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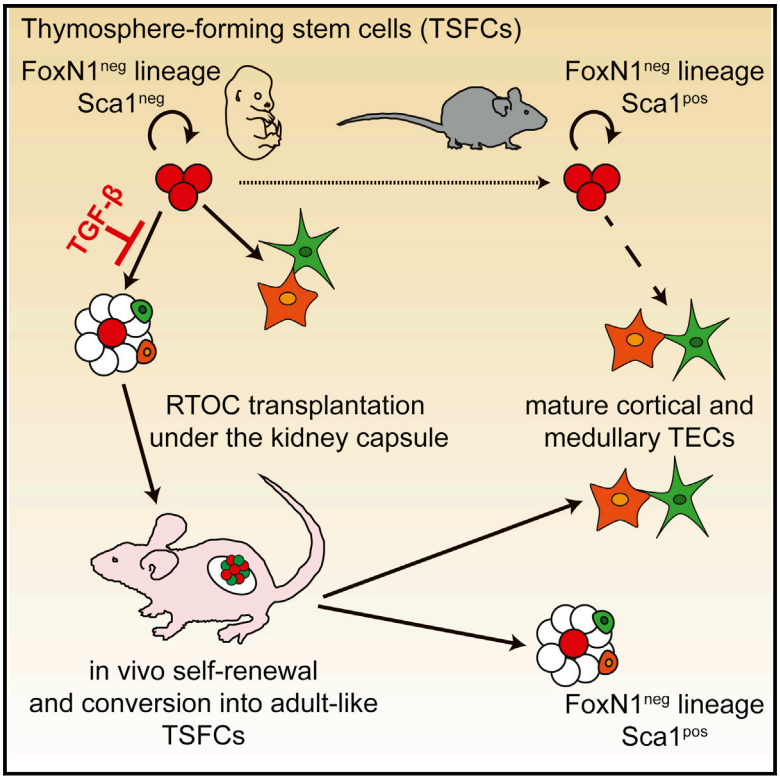
Cell Reports

A Thymic Epithelial Stem Cell Pool Persists throughout Ontogeny and Is Modulated by TGF-β

Graphical Abstract



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In Brief

Ucar et al. address the developmental origins of adult stem cells by using the thymosphere-forming stem cells (TSFCs) of the thymic epithelium. They provide evidence that TSFCs represent a continuous lineage throughout ontogeny and that the fetal and adult stem cell pool might be differentially regulated by TGF-β.

Highlights

- Epithelial thymosphere-forming stem cells (TSFCs) are maintained throughout ontogeny
- Embryonic TSFCs reside in the FoxN1-negative lineage
- Embryonic and adult TSFCs represent a continuous developmental lineage
- Embryonic versus adult TSFCs display differential sensitivity to TGF-β signaling

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A Thymic Epithelial Stem Cell Pool Persists throughout Ontogeny and Is Modulated by TGF-β

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SUMMARY

Adult tissue-specific stem cells (SCs) mediate tissue homeostasis and regeneration and can give rise to all lineages in the corresponding tissue, similar to the early progenitors that generate organs in the first place. However, the developmental origins of adult SCs are largely unknown. We recently identified thymosphere-forming stem cells (TSFCs) in the adult mouse thymus, which display genuine stemness features and can generate the two major thymic epithelial cell lineages. Here, we show that embryonic TSFCs possess stemness features but differ from adult TSFCs in surface marker profile. Our findings support the model of a continuous thymic SC lineage that is maintained throughout ontogeny. TGF-β signaling differentially affects embryonic versus adult thymosphere formation, suggesting that thymic epithelial SC potency depends on both developmental stage and environmental signals. Collectively, our findings suggest that embryonic TSFCs contribute to an adult SC pool and that TSFC plasticity is controlled by TGF-β signaling.

INTRODUCTION

During embryonic development, a handful of potent, fate-committed progenitor cells are responsible for generating the cellular heterogeneity within each organ. After birth, most tissues are left with only a small pool of multipotent stem cells (SCs), which reside at the apex of the tissue hierarchy and determine the rate of organ maintenance, regeneration, and repair. Some tissue-specific SCs support transient periods of extensive proliferation, e.g., hair follicle growth or pregnancy-induced expansion of the mammary epithelium; others, for example, in the intestinal epithelium, bone marrow, or nervous system, mediate continuous tissue homeostasis. Changes in the regenerative potential of adult tissue-specific SCs are likely to contribute to several physiological and pathological processes, such as aging and cancer (Adams et al., 2015).

The developmental origin of adult SCs in most cases is unclear. Although adult tissue-specific SCs retain high potency and even generate mini-organs in vitro, they often have limited functional output during tissue maintenance under physiological conditions in vivo (Donati and Watt, 2015). Moreover, compared to their fetal counterparts, they display differences in potency, division rate, and gene expression profiles (Babovic and Eaves, 2014; Bowie et al., 2007). This raises the question of whether fetal and adult SCs of a particular tissue are related to each other, and if so, which factors might be responsible for the differences in their potency.

The thymus is one of the few organs whose regenerative capacity undergoes a dramatic decline in postnatal life, possibly conducive to a higher incidence of autoimmune disease and higher susceptibility to infection during aging (Chinn et al., 2012). Thymic aging is linked to a decline in thymic epithelial cells (TECs), which represent the major structural component of the thymus and play a pivotal role in T cell development and selection (Anderson and Takahama, 2012). It has been suggested that thymic development and involution are driven by changes in epithelial SC potency; however, lack of SC-restricted markers has limited studies addressing their fetal origins. Our discovery of adult sphere-forming SCs of the thymic epithelium (Ucar et al., 2014) provided an approach to isolate SCs and address the questions of the developmental origin and age-dependent potency of such TEC SCs. Here, we provide evidence that thymosphere-forming stem cells (TSFCs) represent a continuous lineage throughout life, with adult TSFCs being direct descendants of their fetal counterparts. During ontogeny, adult TSFCs change their surface antigen and gene expression patterns and lose their sensitivity to transforming growth factor β (TGF-β). These results suggest a linear relationship between fetal and adult TEC SCs in vivo and show that this SC pool is dynamically regulated by microenvironmental signals in response to tissue needs.

