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Gene Targeting to the Stroma of the Prostate and Bone

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Abstract

Stromal-epithelial interactions mediated by paracrine signaling mechanisms dictate prostate development and progression of prostate cancer. The regulatory role of androgens in both the prostate stromal and epithelial compartments set the prostate apart from many other organs and tissues with regard to gene targeting. The identification of androgen-dependent prostate epithelial promoters has allowed successful gene targeting to the prostate epithelial compartment. Currently, there are no transgenic mouse models available to specifically alter gene expression within the prostate stromal compartment. As a primary metastatic site for prostate cancer is bone, the functional dissection of the bone stromal compartment is important for understanding stromal-epithelial interactions associated with metastatic tumor growth. Use of currently available methodologies for the expression or deletion of gene expression in recent research studies has advanced our understanding of the stroma. However, the complexity of stromal heterogeneity within the prostate remains a challenge to obtaining compartment or cell-lineage specific *in vivo* models necessary for furthering our understanding of prostatic developmental, benign, tumorigenic, and metastatic growth.

Keywords

Prostate; Stroma or Stromal; Mesenchymal; Bone; Osteoblasts; Tissue Recombination
Xenografting; Transgenic Mice; Conditional Knockout

Introduction

The role of mesenchymal tissues in the control of organogenesis has been well established in many organs including the prostate (Wolff, 1968; Cunha, 1972; Slavkin, 1974; Cunha et al., 1980; Kollar and Fisher, 1980; Cunha and Chung, 1981b; Chung and Cunha, 1983; Chung et al., 1984; Saxen et al., 1986). The prostate develops from an ambisexual embryonic rudiment, the endodermal urogenital sinus (UGS). The prostatic urethra and bulbourethral glands in males, the lower vagina and urethra in females, and bladder in both sexes also develops from the UGS. Embryonic connective tissue surrounding the UGS, referred to as urogenital mesenchyme (UGM), expresses androgen receptors and responds to fetal testicular androgens by promoting epithelial development and differentiation. Embryonic development of the prostatic glandular epithelium proceeds from the outward expansion of

cords of urogenital epithelium (UGE) from the UGS into the UGM resulting in the formation of prostatic buds. The prostatic buds elongate and undergo branching morphogenesis and canalization resulting in the formation of prostatic ductal trees. In rodents, the prostatic ductal trees organize into distinct lobes that include the anterior, dorsal-lateral, and ventral prostate. In contrast, the prostatic ductal trees in humans organize into a solid compact gland where distinct sets of ducts result in the organization of the central, transitional, and peripheral zones (reviewed in Thomson and Marker, 2006; Cunha, 2008, this issue).

Stromal tissues, in addition to the basal epithelium, form much of the connective tissue support structures upon and within which prostate epithelia are dependent for survival, growth, and differentiation. UGM derived prostatic stroma specifies prostate epithelial identity and subsequent differentiation into mature secretory epithelium (reviewed in this issue in Cunha, 2008; Matusik, 2008). In turn, the developing prostatic epithelium induces the UGM derived prostatic stroma to undergo smooth muscle differentiation (Hayward et al., 1996b). Components of the adult prostatic stroma include the basement membrane and extracellular matrix (ECM) that is produced by fibroblasts and smooth muscle cells associated with the ductal tissue. The stroma of the prostate also contains blood vessels, nerves, and infiltrating or recruited bone marrow derived cells. Mesenchymally derived cells of the bone, namely osteoblasts and osteoclasts, are also of interest as bone is a frequent metastatic site for prostate cancer. The prostatic and bone stromal tissue components, however, are a mixture of yet undefined states of differentiation. It is undefined because few stromal cell- and tissue- specific markers are known. The knowledge of these specific markers would enable specific targeting of gene expression or knock-down to a specific cell lineage or tissue compartment. Stromal-epithelial interactions are mediated principally by epithelial and stromal secreted cytokines, chemokines, and growth factors (Donjacour and Cunha, 1991). The biology of the ECM, matrix degrading enzymes, as well as endothelial and immune cells contributes significantly to our understanding of stromal-epithelial interactions and the tumor microenvironment. While these topics are not within the scope of this article, these are discussed in other reviews in this issue.

