upper dermis in close proximity to Merkel cells, the neuroendocrine cells of the skin that participate in tactile sensation. It has been suggested that perhaps the unique interactions and cell-cell contacts between the bulge and the arrector pili muscle could be an important factor in maintaining bulge cells in their undifferentiated state (Akiyama et al., 1995).

Within the niche itself, the hair follicle bulge region is also cohabited by cells derived from two neural crest lineages, the melanocyte and the Merkel cell. Melanocytes are involved in the pigmentation of the hair shaft in the mouse and both hair shaft and epidermis in the human and reside within the niche (Nishimura et al., 2002). The function of resident Merkel cells within the hair follicle stem cell niche, however, is entirely unknown. Since these cells have no connection with perifollicular sensory nerve endings, it was suggested some ten years ago that perhaps the Merkel cells serve a stem cell maintenance function rather than the tactile receptor role served by Merkel cells in the skin (Narisawa et al., 1994). With the recent discovery of pluripotent neural crest stem cells within the rat hair follicle (Sieber-Blum and Grim, 2004), it may be time to look inward to neighboring cells in the niche for the answers to questions raised by Blanpain et al. (2004), such as, "What keeps a stem cell a stem cell?"

Perhaps uncovering the secrets of the hair follicle will be facilitated by going back to our roots, when the difference between neural and epidermal cell fates was a simple choice between neighboring cells. The study of this uniquely mammalian stem cell niche provides us with a developmental system rivaling the *Drosophila* germ cell niche (Fuchs et al., 2004) in which to delve deeply into the mysteries of adult stem cell biology in the context of the skin and hair follicle.

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Sculpting Heart Valves with NFATc and VEGF

Heart valves are of vital importance for our moment-to-moment existence, but how they form remains a mystery. In this issue of *Cell*, Chang et al. reveal a novel role for calcineurin, NFATs, and VEGF in valve formation (Chang et al., 2004). Dynamic changes in NFAT/VEGF expression in regional myocardial and endocardial fields and developmental windows orchestrate this complex process.

In contrast to primitive organisms, the heart in mammals contains four chambers. Valve leaflets between the upper and lower heart chambers and the large vessels are critical for the heart to pump the blood to the various parts of the body. In the embryonic heart, a complex interplay of precisely regulated signals is required so that each valve develops at a critical stage and a precise location. Because of this complexity, the process often derails, resulting in valve defects that affect up to one in 100 newborns and represent the most common birth defects in humans.

Soon after the primordial heart forms as an inner endothelial (endocardial) tube into an inner endothelial tube (endocardium) with a surrounding muscular layer (myocardium), endocardial cushions form from expansion of the extracellular matrix (cardiac jelly) in the region between the upper (atrial) and lower (ventricular) heart chambers (Figure 1). At embryonic day 9 (E9), endocardial cells at the atrioventricular canal undergo an endocardial-to-mesenchymal transformation (EMT) during a brief developmental period (E9–E10). In response to signals released from the underlying myocardium, these endocardial cells separate from the endocardium and transform into mesenchymal cells, invading the cardiac jelly. After expansion, these cells further differentiate and reshape the rudimentary valves into slender leaflets. Multiple transcription and growth factors, adhesion molecules, and proteases have been implicated in valve formation (Schroeder et al., 2003), but much of the precise combinatorial code regulating this process remains to be elucidated.

Gene-inactivation studies revealed that NFATc1 (nuclear factor of activated T cells cytoplasmic 1), expressed in the endocardium, is dispensable for EMT but critical for subsequent valve formation (de la Pompa et al., 1998). NFATc1 belongs to a family of calcium-sensitive transcription factors whose activity is dependent on dephosphorylation by calcineurin, an enzyme complex composed of calcineurin A (CnA) catalytic and calcineurin B (CnB) regulatory subunits (Crabtree and Olson, 2002; Schulz and Yutzey, 2004). Loss of *NFATc2*, *c3*, or *c4* alone failed to cause valve abnormalities, but loss of *Cnb1*, pharmacological inhibition of calcineurin signaling by cyclosporine A (CsA), or a combined deficiency of *NFATc3/c4* affected blood vessel development (Graef et al., 2001). At least some of these defects are attributable to upregulation of VEGF, a critical player in angiogenesis (Crabtree and Olson, 2002).

