

# Ground-State Transcriptional Requirements for Skin-Derived Precursors

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Skin-derived precursors (SKPs) are an attractive stem cell model for cell-based therapies. SKPs can be readily generated from embryonic and adult mice and adult humans, exhibit a high degree of multipotency, and have the potential to serve as a patient autologous stem cell. The advancement of these cells toward therapeutic use depends on the ability to control precisely the self-renewal and differentiation of SKPs. Here we show that two well-known stem cell factors, *Foxd3* and *Sox2*, are critical regulators of the stem cell properties of SKPs. Deletion of *Foxd3* completely abolishes the sphere-forming potential of these cells. In the absence of *Sox2*, SKP spheres can be formed, but with reduced size and frequency. Our results provide entry points into the gene regulatory networks dictating SKP behavior, and pave the way for future studies on a therapeutically relevant stem cell.

## Introduction

THE EXTENT TO WHICH molecular control of pluripotency and multipotency are conserved across species has received considerable attention; less focus has been given to the degree of conservation between divergent cell types within a given species. Embryonic stem cells (ESCs) represent the gold standard for examining mechanisms of pluripotency, yet progress toward clinical advances remains slow and research using ESCs is fraught with ethics issues. Induced pluripotent stem cells may one day function as a suitable replacement, but the long-term safety of these cells remains unknown. Given the significant caveats associated with these cells, the continued development of complementary stem cell models with potential for stem cell therapies is important. The neural crest (NC) is an intriguing alternative as a number of the core pluripotency transcription factors expressed in ESCs are also expressed in the NC, including *Sox2* and *Foxd3*. Whether these factors function identically in the NC and in ESCs is unknown.

Neural crest cells (NCCs) are a highly multipotent cell type with broad differentiation potential. The NC is specified in the neurula-stage embryo; these cells undergo an epithelial to mesenchymal transition and migrate along defined paths through the embryo. Their fate depends on their rostral-caudal position of origin in the neural tube, their route of

migration, and final destination (reviewed in [1]). NC defects result in a number of developmental disorders, including CHARGE syndrome, Hirschsprung disease, Waardenburg syndrome, DiGeorge syndrome, congenital heart defects, and craniofacial abnormalities [2–4]. Cell-based therapies may be appropriate for some of these syndromes.

Neural crest stem cells (NCSCs) persist through development, retaining their multipotency in adult organisms. NCSCs can be isolated from a number of embryonic and postnatal derivatives of the NC: dermis of the skin, fetal peripheral nerves, and the fetal and adult enteric nervous system [5–9]. Of these, skin-derived precursors (SKPs) are of particular interest. SKPs are derived from the dermis of rodents and humans, and display a characteristic NC-like gene signature [9]. SKPs generated from the whisker pads of mice are NC-derived and can be lineage-labeled using *Wnt1-Cre* [10], a transgene expressed throughout the majority of the NC [11]. In vitro, SKPs exhibit a highly multipotent phenotype; they can differentiate into neurons, glia, smooth muscle cells, adipocytes, osteoblasts, and chondrocytes [9,10,12–16]. The therapeutic efficacy of these cells has been suggested by rodent transplant studies; undifferentiated SKPs contribute to newly formed bone in a fracture model, “pre-differentiated” SKPs assist in myelination of nerves in a sciatic nerve injury model, and these cells may also serve as an alternative source for cutaneous nerve regeneration

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[12,13,15,17–19]. Importantly, these cells are readily accessible from adult humans and have the potential to serve as a patient-autologous stem cell source for a diverse array of cell-based therapies.

Despite these preclinical advances and the vast knowledge of transcription factor function in the NC [20], little is known about the molecules dictating NCSC self-renewal and multipotency; the “ground state” of these multipotent stem cells has not been widely explored. *Foxd3* and *Sox2* are logical entry points into the genetic regulatory networks governing SKP behavior. *Sox2* expression can be used to prospectively isolate SKPs in addition to other progenitor cells [6,14,21], while loss of *Foxd3* in the NC causes NC-progenitors to lose multipotency and self-renewal ability [22,23]. Null mouse embryos for either *Foxd3* or *Sox2* have virtually indistinguishable phenotypes, with loss of epiblast and an expansion of extra embryonic tissue, and both proteins are required for the establishment of ESCs and trophoblast stem cells (TSCs) [24–26]. Finally, *Foxd3* and *Sox2* are known to antagonistically regulate shared loci in ESCs [27]. Given the prominent role of *Sox2* and *Foxd3* as key regulators of pluripotency in a number of stem cell populations, we examined the consequences of a genetic deletion of *Sox2* in the NC and in NC-derived SKPs, and the role of *Foxd3* in SKPs.

## Materials and Methods

### Mouse lines

The *Wnt1-Cre* transgenic line [28] was used to conditionally modify either the *Foxd3* conditional allele *Foxd3<sup>tm3.Lby</sup>* (called *Foxd3<sup>flox</sup>* here) [23] or the *Sox2* conditional allele *Sox2<sup>tm2Lpev</sup>* (called *Sox2<sup>flox</sup>*) [29] in combination with the reporter allele *Gt(ROSA)26Sor<sup>tm1(EYFP)Cos</sup>* (called *R26R<sup>YFP</sup>*) [30] to visualize the NC lineage. The *Foxd3* null allele *Foxd3<sup>tm1.Lby</sup>* was included in some crosses [25]. The *Foxd3* null green fluorescent protein (GFP) reporter allele *Foxd3<sup>tm2.Lby</sup>* (called *Foxd3<sup>GFP</sup>*) [25] was used to visualize *Foxd3* expression in SKPs. The TM inducible transgenic line *CAGG Cre-ER<sup>TM</sup>* (called CreER) [31] was used for conditional deletions of *Foxd3* or *Sox2* in SKP cell lines. All mouse lines were maintained on a mixed genetic background. All experiments were carried out in accordance with protocols approved by the Vanderbilt University Institutional Animal Care and Use Committee.