A particular focus of recent studies in prostate carcinogenesis has been the functional dissection of the stromal compartment. Genetic targeting of the stroma has been made difficult by stromal heterogeneity. The stroma for any given anatomical site of interest is composed of multiple types of stromal cells, while predominated by either fibroblasts or smooth muscle cells, resulting in internal cellular heterogeneity. The types and ratios of stromal cells present within an anatomical site of interest is regulated by the developmental stage, the state of differentiation of the surrounding cells and tissue, and the normal ongoing homeostatic responses to local paracrine signaling, inflammation, and tissue remodeling and repair. While morphologically similar, stromal cells from a specific cell lineage (fibroblasts for example) isolated from different organ or tissue sites have distinct patterns of gene expression imposed by developmental cell fate programming. Observed variations in gene expression between colonies derived from a single stromal cell lineage, isolated from a specific tissue site, and grown in culture have demonstrated the presence of multiple cell sub-populations with distinct phenotypic and functional characteristics. The detection of

multiple cell sub-populations within a given cell lineage has been demonstrated *ex vivo* by various techniques including flow cytometry, immunohistochemistry, immunofluorescence, and *in situ* hybridization. For example, fibroblasts localized at different levels or locations within the dermis have been shown to have distinct patterns of gene expression by microarray analysis and specific dermal fibroblast sub-populations are differentially activated during the wound healing process (Schor and Schor, 1987; Irwin et al., 1994; Jelaska et al., 1999; Chang et al., 2002; Sorrell and Caplan, 2004; Nolte et al., 2007). Here we review the role of stromal-epithelial interactions in prostate development and tumorigenesis as well as the methodology currently available to target gene expression for studies of the prostatic and bone stromal compartments.

Stromal-Epithelial Interactions in Prostate Development

Prostate development and function is tightly regulated by various autocrine and paracrine factors that include cytokines and chemokines, growth factors like transforming growth factor-beta (TGF- β), and endocrine hormones such as androgens (Hayward and Cunha, 2000; Montgomery et al., 2001). The action of androgens is mediated by the androgen receptor (AR), a member of the steroid hormone receptor superfamily. Testosterone upon cellular entry is converted into 5 α -dihydrotestosterone (DHT) by the enzyme, 5 α -reductase. Upon DHT binding, AR becomes phosphorylated and undergoes conformational changes that release it from heat shock proteins in the cytoplasm. This allows AR to migrate into the nucleus where it interacts with androgen responsive elements found in the promoters of androgen regulated genes (Blok et al., 1996; Brinkmann et al., 1999; Peterziel et al., 1999; Maeda, 2001; Georget et al., 2002). AR in the prostatic epithelial cells (Cooke et al., 1991) up-regulates the expression of various proteins characteristic of the differentiated state including prostate-specific antigen (PSA) and probasin, expressed in humans and rodents respectively (Donjacour and Cunha, 1993; Snoek et al., 1998; Zhang et al., 2004), (Table 1).

Androgens regulate prostate development in part through stromal-epithelial interactions. The neonatal development of prostatic tissue is mediated by androgenic effects within the mesenchyme (Cunha and Chung, 1981a; Cunha and Donjacour, 1989). For example, androgens can induce stromal fibroblasts to express paracrine growth factors, like FGF8 and IGF-1, that stimulate epithelial proliferation (Ruan et al., 1999; Gnanapragasam et al., 2002; Wu et al., 2007a). Androgens can also modulate the activity of other stromally produced paracrine factors, such as EGF, FGF7/KGF, FGF10 and TGF- α , upon the prostatic epithelium (Morton et al., 1990; Reiter et al., 1992; Yan et al., 1992; Bacher et al., 1993; Gray et al., 1995; Sokoloff et al., 1996; Thomson et al., 1997; Itoh et al., 1998; Thomson and Cunha, 1999).

Tissue recombination experiments provide evidence that paracrine signaling from the mesenchymal cells to the epithelial cells in part controls prostate gland development. When allografted with wild-type UGM, prostatic glands with normal glandular architecture but lacking prostatic secretory function developed from epithelia (expressing a non-functional AR) derived from testicular feminized (Tfm) mice (Sugimura et al., 1986; Cunha, 2008, this issue). The resulting prostatic glands were equally sensitive to androgen ablation carried out by castration of the host mice, as compared to the control tissue recombinations generated

with wild-type mesenchyme and wild-type epithelia (Cunha, 2008, this issue). Reciprocal tissue recombinations using Tfm UGM and wild-type epithelia failed to undergo prostatic development (Cunha, 2008, this issue). Together, these data established that stromal androgen-responsiveness is required for glandular development of the prostatic epithelium (Cunha and Lung, 1978; Donjacour and Cunha, 1993). This suggests that the prostatic stroma plays an instructive role in glandular development and influences prostatic responsiveness to androgen ablation (Kurita et al., 2001; Cunha, 2008, this issue).

