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The multi-potentiality of skin-derived stem cells in pigs

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Abstract

Multipotent skin-derived stem cells represent neural-crest derived precursors which have neural and mesodermal potency and can generate neurons, glias, smooth muscle cells and adipocytes. Transcriptional profiling studies show that both intrinsic programs and extrinsic signaling pathways mediate their neural and mesodermal potency. In addition, recent progress implies that skin-derived stem cells may have a broader developmental potency than previously expected, of which is their potential to generate germline cells *in vitro*. In this review, we discuss the transcriptional profiling of multipotency and neural crest-derived characteristics of skin-derived stem cells, and argue for their potential germ-line competency in the view of nuclear and cellular reprogramming.

Keywords

stem cell; skin; chimera; somatic cell nuclear transfer

Introduction

Stem cells are a type of primitive cell arising during embryonic and postnatal development, which have the ability to self-renew and differentiate into multiple functional cell types. A number of pluripotent and multipotent stem cell lines have been derived from preimplantation embryos (1,2), post-implantation epiblast (3,4) and various sources of postnatal animals (5). Mammalian skin is a complex tissue that regenerates dynamically by continuing to turnover during the life of the animal. Thus, on-one-hand, epidermal skin-derived stem cells contribute to balance the epidermal homeostasis in the skin. Such stem cell populations maintain the self-renewing compartments of mammalian skin epidermis: the hair follicle, the sebaceous gland and the interfollicullar epidermis (6). On-the-other-hand, multipotent dermis-derived stem cells provide substantial support for wound healing and regeneration (7,8). A novel type of dermis-derived stem cell termed skin-derived progenitors (SKPs) were first isolated from juvenile and adult mammalian skin by Toma *et al* in 2001 (9,10). They used a serum-free medium (DMEM/F12 (1:1) + B27 + EGF + bFGF) originally developed for neural stem cell culture *in vitro*. The SKPs cells can survive as spheres in suspension culture and the population can double every 2–3 days. Under certain conditions SKPs have multiple lineage potential and can generate neural and mesodermal lineages: neuron, glias, smooth muscle cells and adipocytes (9).

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With properties similar to rodent SKPs, human SKPs were successfully isolated from neonatal foreskin and adult trunk skin (11,12). In contrast to mesenchymal stem cells, SKPs express nestin (neural stem cell marker), p75 (neural crest stem cell marker), fibronection and Sox10. Several lines of evidence show that SKPs are neural crest derived and that they arise during embryogenesis and can persist into adulthood (12–14), thus sharing properties similar to embryonic neural crest stem cells. In pigs, several independent groups reported the isolation of porcine skin-derived progenitors (pSKP) from both embryonic and adult skin (13,15,16). Porcine SKPs express pluripotency-related genes (Oct3/4, Sox2, Stat3 and Nanog) and neural crest marker genes (p75NTR, Sox10, Snail), showing a pattern of marker gene expression similar to human and rodent SKPs *in vitro* (13,15). In addition, individual cells of SKP spheres show heterogeneous expression of specific marker genes, implying a metastable state inside the SKP spheres (13). Porcine SKPs are multipotent and can generate both neural and mesodermal progeny *in vitro.* However, porcine SKPs show distinct transcriptional profiles when compared to neural stem cells in the central nervous system (CNS) and skin-derived fibroblasts, indicating a novel type of multipotent stem cells derived from skin (17). Specifically, porcine SKPs can generate primordial germ cell-like and oocyte-like progeny *in vitro*, showing a promising *in vitro* model to investigate germ-cell formation and gametogenesis (18,19). In this review, we will discuss the neural crest-derived properties, neural and mesodermal differentiation, germline cell potency, and prospective biomedical application of skin-derived stem cells.