### Cell culture

ESCs were cultured as described elsewhere [32]. SKPs were generated and cultured as previously described [9,12]. Briefly, appropriate mice were paired and monitored daily for the presence of a vaginal plug. Noon on the day of an observed plug was considered 0.5 days postcoitum (dpc). In order to generate SKP cultures we used standard methods [12]. Whisker pads or dorsal back skin were dissected from 16.5 to 18.5 dpc embryos. Tissues were digested in 0.1% trypsin/ethylenediaminetetraacetic acid for 30–45 min, mechanically dissociated, and passed through a 40- $\mu$ M cell strainer. Single-cell suspensions were plated in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 3:1 in the presence of 2% B-27 (Invitrogen), 40 ng/mL basic fibroblast growth factor (BD Biosciences), and 20 ng/mL epidermal growth factor (BD Biosciences). For experiments using

the *CreER* transgene, a single dose of 0.2  $\mu$ M 4-hydroxytamoxifen (TM) was added to the culture on day 0. The TM powder was dissolved in 100% ethanol to make a 1 mM TM stock solution and stored at  $-20^{\circ}$ C. To quantify the number of SKP spheres, images of cultures were systematically captured as follows: at least 15 individual 40 $\times$  frames of each culture were taken and the number of spheres per frame was determined. Data are presented as the average number of spheres per frame.  $N=3-5$  for each genotype. For quantification of SKP sphere size, floating spheres were collected, pooled, and imaged. Spheres were outlined in ImageJ and the area was calculated in arbitrary units. At least 65 spheres were counted for each culture. Two-tailed Student's *t* tests were used to determine statistical significance.

### Histology

Embryos were fixed in 4% paraformaldehyde overnight and standard histological procedures used [33]. Antibodies for immunofluorescence were rabbit-anti-*Foxd3* (1:1,000) [26], goat-anti-*Sox2* (1:500; Santa Cruz), and chicken-anti-GFP (1:500; Abcam). Secondary antibodies were from Jackson ImmunoResearch and 4',6-diamidino-2-phenylindole (1:2,000; Molecular Probes) was used to visualize cell nuclei. Images were captured on either a Nikon Eclipse E600 or a Zeiss AxioCam MRC, both using Qcapture software. For quantification of dorsal root ganglia (DRG) size, serial sections of three pairs of DRGs at the level of the cardiac NC were examined for each genotype. DRGs were outlined using ImageJ software and the area calculated in arbitrary units.  $N=2$  embryos for each genotype.

### Quantitative real-time polymerase chain reaction

Total RNA was harvested from established SKP lines using the RNeasy Mini Kit (Qiagen) and genomic DNA removed using Turbo DNase (Ambion). cDNA was prepared using the GoScript<sup>TM</sup> Reverse Transcription System (Promega) and quantitative real-time polymerase chain reaction (qRT-PCR) performed using the GoTaq<sup>®</sup> qPCR Master Mix (Promega) and analyzed on an Applied Biosystems 7900 machine. Primer sequences were as follows: *Foxd3*, 5'-GTCC GCTGGGAATAACTTTCCGTA-3' and 5'-ATGTACAAAGA ATGTCCCTCCCACCC-3'. *Gapdh*, 5'-TGAGCCGGTGTGATATGT-3' and 5'-CCACAGTCTTCTGGGTGGCAGTG-3'. *Sox2*, 5'-GAGGAAAGGGTCTTGCTGGGTTT-3' and 5'-GGT CTTGCCAGTACTTGCTCTCAT-3'. Expression is presented as the difference in threshold cycle number of the transcript of interest relative to *Gapdh*.

## Results

### *Sox2* and *Foxd3* expression in NCCs and NCSCs/SKPs

The function of *Sox2* in the NC has been largely overlooked. Early reports using *in situ* hybridization failed to detect *Sox2* mRNA in NCCs [34]. However, several recent reports described roles for *Sox2* in controlling the behavior of NC stem and progenitor cells: *Sox2* maintains the progenitor status of NCCs fated to become melanocytes [35], and expression of *Sox2* in the NC is essential for peripheral neurogenesis [36]. Given these recent findings, we re-examined

the expression of Sox2 using more sensitive immunofluorescence techniques in combination with an NC-specific lineage label.

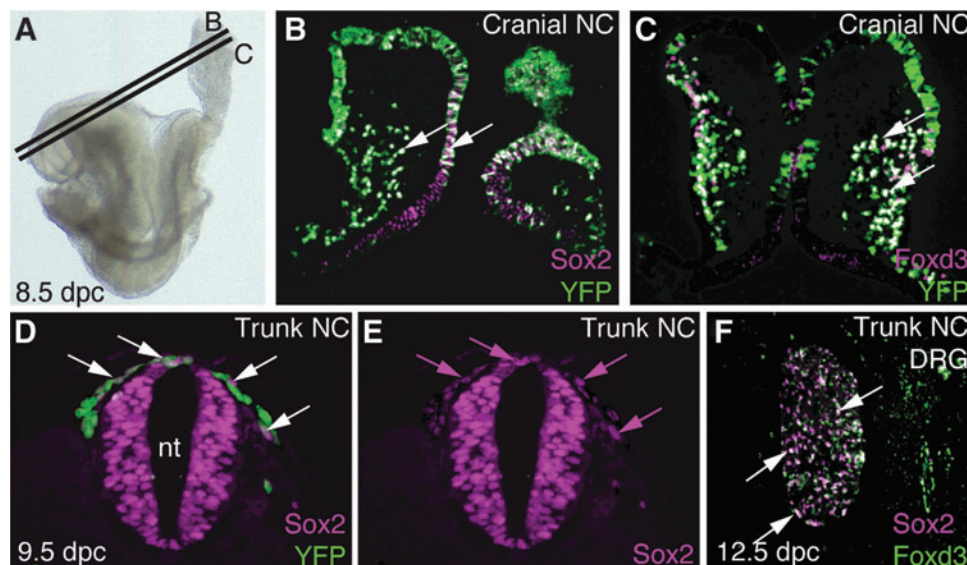
Sox2 protein is detected as early as 8.5 days dpc in pre-migratory and migratory cranial NCCs (Fig. 1A, B). This NC expression pattern is nearly indistinguishable from that of Foxd3 (Fig. 1C), a factor with established roles in the NC in multiple model systems [22,23,37–40]. At 9.5 dpc, Sox2 is expressed at a low level in trunk NCCs as they emigrate from the neural tube (Fig. 1D, E). At 12.5 dpc, Foxd3, and Sox2 are co-expressed in the DRG, an NC-derived structure [41]. Our observations of Sox2 expression in the DRG are consistent with other reports [36,42].