Interestingly, castration induced regression of the prostate results in a detectable long-term increase in TGF- β expression (Kyprianou and Isaacs, 1989; Martikainen et al., 1990; Shidaifat et al., 2007). Induction of a wave of apoptotic death of the prostatic blood vasculature occurs after castration and prior to the regression of the prostatic stroma and epithelial compartments (Kyprianou and Isaacs, 1988; Isaacs et al., 1992; Isaacs et al., 1994; Furuya et al., 1995; Denmeade et al., 1996; Shabisgh et al., 1999; Shabsigh et al., 1999a; Shabsigh et al., 1999b). Together, these data suggest a mechanism whereby increased TGF- β expression may contribute to the initiation and maintenance of prostate regression. Following androgen ablation, interaction of the TGF- β and androgen signaling pathways most likely involves autocrine and paracrine cross-talk involving multiple cytokines and growth factors. Both cooperative and antagonistic interactions of androgen and TGF- β signaling pathways have been reported in the prostatic epithelia (Hayes et al., 2001; Kang et al., 2001; Chipuk et al., 2002), suggesting a possible role for TGF- β signaling in the regulation of androgen responsiveness.

TGF- β isoforms (TGF- β 1, β 2, β 3) have long been established as physiological regulators of prostate growth because of their ability to inhibit cell proliferation and mediate apoptosis (Kyprianou and Isaacs, 1989; Martikainen et al., 1990). TGF- β isoforms exert their effects through binding to the TGF- β type II receptor (Tgfr2) and subsequent recruitment of the type I receptor (Tgfr1) for downstream cytoplasmic signaling through multiple parallel signaling pathways including activation of p38, PI3K/AKT, RhoA/Rock, and SMADs (Massague and Gomis, 2006). SMAD signaling has also been associated with the transcriptional down-regulation of the mitogenic proto-oncogene, c-Myc (Alexandrow and Moses, 1995; Feng et al., 2002; Yagi et al., 2002). The TGF- β type III receptor (Tgfr3), required for TGF- β 2 signaling through Tgfr2, is expressed by the prostatic stroma, but to a lesser extent in the prostatic epithelia (Bhowmick et al., 2004a). The intracellular, extracellular, and microenvironmental contexts determine the outcome of TGF- β signaling in terms of the regulation of differentiation, growth (proliferation and apoptosis), and other biological endpoints (Bierie and Moses, 2006; Stover et al., 2007).

The glandular compartment of the adult human prostate (Figure 1) consists of mature differentiated basal and secretory luminal epithelial cells with few neuroendocrine cells. The epithelial cells are in close contact with an underlying stromal compartment consisting mainly of differentiated smooth muscle cells in extracellular matrix. (For a review about prostate epithelial cell fates, see the reviews by Robert Matusik and Corey Abate-Shen in this issue.) During prostate development, mesenchymal cells progressively differentiate into smooth muscle cells that constitute the adult prostatic stroma. The smooth muscle cells are thought either to differentiate directly from fibroblasts or in a step-wise progression from

fibroblasts to myofibroblastic cells to well-differentiated smooth muscle cells (Hayward et al., 1996a; Hayward et al., 1996b; Hayward et al., 2006). The maturation of the stroma supports differentiation of proliferative immature epithelia into well-differentiated, quiescent adult glandular epithelia. As both prostate epithelial and stromal cells both express AR (Prins et al., 1991), the maintenance of the growth-quiescent state of the adult prostate is directly regulated in part by androgen signaling and reciprocal stromal-epithelial interactions.

Markers frequently used to assess prostate epithelial differentiation (Table 1) include p63 and prostatic acid phosphatase, as well as cytokeratins 5, 8, 14, and 18 (Wang et al., 2001; Wang et al., 2005a). Assessment of prostatic stromal differentiation is more complex as known stromal differentiation markers (Table 2) are often expressed in an increasing or decreasing manner across a continuum of lineage differentiation. For example, prostatic fibroblasts express fibroblast specific protein-1 (FSP-1, S100A4) and vimentin (Sugimoto et al., 2006). Myofibroblasts also express FSP-1 and vimentin as well as some smooth muscle specific proteins, such as desmin, alpha-smooth muscle actin (α SMA), and tenascin (Hayward et al., 1996b; Tuxhorn et al., 2002a). Mature differentiated prostatic smooth muscle cells express these and other smooth muscle specific proteins, such as SM22 and smooth muscle myosin heavy chain (SMMHC), and little to no fibroblast-specific genes like FSP-1 or vimentin (Duband et al., 1993).

