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Cellular and Molecular Interactions Regulating Skeletogenesis

Céline Colnot*

Department of Orthopedic Surgery, University of California, San Francisco, San Francisco, California 94143-0514

Abstract Skeletal development involves complex coordination among multiple cell types and tissues. In long bones, a cartilage template surrounded by the perichondrium is first laid down and is subsequently replaced by bone marrow and bone, during a process named endochondral ossification. Cells in the cartilage template and the surrounding perichondrium are derived from mesenchymal cells, which condense locally. In contrast, many cell types that make up mature bone and in particular the bone marrow are brought in by the vasculature. Three tissues appear to be the main players in the initiation of endochondral ossification: the cartilage, the adjacent perichondrium, and the invading vasculature. Interactions among these tissues are synchronized by a large number of secreted and intracellular factors, many of which have been identified in the past 10 years. Some of these factors primarily control cartilage differentiation, while others regulate bone formation and/or angiogenesis. Understanding how these factors operate during skeletal development through the analyses of genetically altered mice depends on being able to distinguish the effect of these molecules on the different cell types that comprise the skeleton. This review will discuss the complexity of skeletal phenotypes, which arises from the tightly regulated, complex interactions among the three tissues involved in bone development. Specific examples illustrate how gene functions may be further assessed using new approaches including genetic and tissue manipulations. J. Cell. Biochem. 95: 688–697, 2005.

Key words: osteoblast; chondrocyte; endothelial cell; renal capsule transplantation; null mutant mouse; Indian Hedgehog; Runx2; VEGF

Tissue interactions govern most developmental processes, from early patterning events, to cell differentiation, through morphogenesis and finally growth of the many organs in the embryo. Formation of the skeleton is no exception, and these interactions begin during the earliest phases of skeletal development. Before the initiation of limb skeletogenesis, epithelial–mesenchymal interactions control the outgrowth and patterning of the limb bud. Mesenchymal cells then receive unknown signals that induce them to form condensations in specific areas of the limb, which correspond to the future skeletal elements [Hall and Miyake, 2000; Mariani and Martin, 2003]. Cells that are located in the center of the condensation differentiate into chondrocytes, while cells around the condensation elongate and form the perichondrium. Interactions between the cartilage and the perichondrium regulate subsequent steps in chondrogenesis and osteogenesis. The vascular endothelium also plays an important role during long bone development. From the formation of the condensations to the onset of endochondral ossification, the vasculature interacts closely with the cartilage and perichondrium [Eames et al., 2003]. Initially, blood vessels occupy the entire field of the limb bud but they regress at the site of cartilage condensation [Feinberg et al., 1986]. This vascular regression is necessary for normal chondrogenesis to take place [Yin and Pacifici, 2001]. Later during skeletogenesis, blood vessels must invade the perichondrium and the cartilage template to allow ossification and the formation of the marrow cavity [Karsenty and Wagner,

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*Correspondence to: Céline Colnot, Department of Orthopedic Surgery, University of California, San Francisco, San Francisco, CA 94143-0514. E-mail: colnot@itsa.ucsf.edu

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2002]. Cartilage, bone, and vascular development occur concomitantly in both time and space, which makes it a challenge to dissect the cellular and molecular mechanisms regulating skeletogenesis. A growing number of genes have been implicated in bone development, however their precise role in each tissue remains difficult to elucidate. A significant obstacle for understanding precise gene functions in distinct cell types is that many of the genes involved are expressed in multiple sources such as cartilage, bone, and sometimes even in the blood vessels [Colnot and Helms, 2001; Pola et al., 2001]. Therefore, perturbing the function of one molecule may have direct and indirect effects on several aspects of skeletal development. Secreted molecules originating within one tissue may diffuse and influence the development of adjacent tissues making it even more difficult to separate the influence of various molecules on each cell type. Another challenge is to elucidate the precise origins of cells participating in bone development, since they may differentiate from precursor cells generated locally and may be delivered to the developing skeletal elements by the vasculature. This review will illustrate the complex cellular and molecular interactions regulating skeletogenesis after the condensations have formed.

