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Palatogenesis and cutaneous repair: a two headed coin

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Abstract

The reparative mechanism that operates following post-natal cutaneous injury is a fundamental survival function that requires a well-orchestrated series of molecular and cellular events. At the end, the body will have closed the hole using processes like cellular proliferation, migration, differentiation and fusion. These processes are similar to those occurring during embryogenesis and tissue morphogenesis. Palatogenesis, the formation of the palate from two independent palatal shelves growing towards each other and fusing, intuitively, shares many similarities with the closure of a cutaneous wound from the two migrating epithelial fronts. In this review, we summarize the current information on cutaneous development, wound healing, palatogenesis and orofacial clefting and propose that orofacial clefting and wound healing are conserved processes that share common pathways and gene regulatory networks.

Overview

As Paul Martin and colleagues have written: "*Embryos are in the business of building tissue, and wound healing is essentially a process of rebuilding what was damaged. To close a wound, all they do is reactivate the machinery they're using somewhere else in the body anyway*" (Wadman, 2005). In wound repair, and particularly cutaneous wound repair, regeneration of the lost tissue requires migration of mesenchymal cells (fibroblasts, endothelial cells, inflammatory cells) that proliferate, differentiate, and then synthesize, deposit and organize a new extracellular matrix. Epithelialization is initiated during the response to injury and leads to keratinocyte proliferation and migration across the regenerating extracellular matrix. The leading epidermal edges appose, and stratification and differentiation take place to restore the barrier function.

For proper palatal formation to occur, the two palatal shelves elongate as neural crest-derived mesenchymal cells migrate and proliferate, and then dynamically elevate above the tongue. As the two shelves appose, the medial edge epithelium (MEE) of each shelf adhere to each other, in part because of the presence of filopodia on the apical surfaces of these epithelial cells. These edges join to form a single epithelial layer, the midline seam. Finally,

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this seam remodels to allow confluence of the mesenchyme through a combination of processes including cell migration, apoptosis and epithelium-to-mesenchyme transformation (EMT). With the MEE gone, a confluent connective tissue remains and become the secondary palate.

As palatogenesis and wound repair share key cellular behaviors, it is not surprising that conserved embryological and reparative processes involving similar common genes and pathways regulate cutaneous development, wound healing, palatogenesis, and orofacial clefting.

In this review we summarize the current information about the molecular mechanisms involved in normal orofacial and cutaneous development. We propose that wound healing requires many of the same conserved processes necessary for palatal development. We then report on the genetic contribution to orofacial clefting and potential parallels with wound healing.

NORMAL EMBRYOLOGICAL DEVELOPMENT

Orofacial embryogenesis

The face, including the mouth and the nose, form from the oro-pharyngeal region of the early embryo as a result of the complex signaling that occurs between the three primordial cell layers during early embryonic development (Sperber, 2002a). This extremely intricate set of morphogenetic interactions gives rise to the external and internal entrances to the alimentary and respiratory tracts, while functionally separating them to allow normal respiration and deglutition. In human and mice, the growth of placodes yields to pits and processes that fuse to form the nose, the mouth, the lip, the cheeks and the palate.

Early in embryogenesis (before embryonic day (E)10.5 in the mouse and the 6^{th} week in human), the primitive oral cavity consists of a space bound by the forebrain (superiorly), the pericardial cavity (inferiorly) and the first pharyngeal (also called branchial) arch laterally (Sperber, 2002a). The pharyngeal arches are horse-shoe shaped structures that consist of a mesenchymal core covered by an ectodermal epithelium. These arches grow and form embryonic processes that fuse appropriately to turn into the facial and oral structures. The face is derived from seven embryonic processes: i) the medial nasal process, derived from the frontal eminence, that gives rise to the middle part of the nose, including the nasal septum, the middle part of the upper lip, and the primary palate ii, iii) two lateral nasal processes, derived from the frontal eminence, that gives rise to the lateral walls of the nasal cavities iv, v) two maxillary processes, derived from the first pharyngeal arch, that give rise to the upper parts of the cheeks, the upper lip, the maxilla and the secondary palate and vi, vii) two mandibular processes, derived from the first pharyngeal arch, that give rise to the lower part of the face, the lower lip and the mandible (Sperber, 2002b). Chronologically, the external components of the mouth and nose start to form at E9.5 in the mouse, when the nasal placodes develop into pits. Between E10 and E11, the medial and lateral nasal processes extend and meet to form the region around the nostril. A day later, the mandibular processes extend and meet to form the lower jaw and Meckel’s cartilage.

Palatogenesis

The palate functions to separate the oral and nasal spaces to allow respiration, deglutition, and phonation to occur. It is derived from two embryonic parts: the primary palate is the posterior extension of the medial nasal process and forms the four incisors and the most anterior part of the hard palate (Sperber, 2002a). The secondary palate is derived from the maxillary processes and forms the major part of the hard palate and soft palate (Sperber, 2002b). The first sign of development of the murine secondary palate occurs at E11, followed by the vertical growth of the two palatal shelves down the sides of the developing tongue (E12–13). Each shelf consists of a central core of neural-crest derived mesenchymal cells surrounded by an epithelium composed of cuboidal ectodermal cells (resembling keratinocytes of the epidermis) and a surface layer of periderm cells similar to the periderm in the oral cavity and the epidermis (Fig. 1). Recent data shows the critical presence of these periderm cells for proper craniofacial development, as animal models lacking the periderm exhibit oral adhesions and cleft palate (Casey et al., 2006; Richardson et al., 2009; Peyrard-Janvid et al., 2014; Richardson et al., 2014). About a day later, the tongue descends inferiorly as the mandibular arch lengthens to allow the paired palatal shelves to elevate and reorient from a vertical to a horizontal position and start to extend medially. The palatal shelves finally meet at E14 in the midline where they fuse to one another and to the down-growing nasal septum (Murray, 2002). This fusion is initiated in the middle of the shelves and progresses anteriorly and posteriorly to complete palatogenesis. It results in the formation of the oral and nasal cavities, in which each epithelium will have a different fate; the oral epithelium differentiates into keratinizing epithelium whereas the nasal epithelium differentiates into ciliated epithelium of the respiratory tract (Sperber, 2002b).

Palatal development involves the complex choreography of cell migration, proliferation, and differentiation that is closely regulated in a spatiotemporal manner. Any interruption of cranial neural crest (CNC) migration into the palatal shelves, failed palatal shelf elevation (due to intrinsic forces within the palatal shelf or extrinsic forces from the tongue blocking elevation), palatal shelf apposition (due to deficient growth), or failure of the MEE to breakdown, results in a cleft of the palate. Not only are these cellular processes required for the formation of the palate, they are also key in the healing of the palate after repair. This is illustrated by the fact that up to 10% of patients develop wound complications following cleft palate surgical repair.

There are multiple genes required for palatal development and like the embryogenesis of this tissue itself, these genes function in temporal and spatial manners (Bush and Jiang, 2012). During the initial migration of the CNC into the first branchial arch, the mesenchymal cells move in response to soluble signals from the adjacent endoderm. Conversely, the mesenchyme secretes soluble factors, which informs epithelial development. Globally, these genes are part of complex regulatory networks that are conserved among the development of other tissues. However, genes that control the development of the anterior and the posterior palate are not identical. This heterogeneity may in part explain the varied clinical phenotypes of patients with cleft palates.

Multiple transcription and growth factors contribute to the development of the anterior palate (for review, see (Bush and Jiang, 2012)). One of the most critical is the *Msx1* (*Muscle Segment of Homeobox Gene of Drosophila*) gene. It is essential for palatal development and complete abrogation of its transcriptional activity in the mouse causes cleft palate; the shelves are able to elevate, but are severely shortened and can not appose (Vastardis et al., 1996). Further investigations reveal that the *Msx1* gene is required for the expression of other factors, notably the growth factors Bone Morphogenic Protein (Bmp) 2 and Bmp4 in the adjacent palatal mesenchyme and the *Sonic Hedgehog* (*Shh*) gene in the overlying MEE, which then work in a feedback loop to regulate the expression of the *Msx1* gene (Zhang et al., 2002). The interruption of the Msx1-Bmp2-Bmp4-Shh signaling loop leads to decreased cellular proliferation and cleft palate in the mouse. *Msx1* itself is regulated, in part, by the *Hoxa2* (Smith et al., 2009) and the *Foxe1* genes (Venza et al., 2011). Themselves, both of these genes contribute to proper palatogenesis: *Hoxa2* is required for shelf fusion *ex vivo* and mutations in the *FOXE1* human gene cause Bamforth-Lazarus syndrome (OMIM #241850) and contribute to cleft lip and palate. Signaling of the growth factor Fgf10 within the palatal mesenchyme intersects with the *Msx1*-Bmp2-Bmp4 pathway to ultimately regulate the expression of Fgfr2a in the adjacent palatal epithelium (Alappat et al., 2005). These two complementary pathways are critical during the elongation, elevation and fusion of the palatal shelves. During mesenchymal differentiation, cells from the shelves undergo osteoblastic commitment to form the hard palate, requiring activation of the Bmp signaling pathway. Specifically, the loss of Bmpr1a signaling in the CNC causes cleft palate and reduces palatine bone formation (Li et al., 2011).