Interestingly, VEGF also controls valve formation.

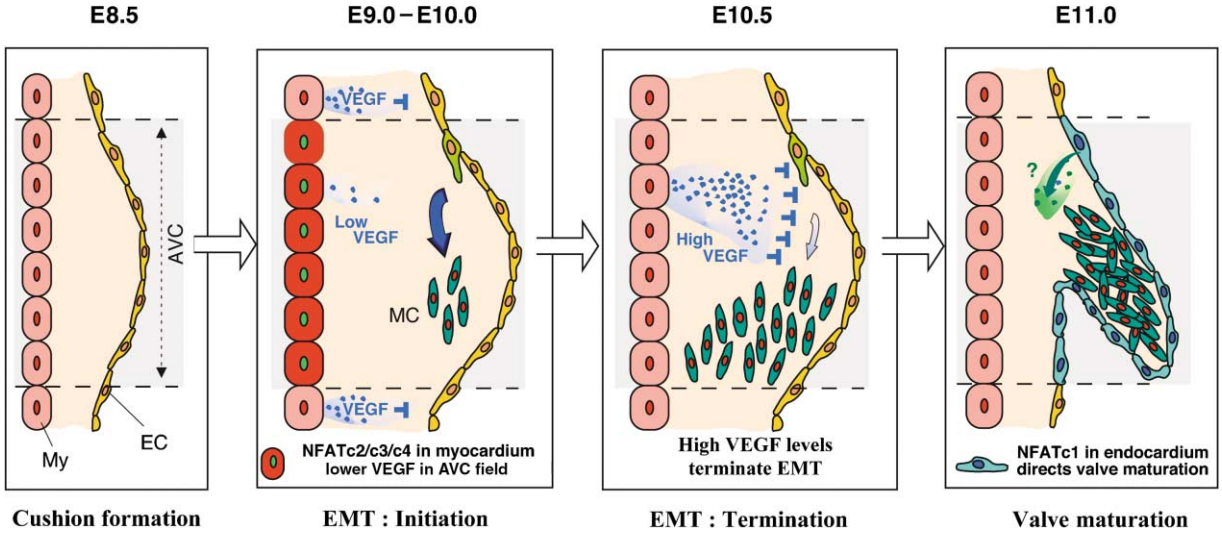

Figure 1. Between E9 and E10, Endocardial Cells Undergo Endocardial-to-Mesenchymal Transformation

VEGF dose-dependently controls endocardial-to-mesenchymal transformation (EMT) in the AVC field: minimal levels at E9.0 are required for EMT, while high levels at E10.5 terminate EMT. By preventing VEGF expression from reaching excessive levels at E9, NFATc2, c3, and c4 in the myocardium allow EMT to proceed. VEGF in the adjacent regions outside the AVC field might suppress EMT. At E11, NFATc1 in the endocardium controls valve maturation, but the signals remain to be determined. EC, endocardium; My, myocardium; AVC, atrioventricular canal; MC, mesenchymal cells.

VEGF binds its receptors, Flt1, Flk1, and the neuropilins, all expressed in the endocardium. Even more striking is that VEGF, while being necessary for initial EMT, subsequently terminates this process (Figure 1). This dual activity of VEGF seems to be strictly dose dependent and dynamically controlled in narrowly defined temporal windows and regional fields. Around the onset of EMT at E9.0, VEGF levels are detectable in the myocardium in and outside the atrioventricular canal (Dor et al., 2001, 2003; Miquerol et al., 2000). Loss of a single VEGF allele or the VEGF164 isoform results in underdeveloped endocardial cushions, chamber malformations, and septation defects (Stalmans et al., 2003), whereas lowering VEGF levels at E9.5 by hyperglycemia or with a soluble Flt1 chimeric protein (sFlt1) prevents EMT (Enciso et al., 2003). Amplification of Flk1 signaling by semaphorin-6D/plexin-A1 also regulates endocardial cushion formation. These findings thus suggest that some VEGF expression is required for endocardial cells to undergo EMT. However, at E10.5, when EMT reaches completion, myocardial VEGF levels in the atrioventricular canal are elevated by >5- to 10-fold (Dor et al., 2001, 2003; Miquerol et al., 2000), raising the question of whether high VEGF levels terminate EMT. Indeed, when myocardial VEGF levels are "artificially" elevated via gene targeting, conditional site-specific overexpression, hypoxia, or supplementation at E9.5 just prior to EMT, heart valve formation is disrupted (Dor et al., 2001, 2003; Miquerol et al., 2000). Thus, low VEGF levels at E9.5 are required for EMT, while high VEGF levels at E10.5 suppress this process.