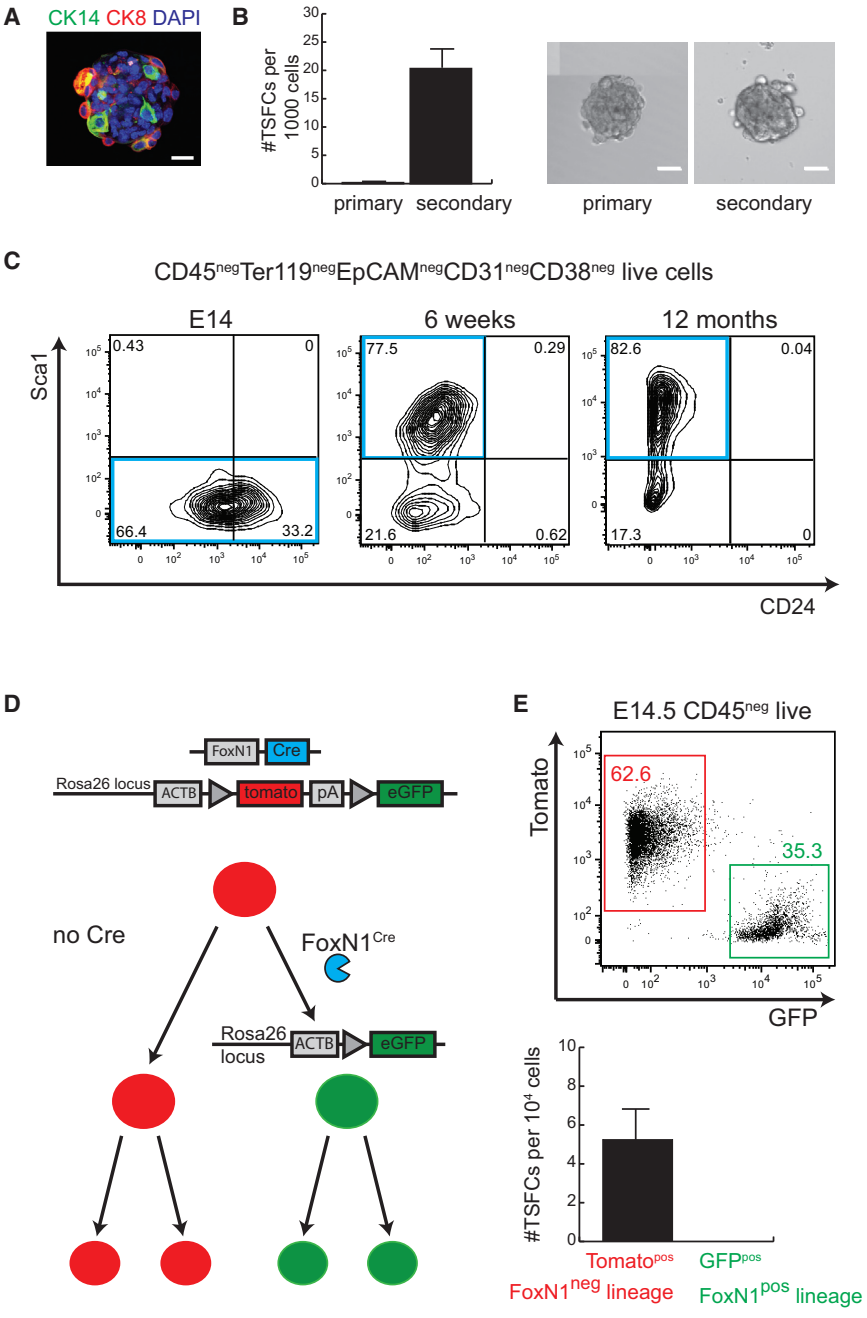


Figure 1. Embryonic TSFCs Display Stemness Features and Change Their Surface Marker Profile during Ontogeny but Remain within the FoxN1^{neg} Lineage

(A) Representative confocal image of a thymosphere derived from E14.5 thymic cells stained for CK8 (cTEC marker) and CK14 (mTEC marker) expression and counterstained with DAPI. Scale bar, 20 μm.

(B) Quantification of the efficiency of E14.5 thymus-derived secondary sphere formation, shown as the number of spheres derived from 10^3 plated cells (mean of two independent experiments ± SD). Typical primary and secondary spheres are shown. Sphere images are cropped areas of whole-well tiled images that were acquired and automatically stitched with Zen software. Scale bar, 40 μm.

(C) Flow cytometry analysis showing the expression of the surface markers Sca-1 and CD24 among CD45^{neg}Ter119^{neg}CD31^{neg}CD38^{neg}EpCAM^{neg} thymic cells derived from different ages (E14.5, young adult, and 12-month-old adult). Blue rectangles indicate subpopulations that contain TSFCs.

(D) Schematic presentation of the FoxN1 lineage-tracing approach.

(E) Sort windows used to purify Tomato^{pos} (FoxN1^{neg} lineage) and GFP^{pos} (FoxN1^{pos} lineage) cells from the embryonic thymi of FoxN1^{Cre} x Rosa26-Tomato-GFP double transgenic E14.5 embryos. Below: quantification of thymosphere formation efficiency of the respective sorted fractions (mean of three independent experiments ± SD).

Data are representative of ≥2 independent experiments. See also Figure S1.

RESULTS

TSFCs Display Stemness Features at E14.5 and Change Their Surface Marker Profile during Ontogeny but Remain FoxN1^{neg}

We have previously shown that TSFCs were present in mouse thymi throughout ontogeny (Ucar et al., 2014). We sought to determine whether embryonic day (E) 14.5 thymus-derived TSFCs display the same stemness features as their adult counterparts. Expression patterns of cytokeratins 8 and 14 (CK8 and CK14, marking cortical and medullary lineage, respectively) on thymospheres derived from E14.5 thymi showed that TSFCs already possess lineage bipotency at E14.5 (Figure 1A). Next, we dissociated primary E14.5 thymus-derived thymospheres and plated the resulting single cells into sphere culture. Similar to adult TSFCs, embryonic TSFCs gave rise to secondary spheres; i.e., they self-renew in culture (Figure 1B).

Adult TSFCs were negative for EpCAM and FoxN1 expression, both of which were upregulated during TEC differentiation (Ucar et al., 2014). To determine whether TSFCs display this immature marker profile characteristic of founder cells throughout development, we analyzed the expression of several surface markers in the TSFCs derived from E14.5 thymi and found that, similar to their adult counterparts, embryonic TSFCs were negative for surface expression of EpCAM and various lineage markers. We identified the surface profile of embryonic TSFCs as CD45^{neg}Ter119^{neg}CD31^{neg}CD38^{neg}EpCAM^{neg} (hereafter referred to as Lin^{neg}). However, embryonic and adult TSFCs differed with respect to Sca1 and CD24 expression. Thus, embryonic TSFCs were Sca1^{neg}CD24$^{neg/pos}$, whereas adult TSFCs

were Sca1^{pos}CD24^{neg} (Figure 1C). We further investigated when the surface profile of TSFCs changed during ontogeny. We found that TSFCs segregated into the Lin^{neg}CD24^{neg}Sca1^{pos} fraction starting 2 weeks postnatally and that they preserved this phenotype at all subsequent ages analyzed (Figure S1). Thus, TSFCs acquire their adult-like surface antigen profile early postnatally and maintain it throughout subsequent life.

To determine whether embryonic TSFCs reside within the FoxN1^{neg} lineage, we used the previously described lineage-tracing mouse model FoxN1^{Cre} x Rosa26-Tomato-GFP, in which FoxN1 promoter-driven Cre expression causes a switch from red to green fluorescent reporter protein expression, marking the FoxN1^{neg} lineage as Tomato^{pos} and the FoxN1^{pos} lineage as GFP^{pos} (Figure 1D). All E14.5 TSFCs resided exclusively in the FoxN1^{neg} lineage (Figure 1E). These results suggest that TSFCs maintain their EpCAM^{neg} and FoxN1^{neg} state throughout ontogeny, arguing that they might represent one continuous lineage.

