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Small Molecules Facilitate Single Factor-Mediated Sweat Gland Cell Reprogramming

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Research

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Abstract

Background: Large skin defect caused severe disruption to the overall skin structure and irreversible damage of sweat gland (SG), resulting in destroy of physiological function of the skin. Reprogramming fibroblasts into sweat gland lineages may provide a promising strategy to obtain the desirable cell types for functional repair and regeneration of damaged skin.

Methods: A direct reprogramming strategy of single factor ectodermal dysplasia antigen (EDA) in combination with small molecule cocktails promoting cell-fate conversion to regenerate SG cells from human dermal fibroblasts (HDFs) was developed. Quantitative PCR (qPCR), flow cytometry, calcium activity analysis, immunocytochemical analyses and starch-iodine sweat tests were used to characterize the phenotype, gene expression and function features of the induced sweat gland cells (iSGCs).

Results: EDA overexpression drove HDFs toward SG lineages, and HDFs transfected with EDA acquired sweat gland cell phenotype in sweat gland conditional medium (SGM). Small-molecule cocktails favoring SG lineages greatly accelerated the SG fate program in SGM-treated HDF-EDA cells and further induced the regeneration of iSGCs. The HDFs-derived iSGCs exhibited similar phenotypical and functional features of native sweat gland cells. Eventually, in vivo transplantation experiment confirmed that iSGCs had the ability to regenerate SG structurally and functionally.

Conclusion: We developed a SG reprogramming strategy to generate functional iSGCs from HDFs by using single factor EDA in combination with small molecules. The generation of iSGCs has important implications for in situ skin regeneration with restoration of sweat glands in the future.

Background

Sweat gland (SG), covering almost the entire body, is a very important skin appendage with the function to maintain the temperature homeostasis by secretion of sweat in response to heat and exercise [1, 2]. However, large skin defect caused by burns, severe trauma and chronic cutaneous wounds could lead to severe disruption to the overall structure and physiological function of the skin, as well as irreversible damage of SG, followed by heat intolerance and dysfunction of thermoregulatory, which is a dramatical threat to the health and quality life of patients. Despite of the great progress of clinical management for skin wound, the restoration of damaged SG is far from being realized, and thus, functional repair of skin wounds with regenerated SG is still a very essential and challenging issue.

It was reported that there were endogenous stem cell populations in sweat gland during embryonic development. However, lineage tracing experiments showed that these precursor/stem cells had limited self-renewal and regeneration potential, exhibited limited response to tissue injury stress, and failed to form sweat glands during wound healing [3]. To overcome these challenges, cellular reprogramming offers an attractive novel strategy for the acquisition of high-quality SG cells in large scale for the functional repair and regeneration of damaged skin tissues. It has been reported that mesenchymal stem cells (MSCs) and epidermal keratinocytes could be converted into induced sweat gland cells (iSGCs) by

the modulation of EDA expression [4-6]. The reprogrammed cells not only expressed SG cell markers CK5, CK10, CK18, CK19, CK14, CEA and AQP5, but also facilitated the restoration of SG in vivo. The enforced expression of lineage-specific factors has been extensively used in the reprogramming strategy [7, 8], however, single factor has not been sufficient for cellular reprogramming [9, 10]. Efforts to use a combinatorial approach for direct lineage conversion requires to be built [9, 11]. Small molecules, as a non-viral and non-integrating approach, have been shown to enhance single factor-mediated reprogramming efficiency [9, 10], such as Repsox (ALK4/5/7 inhibitor), CHIR99021 (GSK3β inhibitor), parnate (LSD1/KDM1 inhibitor, also called tranylcypromine), TTNPB (retinoic acid receptor ligand) and forskolin (adenylyl cyclase activator) [12, 13].

Fibroblasts have been used extensively as the starting cells in most direct reprogramming experiments owing to their availability and potential for in vivo reprogramming [14-16]. In particular, dermal fibroblasts are the one of dominant cell types involved in cutaneous wound repair and regeneration [17]. Accordingly, the reprogramming of dermal fibroblasts is of great significance for in-situ repair and regeneration of the damaged skin. These studies lead us to hypothesize that SG cells can be generated directly from dermal fibroblasts via the combination of EDA overexpression and small molecules.

In present study, we reported that functional iSGCs could be generated from human dermal fibroblasts (HDFs) via a stepwise strategy involving EDA overexpression in combination with defined small-molecule cocktails. We discovered that single factor EDA could prime HDF cells with an intrinsic possibility toward SG lineages. Culturing of the EDA-overexpressed cells under the SG conditional medium enhanced SG lineage conversion. Treatment with additional small molecules facilitated the EDA-mediated SG reprogramming from fibroblasts, resulting in dramatically improved iSGC generation. The iSGCs generated exhibited phenotypical and functional characteristics comparable to native SG cells and restored the damaged skin with functioning SG components in vivo.

Methods

The isolation and culture of human dermal fibroblasts and primary sweat gland cells

Human dermal fibroblasts were isolated from male foreskin specimens (age 15-19 years old). The skin tissue was washed with phosphate buffered saline (PBS) buffer containing penicillin (100 U/mL) and streptomycin (100 μg/mL). After washing, the skin tissue was cut into small pieces (1~2 mm^3) and placed in the petri dish with fibroblasts medium containing high glucose DMEM medium (Gibco) and 10% fetal bovine serum (Gibco). The culture medium was washed and digested with 0.25% trypsin-EDTA for cell passage, and the subsequent experiments were carried out after fibroblasts were transferred to 5-9 passages. Primary sweat gland cells (pSGCs) were isolated from normal breast tissue. The present studies were approved by the Clinical Research Ethics Committee of General Hospital of PLA (Beijing) and written informed consent was obtained from all individuals prior to obtaining samples.