In a series of elegant studies in mice and zebrafish, Chang et al. further unraveled the distinct roles of the NFATc family members and calcineurin signaling in valve formation (Chang et al., 2004). Together, these experiments suggest a model whereby spatial fields of CnB/NFATc signaling, first in the myocardium and thereafter in the endocardium, determine valve initiation and maturation (Figure 1). At E9.0–9.5, CnB activates NFATc2, c3, and c4 in the myocardium and, by regulating the expression of VEGF, allows the overlying endocardium to undergo EMT. But by 10.5, CnB activates NFATc1 in the endocardium, where it provides signals for valve elongation.

Chang et al. deduce their model from several lines of evidence (Chang et al., 2004). A role of calcineurin signaling in the myocardium was supported by findings that EMT was inhibited by treatment of wild-type heart explants with CsA but proceeded normally in explants from embryos, lacking *Cnb1* or *NFATc1* in the endocardium. Furthermore, triple mutant embryos, lacking *NFATc2/c3/c4*, had cushion defects with a hypocellular cardiac jelly. Though expression of *NFATc2*, *c3*, and *c4* in the myocardium remains to be determined, the authors suggest that myocardium-derived *NFATc2*, *c3*, and *c4* cooperate in instructing the endocardium to undergo EMT at E9.5. Evidence for a role of calcineurin signaling in the endocardium was provided by findings that endocardial loss of *Cnb1* did not prevent EMT but impaired subsequent valve elongation—similar to *NFATc1* null embryos (de la Pompa et al., 1998). Moreover, endocardial expression of a *NFATc1* transgene restored valve formation in *NFATc1* null embryos—all indicating that the endocardium provides critical signals for valve elongation, under CnB/NFATc1 control. By using CsA to pharmacologically knock down calcineurin, they further showed that NFATc1 within the endocardial field functions in a precise developmental time window.

Another novel finding is that the authors identified VEGF as a downstream signal of myocardium-derived NFATs. Chang et al. demonstrate that myocardial VEGF levels in mice lacking *NFATc2/c3/c4* or treated with CsA were upregulated prematurely at the onset of EMT

(Chang et al., 2004). As high VEGF levels at E9.5 inhibit EMT, they concluded that the elevated myocardial VEGF levels blocked EMT in these triple mutant embryos. Normalizing myocardial VEGF levels in the CsA-treated embryos by administering sFlt1 restored valve development. Thus, by preventing excessive upregulation of VEGF to levels that would otherwise block EMT, myocardium-derived NFATs allow EMT to proceed.

In their model (Figure 7G in Chang et al., [2004]), Chang et al. suggest that NFATc2, c3, and c4 suppress myocardial VEGF expression within but not outside the field of valve formation. Others documented, however, that myocardial levels are uniform within and outside this field (Dor et al., 2001, 2003; Miquerol et al., 2000). Another outstanding issue is that Chang et al. observed that treatment of wild-type E9.5 heart explants with sFlt1 enhanced EMT, while Enciso et al. reported that a higher dose (than the one used by Chang) of sFlt1 blocked EMT in wild-type heart explants (Enciso et al., 2003). It remains to be explored whether the higher sFlt1 levels blocked all VEGF signaling and thereby eliminated the low "threshold" VEGF levels necessary for EMT, whereas the lower sFlt1 levels removed only some of the excess "inhibitory" VEGF and thereby enhanced EMT.

Now that a distinct role for the various NFATs in valve formation is established, additional questions as to how they function can be addressed. For instance, which other NFAT partners are involved, which signals regulate calcineurin/NFAT, and which downstream targets are affected? The challenge for the future will be to integrate the calcineurin/NFAT pathway together with preexisting molecules, known to modulate valve formation, into a unifying model of EMT and valve maturation.

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