Stromal-Epithelial Interactions in Primary Prostate Cancer

Accumulation of genetic alterations in adult prostatic epithelium promotes carcinogenesis through aberrant changes in gene expression supporting increased epithelial proliferation and evasion from apoptosis. For instance, over-expression of c-Myc (Fleming et al., 1986; Matusik et al., 1987; Jenkins et al., 1997; Ellwood-Yen et al., 2003) and loss of PTEN (Cairns et al., 1997) are thought to be early events in prostate carcinogenesis in comparison to the later stage loss of p53 and pRb (Jenkins et al., 1997; Jarrard et al., 2002; Dong, 2006). Normal prostate epithelial and stromal cells express TGF- β type I and II receptors (Royuela et al., 1998). However, the loss of expression of the TGF- β type I and II receptors in the prostatic epithelium correlates with increased tumor grade in human prostate cancer tissues and poor prognosis (Kim et al., 1996; Kim et al., 1998; Tu et al., 2003). Loss of TGF- β type II receptor in the stroma correlates with poor patient prognosis and the presence of tumor-associated macrophages in colon cancer patients (Bacman et al., 2007), suggesting that stromal loss of Tgfb2 expression may contribute to the progression of other cancers including those of the prostate. Prostatic intraepithelial neoplasia (PIN), a proposed precursor for prostatic adenocarcinoma, is also reported to have similar alterations (Bostwick et al., 1995; Bostwick et al., 1996; Sakr and Partin, 2001).

Just as the stroma has an important role in regulating the development and maturation of the prostatic epithelium, the stroma too plays a key role in the suppression of epithelial transformation. Normal stromal cells have been reported to convert malignant epithelia to morphologically benign lesions *in vitro* (Cooper and Pinkus, 1977; Hayashi and Cunha, 1991). Changes in gene expression in normal stromal cells, however, can promote malignancy in the neighboring unaltered epithelia (Nakamura et al., 1997; Barcellos-Hoff

and Ravani, 2000) and malignant progression in neighboring initiated epithelia (Olumi et al., 1999; Hayward et al., 2001; Phillips et al., 2001). For example, we have previously demonstrated that the knockout of *Tgfb β 2* expression in the stroma promotes the formation of PIN lesions and supports the transformation of adjacent epithelium in prostate and forestomach tissues (Bhowmick et al., 2004a). Another example comes from recent work published by Hill and colleagues in which the inactivation of pRB in the prostatic epithelium of genetically engineered mice resulted in an initial induction of p53 in the epithelium and underlying stroma followed by a selective loss in stromal p53 expression (Hill et al., 2005a; 2005b). This loss in stromal p53 expression resulted in increased proliferation of the prostatic stroma and subsequent increased tumor aggressiveness. In this study, lack of histologic observation of basement membrane breakdown or of stromal cells co-expressing both epithelial and fibroblastic markers supported a paracrine stromal response mechanism as opposed to an epithelial to mesenchymal transition (EMT) (Hill et al., 2005b). A similar promotion in cancer progression and aggressiveness was observed by Kiaris et al. in tissue recombination studies in which MCF7 mammary epithelial cells were recombined and grafted with p53^{+/+} or p53^{-/-} stroma (Kiaris et al., 2005).

The evolution of prostate cancer results ultimately in the establishment of a vicious cycle of disease progression and subsequent metastasis (Sung and Chung, 2002; Cunha et al., 2003; Bhowmick et al., 2004b; Bhowmick and Moses, 2005; Bieri and Moses, 2005). Loss of differentiation in genetically initiated prostatic epithelium results in altered paracrine signaling from the epithelium to the underlying differentiated adult prostatic stroma. In turn, the prostatic stromal cells undergo a reversal in differentiation to become reactive myofibroblasts, promoting malignant progression through altered stromal to epithelial paracrine signaling (Wong et al., 2003). Furthermore, neoplastic prostatic epithelial cells may be able to acquire the ability to assume a fibroblastic phenotype by undergoing EMT, similar to that demonstrated in breast cancer biopsy tissues (Petersen et al., 2003). Another alternative mechanism might be that loss of epithelial and stromal differentiation in the prostate leads to the recruitment of bone-marrow derived cells that subsequently take up residence and differentiate into myofibroblasts in the stroma (Lincoln et al., 1997; Brittan et al., 2002; Direkze et al., 2003; Hashimoto et al., 2004; Brittan et al., 2005; Hayashida et al., 2005; Jabs et al., 2005; Mori et al., 2005; Yamaguchi et al., 2005; Haudek et al., 2006; Asawa et al., 2007; Bellini and Mattoli, 2007; Dupuis et al., 2007; Li et al., 2007; Morimoto et al., 2007).