Neural crest derived properties of skin-derived stem cells

The neural crest cells are a group of transient and multipotent cells which are induced to migrate and give rise to various cell lineages: melanocytes, craniofacial cartilage, bone, smooth muscle, peripheral and enteric neurons and glias. In the past two decades, neural crest stem cells (NCSCs) or neural crest-derived progenitors have been isolated from neural crest explants (20), sciatic nerves (21), dorsal root ganglion (DRG) (22), skin (10,12), enteric ganglia (23–25), cornea (26), carotid bodies (27), whisker pads, bone marrow (28), and heart (29). Neural crest stem cells can generate both neural (neurons and Schwann cells) and mesodermal lineages (adipocytes, chondrogenic cells, osteogenic cells and smooth muscle cells) when stimulated by various extracellular signals (Figure 1) (30). Several lines of evidence suggest that SKP cells are neural crest-derived and show properties similar to neural crest stem cells. It appears that SKPs cells reflect the residual neural crest stem cells in adult skin, whose developmental potential is restricted *in vivo* by the niche it occupies, but could be revealed when cultured *in vitro*. The first evidence comes from the comparison of marker gene expression between neural crest cells and SKP cells. Rodent SKPs express several transcription factors genes (slug, snail, twist, pax3 and sox9) (10) which are involved in the migration and specification of neural crest cells (31). However, p75NTR, which is widely used in identification and isolation of neural crest stem cells by flow cytometry, was either not expressed or undetectable in rodent dorsal and facial SKPs (10), or in human neonatal foreskin SKPs (11). In contrast, multipotent SKP cells from human and mouse trunk skin co-express p75NTR and Sox10 (12), showing that neural crest marker gene expression may be associated with their tissue of origin. In pigs, we found that SKP cells expressed both pluripotency-related genes and neural crest markers (13), further demonstrating the neural crest origin of skin-derived stem cells.

The second evidence arises from the fate mapping approach by which transgenic reporter genes were exclusively expressed in cells of neural crest origin. Fernandes *et al* employed Wnt1-Cre/RosaR26R mice, where β-galactosidase expression was directed by a Wnt1 promoter and was only restricted to the progeny of neural crest stem cells. This demonstrated that follicle dermal papillae contain neural crest-derived cells and SKPs from facial skin are neural crest-derived (10). They also found that neural crest-derived SKP

spheres from facial dermis (whisker papillae) could first be isolated as early as embryonic day 9 and persist into adulthood. Wong *et al* also used Wnt1-Cre/R26R^{LacZ} transgenic mice to conclude that sphere-forming neural crest-derived cells reside in distinct structures of the adult skin and display different intrinsic properties with time and location (12). In whisker follicles of the face, neural crest-derived SKPs appear to be located in many mesenchymal structures; however, in the trunk skin they are restricted to the glial and melanocyte lineages. In addition, these neural crest-derived cells in adult skin have intrinsically different growth factor responsiveness from previously identified neural crest stem cells which exert exclusively neural or glial lineage differentiation in response to bone morphogenetic protein 2 (BMP2) or neuregulin 1 (NRG1) *in vitro* (12,32). Using a combined fate mapping and microdissection approach, Hunt *et al* identified a highly enriched niche of neural crest-derived SKP spheres in the dermal papilla (1,000 enriched compared with whole facial skin and termed papillaspheres) of the hair follicle in the adult skin (33). In addition, Schwann cell precursors are also derived from SKPs in adult skin, further illustrating that the gliogenic SKPs are neural crest derived (34).

The third evidence is from cell transplantation studies. When yellow fluorescent protein (YFP)-labeled SKP spheres were transplanted into the chick neural crest migratory stream *in ovo* at Hamburger-and-Hamilton stage 18, these SKP sphere derived cells migrated into the sympathetic ganglia, spinal nerve, dorsal root ganglion (DRG) and even the dermal layer of the skin, whereas very few cells went into neural tube (10). These results are consistent with the *in vivo* property of $p75^{+}P_{0}^{-}$ neural crest stem cells which can give rise to neurons and glias in peripheral nervous system (PNS) upon transplanting into chick embryos (21). Hence, three lines of evidence suggest that SKP spheres are neural crest-derived precursors that arise from embryogenesis and retain multipotency into adulthood (14).

Transcriptional characterization of porcine skin-derived stem cells: illustrating the neural and mesodermal potential by microarray analysis

