supplemented with 0.1 % polyvinyl alcohol, L-cysteine (0.1 mg/ml), epidermal growth factor (10 ng/ml), 0.91 mM Na-pyruvate, 3.05 mM D-glucose, follicle-stimulating hormone (0.5 mg/ml), luteinizing hormone (0.5 mg/ml), penicillin (75 mg/ml), streptomycin (50 mg/ml) and 10 % follicle fluid. After 42–48 h of culture, the COCs were digested using TCM199 media containing 0.1 % hyaluronidase to free individual oocytes of cumulus. Matured oocytes with extruded polar body were selected under stereo microscope and used for nuclear transfer (NT). The NT, fusion and activation were performed as described (Betthausen et al. 2000). The NT-derived embryos were kept in PZM-3 media at 38.5 °C in humidified air containing 5 % CO₂ for 18–24 h. The surviving embryos were selected and transferred into the oviducts of surrogate sows (Lai et al. 2002).

Genetic screen of transgenic pigs and husbandry

The NT-derived piglets were subjected to PCR screen to detect the presence of transgene using the same primer set used for genetic screen of transgenic PEFs as described above. The transgenic founder pig used for breeding and its offspring individuals were further subjected to Southern blot assay. Southern blot was performed using DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche) as described in manual. Briefly, the DIG-labeled probe was prepared by PCR using the primer pair set DIG-probe-F/DIG-probe-R (DIG-probe-F: 5-CAAGTGCAAGGTCTCCAACA-3; DIG-probe-R: 5-GGATCTTCCAGTGGGATCTG-3; product size: 506 bp), which covered the junction region between hCTLA4Ig CDS and K14 poly(A) signal sequence. Porcine genomic DNAs (>10 μ g) were completely digested at the unique *Eco*RI site, and subjected to electrophoresis in 0.8 % agarose gel with a low voltage (40 v) to thoroughly separate the digested genomic fragments. Thereafter, the genomic DNAs were denatured and transferred to HyBond N+ NC membrane (Amersham), and then hybridized with the DIG-labeled probe. After hybridization, the hybridization signal was detected using the

alkaline phosphatase-conjugated anti-digoxigenin antibody Anti-Dig-AP.

Western blot and quantitative RT-PCR (qRT-PCR) analysis

Total protein samples were extracted from about 50 mg tissues collected from pigs using total protein extraction kit (ProMab) as described in the manual. For western blot (WB), a rabbit anti-human CTLA4 poly-clonal antibody (Abcam) was used as the primary antibody, and the HRP-conjugated goat anti-rabbit IgG antibody as the secondary antibody. The GAPDH protein was detected as internal control using the mouse anti-human GAPDH ploy-clonal antibody (Santa Cruz) and HRP-conjugated goat anti-mouse IgG as the primary and secondary antibody respectively. The WB process was performed as previously described (Jiang et al. 2012). For qRT-PCR analysis, total RNA samples were prepared, treated and reversely transcribed as previously described (Jiang et al. 2012). The primer pair set for qPCR analysis of transgenic hCTLA4Ig expression was hCTLA4Ig-F2/hCTLA4Ig-R2, of which the sequences were 5'-ACTCCGACGGCTCCTTCTT-3' and 5'-TCTTCTGCGTGTAGTGGTTGTG-3' respectively, and the product size was 123 bp. The qPCR primer pair set for the internal control gene GAPDH was GAPDH-F/GAPDH-R, of which the sequences were: 5'-GATCCCGCCAACATCAAAT-3' and 5'-TCACGCCCATCACAACAT-3', and the product size was 166 bp.

Detection of transgenic hCTLA4Ig protein in the sera of transgenic pigs

The levels of CTLA4Ig protein in the sera of transgenic pigs were detected by enzyme-linked immunosorbent assay (ELISA) using the rabbit anti-human CTLA4 poly-clonal antibody (Abcam) attached to ELISA plates as the primary antibody as previously described (Wang et al. 2006; Jiang, et al. 2012).

One-way mixed human lymphocyte culture (MLC) reaction

One-way human MLC system was established as described (Jiang et al. 2012). Briefly, human

peripheral blood mononuclear cells were isolated from two different individuals with no blood relation, which were marked A as reacting cells and B as stimulating cells respectively. The stimulating cells (B) were inactivated by incubating with mitomycin C at a final concentration of 25 µg/ml, and then the mitomycin C-treated stimulating cells (B) and the reacting cells (A) were mixed together in 96-well flat-bottomed plates. After different volumes (10, 20 and 40 µL) of porcine sera were added, fresh culture media was added into MLC systems up to a final volume of 200 µL, and then the MLC systems were cultured at 37 °C, 5 % CO₂, humidified air for 5 d. The MLC system containing only reacting cells was used as a quality control for MLC reaction, and each system had three duplicates. To test cell proliferation level in MLCs, the radioactive substrate 3H-TdR were added into MLC system at a dosage of 1uc per well at 16 h before termination of culture, and the radioactivity of MLC was tested by radio-activity detector after culture was terminated.