Posterior palatal development remains less well understood, but it appears that genes necessary for its development predominantly regulate cellular proliferation. Amongst them, the *Meox2* gene is a transcription factor only detected in the posterior palate. About a third of the mice deficient for *Meox2* display a cleft palate, and unlike previously reported clefts, elevated shelves lack epithelial cells in the medial edge area. Although the molecular mechanism underlying this observation is not known, it apparently results from the breakdown of newly fused shelves (Jin and Ding, 2006). The *Tbx22* gene, a member of the T-box transcription factor family, is another transcription factor specific to the posterior palatal mucosa. Its expression is regulated by Fgf and Bmp signaling and is an integral component of the molecular network that governs palatogenesis (Fuchs et al., 2010). In mice, deletion of the *Tbx22* gene causes submucousal cleft palate that is associated with reduced palatine bone formation and choanal atresia (Pauws et al., 2009). In human, genetic mutations in *TBX22* cause X-linked craniofacial syndrome, X-linked cleft palate and ankyloglossia (CPX; OMIM #303400).

The physical process of the adhesion, fusion and dissolution of the MEE after apposition of the palatal shelves, has undergone a great deal of investigation using DiL experiments as well as *in vitro* and *in vivo* genetic lineage tracing of the epithelium. Of particular interest are the roles of Interferon Regulatory Factor 6 and Tgfβ3 during this process. The expression of the *Irf6* gene intensifies around E13 in the epithelial cells at the tip of the palatal shelves, and even more when the two appose, supporting the notion that the *Irf6* gene is necessary for this process (Knight et al., 2006). However, using a reporter murine model,

Fakhouri *et al* showed that *Irf6* is not required for MEE fusion (Fakhouri et al., 2012). Absence of *Tgfβ3*, a growth factor upstream of the *Irf6* gene, causes cleft palate in the mouse due to the inability of the periderm of one shelf to firmly attach to the opposing shelf (Kaartinen et al., 1995). Reception of Tgfβ signaling during the development of the MEE was necessary for its dissolution as well, suggesting an autocrine Tgfβ signaling pathway during MEE dissolution (Dudas et al., 2004; Xu et al., 2006). Overall, the scientific community has come to agree that three mechanisms are likely acting in concert during MEE dissolution, including apoptosis, EMT, and cellular migration (Bush and Jiang, 2012), and that these mechanisms are likely working in concert with one another rather than each acting independently. Further live imaging studies are warranted to define the fate of MEE cells during this process.

Epidermal development

The ultimate function of the epidermis is to act as a barrier organ system to protect species from external environmental factors and maintain internal homeostasis. It is also a reservoir of stem cells allowing the constant renewal of this tissue for the life of the organism. The developmental processes and pathways involved in epidermal morphogenesis share strong commonality with processes and genes involved in cutaneous repair. As such, we will describe the gene regulatory networks involved in cutaneous embryogenesis to facilitate our understanding of tissue repair, yet focus on molecules with critical roles in palatogenesis.

Though the three germinative layers are established during gastrulation, it is not until after neurulation that the entire outer surface of the developing embryo adopts an ectodermal fate. This single-layered epithelium consists of loosely integrated ectodermal cells containing keratin intermediate filaments (K8, K18, and K19) characteristic of simple epithelia (Dale et al., 1985). Because of their high keratin content, these cells are presumably keratinocytes.

The surface ectoderm takes on an epidermal fate around E8.5 in the mouse and E20–30 in humans, not by default as it was thought for a long time, but under the influence of mesenchymal signals. Although the nature of the inductive signal is largely unknown, data from animal cap transplantation in amphibians and cultured embryonic stem cell differentiation identify the growth factor Bmp4 as the endogenous neural inhibitor/epidermal inducer (Hemmati-Brivanlou and Melton, 1997; Aberdam, 2004). The acquisition of the epidermal fate is associated with the induction of the *p63* gene (Lu et al., 2001; Koster et al., 2004; Koster and Roop, 2007). In fact, the *p63* gene is the first transcription factor that is specific for the epidermal lineage (Green et al., 2003). The importance of *p63* in epidermal induction is underscored by the phenotype of the *p63* knockout mice. Though these mice die shortly after birth, they are born with a single-layered epithelium resembling the surface ectoderm that covers the early embryo, indicating the absence of epidermal specification (Mills et al., 1999; Yang et al., 1999). The *p63* gene directly regulates the *Tfap2c* gene (Koster et al., 2006), a transcription factor previously implicated in the expression of epidermal specific keratin genes, *Krt5* and *Krt14* (Byrne et al., 1994). By E9.5 and up to E10.5 in the mouse and already at E42 in human, *Krt5* and *Krt14* are expressed in the yet still single-layered epithelium (Dale et al., 1985; Byrne et al., 1994). The onset of these two genes reflects a commitment towards the formation of a complex epithelium.

The first step in the process of stratification is a rotation of the plane of cleavage from the vertical position in the single-layer epithelium to a horizontal plane (Smart, 1970). Between E10.5 and E12.5 in the mouse and E35–42 in human, the single-layered epithelium becomes two layers comprised of the basal layer and the periderm (Fig.1). The outermost of the two layers is the periderm, a unique structure that sheds when the epidermis is fully developed underneath. The cells from the periderm are morphologically distinct from the basal layer, with a flattened nucleus and intermediate filaments distributed in small bundles throughout the cytoplasm (Dale et al., 1985). Amongst keratin filaments, K6a, K17, and K19 are expressed in the periderm (Holbrook, 1991; McGowan and Coulombe, 1998; Mazzalupo and Coulombe, 2001). The periderm cells also express Involucrin and Loricrin (Akiyama et al., 1999), which has led to the presentation of the periderm as a stratum corneum substitute during embryonic development. Although its epidermal function is not fully understood, animal models lacking the periderm (i.e. *Irf6*-, *14-3-3σ*-, and *Ikkα*-deficient) exhibit altered epidermal terminal differentiation, suggesting a potential role for this structure in barrier formation (Richardson et al., 2014).

Beneath the periderm is the basal layer (or stratum basale, stratum germinativum), a germinative layer that provides the other epidermal layers with cells, as epidermal differentiation leads to constant shedding and needs to be replenished. The basal layer is characterized by the expression of K5, K14, and TAp63, yet it still expresses K19 (Dale et al., 1985). Transcription factors AP-1, AP-2 and Ets, in addition to p63 are required for the maintenance of K5 and K14 expression in the basal layer (Byrne et al., 1994; Koster et al., 2004; Romano et al., 2009). The rate of basal keratinocyte proliferation is high (Bickenbach et al., 1986), and these cells will, initially, give rise to the intermediate layer, a unique embryonic-specific layer (E13.5 to E14.5 in the mouse and E70–84 in human).

The addition of a fourth layer is the beginning of the appearance of the spinous layer (E14.5 in mouse, E140 in human). The spinous layer is characterized by keratinocytes withdrawing from the cell cycle, down regulating basal cell markers and expressing markers of differentiation, like K1, Involucrin, Irf6 and 14-3-3σ (Fuchs and Green, 1980; Watt, 1983; Ingraham et al., 2006; Richardson et al., 2006; Bazzi et al., 2007). These changes are orchestrated at the transcriptional, cellular and structural levels. Although many of the molecules and their interactions have not fully been elucidated, a role for miR-203, c/EBP, c-Myc, and Notch in the transition from basal to spinous layers has been identified (for review, see (Blanpain and Fuchs, 2009)). Of particular interest to us are the *Irf6*, *Ikkα*, *Receptor-interacting protein kinase (Ripk) 4* and *14-3-3σ* genes. These four genes, when mutated or deleted from the genome in the mouse, lead to animals with a similar phenotype (Hu et al., 1999; Li et al., 1999; Takeda et al., 1999; Holland et al., 2002; Herron et al., 2005; Li et al., 2005; Ingraham et al., 2006; Richardson et al., 2006). Their skin is taut, and close observation of their epidermis reveals an absence of the granular and cornified layers, demonstrating a deficit in proper terminal differentiation. At the same time, ectopic expression of K14 and p63 proteins in the suprabasal layers suggests an expansion of the basal layer. However, the presence of proliferative cells concomitant with K1 expression is reminiscent of the feature of the intermediate layer rather than the spinous layer. Despite their common phenotypes, no genetic interaction between Ikkα and Irf6 or Ikkα and 14-3-3σ

has been identified. Furthermore, *Ikkα*-deficient and *14-3-3σ*-deficient animals were not rescued by mice overexpressing *Ripk-4* under the K14 promoter (Rountree et al., 2010). However, the *Ikkα* gene is a direct target of *p63*, and genetic interaction between *14-3-3σ* and *Irf6* and *p63* and *Irf6* have been demonstrated *in vivo* (Candi et al., 2006; Richardson et al., 2006; Thomason et al., 2010), suggesting that, in part, these genes belong to the same gene regulatory network.

