Discussion

Cutaneous wound healing is a complex biological process that requires cellular interactions between a variety of cells, including fibroblasts, myofibroblasts, smooth muscle cells, endothelial cells, keratinocytes and immune cells. These interactions are mediated by numerous factors such as growth factors, hormones, blood components and second messengers. Several growth factors that are released at the wound site are presumed to be necessary for wound healing. Based on the pathogenesis of wound healing, it has been suggested that chronic wound may be deficient in an orderly production of cytokines (Falanga V et al., 1994). The normal function of growth factors is to attract various cell types into the wound, stimulate cellular proliferation, promote angiogenesis, and regulate synthesis and degradation of extracellular matrix. Clinical results from topical application of growth factors to chronic wounds have not been as dramatic as first hoped. This is unsurprising when one considers the complexity of the wound healing process. To date, only platelet derived growth factor has been licensed for use, for treating non-infected foot ulcers in diabetic patients (Rees RS et al., 1997).

Since the placenta is supplied by maternal blood rich in biomolecules (Muralidhar RV and Panda T, 1999), it is a potent source of these substances. Placental growth and development are critical for fetal growth and development, and are probably regulated by growth factors produced by placental tissue themselves (Reynolds LP et al., 1995). However, it is considered as a waste after parturition. While growth factors and cytokines are key players in the regulation of these events, including mediate the coordinated action of keratinocytes, fibroblasts, endothelial cells and inflammatory cells during wound healing. We expect placenta, which contain various growth factors and cytokines, wound be applied to wound

healing. For a long time, placental extract is familiar to apply on cosmetic. In recently two decades, it has been studies on some clinical disease. In these studies, placenta was extracted by H_2O, alkaline solution (JP patent: 58177918), ammonium sulfate (JP patent: 6234798) and so on. We expect to find out the better extraction condition for placenta extraction. We extracted placenta with different solution, including H_2O, NaCl, $MgCl_2$, and urea at 4°C throughout any processing, and then determined some growth factors relating to wound healing. As compared with all extract solutions, we found 2 M urea would be the better extract solution for growth factors extraction (Table 3). But we didn't know whether they're still active, we proceed to estimate the effects of placental extracts on wound healing *in vitro*. Because fibroblasts, keratinocytes and endothelial cells proliferation and migration play an important role in wound healing, we explore these parameters for effects of placental extracts on wound healing. In our data, placental extracts can stimulate fibroblast proliferation in a dose-dependent manner (Fig. 2A; Fig. 3), in agreement with the report by Wu CH et al. Even, our placental extracts are just 1000-fold less concentration than Wu CH et al. to stimulate the maximal cell response. Besides, keratinocytes and endothelial cells have similar responses with treatment of various placental extracts (Fig. 2B and C). Maybe, the placenta extraction with different solution extracts more bioactive substances, and 4°C throughout any processing keeps most bioactive substances. Moreover, they toke *in vitro* concentration (30 mg/ml H_2O extract) as *in vivo* concentration, and demonstrated placental extracts promote wound healing. We presume that placenta extracts with urea or sodium solution could be better for wound healing *in vivo*.

Treatment with placental extracts promoted keratinocytes and endothelial cells proliferation and migration (Fig. 4A and B), which could result in re-epithelization and angiogenesis, respectively. Cell migration is known to be triggered by constituents of the ECM such as fibronectin and by soluble mediators commonly summarized as mitogens. For

most tissue engineering applications, it is a general requirement that cells adhere to the particular support material or scaffold that is being used. The adhesive contacts between cells and the ECM are important for cellular migration and proliferation, significant parameters of wound healing, as well as the initiation and formation of intercellular adhesion. To explore whether there are interactions between placental extracts and cells, we used cell adhesion assay. In this assay (Fig. 5A, B and C), we observed that porcine placental extracts mediated cell adhesion in a dose-dependent manner. However, we found urea would be a better extract solvent for cell proliferation and migration not for cell adhesion. We consider cell migration and proliferation since it appears to be dependent major on soluble growth factors or other unknown substances in placental extracts, and minor on adhesion molecule or other unknown substances in placental extracts.

In normal wound healing, a network of negative feedback mechanisms activated after successful healing is responsible for the proper termination of the proliferative and fibrotic processes, thus restoring tissue integrity. If these feedback mechanisms fail to operate, however, continuous ECM secretion and deposition will lead to perturbation of normal tissue architecture and endless healing, with the eventual development of tissue fibrosis. Alternatively, such endless healing may also be due to repeated injuries leading to continuous activation of the fibroproliferative response (Eickelberg O et al., 2001). In our studies, cell proliferation is on the downside in the dose over 80-100 μg/ml placental extracts. Afterward we need *in vivo* test to confirm the most appropriate concentration range for wound healing.

In addition to facilitating fetal nourishment, placenta acts as a barrier both to potentially deleterious agents and to contact between their two immune systems. As a consequence, damage to the placenta, even on a relatively small scale, could be very dangerous to the fetus. Therefore, wound repair mechanisms are likely to be of great importance in ensuring that an

intact placental barrier is re-established as soon as possible (Watson AL et al., 1996). Interestingly, fetal wound healing differently from adult wound, in that early gestation fetal wound heal without scar. By late gestation, fetal wound changes to adult wound repair phenotype (Mackool R et al., 1998). The greater increase in MMP-1 and MMP-9 expression demonstrated in scarless wounds (Dang CM et al., 2003). In general, in normal quiescent tissue, MMP-9 associated with acute tissue injury is nonexistent; while MMP-2 associated with daily tissue remodeling is present at low levels (Werb Z et al., 1989; Matrisian LM et al., 1992; Woessner JF et al., 1995). We want to know whether placental extracts could accelerate scarless wound healing. Zymography of culture media showed that protein levels of secreted MMP-9 were no mass production with porcine or placental extracts, but protein levels of secreted MMP-2 increased about 1.5 to 2.5-fold (Fig. 9A and B). Undoubtedly, it's required to make more efforts on MMP and ECM research. We presume that placenta got from normal period of gestation is partial to adult-type wound repair already, and growth factors in placental extracts regulate mechanism of wound healing could trend to adult-type. Our data showed that treatment with placental extracts increased cell adhesion, proliferation and migration *in vitro*. In this case, improved wound healing rate would minimize scar formation. Even though placental extract maybe not accumulate scarless wound healing fully, minimization of scar formation depend on improving the healing rate by bioactivities in placental extracts. Taken together, our study suggests that it is extremely possible to facilitate wound healing on clinical application.

To deserve to be mentioned, we preliminary estimated the effects of both porcine and human placental extracts on cell proliferation. Human placental extract were inferior to porcine placental extracts, but no significantly different. We suggest that it's easier and more convenient to get porcine placental extracts applied on wound healing pharmaceuticals research and development.