INTERPLAY BETWEEN CARTILAGE AND PERICHONDRIUM

Cartilage differentiation influences perichondrium maturation and vice versa. As the initial boundaries between the cartilage and the perichondrium are being established, cells in these two compartments start to differentiate into chondrocytes and osteoblasts, respectively. Whether the initiation of chondrogenesis precedes the initiation of osteogenesis or both occur simultaneously is not known. Subsequent steps of differentiation are clearly synchronized between the two tissues, such as chondrocyte hypertrophy, which is closely linked to the conversion of perichondrium to periosteum [Chung et al., 2001; Colnot and Helms, 2001]. In fact, multiple factors produced by chondrocytes and involved in chondrocyte proliferation, maturation, and hypertrophy have also been shown to regulate the conversion of perichondrium to periosteum [Karsenty and Wagner, 2002]. In return, signals from the perichondrium control the rate of chondrocyte differentiation [Kronenberg, 2003]. As a consequence, removal of the perichondrium has an effect on chondrocyte proliferation and hypertrophy [Long and Linsenmayer, 1998; Colnot et al., 2004].

Various signaling molecules control the interdependent development of cartilage and perichondrium (Fig. 1). A prime example of the molecular signaling between cartilage and perichondrium is illustrated by the Indian hedgehog (Ihh) signaling pathway. Ihh is a secreted molecule produced by pre-hypertrophic and hypertrophic chondrocytes, and may directly and indirectly control both cartilage and perichondrium maturation [Vortkamp et al., 1996]. Ihh can regulate chondrocyte proliferation and differentiation directly via its receptor Patched (Ptch), which is expressed in the cartilage [Lanske et al., 1996; Vortkamp et al., 1998; St-Jacques et al., 1999; Long et al., 2001]. Ihh can indirectly regulate chondrocyte development through induction of intermediate molecules in the perichondrium, where cells also express Ptch. For example, as a result of activating the Hh pathway, Parathyroid hormone related-peptide (PTHrP) is upregulated in the perichondrium and acts on its receptor located in pre-hypertrophic chondrocytes to inhibit hypertrophy [Lanske et al., 1996; Vortkamp et al., 1996]. Other molecules such as transforming growth factor-beta (TGFβ) act as a signal relay between Ihh and PTHrP in the regulation of cartilage hypertrophy [Alvarez et al., 2001, 2002].

Whether Ihh signaling influences perichondrial maturation directly or indirectly is uncertain. By signaling through Ptch in the cartilage and the perichondrium, Ihh not only regulates cartilage differentiation, but also controls the differentiation of osteoblast precursors in the perichondrium. The fact that $Ihh^{-/-}$ long bones never ossify may therefore be due to the direct absence of Ihh signaling in the perichondrium or indirectly to a cartilage defect [St-Jacques et al., 1999]. Several lines of research suggest a direct effect of Ihh on osteoblast precursors. One elegant in vivo study has made use of two null mutant mice, one lacking the *Ihh* gene and the other the *Pthrp-Receptor* (*Pthrp-R*) gene. *Pthrp-*$R^{-/-}$ mice exhibit a growth plate anomaly where the rate of chondrocyte differentiation is accelerated [Lanske et al., 1996]. In chimeric mice composed of wild type and *Pthrp-*$R^{-/-}$ cells, *Pthrp-*$R^{-/-}$ hypertrophic chondrocytes are found in the proliferating zone of the growth