Generation of iSGCs

HDFs between passages 5 and 9 were used for iSGCs generation. The viral particles for iSGCs generation were produced after transfecting 293FT cells using a single pMX retroviral vector coding for EDA (pLV-hefla-EDA-GFP) together with packaging plasmid psPAX2 and envelope plasmid pMD2.G. Lipofectamine^{TM} 2000 was used for further transfection according to the manufacturer's instructions. Puromycin with a final concentration of 0.4ug/ml was added at 48 h post-transfection to obtain stably transfected cells, and puromycin selection was continued for 15 d. And then, HDFs transfected with EDA were cultured in sweat gland conditional medium (SGM) in the presence or absence of distinct small molecules and protein, Repsox (10 μM, Selleck), CHIR99021 (10 μM, Selleck), Isoproterenol (5 μM, Sigma-Aldrich), Retinoic acid (10 μM, Sigma-Aldrich) and BMP4 (20ng/mL, R&D). SGM was consisted of DMEM/F12 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1xB27 (Gibco, 17504044), 1xGlutamax^{TM} (Gibco), human epidermal growth factor (50ng/mL, Sigma-Aldrich), basic fibroblast growth factor (20ng/mL, R&D), penicillin (100 U/mL) and streptomycin (100 μg/mL).

RNA extraction and Quantitative PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen), as recommended by the manufacturer. cDNA was generated by reverse transcription of total RNA (500 ng) with PrimeScript RT reagent Kit (TaKaRa). Quantitative PCR (qPCR) was performed, and SYBR Green Supermix (Bio-Rad) was used for relative quantification of the indicated genes. Quantification of target genes was normalized against the input determined by β-ACTIN. The primers sequences for qPCR are listed in table 1.

FACS analysis

For detection of intracellular antigen, single cells were fixed with 4% paraformaldehyde and permeabilized with PBS containing 0.1% Triton X-100 for 10 min, respectively. Then, cells were blocked with 5% goat serum, and incubated with flow cytometry antibody, including APC-conjugated CK5 (1:100, abcam, ab224984) and PE-conjugated CK18 (1:100, abcam, ab210410). FACStar Plus Flow Cytometer (BD Biosciences) was conducted for further the analysis and quantification of FACS data.

Calcium activity analysis

HDFs, HDF-EDA treated with SGM, iSGCs and pSGCs for calcium activity analysis were harvested and dissociated to single cells with 0.25% trypsin-EDTA (Solarbio), and were suspended in D-hank's free of calcium loaded with Ca^{2+} indicator dyes (calcium orange^{TM}, AM, Invitrogen, C3015) for 30 min. Then, cells were washed three times in D-hank's free of calcium and suspended again with 200ul $CaCl_2$ solution (2mM, Sigma-Aldrich) containing acetylcholine chloride (50uM, Sigma-Aldrich) for immediate analysis and quantification of flow cytometry.

Animals and transplantation

The animal experiment was performed according to the protocols approved by the Ethics Committee at the Fourth Medical Center of PLA General Hospital and carried out in accordance with Institutional

Animal Care and Use Committee (IACUC) guidelines. For establishment of mouse burn model, athymic BALB/c nude mice (female, 8 weeks old) were purchased from SiBeiFu company (Beijing, China). After anesthesia with pentobarbital (100mg/kg), the hint paws of the mice were suffered from second-degree burn to destroy SG in the dermis. At 3 and 5 days after thermal injury, iSGCs (5×10^5 cells in 50μL mSGM) were collected and injected into the paw pads of recipient mice. Simultaneously, 50 μL modified SGM (mSGM) was intradermally injected in the scalded paw pads of vehicle groups. After the injection, mice were monitored daily and sacrificed at indicated days.

Sweat test

The paw pads were first coated with a 2% (W/V) iodine/ethanol solution, followed by starch, and then, after drying, 50 μl acetylcholine chloride (100μM, Sigma-Aldrich) was injected subcutaneously into the paws of mice.

Statistical Analysis

All values were presented as the means ± standard deviation (SD) calculated from the average of at least three biological replicates unless otherwise specified. Statistical analysis was performed using GraphPad prism 8.0. Comparisons between two groups were analyzed by Student's t test. Probability (P) values < 0.05 was considered statistically significant.

Results

EDA alone is not sufficient for reprogramming HDFs into iSGCs

Given the known dominant role of EDA during sweat gland lineage differentiation [18, 19], single factor EDA (pLV-hefla-EDA-GFP) was introduced into HDFs. The greatest number of GFP^+ cells was produced when the transfected cells were enriched by 0.4ug/ml puromycin for 15 days (HDF-EDA), with GFP^+ cells comprising 78.55% of all cells (Fig 1A). Expression of transgenes in the HDF-EDA cells was confirmed by qPCR when compared with the HDFs control (Fig 1B). Although EDA overexpression induced the morphological changes, however, the prominent cell bodies were unlike a typical SG cell morphology (Fig 1C). The qPCR results further showed that the SG cells associated-markers CK5, CK10 and CK18 did not increased significantly in HDF-EDA cells at day 15 when cultured in the fibroblast medium (Fig 1D), indicating that single factor EDA was not sufficient to enable HDFs to acquire phenotype of SG cells.

Generation of iSGCs from HDFs using EDA and SGM

Given these results, the HDF-EDA cells were switched from fibroblast medium to sweat gland conditional medium (SGM) containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). We found that, at day 3 after SGM treatment, HDF-EDA cells with small cell morphology gradually emerged. At day 10, SGM cultures led to a rapid expansion in the number of HDF-EDA cells. Prolonged exposure to SGM until day 15-20, the epidermal-like morphology of the majority of induced cells began to appear (Fig

2A). RNA expression profiling by qPCR revealed a significantly increased levels of key SG genes CK5, CK18 and CEA, but not SG duct cell marker CK10, and luminal marker AQP5 in HDF-EDA cells treated with SGM (Fig 2B). The FACS analysis demonstrated that, after SGM treatment, approximately 4.18±0.04% of the HDF-EDA cells expressed CK5 and 4.36±0.25% expressed CK18 (Fig 2C and 2D). Given the association of growth factor signaling with SG development and regeneration [20, 21], we then performed qPCR experiments to examine the transcriptional changes of EGFR and FGFR2 in SGM conditions. As expected, qPCR revealed the significant increase mRNA levels of EGFR and FGFR2 in HDF-EDA cells treated with SGM (Fig 2E). However, although long-term exposure to SGM upregulated CK5, CK18, and CEA transcription in HDF-EDA cells, only a small proportion of the reprogrammed cells could be induced to express CK5 and CK18, which indicated a partial and inefficient conversion and additional treatments were required to boost the conversion efficiency of iSGCs.