In the past two decades, the genetic program of the “stemness” in multipotent/pluripotent stem cells has been extensively elucidated by high-throughput microarray or next-generation sequencing technologies (35). Recent studies show that the core transcriptional regulatory circuitry centered on the transcription factors Oct3/4, Sox2 and Nanog maintains the transcriptional program required for pluripotency in embryonic stem (ES) cells (36). The importance of transcriptional regulation on maintaining the “stemness” has been further demonstrated by the reprogramming of fibroblast (37) or even terminally differentiated B lymphocytes (38) into induced pluripotent stem (iPS) cells by defined factors (Oct3/4, Sox2, Klf4 and c-myc). However, the transcriptional regulation of somatic stem cells has still been elusive although the transcriptional profiling experiments on hematopoietic stem cells (39), neural stem cells (40), and neural crest stem cells (41,42) have been performed. This unsolved problem is confounded by the variation of transcriptional profiling caused by the noise of different genetic backgrounds and the heterogeneity of ES cells and adult stem cells (43). Different extracellular stimuli may also trigger stem cells to display various transcriptional profiling because transformation or reprogramming is likely to happen during long-term culture (44). Thus, a similar *in vitro* culture system and genetic background would be indispensible to describe the molecular basis of multipotency in porcine skin-derived stem cells.

Transcriptional characterization of neural and mesodermal potency in porcine skin-derived stem cells has recently been dissected (17). The general strategy is summarized in Figure 2. In order to eliminate any potential genetic background difference in describing the neural potency of porcine SKP spheres, neural stem cells (neuropsheres) (45) were isolated from the same fetuses as SKP spheres. Both SKP cells and neural stem cells were cultured in the

same medium (DEME/F12 + B27 + N2 + EGF + bFGF) and they formed spheres after several days' selection. After they formed spheres transcriptional analysis via a pig-specific microarray (46) was performed directly without any further culture. The common highly expressed genes in the SKP spheres and neurospheres are mainly ribosome, tight junction, gap junction, cell communication, calcium signaling, ErbB signaling, JAK-STAT signaling, MAPK signaling *etc*. The differentially expressed genes between SKP spheres and neurospheres are involved in ECM-receptor interaction and the TGF-β signaling pathway. Leukemia inhibitory factor (LIF) or MEK inhibitor treatment results in a distinctive impact on the "stemness" and differentiation-related genes in SKP spheres and neurospheres. Thus it is inferred that the cell-intrinsic genetic program may contribute to the innate "stemness" of tissue origin in SKP spheres and neurospheres in a similar local microenvironment (47).

To decipher the mesodermal potential of porcine SKP spheres, they were induced to differentiate into SKP-derived fibroblast-like cells (SFC) by culture in serum and adhesive dishes. The porcine SKP spheres gradually lost their neural potential but still retained mesodermal properties (17). By microarray and functional annotation clustering analysis of porcine SKP spheres and SFC, 305 genes were found to be up-regulated and 96 genes down-regulated. The down-regulated genes are mostly involved in intrinsic programs such as the *Dicer* pathway and asymmetric cell division; whereas up-regulated genes are more likely to participate in extrinsic signaling pathways such as ErbB signaling, MAPK signaling, ECM-receptor reaction, Wnt signaling, cell communication and TGF-beta signaling pathways. These intrinsic programs and extrinsic signaling pathways may collaborate to mediate the transcription-state transition between SKP spheres and SFC. Together with the neural potential assays, these transcriptional profiling data provide candidate signal pathways that may orchestrate the neural and mesodermal potency of porcine SKP spheres. Further studies would be to test the role of key molecules in signal transduction pathways in the self-renewal and multipotency of porcine SKP spheres by functional experiments.

Neural and mesodermal potency of skin-derived stem cells and their prospective clinical application

Since skin may provide an accessible source for autologous stem cell transplantation, SKP cells would be an idea experimental and therapeutic model for regenerative medicine (48). Strikingly, the neural progeny derived from SKP spheres may provide a new resource of neurons and glias for degenerative diseases in the nervous system. Potential clinical application is suggested by experiments that show that enriched skin-derived AC133+ (a cell surface marker) progenitors can migrate through the forebrain and give rise to astrocytes but not oligodendrocytes after injection into adult brain (49). Similarly, human skin-derived stem cells generate neurons when exposed to hippocampal astrocytes. They stably express neuronal makers (neurofilament and tubulin β-III) and show the presence of voltage-dependent calcium transients (50). This is consistent with Gingras' report that human skin-derived neuronal precursors eventually became terminally differentiated mature neurons *in vitro* (51). In contrast, rodent SKP-derived neuron-like cells can maintain their peripheral phenotypes for more than 5 weeks when transplanted into the brain but still possess an immature electrophysiological profile, implying that further investigation is required to induce them into electrophysiological mature neurons (52). These studies illustrate the prospective clinical application of using SKP-derived neurons for brain therapy.