Skin grafting

Adult transgenic pigs were subjected to euthanasia and hair shaving. Split-thickness skin grafts were made from the full thickness skin peeled from transgenic pig bodies. Skin grafting was performed using rats as the recipients. Briefly, the hair on the back of anesthetized recipient rats was shaved off, and a 1.5 × 1.5 cm² wound was made on the back by shaving off the dorsal skin carefully while leaving the subcutaneous vasculature intact. Porcine skin graft with the size of 1.5 × 1.5 cm² was planted into the wound and sutured with the host skin. To fasten the touch of skin graft with the wound, multilayered gauze pieces with proper size were fixed onto the skin grafts using the same sutures fixing the skin grafts to provide a sustainable pressure. The survival of skin grafts was observed every other day, and the survival time was defined as the first day on which more than 90 % area of skin grafts was necrotic as described (Wang et al. 2008). For histological examination of skin graft survival, on 10 d post grafting, recipient rats were subjected to euthanasia, and the skin graft along with the host skin surrounding the wound was cut off, fixed in 4 % paraformaldehyde and subjected to conventional HE staining.

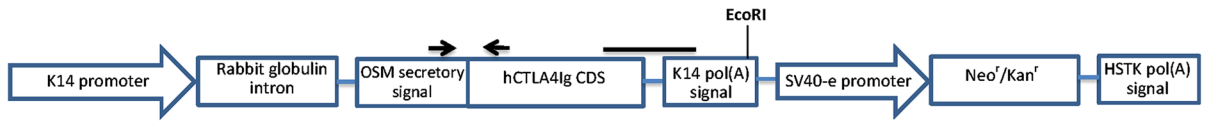


Fig. 1 The schematic structure of transgene vector used in this work. The *arrows* show the positions of primer pair set used for the PCR screen of transgenic pigs, and the *black bar* indicates

the place where Southern blot probe locates. *OSM*: oncostain M, *EcoRI*: the unique cleavage site for Southern blot assay

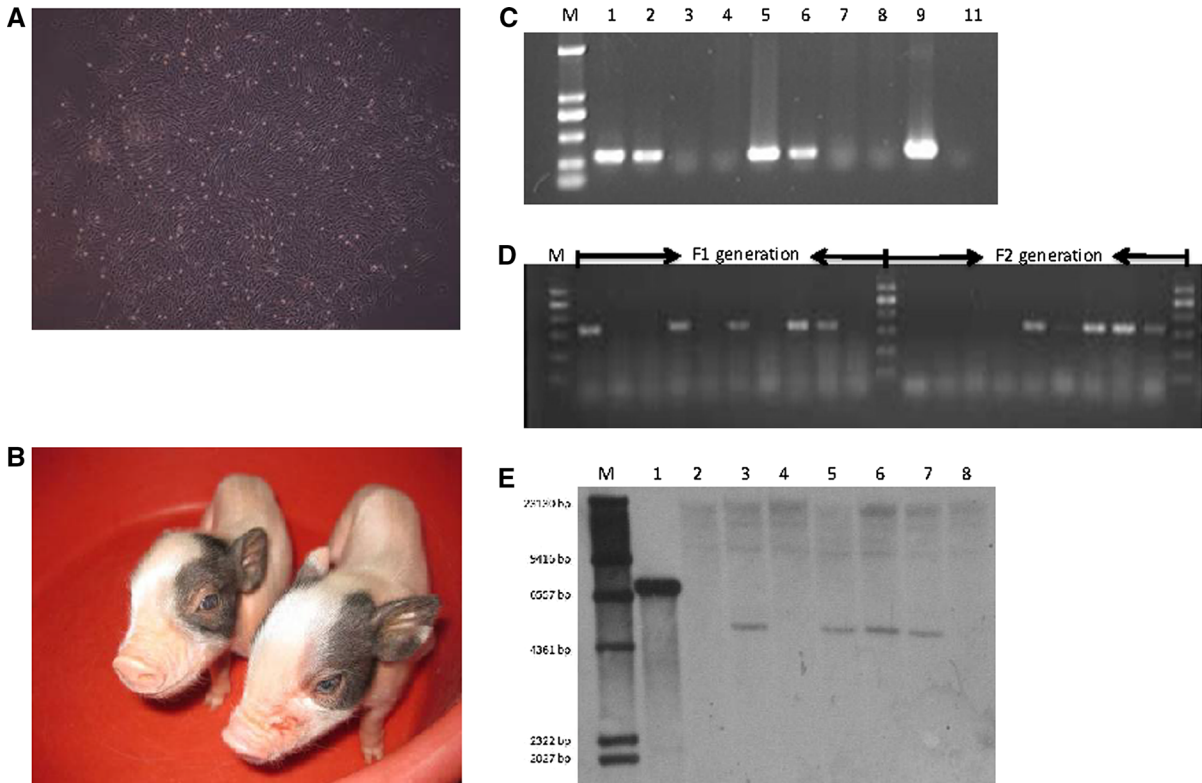


Fig. 2 Production of hCTLA4Ig transgenic pigs by SCNT. **a** The PEF clone transgenic for the pK14-hCTLA4Ig-C1 vector after selection culture with neomycin. **b** The cloned piglets derived from SCNT using transgenic PEFs as nuclei donors. **c** Genetic screen of cloned piglets by PCR; *M*: DNA marker (DL 2,000), *1–8* cloned piglets, *9* transgene plasmids; *10* wild-type