Embryonic TSFCs Give Rise to Both TEC Lineages In Vivo

Next, we investigated the potential of embryonic TSFCs to give rise to both TEC lineages in vivo upon transplantation. Thymospheres derived from thymi of E14.5 FoxN1^{Cre} x Rosa26-Tomato-GFP double transgenic embryos were mixed with wild-type embryonic stroma into re-aggregate thymic organ cultures (RTOCs; Jenkinson et al., 1992), which were then transplanted under the kidney capsule of nude mice (Figure 2A). We hypothesized that the double transgenic Tomato^{pos} (FoxN1^{neg}) spheres will give rise to the FoxN1^{pos} lineage in the transplant. When we analyzed the transplants 5–8 weeks after transplantation, we found that they contained both Tomato^{pos} and GFP^{pos} cells (Figure 2B; Figure S2A), indicating that embryonic sphere-derived cells subsequently underwent further differentiation in the transplant. Although Tomato^{pos} cells showed rather uniform distribution throughout the transplant, GFP^{pos} cells were often found in clusters of 20–140 cells. These clusters spanned cortical and medullary regions and contained cells expressing either cortical (CK8, CD205) or medullary (UEA1, Aire) markers (Figure 2C). These data demonstrate that embryonic TSFCs, which reside in the FoxN1^{neg} lineage, can give rise to FoxN1^{pos} lineage cells in vivo and that such FoxN1^{pos} progeny differentiate to both the medullary and the cortical TEC lineage.

Fetal TSFCs Can Give Rise to Adult TSFCs upon Transplantation

Our analysis of the transplants showed that although some progeny of embryonic TSFCs upregulated FoxN1, EpCAM, and other markers of TEC terminal differentiation, a proportion of that progeny remained Tomato^{pos}, i.e., within the FoxN1^{neg} lineage. We found that these Tomato^{pos} cells were largely EpCAM^{neg} (Figure 2D) and expressed TEC progenitor markers, such as CK5 and DEC205 (Figure S2B). We hypothesized that this Tomato^{pos} cell population was composed of less differentiated cells, possibly including the original TSFCs (because isolated fetal thymospheres were used for RTOCs, all fluorescent cells in the transplant were derived from fetal TSFCs). To determine whether TSFCs persisted and self-renewed in vivo, we isolated Tomato^{pos} cells from 8-week-old transplants and placed them into sphere culture conditions. The Tomato^{pos} cell fraction of the transplants contained TSFCs, which re-formed thymospheres in culture (Figure 2D). No thymospheres could be derived from GFP^{pos} cells. This result indicated that TSFCs persist and/or self-renew in vivo in the transplant.

Next, we asked whether embryonic TSFCs would adapt an adult-like surface marker profile in the transplant. As mentioned earlier, embryonic TSFCs reside in the Sca1^{neg} cell population, whereas adult TSFCs are part of the Sca1^{pos} cell population. To this end, we analyzed the surface antigen profile of Tomato^{pos} cells derived from the transplants and found that they upregulated Sca1 expression (Figure 2E). These results suggest that TSFCs derived from E14.5 thymi that were spiked into RTOCs gave rise to TSFCs with an adult-like surface marker profile 8 weeks post transplantation. EpCAM^{pos} cells also changed their surface expression of Sca1 and CD24 during ontogenic progression. Thus, although at E14.5, most EpCAM^{pos} cells are Sca1^{neg}CD24^{pos}; in the young adult thymus, most of them are Sca1^{pos}CD24^{pos} (Figure 2E). EpCAM^{pos} cells generated by embryonic thymospheres within the transplant also displayed adult-like surface antigen profile. Altogether, these data show that the development of different subpopulations of TECs in the transplanted RTOC followed a physiological pattern. These results indicate that adult TSFCs recovered from transplants are descendants of their transferred embryonic counterparts.

Fetal and Adult Thymus-Derived Thymospheres Differ in Their Gene Expression Profiles

The phenotypic changes that TSFCs undergo during ontogeny are likely to reflect changes in gene expression patterns within thymospheres. We therefore isolated total RNA from pooled thymospheres derived from E14.5 or adult thymic digests and analyzed their transcriptome by whole-genome microarrays. Unsupervised filtering of all genes according to the magnitude of regulation (SD over all four samples) clearly revealed different patterns of gene regulation between fetal and adult thymus-derived thymospheres (Figure 3A; Table S1). Several thymic epithelial markers, including those associated with progenitor cells, were differentially expressed in embryonic versus adult thymospheres. We confirmed these results for a selected set of genes by qPCR (Figure 3B). Furthermore, gene ontology analysis revealed that pathways related to major key biological processes displayed changes between these two stages of ontogeny. We found significant differences in pathways involved in cell proliferation, development and differentiation, cell metabolism, regulation of transcription, and regulation of DNA repair (Figure S3). Most interestingly, we observed significant enrichment of gene expression in several signaling pathways (Figure 3C); in particular, many members of the TGF-β signaling pathway were upregulated in E14.5 versus adult thymospheres (Figures 3C and 3D). These data show that fetal and adult SCs and progenitor cells display different transcriptomes and suggest that the TGF-β signaling pathway is upregulated in the fetal thymospheres.