SKPs are derived from the dermal papillae of mouse hair and whisker follicles [10], and can be prospectively isolated based on Sox2 expression [14]. Although Foxd3 expression has been reported in SKPs [43], it was unknown whether Foxd3 is expressed in the endogenous stem cell niche. Using immunofluorescence, at 12.5 dpc Sox2 was expressed in the NC-derived dermal condensates preceding whisker follicle morphogenesis (Fig. 2A–C). Foxd3 protein was localized to the periphery of the dermal condensates and was co-expressed with Sox2 (Fig. 2A, B, and D). At 17.5 dpc, an age when SKPs are readily generated, Sox2 is expressed in two distinct populations: (1) the NC-derived dermal papilla and dermal sheath, and (2) a non-NC lineage that was previously described as diagnostic of Merkel cells [44]. In contrast, Foxd3 is expressed exclusively in the NC lineage (Fig. 2E). It is not present in the dermal papilla, but was detected more distally in the whisker follicle, where it was co-expressed with Sox2. In cranial NC mesenchyme Foxd3 is expressed independently of Sox2 (Fig. 2F).

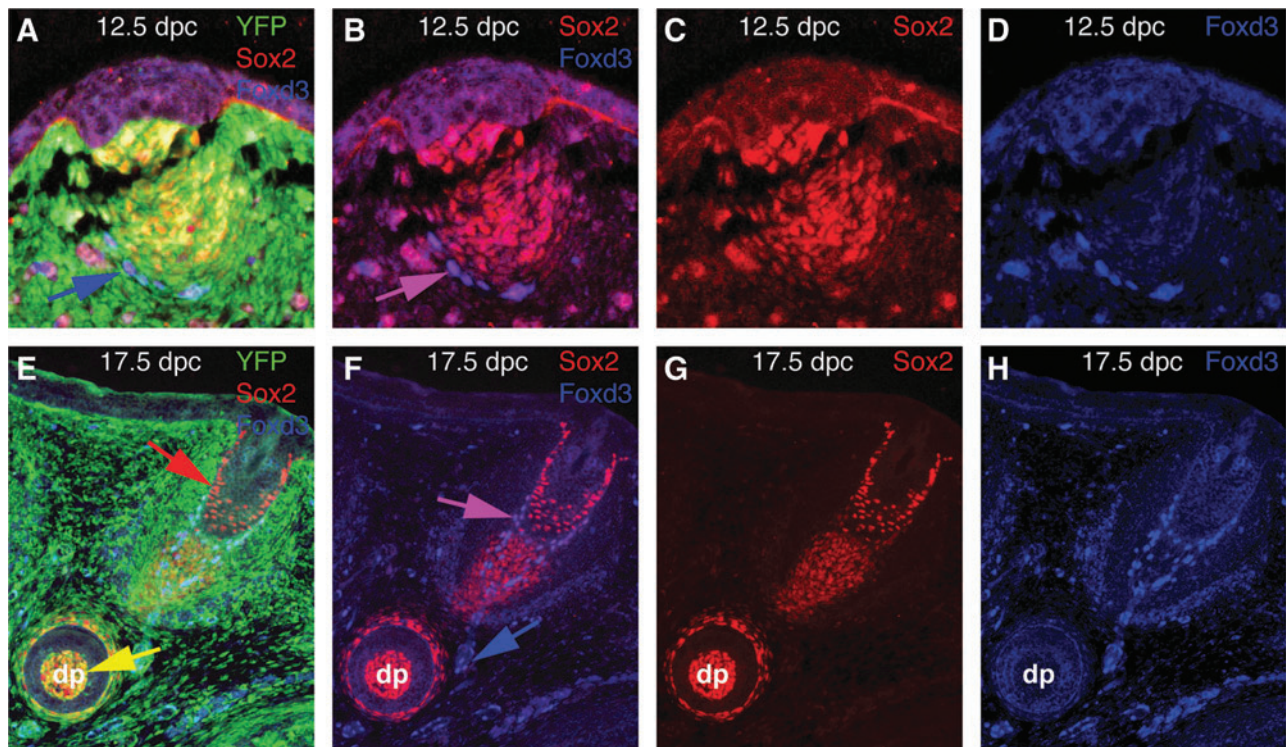
These results suggest the expression of Foxd3 is initiated during SKP establishment and is not present in the progenitors *in vivo*. To confirm this, Foxd3 expression was visualized in SKPs using a *Foxd3<sup>GFP</sup>* allele that has been used for this purpose in other contexts and faithfully represents the endogenous expression of the locus in all contexts tested [45,46] (Fig. 3A, B). This was confirmed by analyzing *Foxd3* transcripts in SKPs using qRT-PCR (Fig. 3C), consistent with previous reports [43]. As a positive control, qRT-PCR was used to show similar levels of Foxd3 transcripts in ESCs, a cell type that requires Foxd3 for self-renewal and inhibition of differentiation [32]. The expression of Sox2 and Foxd3 in NCCs is consistent with the notion that these two transcription factors function in NCSCs. Sox2 and Foxd3 are critical factors in multiple stem cell populations, but their role in NCSCs, and the role of Sox2 in the NC, had not been examined.

#### A *Wnt1-Cre*-driven deletion of Sox2 results in perinatal death, but causes minimal morphological defects

The role of Foxd3 in the NC has been extensively studied; however, the function of Sox2 in the NC is largely unknown. We used *Wnt1-Cre* to drive deletion of Sox2 in the NC. In contrast to previous reports that showed embryonic lethality of these animals [36], mice derived from a *Wnt1-Cre*-driven deletion of Sox2 were born alive ( $n=4$ ). However, these mice did not survive beyond 2 days. *Sox2<sup>flox/flox</sup>; Wnt1-Cre* embryos had no detectable abnormalities in their craniofacial skeletons, nor did they exhibit any congenital heart defects (data not shown). As a potential cause of death, diaphragms were examined for proper innervation. At 16.5 dpc, no