Small molecules facilitated iSGC generation from HDFs

We hypothesized that the induced cells with a combination of small molecules that modulated SG-developmental signaling would promote the SG cells transcriptional program in HDF-EDA cells. In light of above results, we designed the strategy in Figure 3A to systemically reprogram HDFs into iSGCs by combining small molecules, forced transgenic expression of EDA and SGM culture. The results of qPCR demonstrated that, compared to HDFs, there was significantly decreased mRNA level of TGFβ-R1 and increased mRNA levels of LEF1, BMPR1A, β_2-AR and RARα in SG cells (Fig 3B). Therefore, Repsox(R, TGFβ-R1 inhibitor), CHIR99021(C, WNT signaling agonist), Isoproterenol(I, β_2-AR agonist), BMP4(B) and Retinoic acid(R, RARα agonist) were combined as a iSGCs induction of additional condition (RCIBR) in SGM, named modified sweat gland conditional medium (mSGM). 6-8 days post-treatment of mSGM, the iSGCs exhibited SG cells-like morphology (Fig 3C). RNA expression profiling by qPCR revealed the significantly increased levels of CK5, CK18 and CEA than before, and the duct cells marker CK10 and luminal marker AQP5 were also detected (Fig 3D). The FACS analysis further showed that approximately 23.05±2.49% of iSGCs were CK5 positive and 55.79±3.18% were CK18 positive (Fig 3E and 3F). These results demonstrated that small molecules could promote the acquisition of sweat gland cell properties by fibroblasts and improve the reprogramming efficiency greatly.

The functional analysis of iSGCs

The ability to respond appropriately to hormones and transmitters of the autonomic nervous system is one of the most critical functionally characteristics of native sweat gland cells. We therefore sought to determine how HDF-derived induced sweat gland cells respond to muscarinic stimulation. We measured the intracellular Ca^{2+} signals by using an orange fluorescent calcium indicator. As shown in fig 4, addition of the muscarinic agonist acetylcholine chloride (Ach) significantly increased spontaneous calcium transients. The results showed that, compared to HDFs (6.64±0.57%), the intracellular free [Ca^{2+}] intensity of HDF-EDA treated with SGM was increased significantly (12.65±2.07%) but still much less than pSGC (70.59±0.34%). After 6-8 days of mSGM incubation, the intracellular free [Ca^{2+}] intensity in iSGC was

approximately 60.79±7.71% and comparable to that of the pSGC. These results indicated that iSGCs were functionally similar to normal sweat gland cells.

iSGCs engraftment reconstituted damaged skin with fully restored SG functions

To address whether the generated iSGCs were able to restore SG functionally, iSGCs were injected subcutaneously into the hind paws of scalded mouse (Fig 5A). After 21 days, starch-iodine sweat tests on paw pads showed that iSGCs-treated mice responded to the assay by displaying indigo-black dots (Fig 5B). A total of approximately 5.2±1.1% mice exhibited sweat test positive at day 21 after treatment with iSGCs (Fig 5C). The histological analysis results suggested that, like normal group, there were regenerated SG structures in iSGCs-treated mice (Fig 5D), with the expression of SG markers CK5, CK18 and CK14 (Fig 5D). As expected, the skin tissues of mSGM group only showed damaged structures without the SG components and corresponding markers (Fig 5D and 5E). Collectively, these results indicated that iSGCs can promote SG regeneration structurally and functionally in vivo.

Discussion

In the present study, we first investigated the key factors governing cell-fate transition of fibroblasts into sweat gland cells by exploring the kinetics of the direct conversion process. We found that single factors EDA was sufficient to reprogram human fibroblasts into induced sweat gland cells when combined with small molecules cocktails. Systematic characterization demonstrated that the iSGCs exhibited some typical features of SG cells, displaying expression of duct and luminal-specific markers and increased muscarinic reactivity. Notably, we found small molecules that enhance the efficiency of sweat gland cells reprogramming. iSGCs generated by using our method acquired biochemical and functional characteristics of native sweat gland cells in vitro, and importantly, these iSGCs can be transplanted subcutaneously to restore and regenerate sweat gland.

Regeneration of skin appendages is the key to functional repair of large-scale skin defects. Despite of the existence of wound-resident cell, these cells are not sufficient to promote de novo SG morphogenesis [3]. Direct lineage reprogramming could realize the direct induction of functional cell types from one lineage to another lineage without passing through an intermediate pluripotent stage [22], and this technique has been successfully used to regenerate various cells, such as cardiomyocytes and hepatocytes, etc [23, 24]. Therefore, direct lineage reprogramming could become an alternative way to produce sweat gland cells when it comes to the suitable cell source. Fibroblast is the main cell involving in skin injury repair. The achievement of reprogramming fibroblasts into sweat gland cells in vitro would be of great significance for in situ regeneration of sweat gland in the future. Besides, fibroblasts are also widely available and easy to access and culture, and thus, we seek to modulate the fate of fibroblasts into sweat gland cells. Recently, lineage-specific factors-mediated direct conversion technology has been developed, and previous studies have demonstrated that fibroblasts can be directly converted into hepatocyte-like cells [15], melanocytes [8], pancreatic insulin-producing cells [25] and adipocytes [26] by defined factors, etc. Accumulating evidences suggest that EDA plays an essential role in SG morphogenesis [19, 27], and

defective development of SG in hypohidrotic ectodermal dysplasia (HED) could be rescued by EDA administration [18]. Our previous work also demonstrated targeting ectodysplasin promotor by CRISPR/dCas9-effector effectively induced the reprogramming of human bone marrow-derived mesenchymal stem cells and epidermal cells into sweat gland cells [4, 5]. Theses transfected cells not only expressed the related markers (CK5, CK10, CK18, CEA, CK7, CK14, and CK19) of sweat gland cells, but contributed to sweat gland reconstruction in vivo [4, 5]. Therefore, reasonably, EDA could direct induction of the fate of sweat gland. In the present study, we have successfully overexpressed EDA in human dermal fibroblasts and found that a single factor together with the SGM containing growth factor enabled HDF-EDA preliminarily acquired partial SG cell phenotype, with the expression of SG related markers (CEA, CK5 and CK18). But the inductive efficiency was relatively low, only 4.18±0.04% of HDF-EDA cells treated with SGM expressed CK5 and 4.36±0.25% expressed CK18, respectively. Besides, ductal marker (CK10) and luminal marker (AQP5) were not detectable, and muscarinic reactivity was still very different from that of the pSGCs (12.65±2.07% vs 70.59±0.34%). Therefore, there was the need to explore the additional factors that may boost the activation of the SG program.