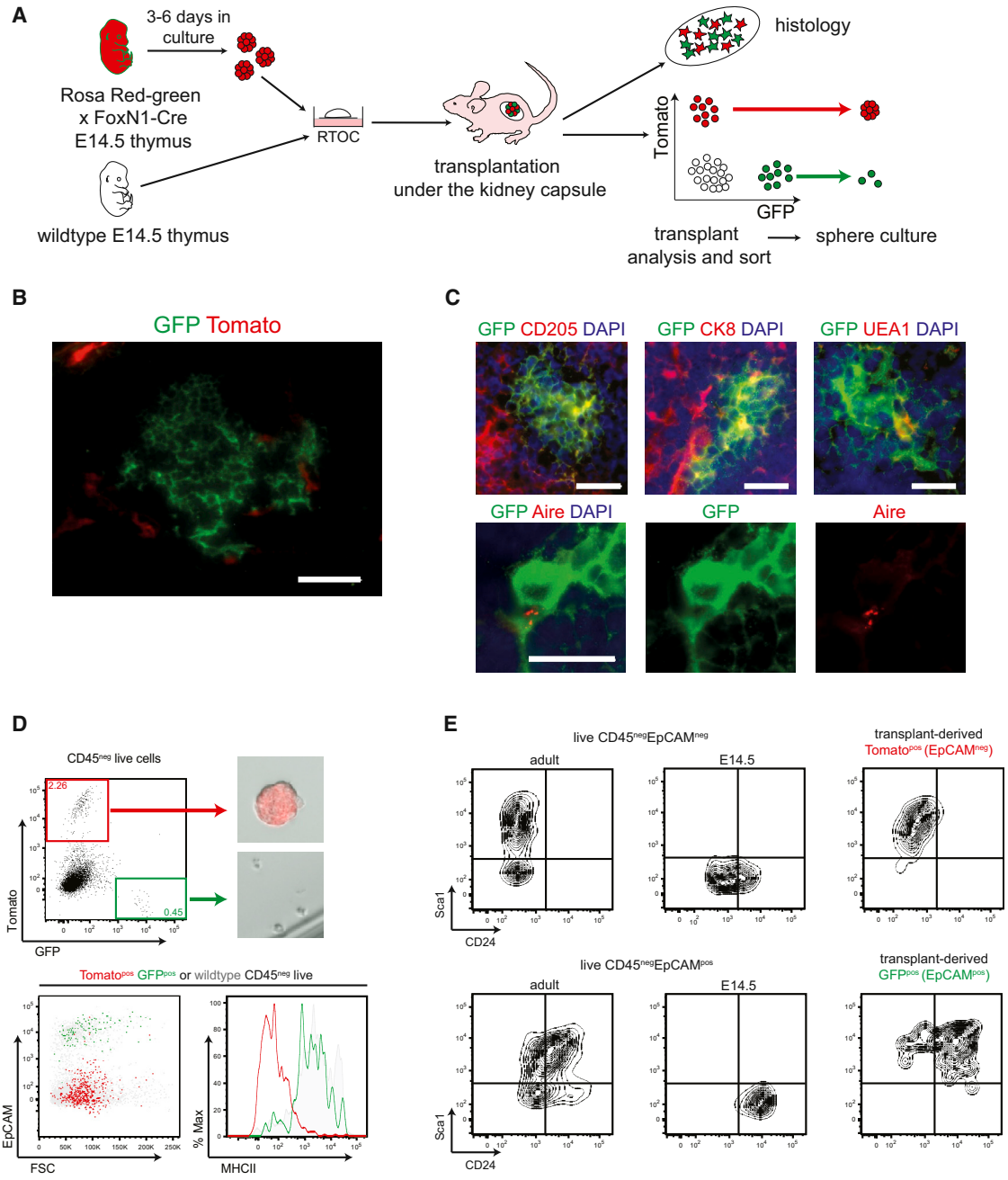


Figure 2. Embryonic TSFCs Give Rise to Both TEC Lineages In Vivo and Generate Adult TSFCs

(A) Schematic presentation of the transplantation experiment and transplant analysis: thymospheres derived from thymic digests of E14.5 FoxN1^{Cre} x Rosa26-Tomato-GFP double transgenic embryos were re-aggregated with wild-type E14.5 thymic stromal cells and transplanted under the kidney capsule of recipient nude mice. Then, 5–8 weeks post-transplantation, transplants were either processed for histology or digested into single cells. In the latter case, Tomato^{pos} and GFP^{pos} cells were sorted and plated in thymosphere culture.

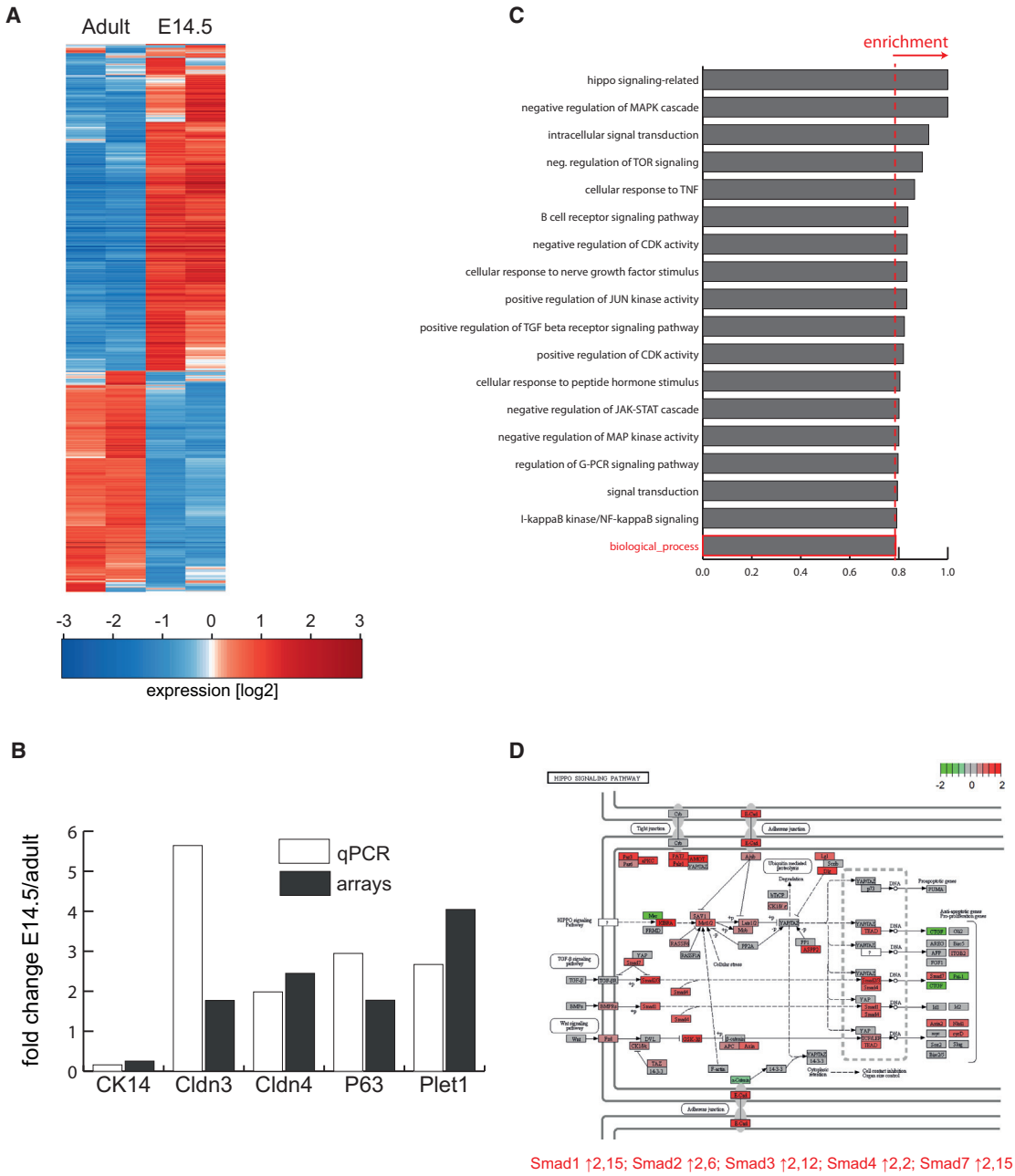
(B) Histological analysis of Tomato^{pos} and GFP^{pos} cell distribution in the transplant. Scale bar, 50 μm.

(C) Histological analyses of transplanted RTOCs containing FoxN1^{Cre} x Rosa26-Tomato-GFP thymospheres 5 weeks post-transplantation. Sections of transplants were co-stained for GFP and CD205, CK8, UEA1, or Aire. Scale bars, 20 μm.

(D) Thymosphere-forming capacity and surface marker profiles of Tomato^{pos} and GFP^{pos} cells derived from the transplants. Red and green rectangles correspond to gates used for sorting cells for sphere culture. Only Tomato^{pos} cells were able to form thymospheres. Images represent cropped areas of whole-well tiled images that were acquired and automatically stitched with Zen software.

(E) Surface marker profiles (Sca-1 and CD24) of Tomato^{pos}EpCAM^{neg} and GFP^{pos}EpCAM^{pos} cells derived from the transplants, compared to the corresponding profiles of embryonic and adult wild-type thymi.

Data are representative of ≥2 independent experiments. See also Figure S2.