Fig. **d** Genetic screen of transgenic individuals of F1 and F2 generations by PCR; *M* DNA marker (DL 600). **e** Transgene integration detected by Southern blot; *M*: DNA molecular-weight marker, *1* transgene plasmids, *2* wild-type pig; *3* the transgenic founder pig, *4–6* offspring individuals of F1 generation, *7–8* offspring individuals of F2 generation

Results

The PEFs used for transfection with the transgene vector pK14-hCTLA4Ig-C1 in this study were derived from a Chinese minipig strain-Bama minipig. Transgenic PEF colonies were generated by electroporation with linearized K14-hCTLA4Ig-C1 plasmid (Fig. 2a), of which the schematic structure was shown in Fig. 1. Using the PEFs transgenic for pK14-hCTLA4Ig-C1 as the donor cells, six male piglets were derived from

SCNT, and four were detected to be transgenic by PCR screen using the genomic DNA samples as templates (Fig. 2c). Two transgenic founder pigs died shortly after birth. The rest two transgenic founders survived (Fig. 2b), but one died after 6 months, while the other one kept healthy. The K14-hCTLA4Ig transgenic pig colony was established and expanded by mating the healthy transgenic founder pig with wild-type sows. About 50 % of the offspring derived from the transgenic founder were detected to be transgenic by

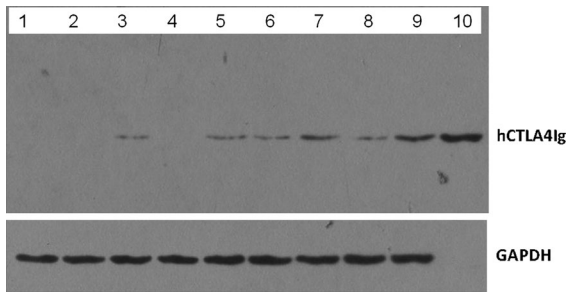


Fig. 3 WB analysis of hCTLA4Ig expression in different organs of transgenic pigs. 1 wild-type porcine skin, 2–7 different organs from transgenic individual of F1 generation (2 liver, 3 heart, 4 lung, 5 kidney, 6 skin, 7 corneal), 8–9 skin and corneal from transgenic individual of F2 generation; 10 purified hCTLA4Ig protein

PCR (Fig. 2d), suggesting that the transgene transmitted through germline in Mendel manner. Transgene integration and germline transmission was further confirmed by Southern blot. As shown in Fig. 2e, the transgene was detected by Southern blot in the founder pig and three of five offspring individuals which were randomly selected from F1 and F2 generations. Only one blot band was observed in the assay, suggesting that the transgene integrated into one genomic site as a single copy, which was consistent with the fact that hCTLA4Ig transgene was transmitted through germline in Mendel manner.

The hCTLA4Ig expression in the transgenic pigs was detected by WB. The K14 promoter-driven hCTLA4Ig expression in pigs was detected in skin, corneal, kidney and heart (Fig. 3), being not exactly confined to skin, which was not consistent with our previous data for transgenic mice (Wang et al. 2005; Jiang et al. 2012). The highest level of hCTLA4Ig expression was detected in corneal, which may be due to the fact that keratins were highly expressed by keratinocytes located in corneal. The transgenic hCTLA4Ig expression was further investigated by qRT-PCR in a relative quantitation manner using the transgenic hCTLA4Ig expression level in the skin of founder pig as the standard. The qRT-PCR data showed that the transgenic hCTLA4Ig was expressed in heart and kidney at levels comparable to that in skin, and in corneal at a remarkably higher level (Fig. 4). Besides, the hCTLA4Ig expression levels in the skins of transgenic individuals randomly selected from F1 and F2 generations were all comparable to that in the

Fig. 4 qRT-PCR analysis of transgenic hCTLA4Ig expression. Using the transgenic hCTLA4Ig expression in the skin of transgenic founder pig as the standard, the transgenic hCTLA4Ig expression was relatively quantitated by qRT-PCR in the skins and other organs or tissues of transgenic individuals randomly selected from F1 and F2 generations (three individuals/each generation). **a** The amplification plot and melt curve of the real-time RT-PCR for the internal control gene GAPDH. **b** The amplification plot and melt curve of the real-time RT-PCR for transgenic hCTLA4Ig. **c** The relative hCTLA4Ig expression levels in the skins and other organs or tissues of transgenic pigs; F0 skin: the skin of transgenic founder pig; F1 skin: the skin samples of transgenic individuals from F1 generation; F2 skin: the skin samples of transgenic individuals from F2 generation

skin of the founder pig, suggesting that the transgenic hCTLA4Ig expression was genetically stable.