Developmentally, SG fate is extremely susceptible to WNT/β-catenin/ Lymphoid enhancer-binding factor 1 (LEF1) signaling pathways. WNT5A and WNT10A could cause accumulation of nuclear β-catenin in the mesenchymal components during cutaneous appendage morphogenesis [20]. It has been reported that WNT10A mutations account for 16% of HED [28]. In addition, there exists an inseparable linkage between the WNT signaling pathway and the EDA/EDAR axis in guiding SwG morphogenesis, for instance, LEF1 could bind to the 1.6-kb DNA sequence upstream of the EDA transcriptional initiation site and further activate the EDA/EDAR signaling pathway [29]. Bone morphogenetic protein (BMP) signaling pathway also mediates a switch in SG fate in skin. The previous study demonstrated that BMPR1A transcripts were markedly elevated in SG-permissive epidermis, and the activation of BMP signaling could determine the formation of SG, while HF morphogenesis would appear in the mouse paws if the BMP signaling was suppressed [20]. In addition, the coculture epidermal stem cell of with embryonic paw pad tissue exhibited glandular structure, and BMP4 concentration was detected in the medium and a BMPR1A inhibitor could effectively block the differentiation [30], further implying the possibility of BMP4 activation in SG regeneration. SG is a peripheral neuroendocrine-control organ. The activation of β-adrenoceptors could open Ca^{2+} channels to elevate intracellular Ca^{2+} concentration and eventually increase sweat secretion. We speculated neuroendocrine signaling could further drive the SG fate, and also found the expression difference of β_2-AR between fibroblasts and primary SG cells. Fibroblasts originate mainly from the mesoderm, and SG is ectoderm organ. Retinoic acid (RA) not only plays an important role in initiating ectoderm development [31, 32], but also participates in the formation of exocrine gland [33, 34]. Moreover, the cooperation of BMP4 with RA could promote the expression of CK18, CK5 and CK14 (the obligate partner of CK5) [35]. TGFβ signaling mediates mesoderm development, the inhibition of TGFβ signaling is necessary for the fate switch from fibroblasts or astrocytes to other cells [36, 37]. It has been reported that the activation of WNT signaling and BMP signaling and inhibition of TGFβ signaling could elevate CK18, CK14 and CK10 [38]. Interestingly, our results also confirmed the difference of above signaling pathway between fibroblasts and pSGCs, which suggested targeted regulation of these

pathways may enhance the efficiency of sweat gland reprogramming. Therefore, we added Repsox (TGFβ signaling inhibitor), CHIR99021 (WNT signaling activator), Isoproterenol (β_2-AR agonists), RA (RARα agonists) and BMP4 (RCIRB) to SGM to consist modified sweat gland conditional medium. After further induction for 6-8 days, cooperation with small molecules, EDA could enable the direct conversion of fibroblasts into iSGCs with high yield. Not only did the expression of CK5, CK18 and CEA be increased further than before, but CK10 and AQP5 were upregulated as well. 23.05±2.49% of the induced cells were CK5 positive and 55.79±3.18% were CK18 positive, respectively. The reactivity to acetylcholine was greatly improved, which was very similar to that of the pSGCs (60.79±7.71% vs 70.59±0.34%).

In vivo transplantation experiment showed that HDF-derived iSGCs could promote SG regeneration, and the emerging SG of iSGCs transplantation group exhibited the structural and phenotypical characteristics closer to normal group, which was consisted of both secretory cells and luminal epithelial layers and represented responsiveness to acetylcholine when engrafted to the paw skin of second-degree burn mice. Collectively, the iSGCs derived from HDF-EDA in mSGM could be a cell source to regenerate SG.

Conclusions

In summary, we established a direct reprogramming strategy for the induction of SG cells regeneration from human dermal fibroblasts in vitro. The genetic approach could drive the fate of desired functional cells during reprogramming, and the combination with the chemical approach could further promote cell-fate conversion. This strategy enables the efficient conversion of human dermal fibroblasts into iSGCs in vitro and permits the generation of desired functional SG for in-vivo treatment.

Abbreviations

HDF: human dermal fibroblasts; SG: sweat gland; pSGCs: primary sweat gland cells; EDA: ectodermal dysplasia antigen; HED: hypohidrotic ectodermal dysplasia; CK5: cytokeratin 5; CK18: cytokeratin 18; CK10: cytokeratin 10; CEA: carcino-embryonic antigen; SGM: sweat gland conditional medium; mSGM: modified sweat gland conditional medium; RA: retinoic acid; EGF: epidermal growth factor; bFGF: basic fibroblast growth factor; BMP: bone morphogenetic protein; ISO: isoproterenol; HED: hypohidrotic ectodermal dysplasia.

Declarations

Acknowledgments

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Authors' contributions

SF J, Y L, XB F and XY S conceived the idea for the study. SF J, Y L and XY S designed the experiments and interpreted the data. SF J, LX Z, JB X, Y L, HT C, YQ L and HH G performed the experiments. SF J, XB

F and XY S wrote the manuscript. The authors read and approved the final manuscript.