Smad1 ↑2,15; Smad2 ↑2,6; Smad3 ↑2,12; Smad4 ↑2,2; Smad7 ↑2,15

Figure 3. Fetal and Adult Thymus-Derived Thymospheres Display Major Differences in Gene Expression

(A) Comparison of gene expression profiles of embryonic versus adult thymus-derived thymospheres. Unsupervised filtering of all genes according to the magnitude of regulation (SD over all four samples) revealed differential gene expression between embryonic and adult thymus-derived thymospheres. The top differentially regulated probes, i.e., 10,000 of 55,821 genes, or around 18% of genes of two biological replicates, are displayed.

(B) Differential expression of TEC markers in E14.5 versus adult thymospheres, as assessed by qPCR and arrays.

(C) Top signaling-related gene sets that displayed enriched regulation between embryonic and adult thymus-derived thymospheres. Bars show the estimated fraction of regulated genes for each gene set. The biological_process gene set at the bottom represents the average regulation over all processes and has been included as a reference; the red line denotes the cutoff based on this set. The top regulated gene set, hippo signaling-related, also comprises the TGF-β pathway.

(D) Map of the top regulated gene set (hippo signaling-related; mmu04390). Upregulation in embryonic thymospheres is displayed in red (log_2 scale). Fold changes of the members of TGF-β signaling pathway are displayed below.

See also Figure S3 and Table S1.

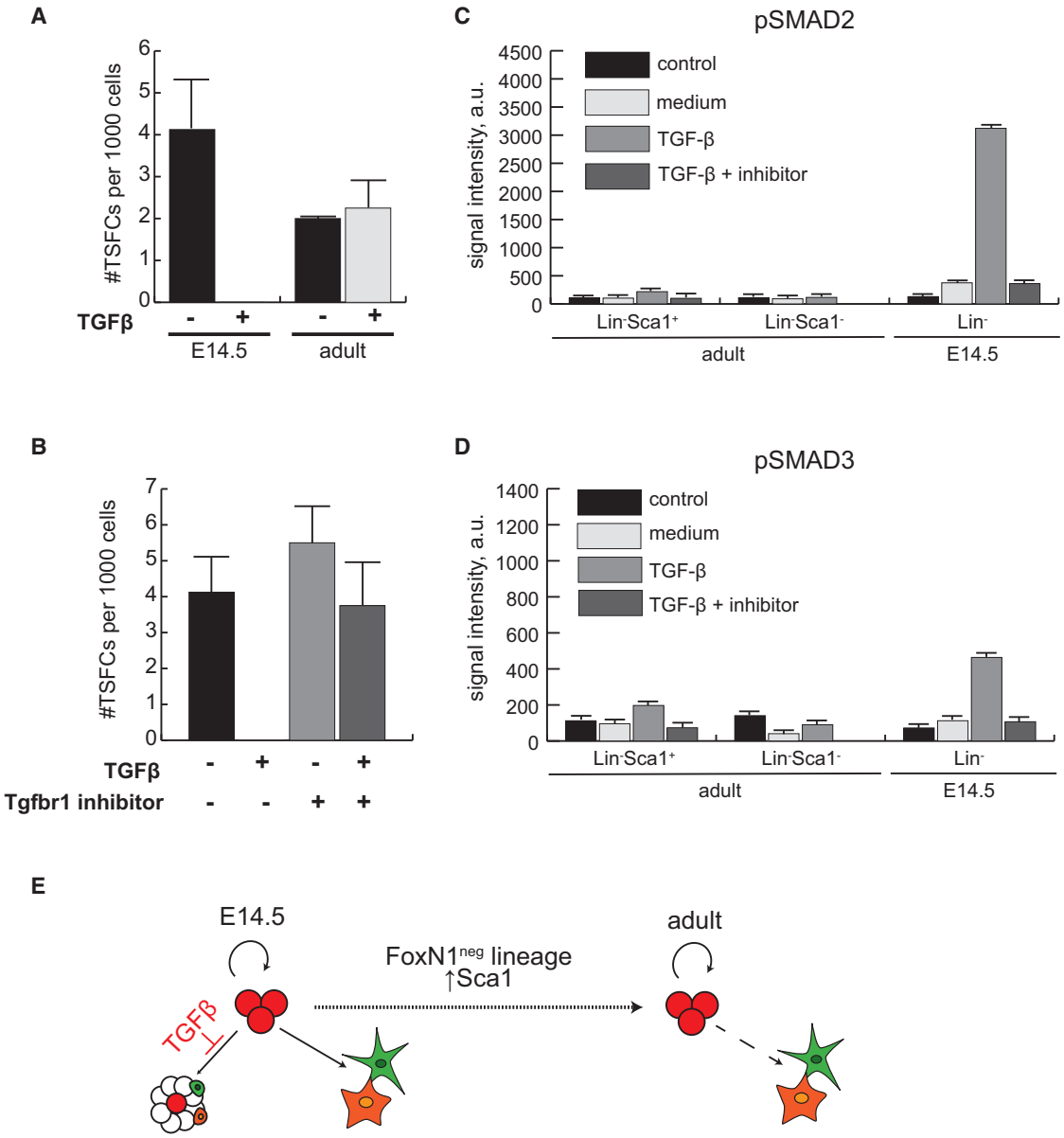


Figure 4. Embryonic and Adult TSFCs and Thymic Cell Populations Display Differential Sensitivity to TGF-β

(A) Embryonic thymosphere formation was inhibited by addition of TGF-β to the culture medium, whereas adult thymosphere formation was not affected.

(B) TGF-β-mediated inhibition of E14.5 thymosphere growth was rescued by the addition of the Tgfbr1 inhibitor LY364947.

(C and D) Adult and E14.5 thymic cell populations exhibited different sensitivity to TGF-β, as measured by SMAD2 (C) and SMAD3 (D) phosphorylation via a Multiplex bead-based assay (a.u.). The effect of TGF-β on the pSMAD2/3 level was abrogated by pre-treatment with the Tgfbr1 inhibitor. Data are shown as mean of n = 3 independent experiments ± SD.

(E) Model of TSFC maintenance during ontogeny.

See also Figure S4.

Responsiveness of Thymic SCs to TGF-β Declines during Ontogeny

TGF-β has been implicated in regulation of TEC development and maintenance (Hauri-Hohl et al., 2014), and it is known to regulate cell growth, proliferation, and SC dormancy in other tissues (Massagué, 2012). Given the differential gene expression of TGF-β signaling components in fetal versus adult thymospheres, we asked whether TGF-β might regulate thymic epithelial SC activity. To this end, we treated TSFCs with TGF-β in thymosphere culture. Surprisingly, TGF-β abrogated the formation of thymospheres derived from E14.5 but not adult thymic digests (Figure 4A). In contrast to TGF-β, activation of the Wnt or BMP4 signaling pathway in culture affected neither fetal nor adult thymosphere formation (Figure S4).