The hCTLA4Ig protein is soluble and secretory which was transported into bloodstream after transgenic expression in mouse skin as described in our previous work (Wang et al. 2006; Jiang et al. 2012). This was also the case when it came to transgenic pigs. By ELISA, hCTLA4Ig protein was detected in the sera of transgenic pigs and the concentrations of hCTLA4Ig protein in the sera of randomly selected transgenic individuals varied between 5–10 ng/ μ L (Table 1). To test the bio-activity of transgenic hCTLA4Ig protein, different volumes (10, 20 and 40 μ L) of sera collected from the transgenic pig with the highest level (9.55 ng/ μ L) of circulatory hCTLA4Ig protein were added into human one-way MLC system consisting of human peripheral lymphocytes collected from two individuals with no blood relation. The commercially purchased purified hCTLA4Ig protein with confirmed and qualified bio-activity were added into MLC systems at different concentrations as positive controls, and the wild-type porcine sera with no hCTLA4Ig protein added as negative controls. As shown in Fig. 5b, the levels of human lymphocyte proliferation in MLCs containing transgenic porcine sera were all significantly lower than those in MLCs containing wild-type porcine sera, indicating that the transgenic hCTLA4Ig protein was biologically active and capable of suppressing human lymphocyte proliferation. The lymphocyte proliferation levels in MLCs containing wild-type porcine sera (negative control) were comparable to those in MLCs without hCTLA4Ig protein or porcine sera (blank control), suggesting that the presence of wild-type porcine sera did not interfere with lymphocyte proliferation in MLC systems