Founding

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Availability of data and materials

The data and materials used in the current study are all available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Tables

Table 1. Primers list for RT-qPCR

Genes	Forward primer	Reverse primer
CK5	GGAGAAGGAGTTGGACCAGTCAAC	CTACCTCCGGCAAGACCTCCAC
CK10	ATGTCTGTTCGATACAGCTCAAG	CTCCACCAAGGGAGCCTTTG
CK18	GTTGACCGTGGAGGTAGATGC	GAGCCAGCTCGTCATATTGGG
CEA	TAAGTGTTGACCACAGCGACCC	GTTCCCATCAATCAGCCAAGAA
AQP5	CGGGCTTTCTTCTACGTGG	GCTGGAAGGTCAGAATCAGCTC
β_2-AR	TTGCTGGCACCCAATAGAAGC	CAGACGCTCGAACTTGGCA
TGFβ-R1	ACGGCGTTACAGTGTTTCTG	GCACATACAAACGGCCTATCTC
LEF1	AGAACACCCCGATGACGGA	GGCATCATTATGTACCCGGAAT
BMPR1A	AGATGACCAGGGAGAAACCAC	CAACATTCTATTGTCCGGCGTA
FGFR2	AGCACCATACTGGACCAACAC	GGCAGCGAAACTTGACAGTG
EGFR	TTGCCGCAAAGTGTGTAACG	GTCACCCCTAAATGCCACCG
RARα	AAGCCCGAGTGCTCTGAGA	TTCGTAGTGTATTTGCCCAGC
EDA	GGACGGCACCTACTTCATCT	TGTAGTTGGTCTTGCCCGTC
β-ACTIN	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT

Figures

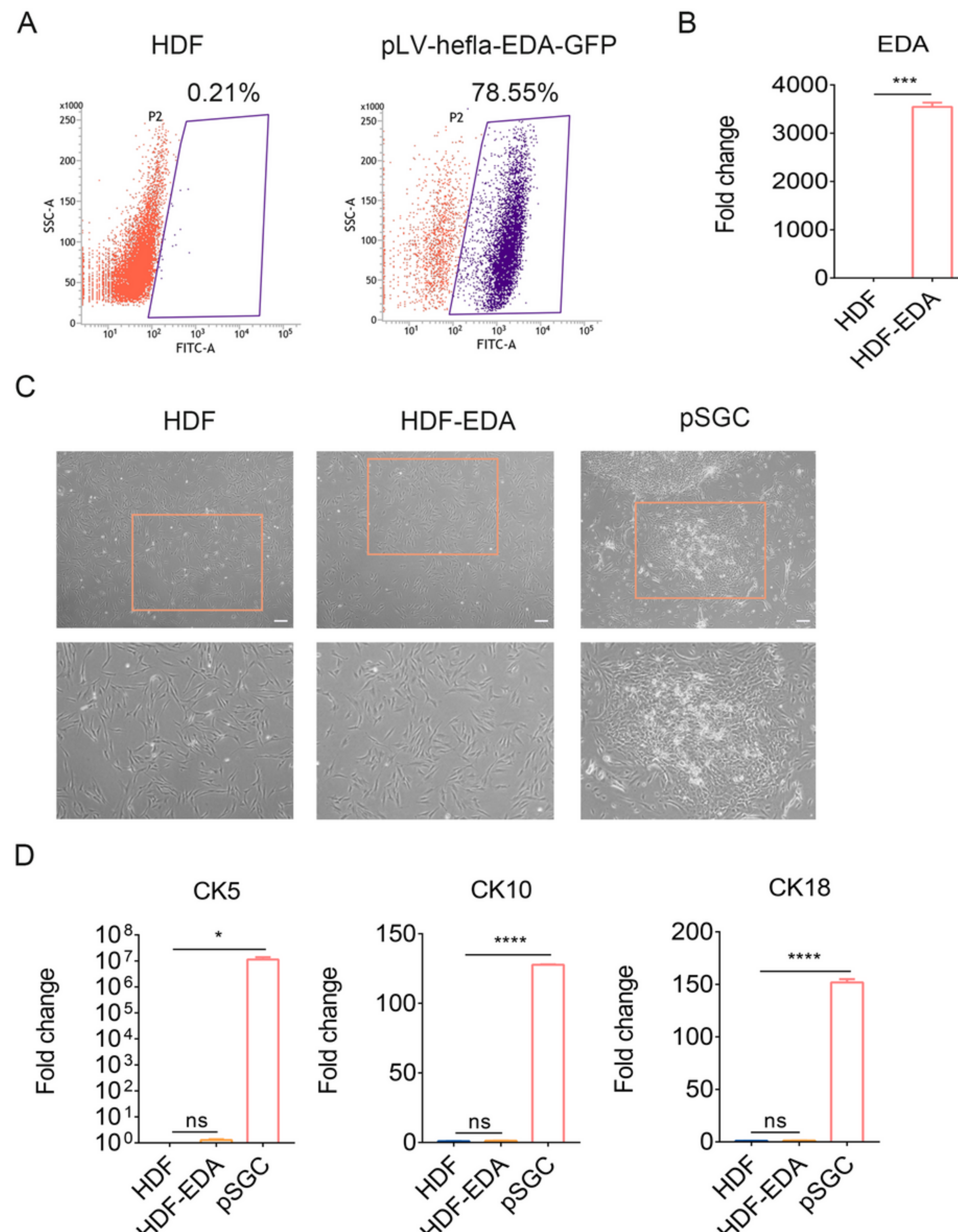

Figure 1

EDA alone is not sufficient for reprogramming HDFs into iSGCs. (A) Flow cytometry quantification of GFP+ cells. Fluorescence intensity of GFP+ cells enriched by puromycin. Human dermal fibroblasts were transfected with EDA at day 15; (B) The confirm of successful transfection with EDA by qPCR analysis; (C) Phase contrast images showing the morphological difference between HDF, HDF-EDA and pSGC. Scale bar = 200 μm. Insets, higher magnification of the boxed areas; (D) qPCR analysis of transcriptional

expression of CK5, CK18 and CK10 in HDF, HDF-EDA and pSGC; The genes showing significant differences in qPCR array assay were presented. Data were mean ± SD and analyzed by two-tailed t-tests, $*P < 0.05$, $**P < 0.01$, $***P<0.001$. ns, not significant.