Stromal myofibroblasts have been referred to by many names including “tumor-associated stroma” (Mueller and Fusenig, 2004; Chung et al., 2005), “reactive stroma” (Tuxhorn et al., 2001; Tuxhorn et al., 2002a; Tuxhorn et al., 2002b), and “carcinoma-associated fibroblasts” (CAFs) (Olumi et al., 1999; Cunha et al., 2002; Cunha et al., 2003). CAFs have been demonstrated to secrete altered levels of a number of cytokines and growth factors including TGF- β (San Francisco et al., 2004; Chung et al., 2005; Joesting et al., 2005; Orimo et al., 2005; Kalluri and Zeisberg, 2006). CAFs derived from human prostate tumors are able to elicit malignant progression in the immortalized non-tumorigenic human prostatic epithelial cell line, BPH-1 (Olumi et al., 1999). This tumorigenic effect can be abrogated by treating animals with a TGF- β neutralizing antibody (2G7) (Ao et al., 2007). Together, these data provide compelling evidence that stromal-epithelial interactions within the prostate

microenvironment regulate and modulate the behavior of normal and malignant prostate epithelial cells.

Bone Stromal Interactions with Metastatic Prostate Cancer

During development, embryonic bone mesenchymal cells differentiate into hematopoietic stem cells (which give rise to the lymphoid and myeloid lineage cells of the blood) or bone-lineage stromal cells (Marieb, 1998). Derived from differentiated macrophages (a myeloid lineage of hematopoietic cells), osteoclasts are found lining the inner surfaces within trabecular bone and function to actively resorb bone (Roodman, 2006). Bone-marrow fibroblasts line the inner surface of the blood marrow cavity. Chondrocytes, which produce cartilage, are found in cartilaginous tissues including the growth plates of long bones in adolescents (Marieb, 1998). Osteoblasts, which actively form bone, are found lining the inner surfaces of trabecular bone (Marieb, 1998). Chondrocytes and osteoblasts are thought to differentiate from a common bone mesenchymal/stromal-lineage precursor (Romero-Prado et al., 2006).

Bone is the most frequent site of prostate cancer metastasis and the major site of tumor burden in most patients with advanced disease. Thereby, complications arising from metastasis of prostate cancer to the bone is most often the primary reason for the morbidity and mortality of prostate cancer patients (Chirgwin and Guise, 2007; Kingsley et al., 2007a; Kingsley et al., 2007b). Expression of bone specific stromal proteins in organ confined prostate cancer, a process termed osteomimicry, may contribute to the ability of migratory prostate tumor cells to successfully seed distant bone metastatic sites (Koenen et al., 1999; Zayzafoon et al., 2004). Further, the ability of the bone stroma to respond to androgens may contribute to the establishment of metastatic prostate tumors in the bone. AR expression is detectable in multiple cell types within the bone microenvironment including bone marrow stromal fibroblasts, chondrocytes, endothelial cells, osteoblasts, osteoclasts, and osteocytes. It is not surprising, therefore, that the expression of multiple genes expressed in the bone is regulated by androgen signaling and that androgens play an important role in the regulation of bone remodeling (Wren, 2005; Xu et al., 2007).

When tumor cells metastasize to bone, another vicious cycle is created between the metastatic tumor cells and normal host bone cells (Mundy, 2002), specifically with osteoclasts and osteoblasts (Figure 2). Tumor cells produce paracrine factors that can stimulate osteoclast or osteoblast activity. Activated osteoclasts in turn cause tumor cells to alter gene expression, behave more aggressively, and cause destructive local bone osteolysis (Guise and Mundy, 1998; Mundy and Yoneda, 1998; Yin et al., 1999; Mundy, 2002). TGF- β , the most abundant growth factor present in bone, is stored in latent form in bone matrix and released in active form during osteolytic bone resorption (Pfeilschifter and Mundy, 1987). Release of TGF- β by osteolysis enhances expression of parathyroid hormone related protein (PTHrP) by tumor cells, which in turn enhances osteoclast activation and subsequent osteolysis as observed in studies of metastatic breast cancers (Guise et al., 1996; Yin et al., 1999). Similar to breast cancer cell lines, some human prostate cancer cell lines can establish osteolytic bone lesions, such as PC3 cells (Sanchez-Sweetman et al., 1998). In general, prostate cancer at the time of the initial establishment of the tumor cells in the bone

results in transient osteolysis followed by predominant osteoblast activation. Activated osteoblasts in turn cause the tumor cells to alter gene expression, behave more aggressively, and cause destructive formation of weaker woven bone. Examples of human prostate cancer cell lines that establish osteoblastic lesions include LNCaP-C4-2B (Lin et al., 2001) and LuCaP 23.1 (Liu et al., 1996). CWR22 cells have been reported to establish both osteolytic and osteoblastic lesions (Andersen et al., 2003). The osteolytic to osteoblastic transition of prostate cancer bone metastasis is thought to be regulated by tumor and bone cell produced factors including TGF- β , Wnt family ligands, and Wnt inhibitors (Keller and Brown, 2004; Hall et al., 2005; Logothetis and Lin, 2005; Hall et al., 2006; Hall and Keller, 2006). Thereby, hijacking of paracrine signaling between osteoclasts and osteoblasts by the tumor cells may contribute to the survival and expansion of metastatic lesions in the bone.