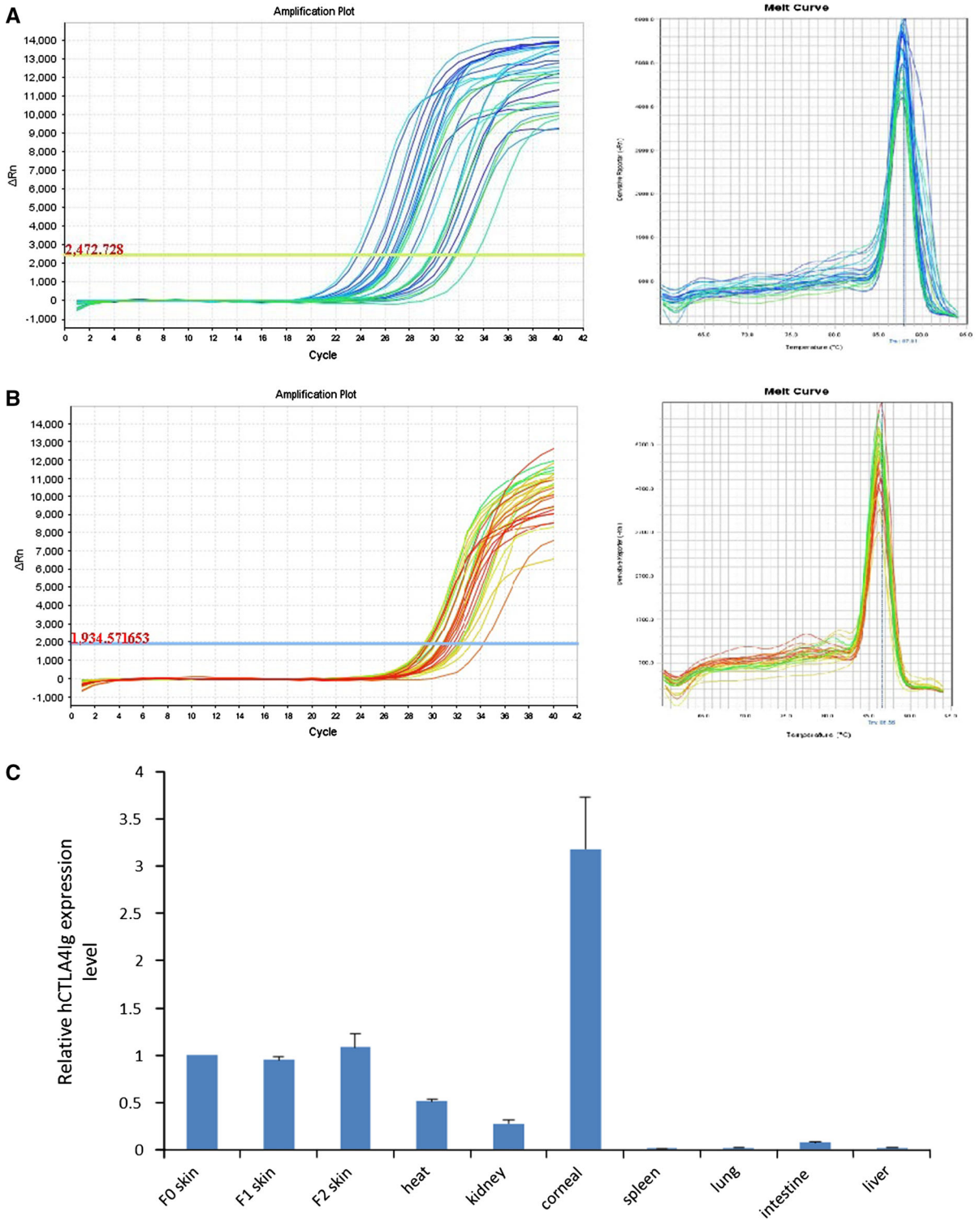


Table 1 Concentrations of hCTLA4Ig protein in sera of transgenic pigs

NO. of transgenic individuals	S49	S43	S34	S29	S39
hCTLA4Ig protein concentrations in sera (ng/ μ L)	5.60755	6.13257	7.138213	9.557639	9.242211

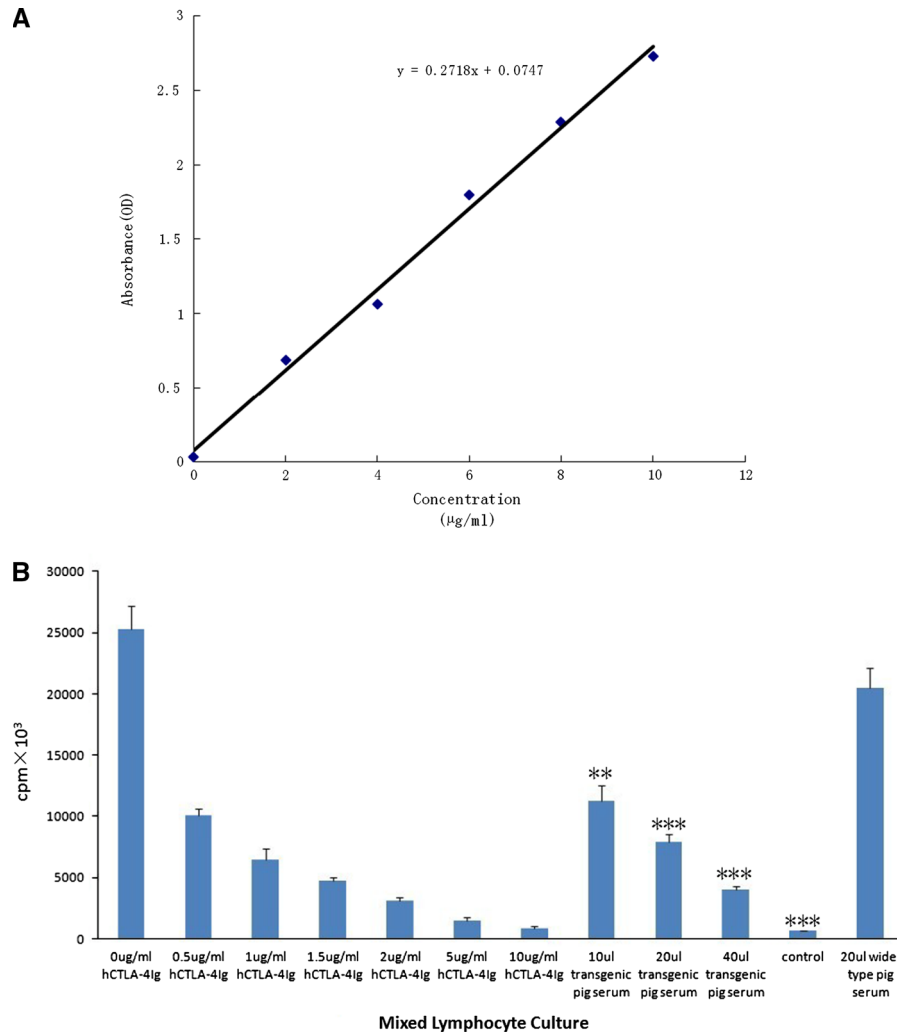


Fig. 5 Transgenic hCTLA4Ig protein in porcine sera suppressed human lymphocyte proliferation in one-way MLCs. **a** The standard curve for detection of circulatory transgenic hCTLA4Ig protein in sera by ELISA. **b** Human lymphocyte proliferation levels in one-way MLC systems containing

purified hCTLA4Ig protein or different volumes of porcine sera. *Asterisk* indicates statistical significance; control: the MLC system containing only the reacting human lymphocytes as a quality control for MLC reaction

remarkably. Besides, the levels of human lymphocyte proliferation in MLCs containing transgenic porcine sera were inversely correlated to the volumes of transgenic sera added, indicating that transgenic hCTLA4Ig protein suppressed human lymphocyte

proliferation in a dose-dependent manner. Moreover, the transgenic hCTLA4Ig protein suppressed human lymphocyte proliferation in MLCs to a similar degree to that of the purified hCTLA4Ig protein at comparable concentrations, indicating that the transgenic