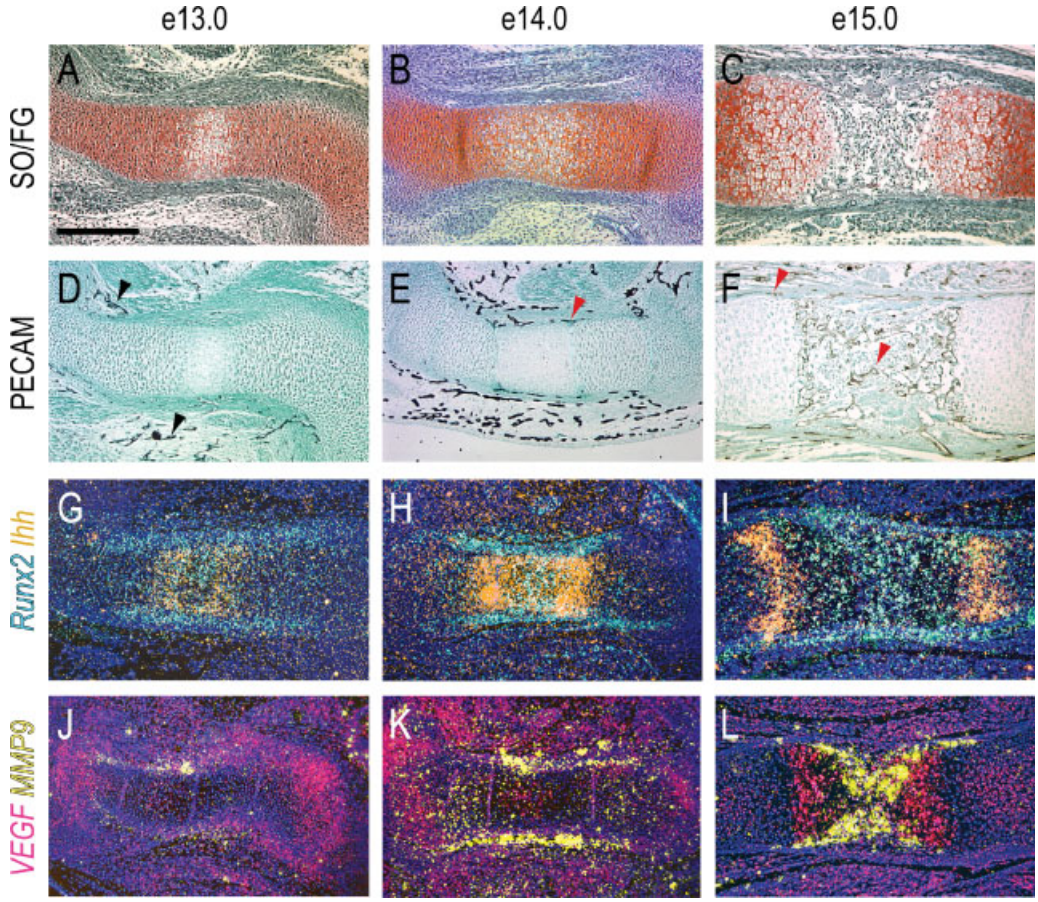

Fig. 1. Organization of blood vessels and gene expression profiles during the initiation of endochondral ossification. **A–C**: Safranin-O/Fast Green (SO/FG) staining on longitudinal section of e13.0, e14.0, and e15.0 mouse stylopod elements. **D–F**: Platelet endothelial cell adhesion molecule (PECAM) immunostaining shows that blood vessels first surround the skeletal elements (e13.0, black arrowheads) and then invade the perichondrium (e14.0, red arrowheads), before invading the hypertrophic cartilage (e15.0, red arrowheads). In situ hybridization signals for (**G–I**) *Indian hedgehog* (*Ihh*, orange), *Runx2* (blue), (**J–L**): *vascular endothelial growth factor* (*Vegf*, purple) and *Matrix metalloproteinase 9* (*MMP9*, yellow) are shown on adjacent sections. Scale bar = 250 μm.

plate because they are still capable of differentiation faster regardless of the wild type surrounding environment [Chung et al., 1998]. These ectopic hypertrophic chondrocytes producing Ihh can induce premature ossification of the adjacent perichondrium. However, if these ectopic hypertrophic cells are devoid of Ihh (*Ihh*;*Pthrp-R*$^{-/-}$ double mutant cells), they cannot stimulate the formation of the bony collar, indicating that Ihh is required locally to induce the differentiation of osteoblasts in the perichondrium [Chung et al., 2001]. Further support for a direct effect of Ihh on osteoblasts was provided by the conditional inactivation of the Ihh signal-transducer *Smoothened* (*Smo*) in the *Col2a1* domain [Long et al., 2004]. Since the *Col2a1* expression domain is not completely restricted to cartilage cells and extends into the perichondrium during the early condensation stages of bone development, *Smo* was also partially inactivated in the perichondrium. Consequently, the inner layer of the perichondrium failed to ossify, indicating a direct requirement for Ihh signaling in the perichondrium for osteoblast differentiation. Nevertheless, this approach did not facilitate an understanding of the role of Ihh on a single tissue, since the inactivation spanned both cartilage and perichondrium.