The effect of TGF-β was TGF-β receptor 1 (Tgfbr1) dependent, because addition of the Tgfbr1-specific inhibitor LY364947 to the cultures rescued the TGF-β-mediated inhibition of embryonic thymosphere growth (Figure 4B). According to the gene expression profiling results, increased responsiveness to TGF-β in embryonic thymosphere formation could not be explained by higher TGF-β receptor expression. However, we observed increased levels of SMAD2, SMAD3, and SMAD4, the key effectors of the TGF-β pathway, in the E14.5 thymospheres (Figure 3D). We thus analyzed TGF-β-mediated SMAD2/3 phosphorylation in TSFC-enriched cell populations isolated from E14.5 and adult thymi. Because SMAD2/3 phosphorylation is dynamically regulated and thus might be lost during the hours-long process of tissue digestion and cell sorting, the analysis was performed on freshly sorted cells, as well as sorted cells treated with TGF-β for 1 hr. Although various embryonic and adult thymic cell populations exhibited comparable background levels of pSMAD2/3, TGF-β stimulation elicited a much higher increase in Tgfbr1-mediated SMAD2/3 phosphorylation in the embryonic cells (Figures 4C and 4D). These data suggest that the negative effect of TGF-β on embryonic thymosphere formation can be due to a relatively higher sensitivity of fetal TSFCs compared to adult TSFCs.

Altogether, our results imply that TGF-β signaling is used as a negative feedback during thymic development to control SC activity (Figure 4E).

DISCUSSION

Determining the relationship between embryonic progenitors that generate organs in toto and corresponding adult SCs responsible for tissue homeostasis and regeneration could lead to a better understanding of the mechanisms underlying the limited regenerative capacity of adult tissues and by implication of the aging process. This issue has been addressed for hematopoietic SCs, where changes in biological potency and gene expression profiles during ontogeny have been well documented (Babovic and Eaves, 2014). Studies of adult SCs in solid tissues rely on different methodologies, making it more difficult to unveil their origin in the embryo (Morrison and Spradling, 2008). Although the developmental origins of adult tissue-specific SCs of most solid tissues remain obscure, they are widely believed to be generated during organ development. A study lends support to this notion by demonstrating that fetal and adult neural SCs have a common origin in early embryonic development (Fuentealba et al., 2015). Our results suggest that the same also holds true for the thymic epithelium, where a sphere-forming population of adult SCs can be traced back to their embryonic counterparts. However, given the possibility that the embryonic TSFC population is heterogeneous, we cannot exclude that different sublineages may be dedicated to either thymic organogenesis or conversion into adult TSFCs.

Studies of adult SCs and their origins in the context of the thymic epithelium have been hampered by the lack of SC-specific markers or assays allowing the delineation of SCs from progenitor cells. Several candidate SC and progenitor cell populations have been described, yet the precise relationship between them remains poorly understood (Hamazaki, 2015; Lopes et al., 2015). The search for exclusive TEC SC markers is ongoing; however, it has been reported that expression or certain lineage markers identify progenitor cells in the embryo, as well as in the adult. A study of medullary TEC (mTEC)-committed SCs documented that SSEA-1^{pos}Cldn3,4^{high} cells maintain the thymic medulla throughout ontogeny (Sekai et al., 2014). Embryonic progenitors derived from a β5t^{pos} lineage have been shown to give rise to both mTEC and cortical TEC (cTEC) lineages during embryonic development and in the adult thymus (Mayer et al., 2015; Ohigashi et al., 2015). Another study demonstrated that Plet1^{pos} cells, which are abundantly present in the embryonic thymus, persist throughout ontogeny, retain their bipotency, and may have stemness features (Ulyanchenko et al., 2016). Here, we used thymosphere formation assay for SC isolation and ontogeny analysis, based on our demonstration that TSFCs display the stemness features of bipotency and self-renewability in the adult state (Ucar et al., 2014) and fetal state (Figures 1A and 1B). Given the lack of a complete marker profile of TSFCs, the extent to which these cells overlap with or are contained within the aforementioned SC candidates remains unclear. Although our findings only pertain to this specific thymic SC population, they are in accordance with previous observations and provide independent experimental evidence of the early separation of the SC lineage in the thymus.

We showed that the E14.5 thymus-derived TSFCs self-renew in vivo and can give rise to adult-like TSFCs upon transplantation. In addition, the progenitors generated from fetal TSFCs within transplants can differentiate to lineage-committed TECs that display adult-like surface antigen profiles. These observations are in line with studies demonstrating that maintenance and repair of the adult thymus in vivo is largely mediated by progenitors generated during embryonic and early postnatal development (Mayer et al., 2015; Ohigashi et al., 2015). Because adult TEC SCs and progenitor cells are capable of generating mature progeny upon transplantation (Ucar et al., 2014; Ulyanchenko et al., 2016; Wong et al., 2014), they likely possess some regenerative potency in vivo. The extent of their natural contribution to steady-state tissue maintenance remains to be determined.

A decline in SC potency during ontogeny may be linked to changes in SC-intrinsic (e.g., gene expression patterns) and/or SC-extrinsic (microenvironment) properties. Our results show that fetal and adult thymic epithelial SCs and progenitor cells display profound differences in gene expression profiles, most strikingly in pathway components related to TGF-β signaling. TGF-β has been implicated as a negative regulator of thymic epithelial development and maintenance (Hauri-Hohl et al., 2008, 2014); however, the exact cellular targets and the time frame of TGF-β action had not been determined. Our data suggest that TGF-β acts predominantly during the embryonic development of the thymus by modulating the activity of fetal TSFCs, whereas adult TSFCs and their immediate progeny remain largely insensitive to TGF-β. As a potent regulator of cell-cycle progression, growth, and differentiation, TGF-β is known to modulate the behavior of SCs by shifting the balance between quiescence and proliferation (Sakaki-Yumoto et al., 2013); furthermore, different SC subsets of the same tissue vary in sensitivity and even show opposite cellular responses (increased proliferation versus induced quiescence)

upon TGF-β administration (Blank and Karlsson, 2015). In the CNS, TGF-β signaling has been shown to control organ size by exerting a negative feedback on neural SCs in the embryonic brain (Falk et al., 2008). Our findings argue that a similar feedback mechanism might be at play in the developing thymus, where the activity of embryonic TEC SCs was inhibited by high concentrations of TGF-β. TGF-β signaling may thus form part of a negative feedback loop during early thymic development to control SC activity (Figure 4E).

Altogether, our results are consistent with direct relatedness between the prenatal and the postnatal TEC SC pool and highlight intriguing changes that these cells may undergo during ontogeny, including cell surface marker expression, gene expression profile, and sensitivity to extrinsic factors. In this regard, it will be important to precisely assess to which extent fetal and adult TSFCs differ functionally, i.e., in thymus organogenesis versus maintenance or reconstitution.

Taking into account that SC isolation using the thymosphere assay may omit any possible non-sphere-forming SC pool or pools, our current findings only apply to TSFCs. Nevertheless, they fully agree with previous studies on SCs in other tissues. Future studies of other candidate TEC SC and progenitor cell populations will expand our knowledge of the precise cellular and molecular mechanisms regulating development and aging of the thymic epithelium. With respect to the poor regenerative ability of the adult thymus and its possible consequences on the maintenance of intact immunity, our results might aid the design of novel approaches to boost adult and aged thymic epithelial SCs and thus restore overall thymic function.