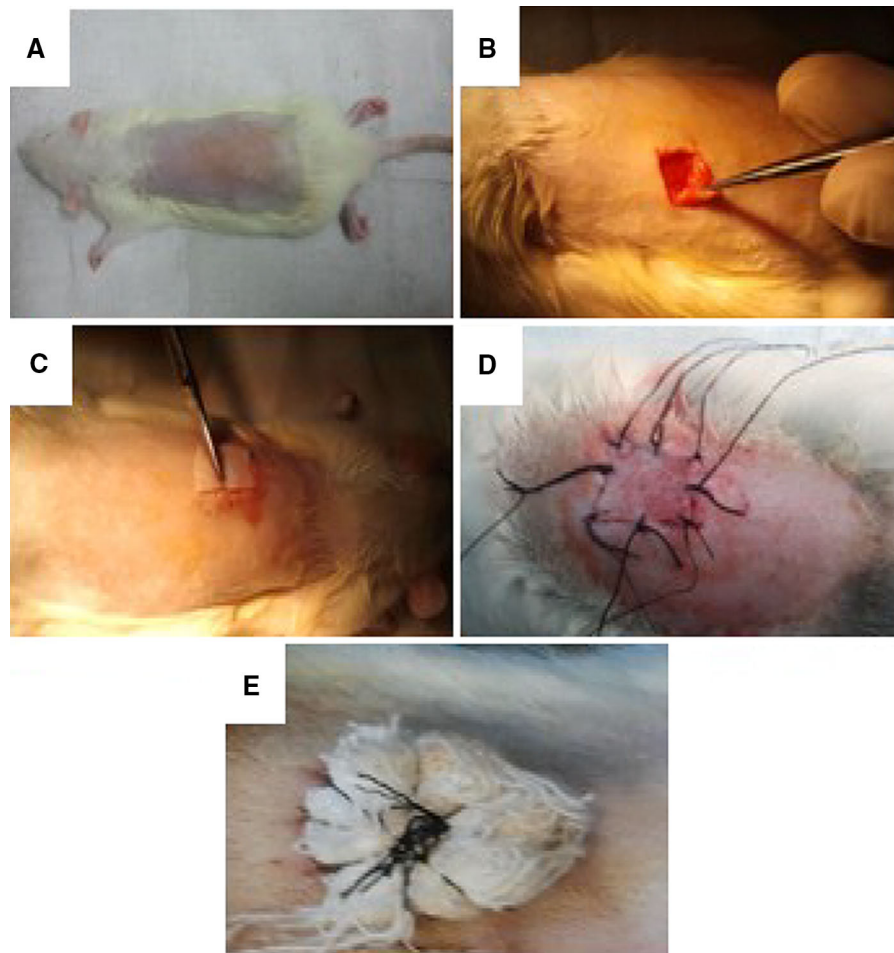


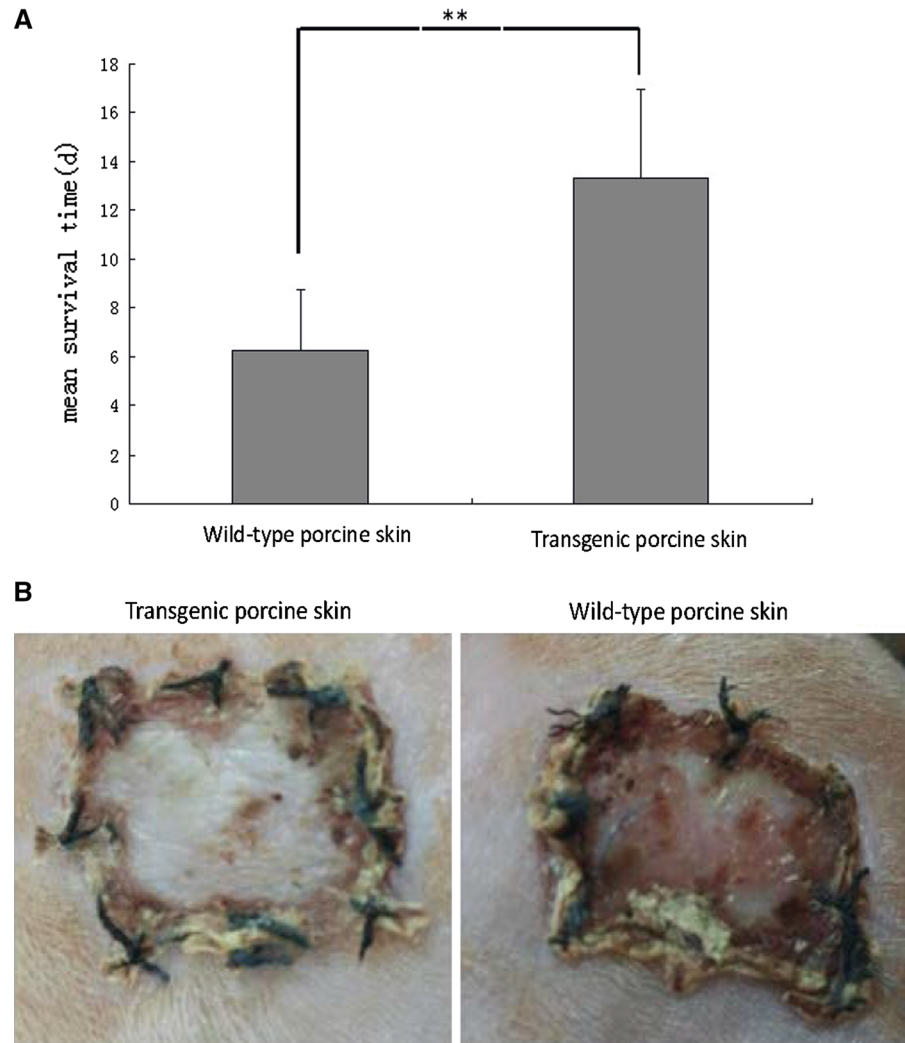
Fig. 6 Steps of skin grafting. **a** Unhairing. **b** Wound-making. **c** Skin grafting. **d** Suture. **e** Dressing-up of the wound grafted with skin