The first granular cells appear during the 6th month of gestation in human (E160) and at about E15.5-E16.5 in the mouse, thereby initiating the events of normal terminal differentiation (Dale et al., 1985; Hardman et al., 1998). The hallmark of the granular layer is the presence of keratohyalin and lamellar granules, each a precursor of unique structures of the stratum corneum. The keratohyalin granules are composed of keratin filaments compacted with Filaggrin and enzymes like Caspase 14, while lamellar granules contain lipid leaflets (Kuechle et al., 2001). An increase in the extracellular calcium concentration leads to the activation of Protein Kinase C, which in turn downregulates K1 and K10, and upregulates Loricrin, Filaggrin, and Transglutaminase (Denning et al., 1995).

The final process in terminal differentiation occurs when cells migrate outward, lose their nuclei and form the cornified layer (or stratum corneum). It is composed of anucleate corneocytes filled with keratin filaments bound to a peripheral cornified envelope composed of cross-linked proteins. Between the corneocytes are intercellular lipid lamellae, indispensable to the barrier function of the skin (Madison, 2003). Numerous proteins, enzymes, and lipids are used to assemble the cornified layer and have been recently reviewed (Candi et al., 2005). Many of the epidermal-specific differentiation genes belong to the epidermal differentiation complex located on murine Chromosome (Chr) 3 and the syntenic region on human Chr 1q21 (Rothnagel et al., 1994). Amongst them are the *Loricrin*, *Involucrin*, *Small Proline-Rich Proteins*, and *Filaggrin* genes, along with the S100 protein family of genes. Another cluster termed the stratified epithelium secreted peptides complex on human Chr 19 and murine Chr 7 was identified (Matsui et al., 2004), containing, amongst others, secreted peptides like Dermokine or Suprabasin (Bazzi et al., 2007). In addition, the ontogeny of the stratum corneum is also regulated at the transcriptional level, with a role for *Kruppel-like Factor 4* (*Klf4*), *Grainyhead-like 3* (*Grhl3*), *Lim-only protein* (*LMO4*), *Ripk-4* and Akt signaling via *Pp2a* (Segre et al., 1999; Yu et al., 2006; O'Shaughnessy et al., 2009; Rountree et al., 2010).

The completion of the morphogenesis of the epidermis leads to a fully functional epidermis at birth that will allow the transition from the aqueous *in utero* to the air terrestrial *ex utero* environment. The embryonic program of differentiation described above is the same as the one taking place throughout adult life when the epidermis continuously renews itself and during cutaneous repair following injury, with the exception that there is no intermediate layer with the basal keratinocytes migrating directly into the spinous layer.

Dermal development

The dermis is the cutaneous tissue below the epidermis and the basement membrane zone, and it consists of connective tissue. The connective tissue layer contains the cutaneous appendages (hair follicles, sebaceous glands, sweat glands), blood vessels and nerves. The

dermis is structurally divided into two areas: the superficial dense papillary dermis and the deeper reticular dermis (Odland, 1991). This connective tissue is constituted by an extracellular matrix (collagen, elastin, glycoproteins, proteoglycans, and glycosaminoglycans) in which the largest population of cells is the fibroblast (Odland, 1991). Other cells found within the dermis include monocytes, macrophages, and dermal dendrocytes. The dermis acts to provide structural integrity to the skin, maintain the appendages, and is a critical component during wound healing.

Broadly, the dermis arises from the mesodermal layer of the embryo. Interspecies tissue recombination experiments defined the embryonic origins of the dermis: the dorsal dermis arises from the somatic dermomyotome, the dermis of the lateral and ventral body originates in the lateral plate mesoderm (Mauger, 1972; Christ and Ordahl, 1995; Fliniaux et al., 2004), and the dermis of the head and neck originates from neural crest cells (Sengel, 1990). At the time when the ectoderm acquires its epidermal fate, the mesodermal component of the skin has not been specified and thus is indistinguishable from the subcutaneous mesenchyme. Dermal specification is first noted histologically by the formation of a homogeneous, loose subectodermal mesenchyme between E9.5 and E13 in the mouse and before 3 months in human (Holbrook, 1991; Dhouailly et al., 2004; Driskell et al., 2013). The dermis becomes specified in a wave that starts in the lateral trunk and moves toward the dorsal midline. This acquisition of dermal fate can be visualized by the expression of two mutually exclusive transcription factors: *Msx1* and *Dermo1*. The presumptive dermal cells first express the *Msx1* gene in the dorsal mesenchyme around E11, before downregulating it upon expression of the earliest known marker of dermal fate, the *Dermo1* gene (also known as *Twist2*). *Dermo1* is first detected around E11 in the lateral trunk and reaches the dorsal midline around E13 (Houzelstein et al., 2000); this pattern of expression correlates with dermal differentiation, hair follicle morphogenesis, and the onset of the first periderm differentiation marker (M'Boneko and Merker, 1988). As cutaneous development continues, the papillary and reticular dermal regions begin to be histologically distinct around E18.5 in the mouse (just before birth) and in the 4^{th}–5^{th} months in human; by P2 (post-natal day 2), the papillary and reticular regions are fully distinguishable from each other. Furthermore, at this time, the lowest part of the dermis, the hypodermis, has formed (Van Exan and Hardy, 1984; Driskell et al., 2013).

Recent molecular analysis of the murine dermis revealed distinct and dynamic expression profiles of the papillary dermis and the reticular dermis (Driskell et al., 2013), with CD26 unique to the papillary dermis and Delta-like homolog 1 (Dlk) specific to the reticular dermis and hypodermis. Flow cytometry sorting of P2 dermal cells and subsequent grafting assays showed that hair follicle formation was dependent on fibroblasts from the papillary dermis, and not from the reticular dermis, demonstrating that these distinct fibroblast lineages have varying developmental potential (Driskell et al., 2013).

Murine models have been used to investigate the molecular pathways of dermal origin and development. Although the *Msx1* gene is detected early during dermal specification, is not necessary for dermal development in the mouse. Of interest, *Msx1*-deficient mice exhibit craniofacial abnormalities, including a complete cleft of the secondary palate (Houzelstein et al., 1997). However, mice deficient for *Dermo1* exhibit a decrease in dermal thickness

caused by tissue atrophy including a reduction of subcutaneous fat (Sosic et al., 2003). One of the most important signaling pathways for specification of all three dermal regions (lateral and ventral body, the dorsum, and the head) is the Wnt/β-catenin signaling in the subepidermal mesenchyme (Atit et al., 2006; Ohtola et al., 2008; Tran et al., 2010). For example, the *Wnt* genes are critical for the survival of early ventrum and flank dermal progenitors and for later fate specification (Ohtola et al., 2008). Further, a subset of En1-positive progenitors in the central dermomyotome are responsive to Wnt signaling to become dorsal dermal fibroblasts (Atit et al., 2006). Finally, cranial mesenchymal cells express the Dermo1 protein, rendering them permissive to Wnt activity, which is required for these cells to adopt a dermal fate (Tran et al., 2010).

As the dermis specifies, it communicates with the epidermis to provide molecular cues for cutaneous appendage morphogenesis (for review on appendage development see (Biggs and Mikkola, 2014)). Briefly, the dermis signals to the epidermis, which responds by forming bulges known as placodes. These placodes signal to the dermis, instructing the fibroblasts to condense into a dermal condensate, which is the precursor to the functional dermal unit of the hair follicle known as the dermal papilla. These specified fibroblasts next communicate to the placode and down-growth of the epidermis and subsequent development of the hair follicle occurs. Finally, the dermis contains matrix proteins, the most abundant being Collagens I, III and Fibronectin. These extracellular matrix proteins are secreted by fibroblasts, providing a scaffold for the fibroblasts to populate, and providing structure and integrity to the skin and the body. These proteins eventually become non-randomly distributed around the skin appendages, lending evidence for their role in the development and/or maintenance of the appendages (Sengel, 1990).

WHEN THINGS GO WRONG

Orofacial clefts

Cleft lip and palate (CL/P) is a complex trait with genetic and environmental triggers that affects about 1 in 800 births (Murray, 2002). During facial formation, the CNC migrates into the frontonasal process, grows inferiorly and the paired maxillary arches grow from the side towards the midline. The medial and lateral nasal processes form from the maxillary and frontonasal process, and they all converge to form the nose and upper lip and primary palate. Failure of CNC migration or inability of the epithelium-covered surfaces to fuse, leads to cleft lip and alveolus. The secondary palate develops as an outgrowth of the maxillary prominences as outline above. Interruption of CNC migration, premature epithelial adherence of the palate to the oral cavity, failure of palatal elevation, or inability of the MEE to dissolve leads to cleft palate. CL/P has a wide variability across geographic origin (Mossey, 2002) suggesting underlying genetic differences in etiology. CL/P differs in frequency by sex and side of clefting. There is a 2:1 male to female ratio and a similar 2:1 left-side:right-side ratio for unilateral clefts. Fogh-Andersen (Fogh-Andersen, 1942) in Denmark first proposed the contribution of genetic factors in clefting, which have subsequently been confirmed by segregation analysis (Marazita, 2002) and twin studies (Mitchell et al., 2002). Evidence from genetic studies, animal models, expression data and environmental correlates suggest a variety of candidate genes for CL/P, with a list of about

300 (Jugessur et al., 2009). These genes have been investigated intensively using candidate gene approaches including direct sequencing and association studies. There is strong evidence to show that at least genetic variants in *IRF6* (Rahimov et al., 2008), *FGFR1, FGFR2* (Riley et al., 2007), *BMP4* (Liu et al., 2005; Suzuki et al., 2009), *FOXE1* (Vieira et al., 2005), *TGFB3* (Lidral et al., 1998), *MSX1* (Jezewski et al., 2003), *MAFB* (Beaty et al., 2010), and at the 8q24 chromosomal locus (Beaty et al., 2010; Mangold et al., 2010) can all result in human clefting (see additional genes in Table 1). Three of these genes, *IRF6, TGFB3* and *BMP4*, have strong evidence to support a role in wound healing as well based on their role in epidermal differentiation (Ingraham et al., 2006; Richardson et al., 2006), scarring (Ferguson and O'Kane, 2004) and spontaneous healing of orofacial clefting (Liu et al., 2005), respectively. Subphenotypes of clefts are increasingly recognized as being important in clinical assessment and gene identification (Marazita, 2007) and include deficiencies of the orbicularis oris muscle, microform clefts such as bifid uvula and submucous clefts and dental anomalies. Deficient wound healing has also been suggested as another manifestation of the underlying contributors to clefting (Suzuki et al., 2009).