EXPERIMENTAL PROCEDURES

Experimental Animals

When not indicated otherwise, E14.5 embryos and 4- to 6-week-old wild-type C57BL/6N mice were used for the isolation of thymic stromal cells. E14.5 embryos were obtained from time-mated C57BL/6N females, with the morning of vaginal plug detection taken as E0.5. NMRI *nude/nude* 6-week-old females (Janvier) were used as hosts in RTOC transplantation experiments. FoxN1^{Cre} mice were kindly provided by T. Boehm of the Max Planck Institute (MP; B6-Tg(Foxn1-cre)1Tbo; Soza-Ried et al., 2008). Rosa-Tomato-GFP mice (B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J; Muzumdar et al., 2007) were kindly provided by R. Sandhoff of the German Cancer Research Center (DKFZ). All animal experiments were approved by the regional authorities (Regierungspräsidium Karlsruhe, #35-9185.81/G-23/14) and performed according to the guidelines of the DKFZ.

Thymic Stromal Cell Isolation and Sorting

Thymic stromal cell isolation from adult animals was performed as previously described (Ucar et al., 2014). Embryonic thymi were digested with collagenase or dispase for 30 min at 37°C. Postnatal thymic digests were depleted of CD45$^+$ cells using CD45 MicroBeads (Miltenyi Biotec). Cell sorting and subsequent analysis were performed on a BD FACSAria cell sorter and FlowJo (Tree Star), respectively. Live imaging of the thymosphere cultures was performed using a motorized inverted Cell Observer Z1 (Zeiss), and image analyses for the quantification of thymospheres were done using Zen software (Zeiss). For counting of thymospheres, we applied a diameter cutoff with a minimum of 50 × 50 μm for two perpendicular axes.

Sphere Culture

Spheres were grown as previously described (Ucar et al., 2014). For TGF-β treatments, sphere medium was supplemented with human recombinant TGF-β (Sigma; final concentration [f.c.] 2.5 ng/mL) and/or LY364947 (Tgfbr1 inhibitor, System Biosciences; f.c. 2.5 μM). Wnt signaling activation in sphere culture was performed by addition of (2'Z,3'E)-6-bromoindirubin-3′-oxime (B.I.O.), a potent GSK3β inhibitor (Cayman Chemical, f.c. 1 μM), to sphere culture medium. BMP4 signaling activation was performed by addition of recombinant BMP4 (Sigma; f.c. 40 ng/mL) to sphere culture medium. Secondary sphere formation assay and whole-mount staining of spheres were performed as described previously (Ucar et al., 2014).

RTOC and Transplantation

Thymic stroma re-aggregates were prepared from fetal thymic stromal cells as previously described (Jenkinson et al., 1992). Briefly, thymi derived from E14.5 C57BL/6N embryos were digested with trypsin-EDTA solution for 5 min at 37°C and mixed with highly enriched thymospheres derived from the Rosa-Tomato-GFP x FoxN1^{Cre} reporter mouse line. The re-aggregates were deposited on a 0.8 μm nucleopore filter (Whatman) placed on a floating sponge in a medium-filled six-well plate. For long-term reconstitution assays, RTOCs were grafted under the kidney capsule of adult NMRI *nude* mice after 1 day of culture (Rodewald et al., 2001; Gill et al., 2002). The transplants were analyzed after 5 and 8 weeks by histology or flow cytometry.

Total Sphere RNA Isolation and Microarray Analysis

Spheres derived from E14.5 and adult primary thymic digests were collected after 7 days in culture, size-selected on 35 μm filters, and lysed in TRIzol reagent (Life Technologies). RNA extraction was performed using the TRIzol/chloroform method; absolute ethanol was used for precipitation at −20°C, and then the RNA pellet was washed twice with 80% ethanol and dissolved in H_2O. RNA concentration and integrity were analyzed by a Eukaryote Total RNA pico Series II chip on a 2100 Bioanalyzer (Agilent). Then, 25 ng of total RNA was used for cDNA synthesis using an Agilent Low Input Quick Amp Labeling Kit.

Expression profiling was performed using Agilent SurePrint G3 Mouse Gene Expression Microarrays according to the manufacturer's instructions. Arrays were scanned with Agilent Scanner G2505C, and the intensity data were imported using Agilent Feature Extraction Software v.10.7.3.1. Background adjustment and quantile normalization were performed with BeadStudio software. All sample expression values are mean values over beads, and group expression values are mean values over the replicates' expression.

qRT-PCR

First-strand synthesis was performed with SuperScriptII and oligo-dT primer. Then, qPCR was performed in triplicate using the Applied Biosystems 7300 light cycler and intron-spanning primers; the results were normalized to housekeeping gene expression levels (β-actin) and analyzed by the ddCt method.

Protein Isolation and Multiplex Bead-Based Assay Analysis

E14.5 and adult primary cells were sorted into 1.5 mL tubes and either lysed directly (control sample) or incubated for 2 hr in modified sphere culture medium (without insulin, epidermal growth factor [EGF], or basic fibroblast growth factor [bFGF]) in the CO_2 incubator before protein isolation. The cells were untreated (medium sample), treated with TGF-β (Sigma; f.c. 1 ng/mL) for the second hour of the incubation (TGF-β sample), or treated with TGF-β for the second hour of the incubation with the pre-addition of the Tgfbr1 inhibitor (LY364947, System Biosciences; f.c. 2.5 μM) 15 min before addition of TGF-β (TGF-β + inhibitor sample).

Whole-cell protein lysates were prepared with 1 × radioimmunoprecipitation assay (RIPA) buffer (see Supplemental Experimental Procedures). Equal amounts of protein from each cell population were analyzed by using the TGF-β Signaling Milliplex kit (Millipore, #48-614MAG) according to the manufacturer's instructions.

Statistical Analysis

Mean and SD were calculated, and Student's t test was used to determine statistically significant differences. For microarray data analysis, t statistics and the 95% confidence interval were used (see the next section for further details).

Microarray Data Analysis

The db2 db and dbWalk identifier conversion tools of the http://biodbnet-abcc.ncifcrf.gov/ database (Mudunuri et al., 2009) were used to convert GenBank accession identifiers (IDs; nucleotide) to Gene Ontology and Kyoto encyclopedia of genes and genomes (KEGG) pathway annotation (KEGG ID and KEGG titles). Because db2 db is only applicable to a limited number of IDs, the optimal conversion paths GenBank Nucleotide Accession → Gene ID → GO ID and GenBank Nucleotide Accession → Gene ID → KEGG Pathway ID as determined by db2 db were used for dbWalk to convert the GenBank accessions from the whole array. The results were downloaded in the Excel format and mapped in MATLAB to the processed data.

A linear model with Intercept and an additional parameter for the fold change between embryonic (E) and adult (A) was applied to estimate regulation (E-A) from the duplicates. Homoscedastic variances for each gene were assumed, and significance was tested based on the t statistics.

Gene set enrichment analysis was performed using the gene set regulation index (GSRI; Bartholomé et al., 2009), which estimates the fraction of regulated genes for a specific gene set.

For displaying significant regulation in the pathway context, the Pathview package (v.1.4.2; Luo and Brouwer, 2013) was applied using the R statistical programming environment (v.3.1.2; http://www.R-project.org/). As a compromise between showing the measured gene regulation for all compounds and filtering non-significant regulation, a 95% confidence level was chosen; i.e., all fold changes with $p < 0.05$ were shown. If several probes on the array mapped to the same compound, the mean of the estimated fold changes was plotted. This pathway analysis was performed for all KEGG pathways (Kanehisa and Goto, 2000), which were annotated in the Signal_transduction or Cell_growth_and_death ontology branches.

ACCESSION NUMBERS

The accession number for the microarray data reported in this paper is GEO: GSE86267.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.027.