hCTLA4Ig protein exhibited a bio-activity comparable to that of the purified hCTLA4Ig protein, which was consistent with our previous data for K14-hCTLA4Ig transgenic mice (Jiang et al. 2012).

Skin grafting from pigs to rats was performed to investigate the impacts of transgenic hCTLA4Ig protein on the survival of porcine skin graft in xenogeneic wounds. Wounds were made by carefully shaving off the rat dorsal skin while leaving the subcutaneous vasculature intact to support the survival of skin grafts as previously described (Wang et al. 2008), and the grafted skins were tightly attached to the wounds by a sustainable pressure provided by the multilayered gauze pieces fixed on the grafts (Fig. 6). Results showed that the survival of hCTLA4Ig transgenic porcine skin grafts were

remarkably prolonged compared to that of wild-type ones derived from the same pig strain (13.33 ± 3.64 vs. 6.25 ± 2.49 days, $P < 0.01$; Fig. 7a). On 10 d post grafting, no remarkable rejection was observed in transgenic porcine skin grafts and the surface temperature of the grafts was normal, while in wild-type skin grafts, severe rejection was observed: the color of skin grafts was darkened, severe necrosis and scarring were observed on the margins of the grafts and the surface temperature of grafts was reduced (Fig. 7b), suggesting that the vasculature supporting skin graft survival was destructed by rejection. By histological examination, on 10 days post grafting, more intensive lymphocyte infiltration was observed in wild-type skin grafts compared to transgenic ones, and microangiopathy also observed

Fig. 7 Survival of transgenic porcine skins grafted into rat wounds. **a** The mean survival times of skin grafts. **b** The skin grafts on 10 d post grafting. Asterisk indicates statistical significance



in wild-type skin grafts, but not in transgenic ones (Fig. 8). These data demonstrated that the transgenic hCTLA4Ig protein was capable of extending porcine skin graft survival on xenogeneic wounds.

Discussion

Skin grafting is primarily required for the treatment of large wounds, and xenogeneic skin, especially porcine skin, is frequently used to cover large wounds as a substitute of human skin in clinic practice of wound care, although different kinds of artificial materials were available for wound coverage. However, the severe immune rejection after transplantation limits their application, and as a

result the survival time of grafted xenogeneic skins without immunosuppression is always too short to meet the clinical needs of burned patients (Konigova et al. 2000). How to overcome the immunological barrier for xenogeneic skin grafting and thereby extend the survival of xenogeneic skin grafts remains to be investigated.

In our previous work, we found that transgenic expression of hCTLA4Ig in skin graft was capable of extending murine skin graft survival on rat burn wounds without extensive immunosuppression (Wang et al. 2008), suggesting that transgenic hCTLA4Ig expression may be an effective and safe method for extending xenogeneic skin graft survival. In this study, we generated transgenic pigs exhibiting hCTLA4Ig expression in skin by SCNT, and a transgenic pig