Dissociating the role of key regulators of endochondral ossification in the cartilage from their role in the perichondrium is also challenging, when these molecules are expressed in both cartilage and perichondrium, such as *Runx2* [Kim et al., 1999]. The most obvious defect of *Runx2*$^{-/-}$ embryos is the absence of bone [Komori et al., 1997; Otto et al., 1997], which was initially attributed to the fact that

Runx2 directly regulates the expression of osteoblast-specific genes such as *Osteocalcin* (*Oc*), *Osteopontin* (*Op*), *Osteonectin*, and *Matrix metalloproteinase 13* [Ducy et al., 1997; Komori et al., 1997; Jiménez et al., 1999]. As *Runx2* expression is not limited to osteoblasts and is also highly expressed in chondrocytes, the existence of a cartilage defect in $Runx2^{-/-}$ embryos was not surprising [Ferguson et al., 1999; Inada et al., 1999; Kim et al., 1999]. Therefore, the lack of bone that is the hallmark of the *Runx2* null phenotype might be caused in part by a defect in cartilage maturation.

A large number of molecules have been identified as components of the interplay between the cartilage and the perichondrium. Members of the bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and Wnt families are expressed by various cell types in developing bones and regulate chondrocyte differentiation and bone growth by acting through their receptors in the growth plate [Deng et al., 1996; Zou et al., 1997; Mancilla et al., 1998; Yazaki et al., 1998; Grimsrud et al., 1999; Weksler et al., 1999; Hartmann and Tabin, 2000; Minina et al., 2001, 2002; Fischer et al., 2002]. These signaling pathways may act in parallel or be interconnected. Indeed, FGFs and BMPs have been proposed to act upstream of Ihh, while Wnt have been shown to act downstream of Ihh [Naski et al., 1998; Liu et al., 2002; Minina et al., 2002; Hu et al., 2005]. The interplays among these molecules remain to be clearly identified.

A THIRD PLAYER: ANGIOGENESIS

Another level of complexity in the tissue interactions regulating long bone development is angiogenesis. The invasion of the perichondrium and cartilage by blood vessels is required for endochondral ossification, as illustrated by the fact that genetic, biochemical, or mechanical disruption of angiogenesis all perturb bone formation [Fenwick et al., 1997; Gerber et al., 1999; Zelzer et al., 2001, 2002; Maes et al., 2002; Colnot et al., 2004]. Angiogenesis permits the degradation of hypertrophic cartilage, a prerequisite for endochondral ossification and the establishment of the marrow cavity. A key angiogenic factor in endochondral ossification is vascular endothelial growth factor (VEGF). VEGF is expressed in both cartilage and perichondrium and is highly upregulated in hypertrophic cartilage prior to blood vessel invasion [Colnot and Helms, 2001; Zelzer et al., 2001]. Inactivation of VEGF inhibits or delays the invasion of perichondrium and hypertrophic cartilage by blood vessels and impairs subsequent steps of cartilage removal and endochondral ossification [Gerber et al., 1999; Maes et al., 2002, 2004; Zelzer et al., 2002, 2004]. By facilitating the proper vascularization of the perichondrium and direct cell–cell interactions between endothelial cells and osteoblasts, VEGF may indirectly regulate osteoblast differentiation [Zelzer et al., 2002; Colnot et al., 2004]. VEGF attracts blood vessels to the primary ossification center, and these vessels deliver important cell types and soluble factors for endochondral ossification. For example, osteoclasts/chondroclasts are recruited to the site of vascular invasion and participate in the initiation of endochondral ossification [Engsig et al., 2000].

The osteoclasts/chondroclasts play a central role in bone remodeling, though their role during bone development is less well understood. Osteoclasts/chondroclasts located at the chondro-osseous junction produce factors regulating bone formation, such as matrix metalloproteinase 9 (MMP9) [Vu et al., 1998]. By cleaving denatured collagens in the cartilage matrix and various other substrates, MMP9 may clear a path for blood vessels and make available growth factors that are sequestered in the matrix. These molecules may themselves regulate chondrocyte and/or osteoblast differentiation, as well as angiogenesis. As a consequence of inactivating *MMP9*, terminal differentiation of cartilage is delayed, further illustrating the effect of perturbing the initiation of angiogenesis and osteoclasts/chondroclasts recruitment on cartilage development [Vu et al., 1998; Engsig et al., 2000].