**FIG. 1.** Sox2 and Foxd3 are expressed in NCCs. (A) Whole-mount bright-field image of an 8.5 dpc embryo. Lines represent the plane of section in B and C. (B) Sox2 (magenta) is expressed in cranial NCCs at 8.5 dpc as indicated by co-expression with the *Wnt1-Cre* activated YFP lineage label (green). White arrows highlight several co-expressing cells (200 $\times$ ). (C) Foxd3 (magenta) expression in cranial NCCs, again white arrows indicate co-expressing cells (200 $\times$ ). (D, E) Sox2 (magenta) is expressed at low levels in trunk NCCs at 9.5 dpc as the cells are exiting the neural tube (white arrows). (E) Shows the Sox2 channel alone. Magenta arrows indicate Sox2 expressing NC cells. Note, the Sox2 expression in the NC is markedly lower than that observed in the neural tube (400 $\times$ ). (F) Foxd3 (green) and Sox2 (magenta) are co-expressed in trunk DRG of a 12.5 dpc embryo (400 $\times$ ). NCCs, neural crest cells; dpc, days post coitum; DRG, dorsal root ganglion; NC, neural crest; nt, neural tube. Color images available online at [www.liebertpub.com/scd](http://www.liebertpub.com/scd)



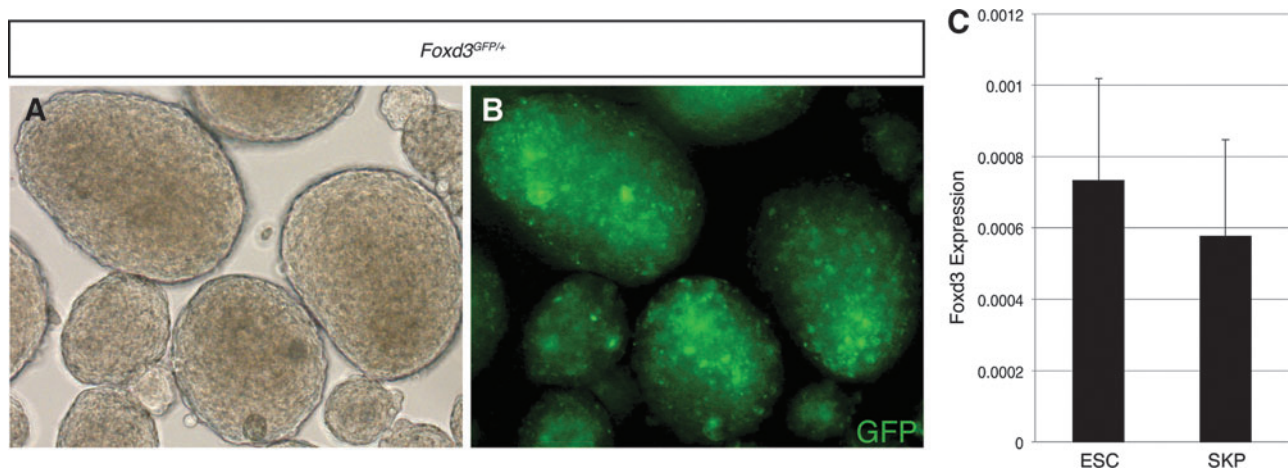
**FIG. 2.** Sox2, but not Foxd3, is expressed in the endogenous SKP niche. (A) At 12.5 dpc, Sox2 (red) is expressed in the dermal condensates preceding whisker follicle morphogenesis. Foxd3 (blue) is expressed on the periphery of the dermal condensates (blue arrow). Expression of both proteins is restricted to the NC lineage (green). (B–D) Panels show the individual channels. Foxd3 (blue) is co-expressed with Sox2 (red) (purple arrow) (200 $\times$ ). (E) At 17.5 dpc, Sox2 (red) is expressed in two distinct lineages: the NC in the dp (yellow arrow) and non-NC surrounding the more proximal part of the whisker follicle (red arrow). Foxd3 (blue) is only expressed in the NC lineage (100 $\times$ ). (F–H) Panels show the individual channels. In the whisker follicle Foxd3 (blue) is co-expressed with Sox2 (red) in the NC-derived cells (purple arrow). In the mesenchyme, Foxd3 is expressed in cells that do not express Sox2 (blue arrow) (100 $\times$ ). SKPs, skin-derived precursors; dp, dermal papilla. Color images available online at [www.liebertpub.com/scd](http://www.liebertpub.com/scd)

defects were seen with the innervation of *Sox2<sup>flox/flox</sup>; Wnt1-Cre* diaphragms as visualized by PGP9.5 immunolocalization to mark neurons. In both *Sox2<sup>flox/flox</sup>; Wnt1-Cre* embryos and *Sox2<sup>wt/wt</sup>; Wnt1-Cre* littermate controls the phrenic nerves ranged the length of the diaphragms, extending to the proximal and distal tips, with numerous secondary branches (Fig. 4A, B). These data suggested that the cause of death is likely not due to respiratory defects. In addition to the NC, the *Wnt1-Cre* transgene is expressed in the developing midbrain and dorsal neural tube, both areas of robust Sox2 expression [28]. Thus, the cause of death may be due to neural tube defects, but at this time remains undetermined. Consistent with a previous report [36] Sox2 expression was absent in the DRGs of 16.5 dpc *Sox2<sup>flox/flox</sup>; Wnt1-Cre* embryos (Fig. 4C, D). We also observed a 47% reduction in the area of the DRGs with a 2.36% standard error of the mean change,  $n=3$  (Fig. 4E, F), again consistent with previous reports [36].

#### *SKPs can be established without Sox2, but at a significantly reduced frequency*

While SKPs can be prospectively isolated based on Sox2 expression [14], the requirement for Sox2 in SKPs had not been investigated. SKPs generated from whisker pads of mice are derived from NC and therefore are lineage labeled with the *Wnt1-Cre* transgene [10]. This Cre was used to determine the requirement for Sox2 in the establishment of SKPs.