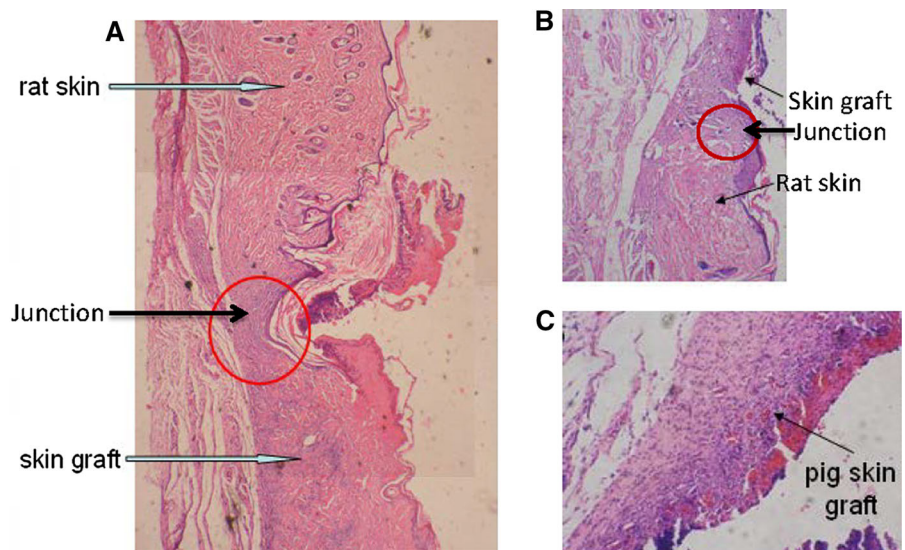
colony was established based on the healthy transgenic founder. The hCTLA4Ig transgene was detected to be single copy-integrated and stably transmitted through gemline in Mendel manner, and similar levels of transgenic hCTLA4Ig expression were detected in the skins of transgenic individuals from both F1 and F2 generations, indicating that a transgenic pig line with a stable hCTLA4Ig expression over generations can be established as a reproducible resource providing transgenic porcine skins. The biological activity of transgenic hCTLA4Ig protein was confirmed by one-way MLC system consisting of human lymphocytes derived from two individuals with no blood relation, and data showed that the transgenic hCTLA4Ig protein exhibited similar biological activity to the commercially purchased purified hCTLA4Ig protein with a confirmed and qualified bio-activity.

By skin grafting from pigs to rats, transgenic hCTLA4Ig expression was shown to extend porcine skin graft survival in xenogeneic wounds remarkably, further indicating that the transgenic hCTLA4Ig protein was biologically active and providing a direct demonstration that transgenic hCTLA4Ig expression was capable of extending porcine skin graft survival in xenogeneic wounds. On 10 days post grafting, transgenic skin graft survived rather well, exhibited reduced lymphocyte infiltration and microangiopathy was not observed, while severe rejection was observed in wild-type porcine skin grafts. Considering the average survival time of wild-type porcine skin grafts in human wounds was usually less than 10 days and

hCTLA4Ig protein should exhibit higher activity in human recipients, these data were of significant clinic implication. However, for clinic application of hCTLA4Ig transgenic porcine skins, further investigation using non-human primates as recipients for skin grafting is needed, for primates, including the human race, exhibited some different physiological characteristics as recipients for xenogeneic organ (tissue) transplantation (grafting) compared to other mammalian species, such as the natural anti-alpha 1,3-Gal antibodies which pre-exist only in primate species (Good et al.1992; Cooper et al.1993).

As far as we know, the transgenic pigs generated in this work are the second transgenic pigs expressing biologically active hCTLA4Ig protein. Martin et al. (2005) produced transgenic pigs expressing hCTLA4Ig specifically in neurons by pronuclear microinjection, and the transgenic hCTLA4Ig protein suppressed human lymphocyte proliferation response against porcine cells. Phelps et al. (2009) generated transgenic pigs extensively expressing porcine CTLA4Ig (pCTLA4Ig) by SCNT, however the transgenic pigs were susceptible to infections, suggesting that conditional transgenic expression of CTLA4Ig should be a better choice. The transgenic pigs produced in this study were healthy and exhibited no signs of susceptibility to infections, although the transgenic hCTLA4Ig expression in the pigs was not only confined to skin, but also detected in heart and kidney at levels comparable to that in skin, and in corneal at a remarkably higher level. The

Fig. 8 Histological examination of skin grafts on 10 d post grafting. **a** Transgenic skin graft (×400). **b** Wild-type skin graft (×200). **c** More intensive lymphocyte infiltration and microangiopathy observed in wild-type skin graft (×400)



ectopic expression of hCTLA4Ig in other organs or tissues (corneal, kidney and heart) suggested that the transgenic pigs generated in this study can also be used as donors for those organ transplantations to investigate the impacts of hCTLA4Ig protein on xenotransplantation.

For further extended survival of porcine skin grafts, other targets, such as alpha 1,3-Gal epitope, porcine MHC I or (and) MHC II expression and human non-polymorphic leukocyte antigen (HLA) molecules such as HLA-G, can be used for genetic modification of pigs in addition to transgenic hCTLA4Ig expression, and the biologically active porcine skin with extended survival derived from the combined genetically modified pigs can be used not only for the coverage of large burn wounds, but also for the difficult-to-heal wounds such as those caused by diabetic ulcer. For the coverage of difficult-to-heal wounds, the biologically active skin had advantages over other kinds of materials in many aspects such as natural physiological characteristics, full bio-compatibility, plentiful supply and convenience in usage, once the strong immune rejection after grafting were effectively controlled. In addition, the combined genetically modified pigs can be used as donors not only for skin grafting, but also for other organ xenotransplantation, for the transgenic hCTLA4Ig expression was also detected in other organs besides skin.

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