Targeting Gene Deletion or Expression to Stromal Compartments of the Prostate and Bone

Stromal-epithelial interactions are essential for normal prostate development and contribute significantly to the process of prostate carcinogenesis at the primary and metastatic sites. Research over the past four decades has elucidated much detail about the gene expression and signal transduction of normal and neoplastic epithelia, however, much less is known about the stromal compartment. The accumulation of data supporting the ideas of cancers as “wounds that do not heal” (Dvorak, 1986) and the “seed and soil” mechanism for cancer metastasis (Paget, 1889; McCawley and Matrisian, 2001; Fidler et al., 2002) has also illuminated the need to increase our understanding of normal tissue and tumor microenvironments (Matrisian et al., 2001; Cunha and Matrisian, 2002).

Many mouse models are available for the study of prostate cancer and prostate cancer metastasis (reviewed in Singh and Figg, 2005). However the utility of these models to study stromal-epithelial interactions and the tumor microenvironment is often severely limited. Prostate epithelial and/or stromal cells (with or without genetic modification) can be injected orthotopically into the lobes of the mouse prostate. Left ventricular intra-cardiac and intra-tibial injections can be accomplished and used to study bone metastasis (Wang et al., 2005b). The characteristics of a particular prostate cancer cell line, however, will ultimately determine the utility of these models in terms of viable take rates to the bone. Metastatic progression of prostate cancer in bone may also be studied using a model in which bone tumors are established by grafting prostate cancer epithelia or stroma along with pieces of human fetal bone into male severe combined immunodeficient (SCID) mice. Establishment of prostate cancer cells in bone can be monitored *in vivo* through utilization of imaging modalities such as x-ray, micro-computed tomography (microCT), magnetic resonance imaging (MRI), and bioluminescence. Taken together, the low-take rates of the models preclude their more widespread use in experiments aimed at studying changes in gene expression in the prostatic epithelial and prostatic or bone stromal compartments (Nemeth et al., 1999).

Transgenic approaches for modulation of gene expression in prostate epithelia are well established (reviewed by Michael Shen in this issue). In addition, multiple prostate cancer transgenic mouse models have been generated and reviewed previously (Kasper and Smith,

2004; Abdulkadir and Kim, 2005; Kasper, 2005). Methods for *in vivo* targeted gene expression or deletion in stromal compartments as opposed to epithelial compartments, however, have been developed more recently taking advantage of rapidly developing improvements in conditional Cre/LoxP transgenic mice and RNA interference (RNAi).

Conditional Gene Knockout in Mice

Targeted gene insertion or deletion by homologous recombination into the mouse genome (Figure 3A) has been crucial to the elucidation of the functional roles for endogenously expressed genes and the signaling networks to which they contribute. While specific for a gene locus of interest, constitutive knockout of a gene(s) of interest frequently results in embryonic lethality where the gene(s) are critical for one or more stages of development. Introduction of the use of Cre and FLP_e recombinase mediated excision of loxP or FRT flanked expression cassettes, respectively, increased the ability to obtain the desired mouse model through improved yield by reducing lethality phenotypes (Branda and Dymecki, 2004; Sorrell and Kolb, 2005; Feil, 2007). Adenoviral Cre (Ad-Cre) and cell-permeable Cre are effective measures for the *in vitro* knockout or inversion of floxed genes or gene cassettes (Anton and Graham, 1995; Jo et al., 2001; Joshi et al., 2002; Patsch and Edenhofer, 2007). Utility of Ad-Cre and cell-permeable Cre *in vivo* is limited to accessible compartments where non-specific transduction of Cre into adjacent tissues will not hamper the experimental outcome. Crossing of mice bearing floxed allele(s) to mice expressing the Cre transgene improved knockout cell-specificity, but was dependent on the time and regulation of promoter activity (Figure 3B). Examples include androgen-dependent promoters active in the prostate epithelia such as NKX3.1-Cre (personal communication with Michael Shen, UMDNJ-Robert Wood Johnson Medical School) and ARR2Pb-Cre (based on the probasin promoter) (Wu et al., 2001), which are expressed early or late in prostate development respectively. As no prostate stromal specific promoters are yet available, various stromal promoters have been used with limited success in prostate studies.