Surgical repair of orofacial clefts

The standard of practice for children born with cleft lip and palate is surgical repair. Usually within the first six months of life, the cleft lip is repaired while the palate is usually repaired between 6 to 18 months of age (Hurwitz and Kathju, 2003). Secondary corrections are often required to improve the function and appearance of the lip. Secondary lip repairs may occur before 5 years of age or later, and corrections of nasal deformity around 14–18 years of age. Alveolar bone grafting improves the bony support of the cleft-adjacent teeth and allows the closure of oronasal and nasolabial fistulae. This procedure is commonly performed around 6–9 years of age. Among reported post-operative complications are oropharyngeal infection, upper respiratory tract infection, airway obstruction, feeding difficulties, flap dehiscence and palatal fistula (Moore et al., 1988). The latter two are particularly relevant as they directly relate to wound healing. They both consist of the reopening of the closed wound along the surgical suture line. The incidence of these post-operative complications varies from 10–30% reported for palatal fistula (Cohen et al., 1991; Muzaffar et al., 2001) up to 63% (Gosman, 2007). A retrospective chart study performed at the University of Iowa suggests that the *IRF6* genotype of the individual may be a determinant factor as patients with Van der Woude syndrome (OMIM #119300, haploinsufficient for *IRF6*) are more likely to have a poorer surgical outcome compared to patients with isolated CL/P with the same degree of clefting (Jones et al., 2010). Using a novel digital imaging system to quantitatively characterize the cleft scar, we identified genetic variants in *RhoA GTPase Activating Protein 29 (ARHGAP29)* and *TGFβ3* as associated with poor scarring (Smith et al., 2014). Expanding the identification of biomarkers (like *IRF6*) could inform craniofacial surgeons on the risks for such post-operative complications, and may suggest rational interventions.

There have been very few alternatives or adjuvants to the classical surgical repair reported above. The prospect of fetal surgery has been driven by the potential advantages of scar-less healing (due in part to an increased TGFβ3:TGFβ1 ratio in mesenchymal tissues (Schrementi et al., 2008)), repair of the primary deformity, prevention of secondary deformities, prevention of facial growth disturbance due to scarring and normal facial

appearance of the infant when presented to the parents at birth. Another alternative surgical adjuvant to surgery is the use of recombinant protein in replacement of autologous bone graft for alveolar repair. Particularly, recombinant human BMP2 has proven to be an efficient substitute in patients with cleft (Chin et al., 2005). The use of mesenchymal stem cells with platelet-rich plasma has also been used as an adjuvant during palatal repair and alveolar bone grafting (Behnia et al., 2012). Collectively, despite the fact that many patients with CL/P suffer complications and require revision surgery, very few options are currently available to them, yet are critically needed.

Cutaneous wound healing

Injury to the skin initiates a cascade of events that, when executed properly, leads to tissue repair (for review, (Singer and Clark, 1999; Werner and Grose, 2003; Shaw and Martin, 2009)). This repair process is artificially compartmentalized into three phases: inflammation, proliferation, and maturation. None of these phases correspond to a precisely defined period of time, and all phases overlap to a certain degree.

Inflammation—Upon wounding, both the skin and the underlying vasculature are injured. The mechanical damage to cells results in a rapid flash of calcium that travels from the wound margin outward and ebbs back toward the wound margin, resolving within minutes (Razzell et al., 2013), yet is sufficient to activate DUOX, and leads to the production of hydrogen peroxide (Niethammer et al., 2009). Reactive oxygen species as well as adenosine triphosphate serve as attractants to induce extravasation of circulating leukocytes, mostly neutrophils, to the site of injury (Hattori et al., 2010; McDonald et al., 2010). A blood clot forms within minutes to restore homeostasis and to secrete mediators of wound healing that attract and activate neutrophils, macrophages and fibroblasts (Singer and Clark, 1999).

Neutrophils extravasate from damaged blood vessels and are the first cells to arrive at the wound site. They are responsible for protecting and cleaning the area of foreign particles and bacteria. In addition, it has been proposed that neutrophils contribute to the resolution of the fibrin clot and provisional extracellular matrix (ECM), to the promotion of angiogenesis, and to epithelialization. After the initial influx of neutrophils, monocytes are recruited to the injured site by chemoattractants such as ECM protein fragments, TGFβ1, PDGFs, and Monocyte Chemoattractant 1, amongst many others (Singer and Clark, 1999; Werner and Grose, 2003). Monocytes appear in two waves: the first one is immediate, resulting from ‘inflammatory monocytes’ while the second consists of tissue resident-derived monocytes that arrive after homing to inflammation (Novak and Koh, 2013). Once outside the circulation, monocytes become activated and differentiate into macrophages. Macrophages are responsible for clearing matrix and cell debris, including spent neutrophils (Eming et al., 2007). Inflammatory cells release cytokines and growth factors and also generate nitric oxide and reactive oxygen species. Together, they clean the wound bed and prepare it for epithelialization, neovascularization and fibroblast migration. These reactive oxygen species also act as signaling molecules to promote wound healing including release of cytokines, angiogenesis, cell motility, and ECM formation (for review see (Sen, 2003)).

Keratinocytes also contribute to this initial phase of tissue repair. They produce and secrete a large number of cytokines, including interleukins, growth factors, colony stimulating factors and chemokines, which can influence local fibroblasts, endothelial cells and regulate the inflammatory response (Werner and Grose, 2003). They also express receptors from the Toll-like family, which are known to mediate the innate and adaptive immune responses. Of note is the prominent role for TLR4 in the production of interleukins and growth factors and ultimately to proper tissue repair (Chen et al., 2013)

The requirement for inflammatory cells in wound repair remains controversial (Martin and Leibovich, 2005). Some studies report faster epithelialization in mice lacking innate immune cells (Dovi et al., 2003; Cooper et al., 2005), others unchanged healing (Martin et al., 2003), while models lacking macrophages demonstrate delayed tissue repair (Goren et al., 2009; Mirza et al., 2009). However, it becomes evident that the disappearance of inflammatory cells is required for healing to proceed, and that the persistence and/or the dysfunction of innate immune cells leads to impaired healing as exemplified by chronic inflammation in diabetic foot ulcers and chronic wounds (Khanna et al., 2010; Pukstad et al., 2010; Miao et al., 2012).

Epithelialization—Epithelialization of the wound is the phase during which the injury is resurfaced by new epithelium derived from both keratinocyte migration at the wound edge and proliferation behind the wound front (for review, see(Coulombe, 2003)). Both of these processes are critical to epithelialization, as chronic wounds exhibit defective keratinocyte migration and ectopic proliferation (Stojadinovic et al., 2008).

Basal keratinocytes normally rest on the basement membrane, a structure composed of Laminin, Entactin, Proteoglycans, and Type IV Collagen (Marinkovich et al., 1993). Wounding destroys this membrane and during epithelialization, keratinocytes migrate on a provisional matrix provided by the clot, such as dermal collagen, unprocessed Laminin 332, and Fibronectin (Nguyen et al., 2000). Keratinocytes themselves secrete unprocessed Laminin 332 at the wound edge, promoting their own migration via Integrin $\alpha 3\beta 1$ binding. Once the $\alpha 3$ chain of Laminin 332 is processed, the $\alpha 6\beta 4$ Integrin, also present on the surface of keratinocytes, binds to processed Laminin 332 and mediates stable adhesion to the basement membrane (Usui et al., 2008). As a consequence, the keratinocytes must change their cell-substrate adhesive properties when migrating over the wound. Integrin $\alpha 6\beta 4$ relocates from the hemidesmosomes to the lamellipodia of the keratinocyte's leading edge, allowing $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins to maintain the directional migratory trajectory by intracellular association with Rac1 (Choma et al., 2004; Pullar et al., 2006).