In addition, SKP-derived progeny also play a role in the regeneration of injury in the peripheral nervous system. Skin-derived stem cells can improve functional nerve regeneration after sciatic nerve resection when transplanted into resorbable guides (53). Similarly, rodent SKPs can generate myelinating Schwann cells for an injured peripheral nerve, promoting remyelination and functional recovery after contusion spinal cord injury

(54). These SKPs produced Schwann cells which proliferated and induced myelin protein when in contact with sensory neuron axons. Furthermore, SKPs-derived Schwann cells can myelinate CNS axons or injured peripheral nerves in either wild type or *shiverer* mutant mice, thus providing an accessible source of myelinating cells for nervous system injury and dysmyelinating disorders (55). Nevertheless, SKPs or their derivatives are proposed to be the cell origin of dermal neurofibroma through loss of Nf1 tumor-suppressor gene (56). Furthermore, it is still not known whether SKPs are tumorigenic and can give rise to teratomas *in vivo*. Thus more comprehensive tests should be performed before any clinical application.

Another clinical application may be a result of the mesodermal potency of SKP cells. Mouse SKP cells can produce muscle progenitors and differentiated muscle cells both *in vitro* and *in vivo* (57). Both rodent and human SKPs generate skeletogenic cell types: chondrocytes and osteocytes *in vitro*, and can be induced into an osteogenic lineage when transplanted into injured bone marrow (58). This skeletal and osteogenic potency was also reported in porcine skin-derived stem cells (16,59). Interestingly, mouse skin-derived stem cells can be converted into insulin-producing cells *in vitro* (60), indicating another possible source of stem cells for cell-transplantation therapy of diabetes.

Germline potency and cellular reprogramming in porcine skin-derived stem cells

Although it is still controversial about the plasticity of adult/somatic stem cells (61), several studies reported a very broad developmental potential of somatic stem cells. For example, adult neural stem cells can contribute to chicken and mouse embryos and give rise to cells of all three germ layers (62). Bone marrow-derived multipotent adult progenitors cells (MAPCs) are also capable of generating cells of mesenchyme, neuroectoderm and endoderm *in vitro* and contributing to most somatic tissue when injected into blastocysts and engrafted (63). For skin-derived stem cells, epidermal stem cells have the ability to produce cells of ectodermal, mesenchymal, and neural crest-derived tissues when injected into day 3.5 C57BL/6 mouse blastocysts (64). In particular, hair follicle dermal stem cells are capable of repopulating the hematopoietic system after transplantation into lethally irradiated recipient mice (65). Porcine skin-derived stem cells are demonstrated to have the intrinsic ability to differentiate into oocyte-like and primordial germ-like cells *in vitro* (18,19). The oocyte-like cells can spontaneously develop into parthenogenetic embryo-like structures. However, they failed to be fertilized by sperm *in vitro* because of their unstable structure. Until now this germline potential of skin-derived stem cells has not been confirmed by other independent groups even though porcine SKP cells can potentially integrate into the genital ridge *in vivo* when injected into peri-morula embryos (66). A more stringent assay, such as production of a chimeric animal should be performed to test the germline potency of porcine skin-derived stem cells.

In theory, skin-derived stem cells have the possibility of producing germline cells based on the following strategies (Figure 3). First, somatic cells can be reprogrammed into a totipotent embryonic state and produce offspring by using somatic cell nuclear transfer technology (67). Skin-derived stem cells have been used as donor cells to generate live offspring (68). Somatic adult cells, even terminally differentiated B lymphocytes, can be reprogrammed into iPS cells by defined factors *in vitro* (37). The iPS cells have the ability to produce chimeric mice when injected into early blastocysts and even produce completely iPS-derived live offspring after tetraploid complementation (69). The original somatic cells, of course, develop into germline cells in either chimeric or completely iPS-derived mice. Second, both male (70,71) and female gametes (72) can be derived from embryonic stem cells *in vitro*, and the ES cell-derived sperm can produce live mice. It may be possible to

reprogram skin-derived stem cells into an intermediate pluripotent state and then differentiate them into gametes *in vitro*. Third, direct cell reprogramming may be an alternative strategy to interconvert cell states between different cell types. Recent progress includes directly converting mouse embryonic fibroblast cells into functional neurons by defined factors *in vitro* (*Ascl1*, *Brn2* and *Myt1l*) (73) and *in vivo* reprogramming of adult pancreatic exocrine cells to β-cells by a combination of three factors (*Ngn3*, *Pdx1* and *Mafa*) (74). Thus it is conceivable to convert skin-derived stem cells into germline cells by unknown factors in metaphase II oocytes and early embryos.