First, to confirm Sox2 was deleted in the endogenous SKP niche, whisker pads were examined from 17.5 dpc *Sox2<sup>flox/flox</sup>; Wnt1-Cre* embryos. Sox2 was no longer detected in the NC-derived portion of the whisker follicle (Fig. 5E), although Sox2 expression was unchanged in the non-NC portion. We observed no qualitative differences in the morphology of the follicles at a macroscopic or histological level. Co-expression of Sox2 and Foxd3 was completely lost in *Sox2<sup>flox/flox</sup>; Wnt1-Cre* whisker pads (Fig. 5F). SKPs generated from whisker pads of *Sox2<sup>flox/flox</sup>; Wnt1-Cre* embryos were modestly reduced in number compared to littermate controls; this difference was not statistically significant (Fig. 6A–D, quantified in Fig. 6E). Sox2 transcripts were reduced  $\sim 14$ -fold in *Sox2<sup>flox/flox</sup>; Wnt1-Cre* SKPs compared to *Sox2<sup>wt/wt</sup>; Wnt1-Cre* littermate control SKPs (Fig. 6F). To extend this line of investigation, we used a TM inducible *CreER* to conditionally delete Sox2 from SKPs during establishment of the cultures. Cells from *Sox2<sup>flox/flox</sup>; CreER* embryos treated with a single dose of 0.2  $\mu$ M TM on day 0 of culture resulted in an 66% reduction in the number of spheres compared to untreated *Sox2<sup>flox/flox</sup>; CreER* cells from the same embryo (Fig. 6G–K). TM treatment of cells from *Sox2<sup>flox/flox</sup>* embryos lacking the inducible Cre transgene showed no difference in the number of spheres generated compared to untreated cells from the same embryo. Additionally, spheres derived from TM-treated *Sox2<sup>flox/flox</sup>; CreER* cells were reduced in size by 72%



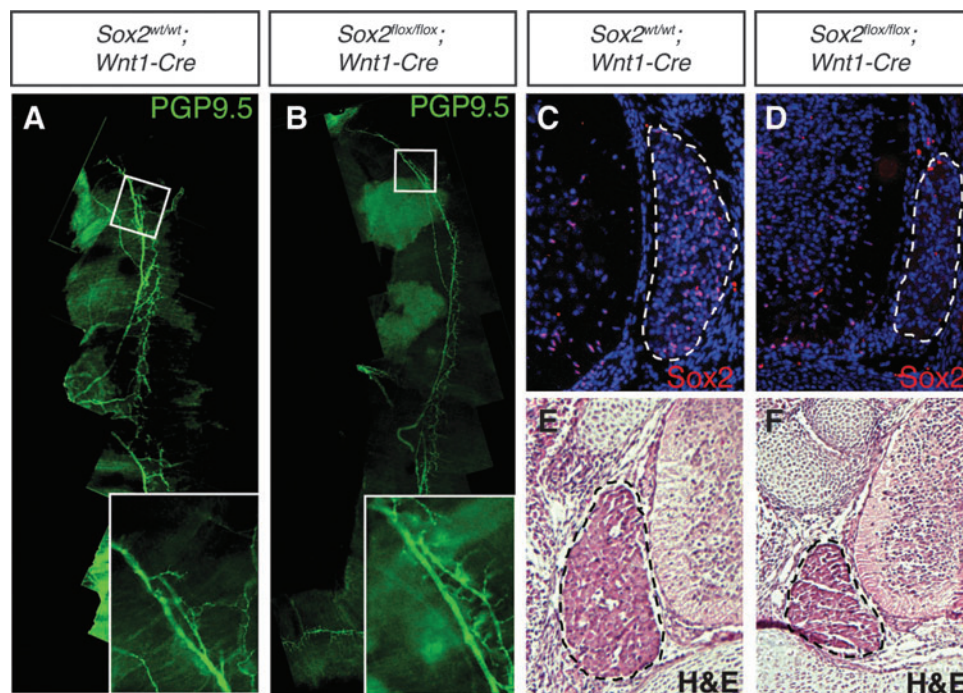
**FIG. 3.** Foxd3 is expressed in SKPs. (A, B) Bright-field and fluorescent images of SKPs carrying the *Foxd3*<sup>GFP</sup> allele (200×). (C) *Foxd3* transcripts were detected by quantitative real-time-polymerase chain reaction in SKPs. *Foxd3* transcript levels in embryonic stem cells (ESCs) are included for comparison. Color images available online at [www.liebertpub.com/scd](http://www.liebertpub.com/scd)

compared to untreated cells from the same embryo (Fig. 6L). These results demonstrate that SKPs are able to form in the absence of Sox2, but do so at a significantly reduced capacity.

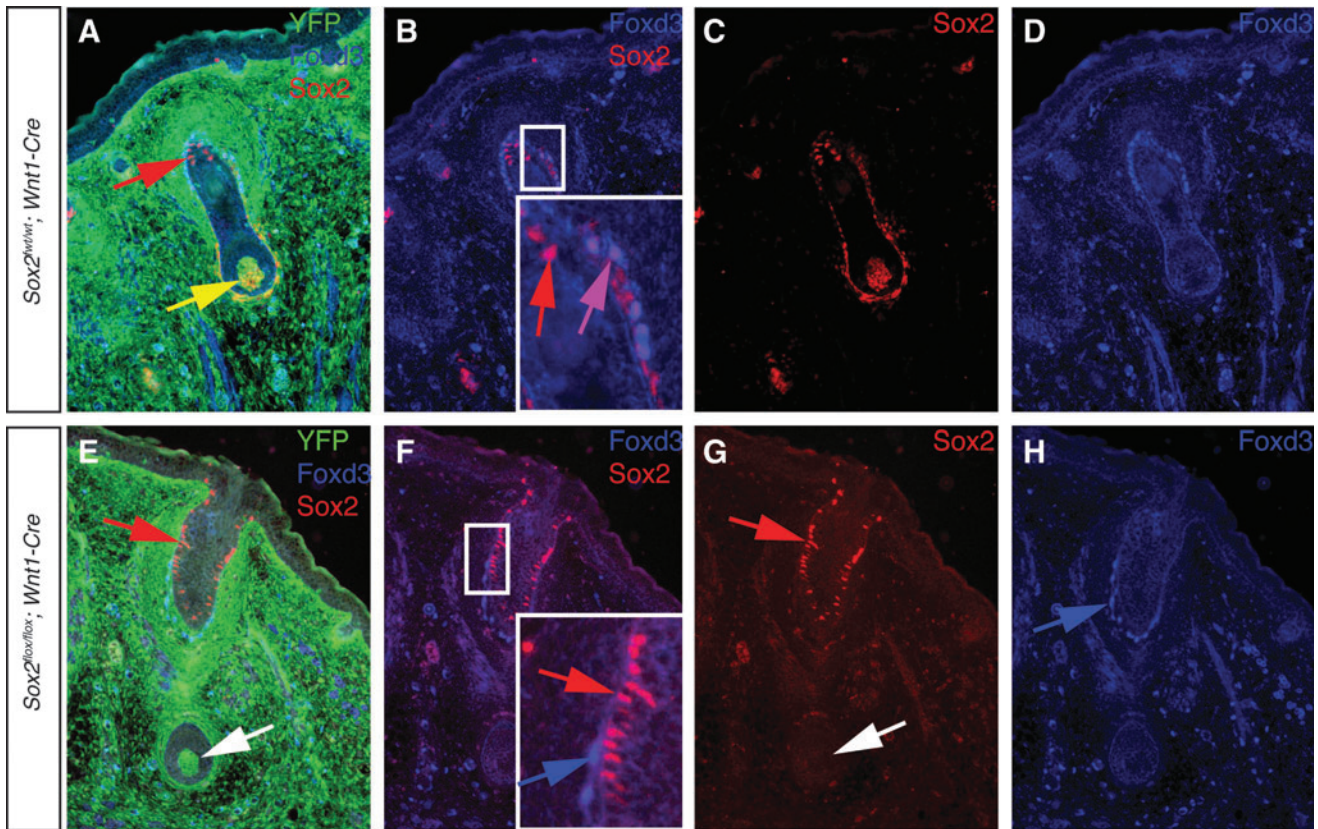
#### *Foxd3* is required for the establishment of SKPs