Exactly which keratinocytes participate in wound epithelialization has been controversial, with studies supporting a sliding model (Radice, 1980), a rolling model (Krawczyk, 1971; Paladini et al., 1996), or a combination of both (Usui et al., 2005). The sliding model posits that the basal keratinocytes release their hemidesmosomal attachments and maintain their desmosomal attachments. They migrate, pulling with them their neighbors and those post-mitotic cells attached above them. The rolling model posits that the suprabasal cells 'roll over' the basal keratinocytes, undergoing shape changes and releasing their desmosomes to fill in the hole. Indeed, keratinocytes in their early stages of differentiation are thought to

contribute to tissue repair (Mannik et al., 2010). The third model is a combination of the rolling and sliding models, as demonstrated by ectopic expression of K14 and down-regulation of K10 expression in suprabasal cells (Usui et al., 2005). A recent study using cultured human skin equivalents and time-lapse fluorescent labeling found that none of the previously proposed models of epithelialization were true. In fact, they demonstrate that basal cells migrate below the suprabasal cells, which remain in place to act as a shield (Safferling et al., 2013). In this study, proliferating cells in the unwounded tissue, and not the advancing epithelial leading edge, constitute the supply of new cells covering the wound. Although this study provides crucial information as to which cells are participating in wound healing, this *in vitro* model lacks cutaneous appendages and stem cell niches, two sources of contributing keratinocytes during tissue repair (Ito et al., 2005; Lau et al., 2009). Collectively, it raises the question as to whether this model applies to *in vivo* situations.

At the single cell level, it is the spatial and temporal reorganization of the filamentous (F-) actin network within the cell and the dynamics of cellular junctions that allows cellular migration (Kardash et al., 2010). The family of small Rho-GTPases, including RhoA, Rac and Cdc42, is the master regulator of the cytoskeleton dynamics and has been extensively studied *in vitro* and *in vivo*, with specific roles for each of the enzymes in lamellipodia and filopodia formation as well as overall migration (for review, see (Burridge and Wennerberg, 2004; Hall, 2005). In the context of cutaneous wound healing, RhoA is essential for wound closure by modulating actin cables (Wood et al., 2002), and epidermal-specific deletion of RhoA decreased directed cellular migration (Jackson et al., 2011).

In monolayer epithelial sheets, a finite number of cells are mobilized to close a scratch, and the mobilization of distant cells is independent of the advancement of the leading cells (Matsubayashi et al., 2011). However, in the *Drosophila* embryo wound model, the cells away from the wound edge stretch toward the wound and show actomyosin-dependent shrinkage of cell-cell junctions with their neighbors to drive cell intercalation events and thus close the wound (Razzell et al., 2014).

Numerous factors regulate the proliferation and migration of keratinocytes over an open wound. Amongst them, a multitude of transcription factors have been described playing a critical role in this process, including AP-1, c-jun/c-fos, PPARβ/δ, c-Myc, E2F-1, Egr-1, Grhl3, HoxA3, HoxD3, Smad2, Smad3, and Stat3, to name just a few (Schafer and Werner, 2007). We recently added Irf6 to the list (Biggs et al. 2014). Rather than enumerate their function in epithelialization, we will elaborate on their role in tissue repair in the context of the potential parallels with palatogenesis at the end of this review.

While the epidermis is undergoing the changes described above to cover the wound, the dermis is also active. It contributes to the closing of the wound by pulling the edges of the wound closer together. First, the fibrin clot is replaced by a new stroma called granulation tissue. Participating cells include migrating fibroblasts from wound margins, circulating fibrocytes, bone marrow progenitor cells, and adipocytes (Hinz, 2007; Schmidt and Horsley, 2013). It is also the time when angiogenesis occurs, leading to the formation of new capillaries. Concomitantly, fibroblasts acquire contractile properties through the inherent formation of actin stress fibers in their cytoplasm. Furthermore, secretion of TGFβ1 and

Periostin from the ECM, along with mechanical stress, induce the differentiation of fibroblasts into myofibroblasts (Hinz, 2007; Elliott et al., 2012). Through the action of both wound fibroblasts and myofibroblasts, collagen fibers are synthesized, aligned and bundled, leading to the contraction of the connective tissue (Hinz, 2007). The first fibroblasts to migrate to a wound originate from both the reticular dermis and the hypodermis. Subsequently, the papillary dermis replaces the reticular dermis during epithelialization (Driskell et al., 2013). Migration of the fibroblasts into the wound bed is dependent on the presence of adipocytes (Schmidt and Horsley, 2013). Interestingly, the absence of adipocytes in the wound and subsequent absence of fibroblasts was not detrimental to the contraction of the wound, nor to the epithelialization at first; however, during dermal remodeling, the size of the wound bed increased and the new epithelium reopened (Schmidt and Horsley, 2013), supporting cross-talk between mesenchymal and epithelial cells during tissue repair. Together, these studies provide a rationale for the rapid adipocyte deposition and the lack of hair follicles in newly epithelialized wounds (Ito et al., 2007; Driskell et al., 2013; Schmidt and Horsley, 2013).

Maturation—Upon completion of epithelialization, cellular proliferation and neovascularization cease, scar tissue forms and the wound enters the maturation phase, which lasts several months. Keratinocytes begin again their program of differentiation and granulation tissue turns into scar tissue through the continuous turnover of collagen. Collagen degradation is mediated by matrix metalloproteinases that are secreted by macrophages, epidermal and endothelial cells, and fibroblasts. Wounds slowly regain tensile strength over the ensuing months but never reach the same breaking strength as pre-wound. In fact, wounds are only 70% as strong as normal skin (Levenson et al., 1965). In addition, in human, a scar remains and hair follicles never repopulate the healed tissue (Martin, 1997; Gurtner et al., 2008). However, in the mouse, very large wounds, as well as wounds in which β-catenin was activated in the epidermis before wounding did show *de novo* hair follicle formation (Ito et al., 2007; Driskell et al., 2013). This suggests different regenerative capacities between the mouse and human.

The scar is the most visible remnant of the cutaneous wound with a wide range of phenotypes, yet healing occurs without a scar before birth. Murine embryos up to E15.5 exhibit scar-less wound healing (Fig. 1), presumably due to immature innate immune cells and the absence of an inflammatory response (Hopkinson-Woolley et al., 1994; Wulff et al., 2012; Chen et al., 2014).

Resolution of inflammation is a critical step for the wound to heal, and its failure results in chronic wounds. The flux of calcium rapidly returns to homeostatic levels through a yet-to-be discovered mechanism while neutrophil –produced myeloperoxidase is required to dampen the production of superoxides (Pase et al., 2012). Further clearing of inflammatory cells occurs through apoptosis of leukocytes and macrophage phagocytosis of neutrophils (Haslett, 1992). Macrophages themselves are deactivated by anti-inflammatory cytokines, glucocorticoids, cell-cell contacts and phagocytosis. In addition, some neutrophils and macrophages return to the vasculature and/or emigrate via lymphatic vessels (Mathias et al., 2006).

Differential wound healing: cutaneous versus oral wound healing

Several studies have demonstrated a marked difference between cutaneous and oral wound healing, most of which concluding that oral wounds heal with much less scarring than cutaneous wounds (Wijdeveld et al., 1991; Larjava et al., 2011). As wound healing can contribute to significant fibrosis and hypertrophic healing, comparing oral with cutaneous wound healing could provide insights into potential therapeutic avenues.

The main environmental difference between cutaneous and oral wound healing is that oral wounds are healing in the presence of saliva. Saliva contains many antibodies, growth factors, and chemokines, including vascular endothelial growth factor (VEGF) (Keswani et al., 2013), epidermal growth factor (EGF) (Noguchi et al., 1991), and basic fibroblastic growth factor (bFGF) (Kagami et al., 2000). Additional factors, including salivary antimicrobial peptide (AMP) and salivary leptin are thought to enhance oral wound closure (Groschl et al., 2005; Oudhoff et al., 2009). The human body makes over a liter of saliva per day, bathing the healing wounds in these, and many more growth factors which are thought to reduce the inflammation, and thus reduce the formation of scar following tissue repair.

Additional intrinsic differences in cutaneous vs oral wound healing include the following observations: i) Greater response to inflammatory stimuli evidenced by high IL-6 and TNF-α (Li et al., 1996), ii) Greater and prolonged expression of Tenasin-C in palatal wounds (Wong et al., 2009), iii) Reduced presence of mast cells, macrophages and neutrophils in oral wounds compared to cutaneous wounds (Kischer et al., 1978; Szpaderska et al., 2003; Mak et al., 2009), iv) Increased levels of Tgfβ3 (antifibrotic) and reduced levels of Tgfβ1 (profibrotic) in oral wounds (Schrementi et al., 2008; Eslami et al., 2009) and v) Increased angiogenesis and gingival-derived mesenchymal stem cells proliferation following oral injury (Zhang et al., 2010).

Collectively, these critical differences between cutaneous and oral wound healing could serve as targets to modulate wound healing in the future, including the use of Tgfβ3 and/or stem cell therapies.

Craniofacial development and cutaneous repair: similar, yet different, conserved mechanisms

It has been proposed and discussed elsewhere that embryogenesis of one tissue or organ and the repair of this same tissue during adulthood share common molecular mechanisms and gene regulatory networks (Ferguson et al., 1998; Wood et al., 2002; Martin and Parkhurst, 2004; Mitsiadis and Rahiotis, 2004; Aller et al., 2012). The parallel between the embryogenesis of one tissue and the repair of a different tissue, however, has not been addressed. Our first observation that patients with Van der Woude syndrome had worse surgical outcome, based on surgical wound healing complications, compared with patients with non-syndromic CL/P lead us to propose that IRF6 plays a role both in craniofacial development and cutaneous wound healing. As we confirmed our hypothesis both clinically and experimentally (Jones et al., 2010; Biggs et al., 2014), we postulated that this concept may be more global and that additional molecules would share commonalities with craniofacial development and wound healing.