Conditional Knockout Utilizing Stromal Specific Cre Mice

Molecular regulation of Cre expression has allowed the creation of more specific and informative mouse models. Developmental stage specific gene promoters and tetracycline regulated (TET-on or TET-off) promoters have been used successfully to regulate the temporal induction of expression of a transgene of interest *in vivo* (Figure 3). Examples of such transgenes include Cre (Lewandoski, 2001) and targeting inserts for RNA interference (RNAi) mediated gene knockdown (Dickins et al., 2007). Similarly, use of tissue specific promoters allows for restricted spatial expression to the compartment (tissue or organ) or cell lineage of interest, such as the prostate or bone stroma (Table 2). Promoters for collagen type 1 alpha 1 (Col1a1, 2.3 kb or 3.7 kb promoters) (Dacquin et al., 2002; Kim et al., 2004; Liu et al., 2004), collagen type 1 alpha 2 (Col1a2) (Zheng et al., 2002; Florin et al., 2004), and FSP-1/S100A4 (Iwano et al., 2001; Iwano et al., 2002; Bhowmick et al., 2004a; Inoue et al., 2005) can be used to target gene deletion or expression to fibroblasts, myofibroblasts, and smooth muscle cells (which constitute the stromal compartment) of the prostate. Fibroblasts can also be targeted utilizing the vimentin promoter and non-cardiac smooth muscle cells using the promoters for alpha-smooth muscle actin (α SMA) (Miwa et al., 2000;

Wu et al., 2007b), SM22/transgelin (Kuhbandner et al., 2000; Holtwick et al., 2002), and SMMHC (Regan et al., 2000).

FSP-1 is expressed early in development at embryonic day nine (Iwano et al., 2001; Iwano et al., 2002) and is inherently upstream of the mesenchymal differentiation lineage being expressed in precursor cells of the hematopoietic system, osteoblasts, and the mesenchyme of nearly every tissue examined including the prostate (Klingelhofer et al., 1997; Bhowmick et al., 2004a). The wide distribution of FSP-1 expression enables the study of the role of stromal genes in many tissues. Of interest, the FSP-1 gene is not expressed in the lineage of all cells of the prostate stroma (or other tissues), but rather a fraction close to 30-50% of the mature stromal cells. This observation highlights the heterogeneity of the stromal compartment within organs and enables the study of juxtacrine signaling within the stromal compartment. Importantly, FSP-1 promoter regulated gene targeting is an example of how targeting by other familiar stromal promoters such as vimentin or SM22 will not be tissue specific, but also only affect a fraction of the stromal compartment (Sugimoto et al., 2006). The latter trait may enable mouse viability from redundancy by other stromal cells not affected. Expression of FSP-1 visualized by immunofluorescence of a green fluorescence protein (GFP) reporter in tissues including the bladder, prostate, and uterus illustrates both stromal specificity and stromal heterogeneity (Figure 4) (Bhowmick et al., 2004a).

Previously, we generated a conditional mesenchymal (stromal) Tgfr2 knockout mouse using FSP-1 Cre-mediated recombination (Tgfr2^{fspKO}) (Bhowmick et al., 2004a). While prostates from control Tgfr2^{FloxE2/FloxE2} mice were morphologically normal, prostates from Tgfr2^{fspKO} mice developed stromal and epithelial hyperplasia with foci of hyperchromatic nuclei with atypia by 5-7 weeks of age. These PIN lesions occurred predominantly in the anterior and dorsolateral lobes of the prostate. The Tgfr2^{fspKO} mice lacked nuclear phosphorylated Smad2 immunostaining indicative of loss of TGF- β signaling, as compared to floxed controls. Invasive squamous cell carcinoma of the forestomach consistently developed in the Tgfr2^{fspKO} mice, which die prematurely by eight weeks of age. The PIN lesions were reported to result from the reduction in the expression of p21 and p27 (cdk inhibitors) and an increase in the levels of c-Myc and phospho-c-Met receptor (determined to be a consequence of increased HGF secretion by the stroma). In addition, the forestomach epithelial tissues of the Tgfr2^{fspKO} mice developed squamous cell carcinoma through a presumed similar mechanism as the pre-neoplastic lesions of the prostate. In summary, these studies demonstrated that blocking TGF- β signaling in the stromal compartment relieved the cell cycle inhibition in the epithelium and enabled proliferation and transformation of the adjacent epithelial compartment in prostate and forestomach tissues. Due to the early lethality of the Tgfr2^{fspKO} mouse model, it has proven to be useful in studying initiating factors involved in tumorigenesis and less useful for studying subsequent tumor progression. Tissue rescue of the Tgfr2^{fspKO} prostates under the renal capsule of syngenic mice for seven months have shown further progression of the PIN lesions to adenocarcinoma (unpublished data).