*Foxd3* is one of the earliest markers of NC [37,38,40,47–50] and its role in NC maintenance has been well documented

[22,23,38,51]; however, its role in NCSC/SKPs was unknown. As described above for Sox2, we tested the requirement for Foxd3 in establishment of SKPs. In control embryos heterozygous for Foxd3, all SKPs were derived from NC as shown using the *Wnt1-Cre; R26R*<sup>YFP</sup> lineage-label (Fig. 7A–C). In contrast, although numerous spheres were formed from whisker pads of *Foxd3*<sup>flx/flx</sup>; *Wnt1-Cre* embryos (Fig. 7D–F), none of the resulting spheres were derived from NC, evidenced by a lack of



**FIG. 4.** *Sox2*<sup>flx/flx</sup>; *Wnt1-Cre* embryos showed normal innervation of the diaphragm, but reduced DRG size. (A, B) PGP9.5 (green) staining revealed no difference in the innervation of the diaphragm at 16.5 dpc in control versus *Sox2*<sup>flx/flx</sup>; *Wnt1-Cre* embryos, suggesting that respiratory defects are not the cause of death for the *Sox2*<sup>flx/flx</sup>; *Wnt1-Cre* mice. The white box denotes the area shown in higher magnification at the bottom of the panel (100× composite, inset 400×). (C, D) Sox2 protein (red) was present in the DRGs of 16.5 dpc control embryos, but not detectable in the DRGs of *Sox2*<sup>flx/flx</sup>; *Wnt1-Cre* littermates. DRGs are outlined in white (200×). (E, F) Hematoxylin and eosin (H&E) staining revealed DRGs from *Sox2*<sup>flx/flx</sup>; *Wnt1-Cre* 16.5 dpc embryos are reduced in size compared to controls, DRGs are outlined in black (200×). Color images available online at [www.liebertpub.com/scd](http://www.liebertpub.com/scd)



**FIG. 5.** Sox2-expressing cells remain in whisker follicles of *Sox2<sup>flox/flox</sup>; Wnt1-Cre* embryos. (A–D) Sox2 (red), Foxd3 (blue), and YFP (green) expression in a control 17.5 dpc whisker pad. Red arrow indicates Sox2 expression outside the NC, yellow arrow indicates Sox2 expression in the NC lineage. Foxd3 is expressed in the NC and co-expressed with Sox2 (magenta arrow) (100 $\times$ ). (E–H) In *Sox2<sup>flox/flox</sup>; Wnt1-Cre* embryos, Sox2 (red) expression is lost in the NC lineage (white arrow). Outside the NC, Sox2 expression is unchanged. Foxd3 (blue) and Sox2 (red) are no longer co-expressed in the NC lineage. Red arrow indicating a cell expressing Sox2, blue arrow indicating a cell expressing Foxd3 (100 $\times$ ). Color images available online at [www.liebertpub.com/scd](http://www.liebertpub.com/scd)

YFP-positive spheres (Fig. 7E). Additionally, *Foxd3* transcripts were detected at levels comparable to littermate controls (Fig. 7G). These data suggest that when Foxd3 is deleted, NC-derived SKPs are not established, yet spheres were generated from an as-yet-undefined non-NC source.

To test the requirement for Foxd3 in the establishment of SKPs independently, SKPs were generated from *Foxd3<sup>flox/flox</sup>; CreER* embryos. A single dose of 0.2  $\mu$ M TM completely abolished the sphere-forming potential of these cells (Fig. 7H, I), while untreated cells and *Foxd3<sup>flox/flox</sup>* cells lacking the inducible Cre transgene treated with 0.2  $\mu$ M TM behaved identically to control cells (Fig. 7G). These results indicate that Foxd3 is required for establishment of SKP cultures.