The initial thought that one could generate a catalog of shared genes and regulatory network between cleft lip and palate and cutaneous wound healing is somewhat utopic. If the genetic contribution to proper palatogenesis is unquestionable, and the list of genes involved in cleft lip and palate established at about 300 (Jugessur et al., 2009), it has been challenging to generate a workable list of genes involved in wound healing, mainly because of the complexity of the process and the difficulties of obtaining relevant human samples. Nevertheless, a few studies have performed gene expression profiling of cutaneous wound healing (Deonarine et al., 2007; Roupe et al., 2010; Nuutila et al., 2012), or identified a genetic signature unique to non-healing wounds (Charles et al., 2008), yet none provided access to the full list of differentially-regulated genes. Using available information from these studies, we estimated the percentage of reported wound-healing genes that shared a role in CL/P. The results, presented in Table 2, show about 10% commonality in the gene regulatory network between the two processes. To extend this comparison further, we established a list of our top 35 candidate genes for CL/P and asked whether these genes were expressed in cutaneous tissues along with a potential role in wound healing (Table 1). About 50% of them shared a known role in both craniofacial development and wound repair, confirming strong commonality between the two biological processes. The notable difference in shared percentage of genes (Table 1 vs Table 2) may be due to the fact that CL/P occurs during embryonic development involving a broad gene regulatory network, while adult wound healing engages already differentiated tissues and a more narrow and differentiation-oriented gene regulatory network. From these two tables, we chose to discuss a few genes as part of regulatory networks involved in cellular migration and proliferation, two of the most relevant biological mechanisms critical to both the development of the palate and the healing of the skin.

The first gene regulatory network involves *TGFβ3*, *IRF6*, *ARHGAP29*, and *GRHL3*, all of which regulate cellular migration by modulating the actin cytoskeleton. Tgfβ3 induces rapid activation of RhoA GTPase and EMT *in vitro* (Kaartinen et al., 2002) and mice deficient for *Tgfβ3* exhibit a cleft palate (Kaartinen et al., 1995; Proetzel et al., 1995). Tgfβ3 was also required for epithelialization and granulation tissue maturation in a murine model of cutaneous wounds, while dispensable for keratinocyte migration *in vitro* (Le et al., 2012). In humans, genetic variants in *TGFβ3* are associated with CL/P (Lidral et al., 1998), and clinical trials using prophylactic TGFβ3 on cutaneous incisions demonstrated reduction of scarring compared to untreated incisions (Ferguson et al., 2009). Mice deficient for *Tgfβ3* also show reduced level of expression of Irf6, both in the medial edge epithelium (Xu et al., 2006) and in the skin (Le et al., 2012), supporting the model that *Irf6* is downstream of *Tgfβ3*.

IRF6 plays an unquestionable role in palatogenesis and human clefting disorders (Kondo et al., 2002; Zucchero et al., 2004; Ingraham et al., 2006; Richardson et al., 2006; Rahimov et al., 2008; de Lima et al., 2009). Recently, we reported a novel function for the Irf6 protein in regulating RhoA GTPase-dependent cellular migration mediated by Arhgap29, a specific inhibitor of RhoA (Biggs et al., 2014). In keratinocytes, lack of *Irf6* promotes the formation of actin stress fibers due to increased active RhoA in the cells and decreased level of Arhgap29 protein. Interestingly, the *ARHGAP29* human gene was identified as the etiologic

gene for the CL/P locus identified by genome-wide association on chromosome 1p22 (Leslie et al., 2012). A genetic variant in *ARHGAP29* was also associated with poor scar outcome in patient with CL/P (Smith et al., 2014), demonstrating the conservation of pathways regulating tissue repair and orofacial clefting.

Using the zebrafish as a model for craniofacial development, de la Garza *et al*. identified *grhl3* as a direct target of *irf6* (de la Garza et al., 2013). *Grainyhead* was discovered in *drosophila* as a critical regulator of the repair of the cuticle (Mace et al., 2005). Grhl3 is expressed in several epithelia, as well as in the periderm of the skin and of the oral cavity, and its expression is altered in the periderm of *Irf6*-deficient mice. The *Grhl3* gene is required for proper craniofacial development as mice deficient for this transcription factor exhibit cleft palate and oral adhesion (Peyrard-Janvid et al., 2014). Furthermore, patients with Van der Woude syndrome, who were lacking a detectable mutation in *IRF6*, harbored mutations in *GRHL3* (Peyrard-Janvid et al., 2014). Like *Irf6*, the *Grhl3* gene is also required for proper migration of keratinocytes. However, compared to *Irf6*, the absence of *Grhl3* led to decreased level of active RhoA and absence of actin stress fibers (Caddy et al., 2010). In fact, *RhoGEF19*, an activator of RhoA, was identified as the direct target of *Grhl3*. It is therefore interesting to think that two genes (*Irf6* and *Grhl3*), one a direct target of the other, both regulate Rho activity in opposite directions to give the same phenotype (migration defect and Van der Woude syndrome). It exemplifies how cellular migration and cytoskeletal dynamics are tightly controlled with positive and negative regulators.

The second gene regulatory network involves *WNT*, *TP63*, *IRF6*, *TFAP2*, and *c-MYC*, all of which regulate cellular proliferation. Several studies have demonstrated that mutations in *WNT* genes are associated with CL/P. The Wnt pathway regulates orofacial development via multiple downstream targets, including *Tgfß3*, *Lrp6*, *Irf6*, and *p63* (Song et al., 2009; He and Chen, 2012; Kurosaka et al., 2014). It is equally essential for cutaneous development, particularly for the maintenance of stem cells (Blanpain and Fuchs, 2009; Lim and Nusse, 2013; Lim et al., 2013). During wound healing, Wnt signaling affects both dermal and epidermal compartments (Amini-Nik et al., 2014). Interestingly, it contributes to the *de novo* regeneration of hair follicles in large cutaneous wounds (Ito et al., 2007) and enhanced Wnt signaling promotes cutaneous healing in a murine ear model (Whyte et al., 2013), suggesting a potential therapeutic application for this secreted protein.

p63 and *Irf6* operate downstream from Wnt signaling and within a regulatory loop to coordinate epithelial proliferation and differentiation during normal epidermal and palatal development (Mills et al., 1999; Yang et al., 1999; Ferretti et al., 2011). Disruption of this pathway because of mutations in *TP63* or *IRF6* causes CL/P in mice and humans (Brunner et al., 2002; Moretti et al., 2010; Thomason et al., 2010) and mutations in *P63* causes a variety of ectodermal dysplasias (Brunner et al., 2002). Although mice deficient for *p63* exhibited impaired wound healing (Koster et al., 2007), two patients with ankyloblepharon ectodermal defects-cleft lip and palate syndrome (AEC) appeared to heal properly following surgical repair of their cleft (Cabiling et al., 2007).

Tfap2 is a direct target of *p63* (Koster et al., 2006) and binds to an enhancer region of *Irf6* containing a genetic variant associated with CL/P (Rahimov et al., 2008). Human mutations

in *TFAP2* causes branchio-oculo-facial syndrome (OMIM #113620, (Milunsky et al., 2008)), and *Tfap2*-deficient mice fail to close their cranial neural tube (Zhang et al., 1996). Epidermal-specific removal of *Tfap2α* and/or γ causes disruption of proper epidermal differentiation (Guttormsen et al., 2008; Wang et al., 2008; McDade et al., 2012), yet a role for this family of transcription factor in cutaneous repair remains to be discovered.

c-Myc is also a downstream target of the *p63* gene (Wu et al., 2012). It regulates about 15% of all genes (Gearhart et al., 2007) and may be considered the universal regulator of cellular proliferation. Targeted expression of c-Myc in the epidermis alters keratinocyte proliferation and differentiation, and regulates epidermal homeostasis by depleting the stem cells from the interfollicular compartment (Waikel et al., 1999; Waikel et al., 2001; Frye et al., 2003). It has been found in keratinocytes of the leading edge of chronic wounds, where it inhibits epithelialization (Stojadinovic et al., 2005). c-Myc is activated by Tgfβ during growth of the palatal shelves, and neural crest cell deficiency of *c-Myc* causes skull frontal bone and middle ear ossicle developmental defects (Wei et al., 2007). Interestingly, *c-Myc* is physically the closest gene to the Chr. 8q24 cleft susceptibility locus, a 640 kb gene desert chromosomal location which was identified by genome-wide association studies in a population of European ancestry (Birnbaum et al., 2009; Beaty et al., 2010). A recent study in the mouse demonstrates that this interval contains long-range enhancers controlling *c-Myc* expression in the developing face (Uslu et al., 2014). Deletion of this interval or introduction of genetic variants associated with CL/P at this locus leads to alteration of facial morphology and low penetrance of CL/P in the mouse, supporting the hypothesis that the Chr. 8q24 susceptibility locus regulates *c-Myc* during craniofacial development.