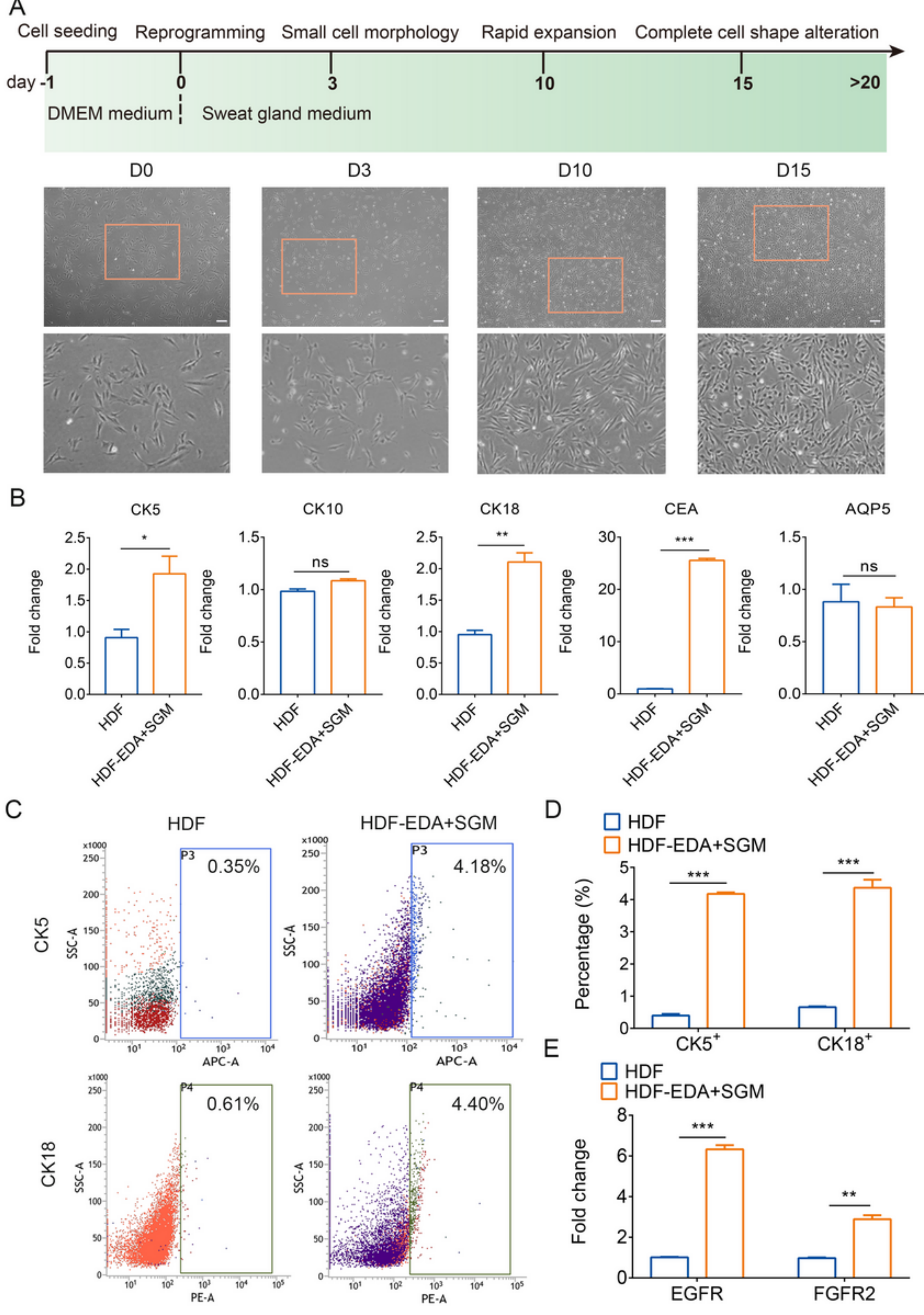

Figure 2

The acquisition of SG cells phenotype by forced transgenic expression of EDA and SGM culture. (A) Phase contrast images showing the morphological changes of HF-EDA in SGM at day 0, day 3, day 10

and day 15. Scale bar = 200μm. Insets, higher magnification of the boxed areas; (B) qPCR analysis of transcriptional expression of CK5, CK10, CK18, AQP5 and CEA in HDF and HDF-EDA cultured in SGM after 15 days of induction; (C, D) Flow cytometry quantification of CK5 positive cells and CK18 positive cells of HDF-EDA in SGM; (E) qPCR analysis of EGFR and FGFR2 mRNA levels in HDF-EDA cultured in SGM. Data were mean ± SD and analyzed by two-tailed t-tests, *P < 0.05, **P < 0.01, ***P<0.001. ns, not significant.