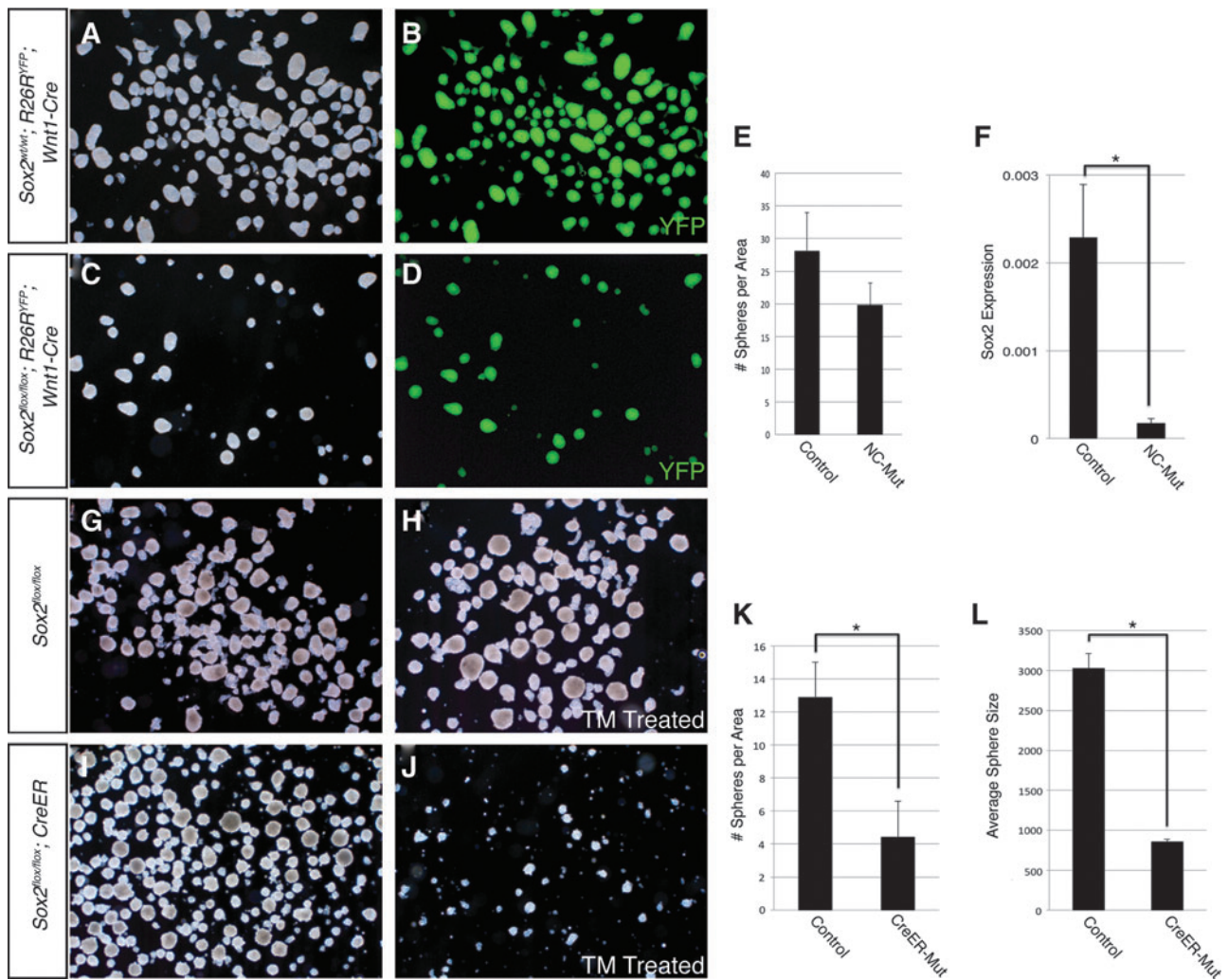
## Discussion

Sox2 and Foxd3 are key regulators of pluripotency and multipotency in several different stem cell populations [24–26]. Our findings suggest these factors function in a similar manner in NCSCs. As with ESCs and TSCs there is an absolute requirement for Foxd3 in the establishment of SKP cell lines. We found that SKP spheres are formed in the absence of Sox2, but at a severely diminished frequency.

Given the robust expression of Sox2 in the endogenous SKP niche, and the apparent absence of Foxd3 in the dermal

papilla, it is somewhat surprising that deletion of Foxd3 from SKPs exhibits a more robust phenotype. Foxd3 is one of the earliest markers of the NC. Expression of Foxd3 is highest in uncommitted NC stem and progenitor cells, and is diminished as cells lose multipotency and differentiate [47,49]. Our observation that Foxd3 expression must be re-acquired in order to establish SKP cultures further confirms its role as a key factor controlling stem cell behavior.

There are a number of possibilities to explain the differences we describe between deletion of Sox2 with the *Wnt1-Cre* versus the *CreER* transgene. Sox2 is a prospective marker of SKP progenitor cells [14], yet in *Sox2<sup>flox/flox</sup>; Wnt1-Cre* embryos, a population of Sox2-expressing cells remains in the whisker follicle. It is currently unknown whether these cells outside the NC are able to contribute to SKP formation. Additionally, SKP spheres are isolated by serial transfer of the floating spheres from the underlying adherent population of cells. However, these adherent cells are always re-established at each passage. The relationship between these support cells and SKPs is not understood and there could be a requirement for Sox2 in the support cells. These cells might not be targeted using the *Wnt1-Cre* and Sox2 might retain functionality in these cultures. The more dramatic phenotype seen with *Sox2<sup>flox/flox</sup>; CreER* SKPs could account for both these possibilities. Alternatively, the difference could be due