VEGF was first characterized as a mediator of angiogenesis, but other factors that were initially identified to influence chondrocyte and osteoblast differentiation have subsequently been shown to regulate angiogenesis. In addition to defects in cartilage and perichondrium, $Runx2^{-/-}$ and $Ihh^{-/-}$ embryos have a deficiency in vascular invasion. The bases for the vascular phenotypes in each of these mouse strains are different [St-Jacques et al., 1999; Zelzer et al., 2001]. $Runx2^{-/-}$ mutant cartilage elements do not become invaded by blood vessels because the late stages of chondrocyte hypertrophy are blocked, which is illustrated by the lack

of *Vegf* expression [Zelzer et al., 2001]. Conversely, $Ihh^{-/-}$ chondrocytes can undergo late hypertrophy, terminal differentiation and induce angiogenesis, however the $Ihh^{-/-}$ vasculature is unable to expand and support the formation of a marrow cavity [Colnot et al., 2005]. The resulting bone phenotypes observed in $Runx2^{-/-}$ and $Ihh^{-/-}$ mutants consequently result from a combination of defects in the cartilage, the perichondrium, and the vasculature. Altogether, these findings show that key regulators in long bone development are difficult to discern independently in separate tissues or cell types. These factors can be viewed as coordinators of the development of the three tissues that comprise skeletal elements.

DISSECTING APART CHONDROGENESIS, OSTEOGENESIS, AND ANGIOGENESIS

In order to further dissect the role of these coordinators of skeletal development, a number of new approaches are being explored. The examples cited above illustrate how genetic mutations affecting bone development can have pleiotropic effects and generate phenotypes difficult to elucidate. Inactivation of genes in a tissue specific manner will help separate the various roles of molecules not only during skeletogenesis, but also in the development of numerous organs during early embryogenesis. For example, conventional inactivation of *Vegf* causes early embryonic lethality, preventing the analysis of its function in late developmental stages when it is required for skeletogenesis [Carmeliet et al., 1996, 1999; Ferrara et al., 1996]. To target *Vegf* inactivation to the skeleton, the floxed-*Vegf* allele was excised using the CRE enzyme expressed under the control of the human *Collagen2a1* (*Col2a1*) promoter [Haigh et al., 2000]. This led to the deletion of *Vegf* wherever the *Col2a1* promoter was active, however, expression was not restricted in the cartilage. *Col2a1* expression is also observed in the developing eye, epidermis, and heart, hence, *Col2a1-CRE* mediated deletion of *Vegf* occurred in these tissues as well as in chondrogenic tissues [Cheah et al., 1991; Haigh et al., 2000]. In fact, defects in the heart and blood vessels caused lethality at mid-gestation. The embryos that survived until e17.5 exhibited skeletal defects reminiscent of the phenotypes of both $MMP9^{-/-}$ mice and neonatal mice treated with soluble VEGF-Receptor (mFlt-IgG), where hypertrophic chondrocytes accumulated in the growth plate [Vu et al., 1998; Gerber et al., 1999]. These results demonstrated the importance of VEGF in regulating growth plate angiogenesis that is necessary for the removal of hypertrophic chondrocytes, though they could not assess a possible role of VEGF in chondrocyte differentiation. Inactivation of *Vegf* has now been carried out using another, presumably more restricted, *Col2a* promoter and induced massive cell death of chondrocytes in the epiphysis of developing long bones, revealing an important role for VEGF in chondrocyte survival [Zelzer et al., 2004].