Conclusion

Multipotent skin-derived stem cells have neural and mesodermal lineage potency and represent a prospective stem cell source for autologous cell transplantation. They are neural crest-derived but display intrinsic transcriptional profiling reflecting their tissue origin and developmental stage. Endogenous SKP can be isolated from embryonic skin and they persist into adulthood. It seems that these skin-derived stem cells have a broader developmental potency including generating primordial germ cells and gamete-like cells. Recent progress on nuclear and cellular reprogramming implies that it is possible to convert skin-derived stem cells into germline cells or any other cell type of interest. However, rigorous cellular and transplantation tests are needed before any clinical application because SKP cells are considered to be the cell origin of particular tumors in the nervous system. Therefore, further studies are needed to assess their developmental potency of skin-derived stem cells and illustrate the molecular basis which establishes and maintains the multipotency.

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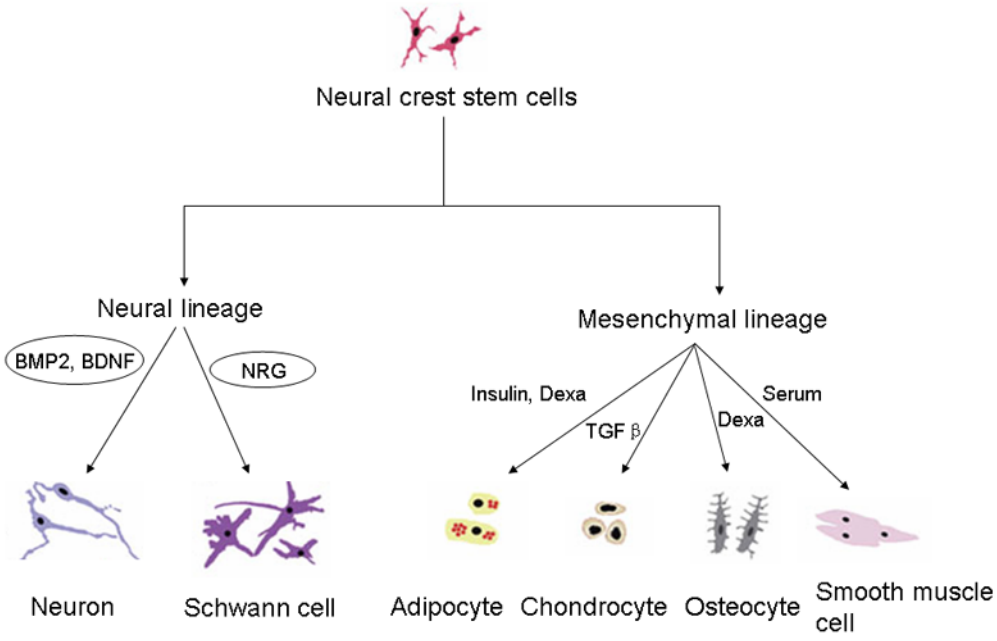

Figure 1.
The developmental potential of neural crest-derived stem cells. Neural crest stem cells can generate neural (neurons and glias) and mesodermal progeny (adipocytes, chondrocytes, osteocyte and smooth muscle cells) when various extracellular signals are present. BMP2: bone morphogenetic protein 2; NRG: neuregulin; TGF-β: transforming growth factor-β; BDNF: brain derived neurotrophic factor; Dexa: dexamethasone.