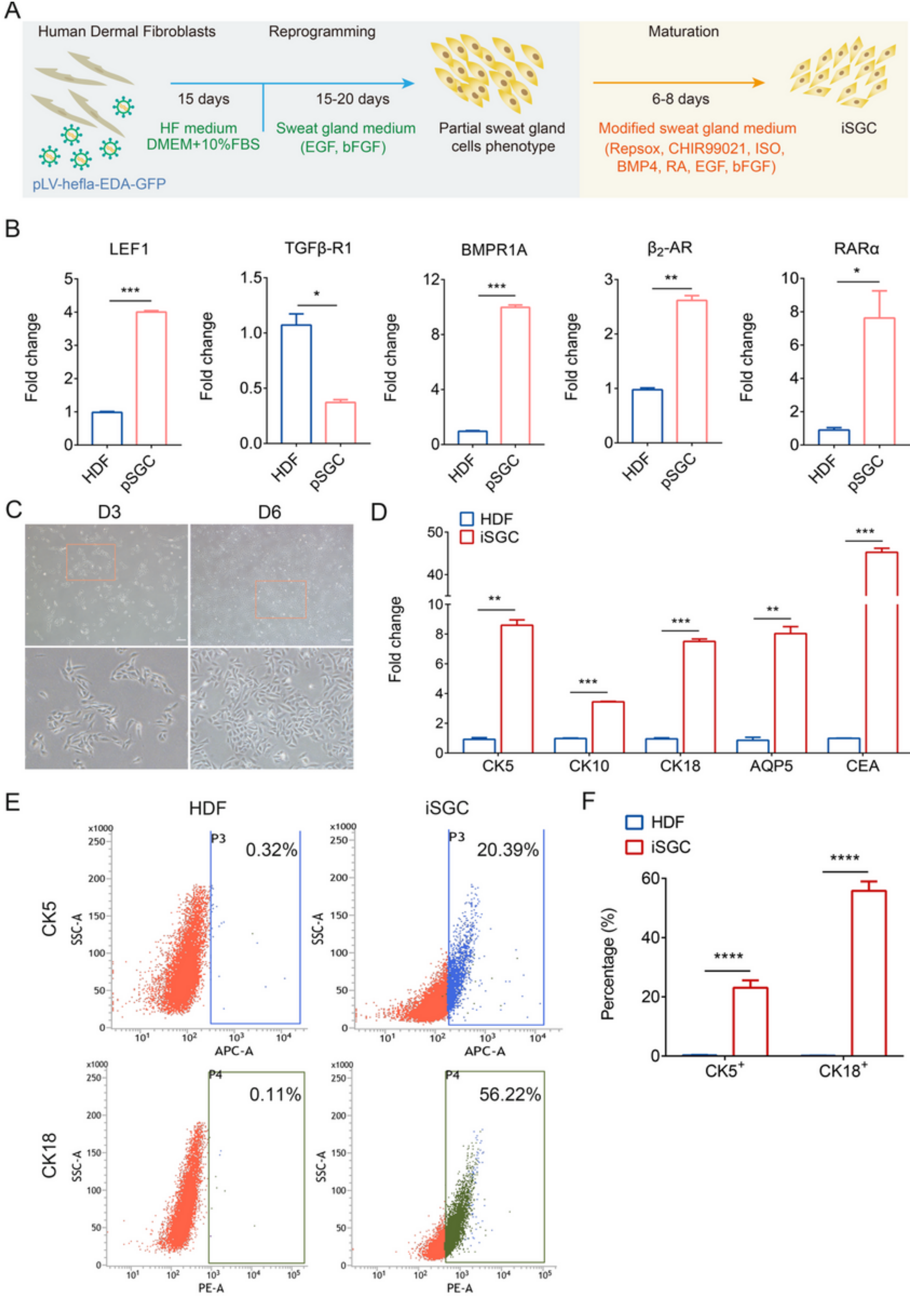

Figure 3

The conversion of HDF into iSGC with small molecules. (A) Scheme of reprogramming strategy procedure. HDF were transfected with EDA and plated in fibroblasts medium, then cells were transferred into SGM for 15-20 days, and eventually HDF-EDA in SGM were further transferred into modified sweat gland medium (mSGM) for 6-8 days; (B) qPCR analysis of transcriptional expression of TGFβR1, LEF1, BMPR1A, β2-AR and RARα in HDF and pSGC; The genes showing significant differences were presented; (C) Phase contrast images showing the morphological changes of iSGC in mSGM at day 3 and day 6. Scale bar = 200μm. Insets, higher magnification of the boxed areas; (D) qPCR analysis of transcriptional expression of CK5, CK10, CK18, AQP5 and CEA in HDF and iSGC after 6-8 days of induction; (E, F) Flow cytometry quantification of CK5 positive cells and CK18 positive cells of iSGC. Data were mean ± SD and analyzed by two-tailed t-tests, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P<0.001$.