Differences remain between the closure of a cutaneous wound and the embryogenesis of the palate. Do palatal epithelial cells display an “activated” phenotype identical to keratinocytes migrating during epithelialization? Are the signals sensed by keratinocytes touching each other at epithelial closure similar to signals sensed by epithelial cells from the palatal shelves touching each other and fusing? What drives palatal shelf movement – do cells “rise up” like a bridge, or treadmill or push like in collective cell migration? Answers to these questions will provide further insights into cutaneous wound healing and craniofacial development. Ultimately, as these two biological processes share common pathways and gene regulatory networks, a deeper understanding of one will inform the other to develop new therapeutic strategies.

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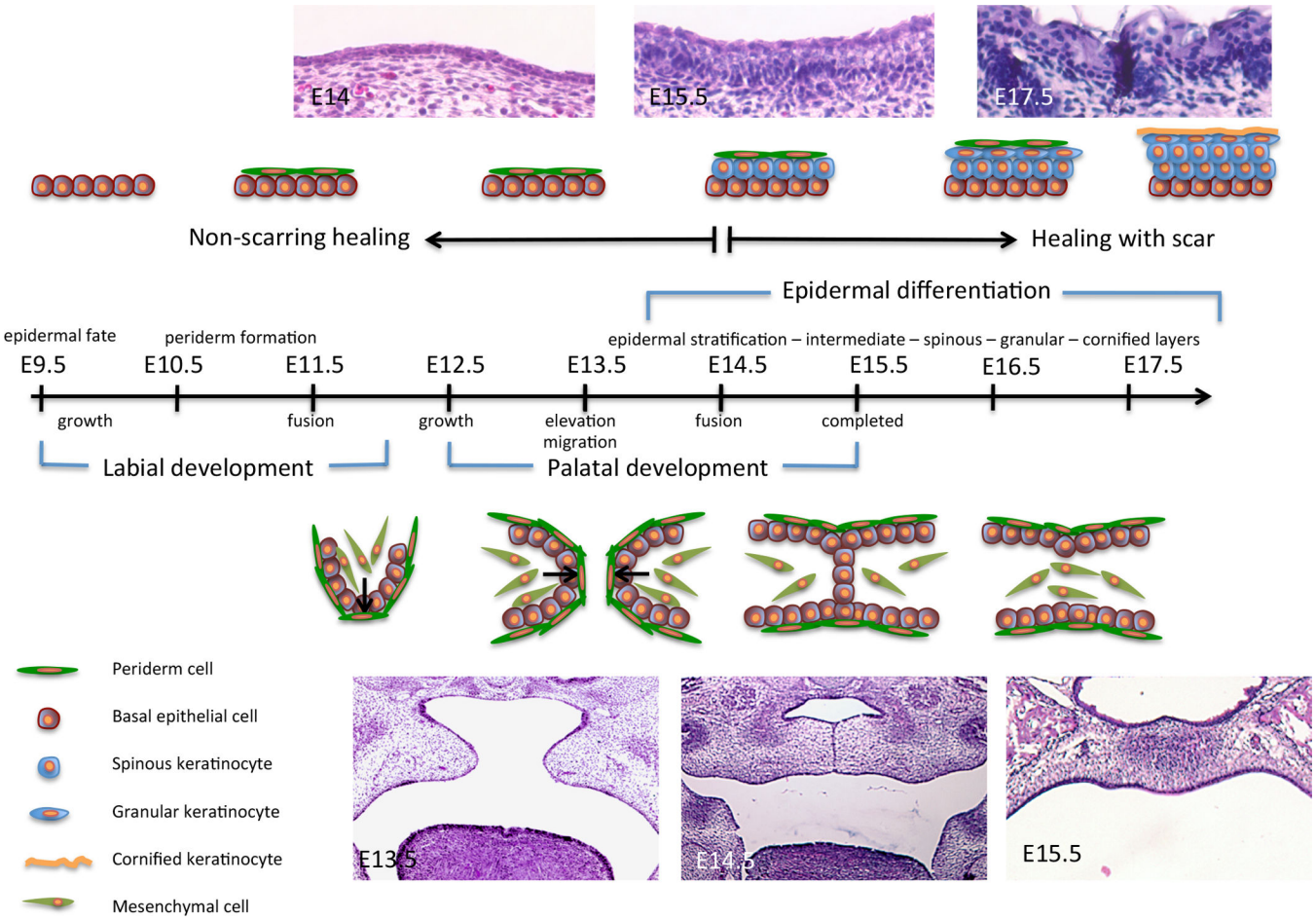

Figure 1.

Embryonic development landmarks of the epidermis and the palate in the mouse. Embryonic time scale from E9.5 to E18.5 is represented. Events related to epidermal development are depicted above the time scale. Events related to palatogenesis are depicted below the time scale.

Table 1

Genes associated with clefting[a] and their known role in cutaneous wound healing

Gene name	Expression in face	Role in CL/P	Expression in skin	Role in Wound Healing
RhoGTPase activating protein 29 (ARHGAP29)	In the oral epithelium (Leslie et al., 2012)	Associated with CL/P (Leslie et al., 2012)	In both dermis and epidermis (Biggs et al., 2014)	Genetic variant associated with poor scarring (Smith et al., 2014)
Bone Morphogenetic Protein 4 (BMP4)	In the epithelium, then the mesenchyme of the facial primordial (Francis-West et al., 1994)	Mutations in patients with cleft lip (Suzuki et al., 2009). Conditional knockout have cleft lip that spontaneously heal (Liu et al., 2005)	In the hair follicle, fibroblasts, melanocytes and keratinocytes (Yaar et al., 2006; Plikus et al., 2008)	Role in bone repair, no direct studies on cutaneous wound healing
Cystein-Rich Secretory Protein LCCL Domain Containing 2 (CRISPLD2)	In developing oropharynx and nasopharynx at e13.5, mandible at e14.5, and cartilage primordial of the nasal bones, palate, and tooth germs at e17.5 (Chiquet et al., 2007)	Associated with CL/P (Chiquet et al., 2007)	Not reported.	Not reported.
Fibroblast Groth Factor 8 (FGF8)	In medial and lateral nasal processes of e10.5 and e11.5 mice (Thomason et al., 2008)	Associated with CL/P (Riley et al., 2007)	Not reported.	In amphibian wound blastema (Endo et al., 2000)
Fibroblast Growth Factor Receptor 1 (FGFR1)	In the palatal epithelium (Britto et al., 2002)	Associated with CL/P (Riley et al., 2007)	In dermis and epidermis (Takenaka et al., 2002)	Upregulated in wound healing (Komi-Kuramochi et al., 2005)
Fibroblast Growth Factor Receptor 2 (FGFR2)	In the palatal epithelium (Britto et al., 2002)	Associated with CL/P (Riley et al., 2007)	In the basal layer of the epidermis (Takenaka et al., 2002) Knockout mice have thinner skin (Revest et al., 2001)	No change in expression during murine cutaneous wound (Komi-Kuramochi et al., 2005)
Forkhead/wingled-helix domain transcription factor (FOXE1)	In oral epithelia (palatal, nasal) (Dathan et al., 2002)	Associated with CL/P (Vieira et al., 2005) Knockout mice have cleft palate	In the epidermis and hair follicles (Eichberger et al., 2004)	Not evaluated
GLI-Kruppel family member GLI2 (GLI2)	In the palatal shelves (Mo et al., 1997)	Associated with CL/P (Vieira et al., 2005)	In the epidermis and hair follicles (Eichberger et al., 2004)	Not evaluated
Grainyheald like-3 (GRHL3)	In the oral cavity (Auden et al., 2006).	Mutations cause VWS (Peyrard-Janvid et al., 2014) Mice have abnormal periderm with oral adhesions and low penetrance cleft (Peyrard-Janvid et al., 2014)	In the periderm, ectoderm, and throughout the epidermis (Auden et al., 2006; de la Garza et al., 2013)	Embryonic wounds open after 24 hr assay (Caddy et al., 2010)
Interferon Regulatory Factor 6 (IRF6)	In the medial edge epithelium, palatal epithelium (Knight et al., 2006)	Mutations cause VWS and PPS (Kondo et al., 2002). Knockout mice have orofacial anomalies (Ingraham et al., 2006)	In the spinous layer of the epidermis (Ingraham et al., 2006; Richardson et al., 2006)	Patients with VWS have increased likelihood of worse surgical outcome (Jones et al., 2010)
Isthmin 1 (ISM1)	In oral mucosa (Valle-Rios et al., 2014)	Not reported	In human skin (Valle-Rios et al., 2014)	Not reported
Jagged 2 (JAG2)	In the oral epithelium (Casey et al., 2006)	Associated with CL/P (Vieira et al., 2005) Knockout mice have	In fibroblasts	Not reported.