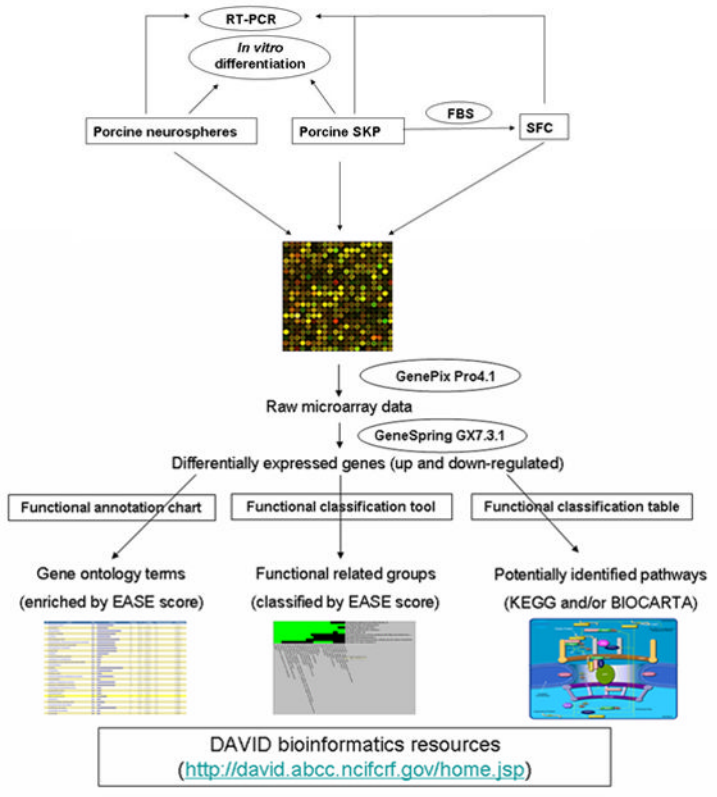

Figure 2.

Strategy for deciphering the neural and mesodermal potency of porcine skin-derived stem cells by microarray analysis and functional annotation clustering. Porcine SKP spheres and neurospheres were isolated from the same fetuses and cultured in the same medium. SKP spheres differentiate into SKP-derived fibroblast-like cells (SFC) with serum. Reprinted with permission from (17). The publisher for this copyrighted material is Mary Ann Liebert, Inc. publishers.

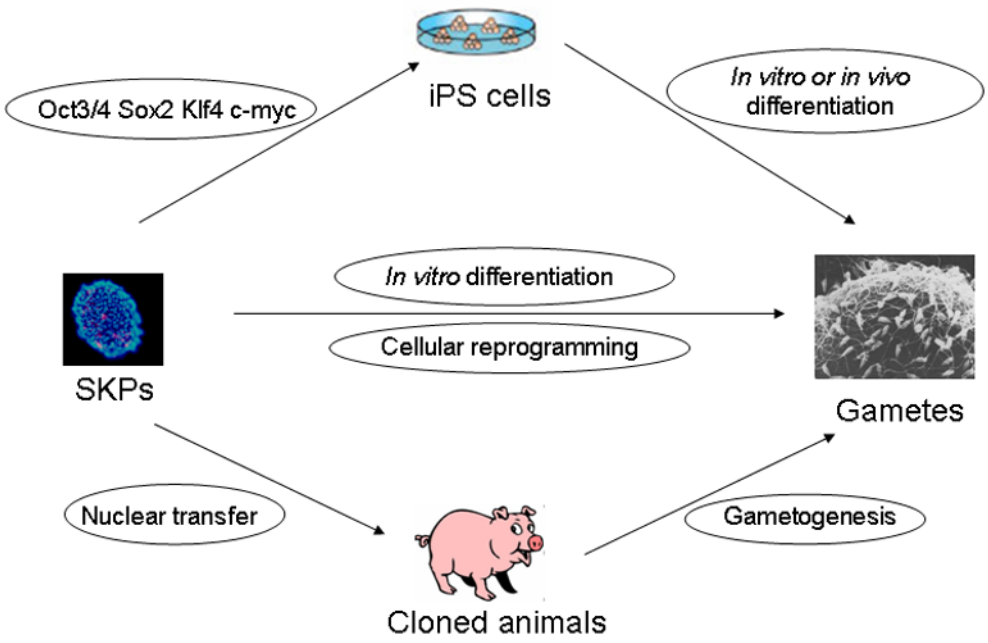

Figure 3.

Prospective strategies to reprogram porcine skin-derived stem cells into germ-line cells. Porcine SKPs can generate oocyte-like cells *in vitro* (middle panel). Alternatively, they may be used as donor cells for nuclear transfer to produce cloned animals which can generate normal gametes *in vivo* (lower panel). Finally they might be reprogrammed into iPS cells by defined factors and then derive gametes by *in vitro* induction or *in vivo* chimera production and tetraploid complementation (upper panel).