AUTHOR CONTRIBUTIONS

O.U., K.L., D.D., S.M., L.B., and C.S. designed and performed the experiments. C.K. and J.T. designed and performed microarray analysis and pathway analysis. O.U., K.L., D.D., S.M., M.A.T., T.G.H., U.K., and B.K. analyzed and interpreted results. O.U. and B.K. directed the study and wrote the manuscript, and all authors read and approved the manuscript. O.U. and B.K. are jointly responsible for all data, figures, and text and for adhering to all editorial and submission policies and good publication practice.

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REFERENCES

Adams, P.D., Jasper, H., and Rudolph, K.L. (2015). Aging-induced stem cell mutations as drivers for disease and cancer. Cell Stem Cell *16*, 601–612.

Anderson, G., and Takahama, Y. (2012). Thymic epithelial cells: working class heroes for T cell development and repertoire selection. Trends Immunol. *33*, 256–263.

Babovic, S., and Eaves, C.J. (2014). Hierarchical organization of fetal and adult hematopoietic stem cells. Exp. Cell Res. *329*, 185–191.

Bartholomé, K., Kreutz, C., and Timmer, J. (2009). Estimation of gene induction enables a relevance-based ranking of gene sets. J. Comput. Biol. *16*, 959–967.

Blank, U., and Karlsson, S. (2015). TGF-β signaling in the control of hematopoietic stem cells. Blood *125*, 3542–3550.

Bowie, M.B., Kent, D.G., Dykstra, B., McKnight, K.D., McCaffrey, L., Hoodless, P.A., and Eaves, C.J. (2007). Identification of a new intrinsically timed developmental checkpoint that reprograms key hematopoietic stem cell properties. Proc. Natl. Acad. Sci. USA *104*, 5878–5882.

Chinn, I.K., Blackburn, C.C., Manley, N.R., and Sempowski, G.D. (2012). Changes in primary lymphoid organs with aging. Semin. Immunol. *24*, 309–320.

Donati, G., and Watt, F.M. (2015). Stem cell heterogeneity and plasticity in epithelia. Cell Stem Cell *16*, 465–476.

Falk, S., Wurdak, H., Ittner, L.M., Ille, F., Sumara, G., Schmid, M.T., Draganova, K., Lang, K.S., Paratore, C., Leveen, P., et al. (2008). Brain area-specific effect of TGF-beta signaling on Wnt-dependent neural stem cell expansion. Cell Stem Cell *2*, 472–483.

Fuentealba, L.C., Rompani, S.B., Parraguez, J.I., Obernier, K., Romero, R., Cepko, C.L., and Alvarez-Buylla, A. (2015). Embryonic origin of postnatal neural stem cells. Cell *161*, 1644–1655.

Gill, J., Malin, M., Hollander, G.A., and Boyd, R. (2002). Generation of a complete thymic microenvironment by MTS24(+) thymic epithelial cells. Nat. Immunol. *3*, 635–642.

Hamazaki, Y. (2015). Adult thymic epithelial cell (TEC) progenitors and TEC stem cells: models and mechanisms for TEC development and maintenance. Eur. J. Immunol. *45*, 2985–2993.

Hauri-Hohl, M.M., Zuklys, S., Keller, M.P., Jeker, L.T., Barthlott, T., Moon, A.M., Roes, J., and Holländer, G.A. (2008). TGF-beta signaling in thymic epithelial cells regulates thymic involution and postirradiation reconstitution. Blood *112*, 626–634.

Hauri-Hohl, M., Zuklys, S., Holländer, G.A., and Ziegler, S.F. (2014). A regulatory role for TGF-β signaling in the establishment and function of the thymic medulla. Nat. Immunol. *15*, 554–561.

Jenkinson, E.J., Anderson, G., and Owen, J.J. (1992). Studies on T cell maturation on defined thymic stromal cell populations in vitro. J. Exp. Med. *176*, 845–853.

Kanehisa, M., and Goto, S. (2000). KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res. *28*, 27–30.

Lopes, N., Sergé, A., Ferrier, P., and Irla, M. (2015). Thymic crosstalk coordinates medulla organization and T-cell tolerance induction. Front. Immunol. *6*, 365.

Luo, W., and Brouwer, C. (2013). Pathview: an R/Bioconductor package for pathway-based data integration and visualization. Bioinformatics *29*, 1830–1831.

Massagué, J. (2012). TGFβ signalling in context. Nat. Rev. Mol. Cell Biol. *13*, 616–630.

Mayer, C.E., Žuklys, S., Zhanybekova, S., Ohigashi, I., Teh, H.Y., Sansom, S.N., Shikama-Dorn, N., Hafen, K., Macaulay, I.C., Deadman,

M.E., et al. (2015). Dynamic spatio-temporal contribution of single β5t+ cortical epithelial precursors to the thymus medulla. Eur. J. Immunol. *46*, 846–856.

Morrison, S.J., and Spradling, A.C. (2008). Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell *132*, 598–611.

Mudunuri, U., Che, A., Yi, M., and Stephens, R.M. (2009). bioDBnet: the biological database network. Bioinformatics *25*, 555–556.

Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. Genesis *45*, 593–605.

Ohigashi, I., Zuklys, S., Sakata, M., Mayer, C.E., Hamazaki, Y., Minato, N., Hollander, G.A., and Takahama, Y. (2015). Adult thymic medullary epithelium is maintained and regenerated by lineage-restricted cells rather than bipotent progenitors. Cell Rep. *13*, 1432–1443.

Rodewald, H.R., Paul, S., Haller, C., Bluethmann, H., and Blum, C. (2001). Thymus medulla consisting of epithelial islets each derived from a single progenitor. Nature *414*, 763–768.

Sakaki-Yumoto, M., Katsuno, Y., and Derynck, R. (2013). TGF-β family signaling in stem cells. Biochim. Biophys. Acta *1830*, 2280–2296.

Sekai, M., Hamazaki, Y., and Minato, N. (2014). Medullary thymic epithelial stem cells maintain a functional thymus to ensure lifelong central T cell tolerance. Immunity *41*, 753–761.

Soza-Ried, C., Bleul, C.C., Schorpp, M., and Boehm, T. (2008). Maintenance of thymic epithelial phenotype requires extrinsic signals in mouse and zebrafish. J. Immunol. *181*, 5272–5277.

Ucar, A., Ucar, O., Klug, P., Matt, S., Brunk, F., Hofmann, T.G., and Kyewski, B. (2014). Adult thymus contains FoxN1(-) epithelial stem cells that are bipotent for medullary and cortical thymic epithelial lineages. Immunity *41*, 257–269.

Ulyanchenko, S., O'Neill, K.E., Medley, T., Farley, A.M., Vaidya, H.J., Cook, A.M., Blair, N.F., and Blackburn, C.C. (2016). Identification of a bipotent epithelial progenitor population in the adult thymus. Cell Rep. *14*, 2819–2832.

Wong, K., Lister, N.L., Barsanti, M., Lim, J.M., Hammett, M.V., Khong, D.M., Siatskas, C., Gray, D.H., Boyd, R.L., and Chidgey, A.P. (2014). Multilineage potential and self-renewal define an epithelial progenitor cell population in the adult thymus. Cell Rep. *8*, 1198–1209.