Other targeted gene recombinations revealed the distinct functions of the three *Vegf* isoforms. While the matrix bound isoform of the protein, VEGF188, is essential for metaphyseal vascularization; the soluble form, VEGF120, regulates chondrocyte survival and epiphyseal cartilage angiogenesis [Maes et al., 2002, 2004; Zelzer et al., 2004]. VEGF164 can be both soluble and matrix bound, therefore, mediating both functions; and may also act directly on chondrocytes through the Neuropilin-1 receptor [Soker et al., 1998; Maes et al., 2004; Zelzer et al., 2004]. Although, genetic manipulations have allowed the separation of VEGF functions on chondrocytes versus blood vessels, its in vivo effect on osteoblasts remains to be pursued. VEGF may directly regulate osteoblast differentiation since VEGF receptors are expressed not only on endothelial cells and chondrocytes, but also on osteoblasts [Deckers et al., 2000; Street et al., 2002; Zelzer et al., 2002]. Inactivating the VEGF receptors in osteoblasts only would address this question.

When using conditional gene inactivation approaches, detailed evaluation of the specific tissues/cells displaying CRE-recombination will be crucial in order to carry out analyses of skeletal phenotypes at the cellular level, in particular to identify cell autonomous mechanisms. Variability in the extent of the domain where CRE is active in vivo may exist depending on the promoter fragment and will have to be addressed. Although, some promoters may not provide restricted expression to single tissues or cell types, a fragment of the same promoter may do so. The examples of the conditional inactivation of *Smo* or *Vegf* in the *Col2a1* domain discussed above demonstrate the difficulty in finding promoters exhibiting the required specificity. During later stages of development,

tissue boundaries are delimited by gene expression patterns, however, these boundaries are established progressively throughout development. For example, *Col2a1*, which is exclusively found in cartilage in late stages of skeletogenesis, is not restricted to the chondrogenic lineage at earlier stages. When *Col2a1* is first upregulated in cartilage condensations, the separation between the cartilage core of the condensation and the perichondrium is not complete. Therefore, the target gene may likely become inactivated in cells forming the cartilage template as well as in cells contributing to the perichondrium. Similarly, *Collagen type 1* is restricted to the perichondrium when the process of endochondral ossification is well advanced, but is expressed in a more diffuse and broad pattern throughout limb mesenchyme at early stages. The use of inducible tissue specific promoters may help overcome this problem, which can be activated once the gene expression patterns become more tightly restricted. Additionally, tissue specific markers are not widely available. While some molecules become restricted to cartilage, such as *Collagen type 2 or 10*, no perichondrial specific marker is available. *Collagen type 1*, *Neuropilin-2*, *Patched*, *Gli1*, *Runx2*, and *Osteopontin* are all potential candidates, but closer examination reveals that none are restricted enough. Known perichondrial markers such as *Runx2* and *Osteopontin* are also found in late hypertrophic chondrocytes. Other markers such as *Osteocalcin* are expressed only in a subset of cells in the perichondrium, the differentiating osteoblasts.

The lack of strict lineage or tissue specific markers to exploit tissue-specific gene inactivation using CRE recombinase may be circumvented in future investigations by using a combination of gene inactivation and tissue manipulation to dissect the molecular basis of skeletal tissue interactions. Renal capsule transplantation has previously been used to characterize tissue interactions that govern the development of teeth, lungs, and the urogenital tract [Norman et al., 1986; Ferguson et al., 2001; Vu et al., 2003]. Similarly, cartilage templates can be dissected from mouse embryos and transplanted to the renal capsule of syngenic mice, where all aspects of bone development are recapitulated [Colnot et al., 2004]. Using this system, embryonic tissues can be manipulated and their development can be followed through late stages of endochondral ossification, such as vascular invasion, a feat that cannot be achieved in culture. Mutant skeletal elements may be harvested and placed in a wild type host or vice versa, which create chimeric situations. In these cases, the cartilage and perichondrium are from one genetic background while the vasculature, provided by the host environment, is from another genetic background (Fig. 2). Furthermore, cartilage and perichondrium from mutant embryos can be separated and recombined before transplantation to the host kidney capsule. These strategies may better elucidate the function of molecules that synchronize cartilage differentiation, perichondrial maturation, and vascular invasion.