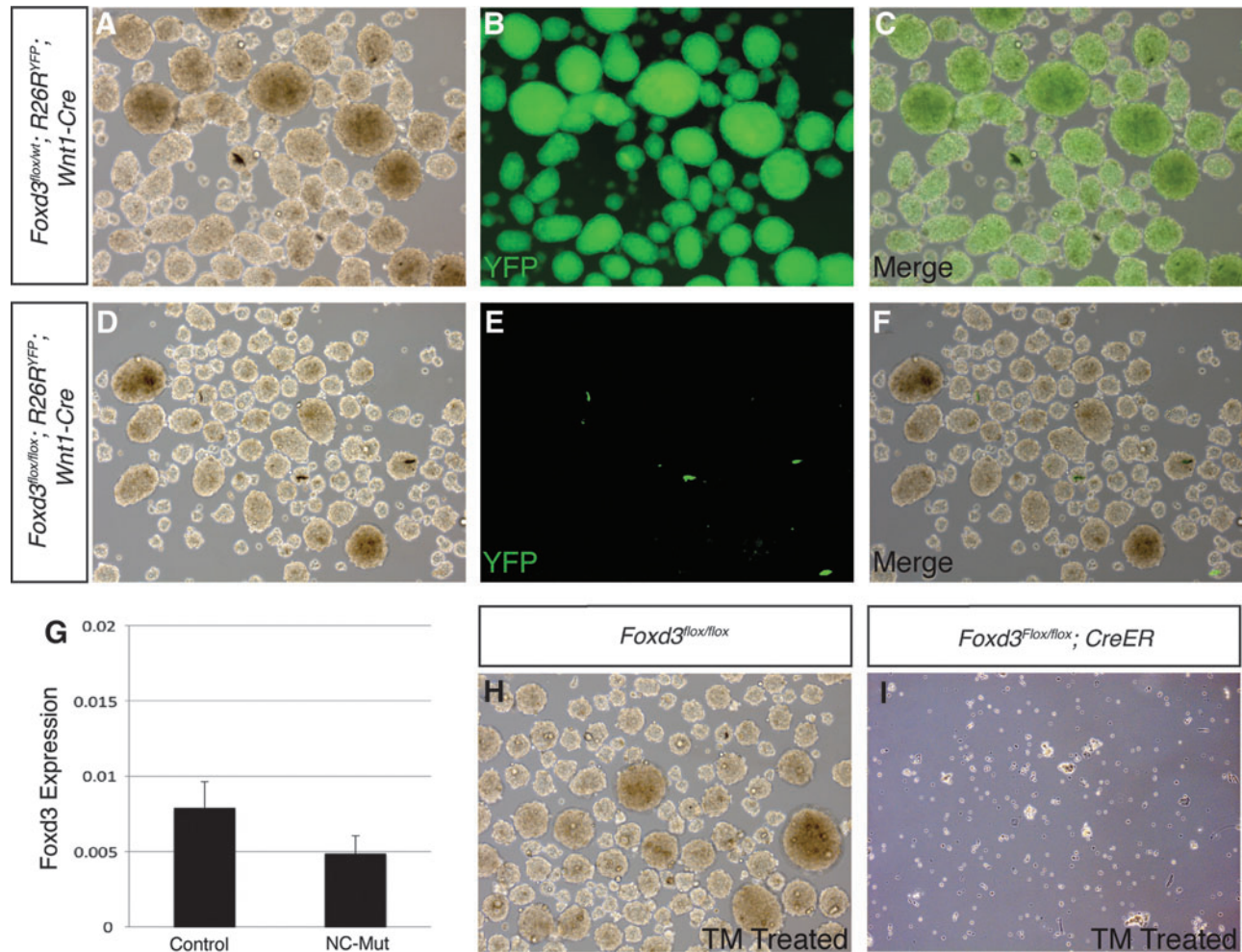
**FIG. 6.** Deletion of Sox2 results in reduced sphere forming potential. (A, B) Bright-field and fluorescent images of SKPs derived from the whisker pads of a 17.5 dpc control embryo (40 $\times$ ). (C, D) SKPs derived from *Sox2<sup>flox/flox</sup>; Wnt1-Cre* embryos are NC-derived and modestly reduced in number (40 $\times$ ). (E) Quantification of the number of spheres formed when Sox2 is deleted with *Wnt1-Cre* ( $P=0.29$ ). (F) Sox2 transcripts were reduced  $\sim 14$ -fold in *Sox2<sup>flox/flox</sup>; Wnt1-Cre* SKPs as compared to littermate controls. This difference is statistically significant ( $*P<0.05$ ). (G–J) Inducible deletion of Sox2. In (F) and (G) a control cell line shows no difference in sphere forming potential when treated with TM. When the cell line carrying the inducible Cre transgene is treated with TM to delete Sox2 we observed a significant reduction in the number of spheres (40 $\times$ ). (K) Quantification of the number of spheres formed when Sox2 is deleted using TM to activate the *CreER* transgene ( $*P<0.05$ ). Data is presented in average number of spheres per area. (L) Quantification of the average sphere area from *Sox2<sup>flox/flox</sup>; CreER* untreated and TM treated cultures. Data are presented in arbitrary units ( $*P<0.05$ ). TM, tamoxifen. Color images available online at [www.liebertpub.com/scd](http://www.liebertpub.com/scd)

to the timing of deletion of Sox2. Sox2 is deleted much earlier in the embryo using the *Wnt1-Cre* as opposed to the *CreER*, and it is possible the SKP precursor cells have sufficient time to compensate for the loss of Sox2 while still in the embryo.

A unique feature of SKPs is that these transcriptionally similar stem cells can be generated from divergent embryonic origins: NC in the facial dermis versus mesoderm in the dorsal skin [43,52]. Recent work demonstrated that mesodermally derived SKPs are able to generate Schwann cells in vitro, a cell type thought to be exclusively derived from the NC [53]. Our findings are consistent with the possibility that cells of mesodermal origin may compensate for the lack of NC precursors in *Foxd3<sup>flox/flox</sup>; Wnt1-Cre* embryos in vivo. Given the size, importance, and highly regenerative nature of

the skin, it is perhaps not surprising that multiple mechanisms exist to generate the multipotent progenitor cell types in the skin.

In ESCs, Sox2 and Foxd3 regulate several shared loci, with Sox2 maintaining DNA in an open chromatin conformation, while Foxd3 actively represses transcription of these shared target genes [27]. This antagonistic relationship is hypothesized to keep target genes in a poised state, able to respond to differentiation cues in a rapid manner. It is currently unknown whether this relationship is conserved in NC progenitors or in SKPs. In fact, little direct regulatory information exists regarding the gene regulatory networks of NC and NCSCs. SKPs provide an abundant cell source for addressing these questions.



**FIG. 7.** Foxd3 is required for establishment of SKPs. (A–C) Bright-field and fluorescent images of SKPs derived from the whisker pads of a 17.5 dpc control embryo. SKPs from control embryos are derived from the NC as demonstrated by the lineage label and the observation that all spheres are fluorescent (100 $\times$ ). (D–F) SKPs derived from the whisker pads of *Foxd3<sup>lox/lox</sup>; Wnt1-Cre* embryos are not derived from the NC, revealing an unknown source of compensation (100 $\times$ ). (G) Foxd3 transcript is still detected and not significantly changed in *Foxd3<sup>lox/lox</sup>; Wnt1-Cre* SKPs. (H–I) Conditional deletion of Foxd3 on day 0 of SKP culture using the TM-inducible *CreER* transgene completely abolished the sphere forming potential of the SKP precursor cells (100 $\times$ ). Untreated cells behaved identically to controls (data not shown). Color images available online at [www.liebertpub.com/scd](http://www.liebertpub.com/scd)

## Conclusion

Being able to effectively control the self-renewal, proliferation, and differentiation of SKPs is key to advancing these cells toward clinical use. Toward this goal, we have demonstrated here that two well-known stem cell proteins, Foxd3 and Sox2, are important regulators of the stem cell properties of SKPs. This study provides the framework to move forward our understanding of the molecular controls of self-renewal and multipotency in a potentially therapeutic stem cell.

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## Author Disclosure Statement

We declare no potential conflicts of interest.

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