Gene name	Expression in face	Role in CL/P	Expression in skin	Role in Wound Healing
		craniofacial defects (Jiang et al., 1998)		
LIM homeobox 8 (LHX8)	In mesenchyme of maxillary and mandibular processes during palatogenesis (Zhao et al., 1999)	Associated with CL/P (Vieira et al., 2005) Knockout mice have cleft palate (Zhao et al., 1999)	Not reported.	Not reported.
v-Maf musculo aponeurotic fibrosarcoma (MAFB)	In the oral epithelium (Beaty et al., 2010)	Associated with CL/P (Beaty et al., 2010)	In the suprabasal layer of the epidermis (Ogata et al., 2004) and hair follicles (Miyai et al., 2010)	Not reported
Muscle Segment Homeobox Gene of Drosophila (MSX1)	Restricted to the anterior of the first upper molar site in the palatal mesenchyme (Zhang et al., 2002)	Associated with CL/P (Jezewski et al., 2003) Knockout mice have cleft palate (Satokata and Maas, 1994)	In embryonic and adult skin, epithelial derived structures (Stelnicki, 1997) (Vieira et al., 2005) In the hair follicle (Ma et al., 2003)	Not evaluated
Muscle Segment Homeobox Gene of Drosophila (MSX2)	In the developing palate (Winograd et al., 1997)	Point mutations associated with CL/P (Vieira et al., 2005) Knockout mice have a cleft palate (Winograd et al., 1997)	In the hair follicle (Ma et al., 2003)	Not evaluated
Paired box 7 (PAX7)	Not reported	Maternal SNPs associated with CL/P (Beaty et al., 2013; Butali et al., 2013)	Not reported	Lineage tracing show 25% of dermal cells in the healing wound Pax7 expressing progeny (Amini-Nik et al., 2011)
Paired box 9 (PAX9)	In the lingual epithelium, medial edge epithelium, and palatal mesenchyme (Peters et al., 1998)	Associated with CL/P Knockout mice exhibit a cleft palate (Peters et al., 1998)	Not expressed in the epidermis (Peters et al., 1998)	Not reported.
Platelet-derived Growth Factor C (PDGF C)	In the epithelium of the palatal shelves (Ding et al., 2004)	Associated with CL/P (Choi et al., 2008) Knockout mice have cleft palate (Ding et al., 2004)	In basal layer of the epidermis and hair follicle (Ding et al., 2000)	Promotes wound healing (Gilbertson et al., 2001)
Patched Homolog 1 (PTCH1)	In neural crest-derived mesenchyme of facial prominences (Metzis et al., 2013)	Associated with CL/P (Mansilla et al., 2006) Neural crest specific knockout exhibit cleft lip (Metzis et al., 2013)	In dermal papillae and a few follicular and interfollicular keratinocytes (Morgan et al., 1998)	Absence of Ptch1 in basal keratinocytes results in formation of basal cell carcinomas in healing wounds (Kasper et al., 2011)
Poliovirus Receptor-Related 1 (PVRL1, nectin-1)	In the medial edge epithelium (Suzuki et al., 2000)	Mutations in human cause autosomal recessive ectodermal dysplasia associated with clefting (CLPED-1) (Suzuki et al., 2000)	In all the layers of the epidermis, stronger in the spinous layer; regulates loricrin expression via ERK pathway (Wakamatsu et al., 2007)	Not reported.
Poliovirus Receptor-Related 2 (PVRL2)	Not reported.	Associated with CL/P (Nikopensius et al., 2011)	Not reported.	Not reported.
Receptor-Interacting Serine-threonine Kinase 4 (RIPK-4)	Not reported	Mutations cause Bartsocas-Papas Syndrome (Mitchell et al., 2012) and recessive form of popliteal	In epidermis and necessary for proper epidermal differentiation (Holland et al., 2002; Adams et al., 2007; Rountree et al., 2010).	Downregulated within 1 hour of wounding and remains low throughout healing process (Adams et

Gene name	Expression in face	Role in CL/P	Expression in skin	Role in Wound Healing
		pterygium (Kalay et al., 2012)		al., 2007), regulator of inflammation (Rountree et al., 2010)
Receptor-like Tyrosine Kinase (RYK)	In lingual and palatal mesenchyme (Halford et al., 2000)	Associated with CL/P (Watanabe et al., 2006) Knockout mice have cleft palate (Halford et al., 2000)	In the basal layer of the epidermis and hair follicles (Serfas and Tyner, 1998)	Not reported.
SATB homeobox 2 (SATB2)	In the developing palate (Fitzpatrick et al., 2003)	Association with CL/P (Fitzpatrick et al., 2003)	Not known	Not evaluated
SKI-protooncogene (SKI)	At low levels in all tissues, but increased expression in cranial neural cells (Berk et al., 1997)	Associated with CL/P (Vieira et al., 2005) Knockout mice exhibit exencephaly and cleft lip and palate (Berk et al., 1997)	Not expressed in normal skin (Liu et al., 2006)	Upregulated after wounding during epithelialization and maturation phase in fibroblasts and less so in epithelial cells (Liu et al., 2006)
Sonic Hedgehog (SHH)	In the ectoderm of the facial primordial (Hu and Helms, 1999)	Mutations cause CL/P (Orioli et al., 2002; Porter, 2006)	In the hair follicle (Chuong et al., 2000)	Promotes wound healing (Le et al., 2008)
Sprouty Homolog 2 (SPRY2)	In the epithelium and mesenchyme of the developing palate (Matsumura et al., 2011)	Point mutations may cause CL/P (Vigano et al., 2006) Knockout mice have a cleft at low penetrance (Matsumura et al., 2011)	Not reported	Downregulates angiogenesis during resolution of wound healing (Wietecha et al., 2011)
T-Box 10 (TBX10)	In the hindbrain but not in the face (Bush et al., 2003)	Associated with CL/P (Vieira et al., 2005) Spontaneous mutant 'Dancer' (Dc), which is the result of ectopic Tbx10 has a cleft palate (Trasler et al., 1984)	Not reported	Not reported
Transcription Factor AP2 (TFAP2a)	In the facial mesenchyme (Byrne et al., 1994; Moser et al., 1997)	Mutations cause branchio-oculo-facial syndrome (Milunsky et al., 2008) Knockout mice have severe craniofacial anomalies (Schorle et al., 1996)	Alpha and gamma in the epidermis and hair follicle (Byrne et al., 1994)	Not evaluated
Transforming Growth Factor Beta 3 (TGFB3)	In the medial edge epithelium (Fitzpatrick et al., 1990)	Mutations and association with CL/P (Lidral et al., 1998) Knockout mice have cleft palate (Kaartinen et al., 1995)	In all layers of the epidermis (Levine et al., 1993)	Important for scarless wound healing (Ferguson and O'Kane, 2004). Recombinant form in clinical trial (Ferguson et al., 2009).
Tumor Protein 63 (TP63)	In the basal cells of the ectoderm in nasal processes, medial edge epithelium, palatal epithelium (Thomason et al., 2008)	Mutations and association with CL/P (McGrath et al., 2001) Knockout mice have orofacial anomalies (Mills et al., 1999; Yang et al., 1999)	In the basal layer of the epidermis (Koster et al., 2004)	No wound problem in cleft repair of p63 ectodermal dysplasia (Cabiling et al., 2007) Splice variant specific function based on spatiotemporal expression in murine wounds (Bamberger et al., 2005)
Ventral anterior homeobox 1 (VAX1)	In the first branchial arch and palate (Zhao et al., 2010)	Associated with CL/P (Mangold et al., 2010; Butali et al., 2013)	Not reported	Not reported

Gene name	Expression in face	Role in CL/P	Expression in skin	Role in Wound Healing
Wingless type 5a (WNT5a)	In the mesenchyme of palatal shelves at e13.5 in a gradient from high to low in an anterior-posterior direction (He et al., 2008)	SNPs associated with NSCLP (Chiquet et al., 2008) Knockout mice have cleft of secondary palate (He et al., 2008)	In the dermis and inner and outer root sheaths of mature hairs (Reddy et al., 2001)	Upregulated in the mesenchyme after epithelialization (Fathke et al., 2006)
Wingless type 9b (WNT9b)	In the maxillary, medial and lateral nasal ectoderm from e9.5 through e11.5 (Lan et al., 2006)	Associated with CL/P (Chiquet et al., 2008) Knockout mice have cleft palate with incomplete penetrance (Carroll et al., 2005)	Not expressed in skin (Reddy et al., 2001)	Not reported

[a]These 35 genes are not a comprehensive list of clefting genes, but rather a list of genes that are deemed most important and/or most interesting genes as research in the field of craniofacial research moves forward. Its strength is that it was elaborated based on the contribution of these genes either in craniofacial development, in the function of craniofacial structures or in human genetics.

Table 2

Comparison of overlapping genes between wound healing and CL/P

Ref	Wound healing genes[a]	Wound healing and clefting genes[b]
Charles et al (Charles et al., 2008)	58	7 (12%)
Deonarine et al (Deonarine et al., 2007)	250	18 (7%)
Nuutila et al (Nuutila et al., 2012)	42	4 (9%)
Eming et al (Eming et al., 2010)	60	4 (7%)
Roupé et al (Roupe et al., 2010)	61	3 (5%)

[a] Number of genes reported up or down regulated in each of the cited wound healing studies

[b] From the genes reported in the previous column, we filtered for genes that have a role in human clefting and/or exhibit a cleft palate in a mouse model deficient for that gene. Data is also expressed in percentage and shown under parenthesis.