ORIGINS OF SKELETAL STEM CELLS DURING DEVELOPMENT

More detailed cell lineage analyses in wild type and mutant contexts will also be performed, thanks to the identification of tissue specific promoters and the use of tissue recombination approaches. For example, the renal capsule transplant system can also be used to follow cells in vivo to elucidate the contributions of the perichondrium and the skeletal vascular endothelium to endochondral ossification (Fig. 2) [Colnot et al., 2004]. In this case, tissue manipulation can be combined with a genetic approach to follow the fate of cells derived from the perichondrium and from the vasculature. This approach brings a better understanding of the sequence of events leading to initial vascular invasion of hypertrophic cartilage by demonstrating that the first wave of endothelial cells found in the ossification center comes from the perichondrium. A second wave of endothelial cells is derived from vessels surrounding skeletal elements [Colnot et al., 2004]. These results build a context in which to study the molecular regulation of skeletal angiogenesis and the interactions between blood vessels and bone cells.

The osteoblast lineage remains the most intricate to study. Multiple sources of osteoblasts have been proposed in the literature without bringing a clear idea about their exact origin(s) during long bone development. In the limb bud, mesenchymal cells participating in the cartilage condensations appear to be the main source of osteoblasts [Bruder and Caplan, 1990; Nakahara et al., 1990]. Osteoblasts may also arise from satellite cells residing in the surrounding soft tissues [Asakura et al., 2001]