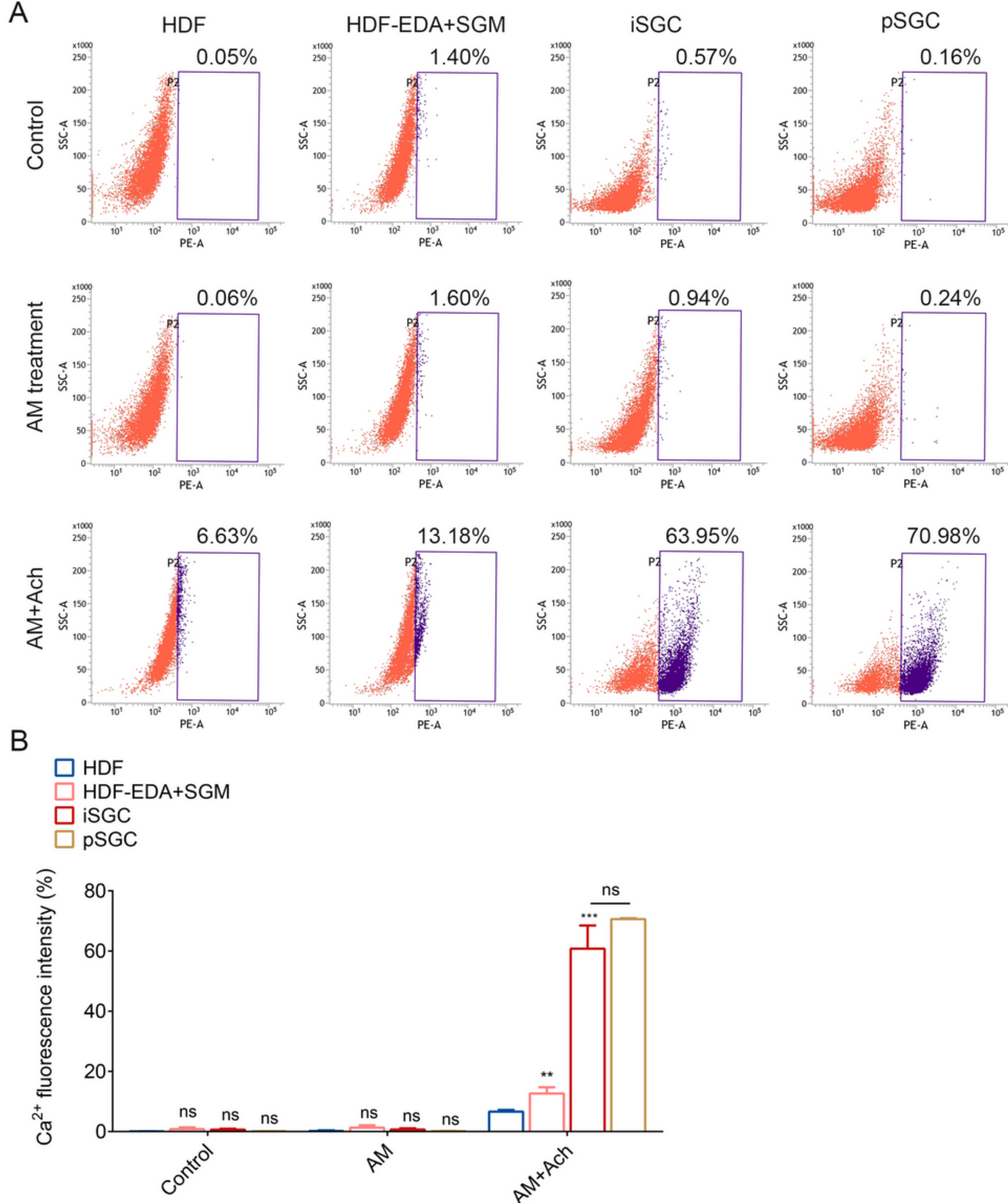

Figure 4

The functional analysis of iSGC. (A) Calcium activity analysis was used to assess the reactivity to acetylcholine. (B) The data presented the intracellular free [Ca2+] intensity of iSGCs was higher than HF-EDA in SGM and similar to that of the pSGC, 60.79±7.71%, 12.65±2.07% and 70.59±0.34%, respectively.

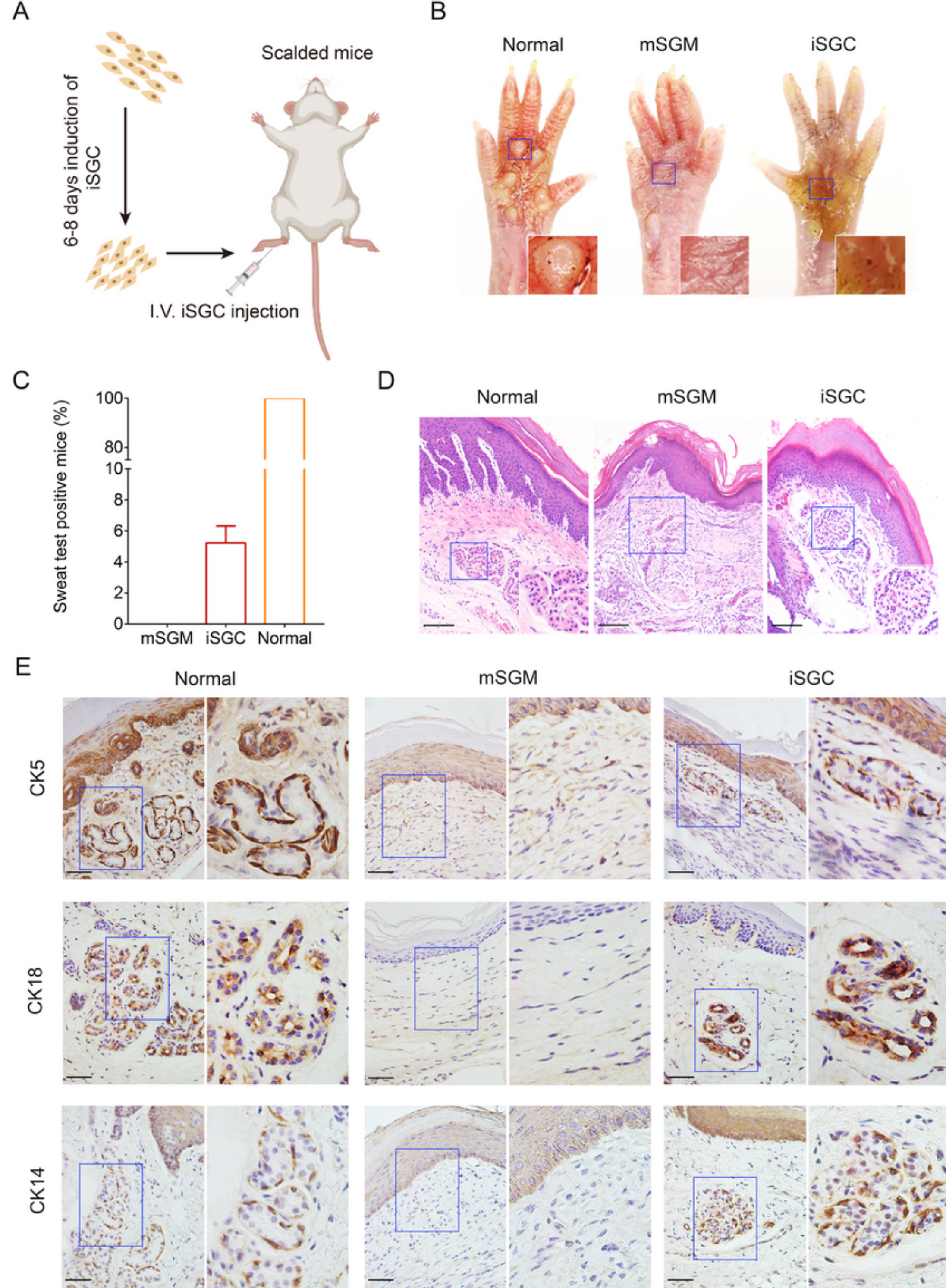

Figure 5

Engraftment of iSGC functionally restored SG. (A) Schematic diagram representing the experimental procedure; (B) Starch-iodine sweat tests on paw skin of thermal-injured mice showed that paws of iSGC-treated mice and normal mice responded by displaying indigo-black dots at day 21 after transplantation; (C) The positive rate of sweat test of iSGC-treated group, 5.2±1.1% of the recipient mice; (D) H&E staining was conducted to visualize normal group and mSGM- and iSGC-treated wounds at day 21 post-injury.

Emerging glandular structures were seen in the dermis of iSGC-treated mice and normal mice. Scale bar = 50μm. Insets, higher magnification of the boxed areas; (E) The SG markers CK5, CK18, and CK14 were assessed by immunohistochemical (IHC) analysis to examine the SG formation. The results showed that, like natural paw shin, the iSGC-treated group could form SG-like structures with positive staining for the SG markers, while no SG regeneration was observed in mSGM-treated group. Scale bars =50μm. Insets, higher magnification of the boxed areas;