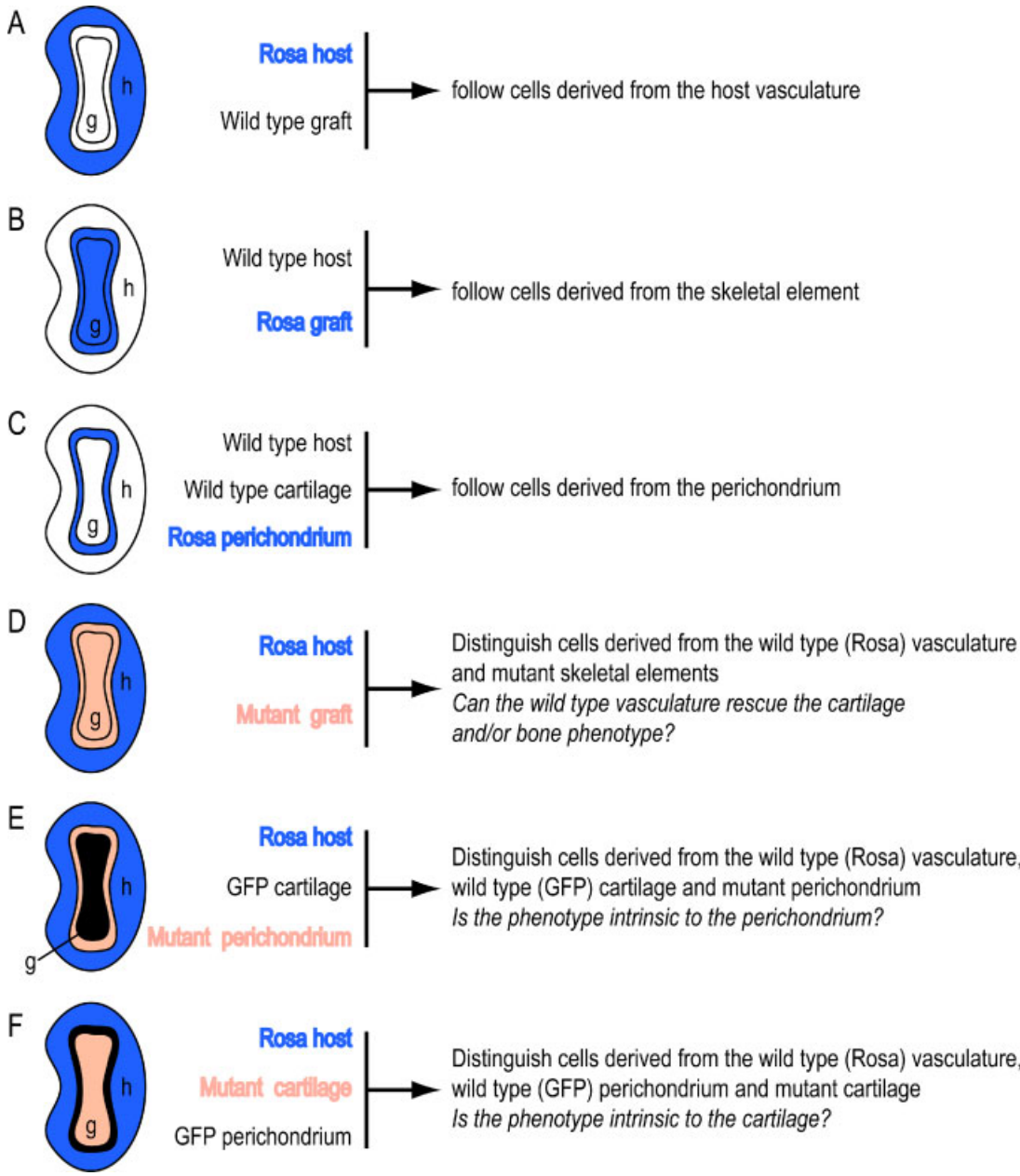

Fig. 2. Examples of tissue recombinations and genetic manipulations that can be used to separate the role of cartilage, perichondrium, and vasculature during bone development. The renal capsule transplant system allows us not only to perform cell lineage analyses (**panels A–C**), but also to examine the underlying cellular mechanisms behind complex skeletal phenotypes by separating interdependent tissues from transgenic embryos and evaluating the contribution of each tissue to the phenotype (**panels D–F**). Rosa refers to transgenic mice carrying the *LacZ* transgene encoding the beta-galactosidase enzyme, expressed under a ubiquitous promoter. GFP, Green Fluorescent Protein. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

or from the vasculature [Schor et al., 1995; Doherty et al., 1998]. Within the blood vessels, pericytes, endothelial cells, or even circulating stem cells may differentiate or transdifferentiate into osteoblasts [Kuznetsov et al., 2001; Qi et al., 2003]. Studies using the renal capsule system indicate that the perichondrium is the primary source of osteoblasts in the cortex and trabeculae of long bones [Colnot et al., 2004]. The vasculature may therefore contain a pool of osteoblast precursors, which is established later during development and play a role during bone growth, remodeling, and repair. Another possibility is an additional contribution of the

cartilage as a source of trabecular osteoblasts [Bruder and Caplan, 1990; Nakahara et al., 1990]. Chondrocyte transdifferentiation into osteoblasts remains elusive, although several studies suggest that this phenomenon occurs [Kahn and Simmons, 1977; Roach, 1992; Roach et al., 1995; Bianco et al., 1998].

UNDERSTANDING EARLY MANIFESTATIONS OF SKELETAL PHENOTYPES

The majority of bone phenotypes are analyzed at late embryonic stages, when the interactions between the cartilage, perichondrium, and vasculature are already intricate. Another way to unravel complex skeletal phenotypes may be to study early stages of skeletogenesis during the formation of the cartilage condensations. Take again $Ihh^{-/-}$ mutants for example, they not only exhibit a cartilage phenotype, but they also exhibit ossification and vascular phenotypes. As discussed above, the cumulative phenotype could be a consequence of a primary cartilage defect or *Ihh* may regulate osteoblast and endothelial cell differentiation independently. Alternatively, the primary defect in these mutants may arise from earlier stages in development when $Ihh^{-/-}$ embryos fail to form a distinct perichondrium (Colnot et al., in press). Indeed, Ihh is upregulated at very early stages in cartilage condensations. Similarly, *Runx2* is found in mesenchymal cells of some skeletogenic condensations [Otto et al., 1997; Lengner et al., 2002; Eames and Helms, 2004], where its role is still unclear. *Runx2* might control some earlier stages in skeletogenesis during mesenchymal condensation. It will be important to revisit some of the known skeletal phenotypes in order to look for the first detectable defects that may induce later defects at the level of the growth plate or during bone growth. Since little is known about the initial steps of skeletogenesis, these analyses may also bring new insights into this area of bone development.

In conclusion, several avenues can be explored to further understand the molecular bases of skeletal development. Advances in genetic manipulation, the identification of more tissue, and cell specific markers of skeletogenesis, to generate new mouse models in combination with the use of tissue manipulations will help refine the functions of genes and their interactions with multiple factors involved in chondrogenesis, osteogenesis, and angiogenesis.

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