If genes essential in early development are altered using a promoter driving Cre expression, such as FSP-1-Cre, it can result in early lethality of the mouse models often precluding analysis of the tissue of interest. Such early developmental gene promoters, not surprisingly,

have broader cell specificity. Unpublished observations suggest additional FSP-1-Cre activity in the bone, which is of interest as osteoblasts are of mesenchymal origin. The Col1a2-Cre mice, developed by de Crombrughe and colleagues, is expressed by many fibroblastic cell types (more restricted than FSP-1) and have the added advantage of expressing a Cre-ER fusion protein (Zheng et al., 2002). The modified estrogen receptor provides a tight tamoxifen-regulation of Cre nuclear localization, thus allowing both temporal and tissue specific Cre activity. Since tamoxifen is only needed briefly for Cre-mediated DNA recombination activity, any unwanted side-effects of tamoxifen can be avoided if the following experiments are performed about five to seven days following Cre activation. While use of tamoxifen to activate Cre-ER mediated gene knockout in unborn fetal mice is routinely successful, the abortigenic effects of tamoxifen may hamper the ability to obtain live born mouse pups (Kuhbandner et al., 2000; Zheng et al., 2002; Nakamura et al., 2006). Administration of progesterone with tamoxifen may improve yield of living Cre-ER knockout mice by helping to counteract the abortigenic effects of tamoxifen (Nakamura et al., 2006). However, the limiting factor in more widespread use of Cre-ER is that there are only a few Cre-ER mouse models currently available for targeting the stromal compartment.

Mesenchymal derivatives of bone may also be targeted for genetic deletion or expression (summarized in Table 2). Chondrocytes and osteoblasts, which constitute bone stroma, can be targeted using Col1a1, (Dacquin et al., 2002; Kim et al., 2004; Liu et al., 2004), Dermo-1 (Yu et al., 2003), FSP-1 (Iwano et al., 2001; Iwano et al., 2002; Bhowmick et al., 2004a; Inoue et al., 2005), and Sox9 promoters (Akiyama et al., 2005). The promoter for collagen type 2 alpha 1 (Col2a1) can be used to target chondrocytes as Col2a1 is not reported to be expressed by osteoblasts or osteoclasts (Long et al., 2001; Sakamoto et al., 2005; Nakamura et al., 2006). The promoter for Prx1, a marker of the undifferentiated osteoblast precursor, has been used for Cre-mediated knockout of gene expression in chondrocytes (Logan et al., 2002). The promoter for Osteocalcin (OC), a marker of differentiated osteoblasts positively regulated by Runx2, has been reported to allow Cre-mediated knockout of gene expression in chondrocytes and osteoblasts (Zhang et al., 2002). Osteoclasts have been targeted using promoters including cathepsin K (CTSK) and tartrate resistant acid phosphatase (TRAP) (Chiu et al., 2004). The CD11b-Cre mice targets cells of myeloid of origin, which include osteoclasts, granulocytes (basophils, eosinophils, and neutrophils) and monocyte/macrophage lineages (Ferron and Vacher, 2005). Endothelial cells can be targeted using Tie1 (Song et al., 2007) or Tie2 promoters (Kisanuki et al., 2001), the latter of which also has been reported to be expressed in macrophages. The genetic parallels of the bone and prostate stroma associated with gene targeting may suggest similarities in biological regulation and thus is likely not coincidental that prostate cancer metastasizes to the bone.

Conditional Knockout Utilizing RCAS TVA/TVB Mice

A potentially cost effective and efficient alternative to conventional mouse models, that frequently require extensive breeding of transgenic and conditional gene alterations in order to study synergistic interactions, is the utilization of the RCAS system for targeted gene expression. Developed originally by Hughes and colleagues and later adapted by the Varmus and Holland laboratories, the RCAS system takes advantage of expression of TVA, a

membrane-bound receptor for the avian leukosis virus sub-group A (ALV-A) retroviruses, not expressed in rodents or humans, encoded by the *TVA* gene (Bates et al., 1993; Federspiel et al., 1994; Holland et al., 1998a; Holland et al., 1998b; Holland and Varmus, 1998; Fisher et al., 1999). Ectopic expression of the *TVA* gene into mammalian cells permits ALV-A infection, viral entry and chromosomal integration, and expression of genes carried by RCAS vectors. High-titer viral propagation can occur in the packaging avian cell line, DF-1, (Himly et al., 1998; Schaefer-Klein et al., 1998) or chicken embryonic fibroblasts (CEFs). As ALV-A viruses are not infectious to mammalian cells, this prevents cell-to-cell spread of the virus and reduced probability of an immune response by the host because the targeted cells of interest are not producing viral packaging proteins.

Due to the poor expression of ALV-A env gene in infected TVA+ mammalian cells, the cells remain susceptible to super-infection with RCAS vectors, allowing for the transfer of multiple genetic lesions and/or histologic markers into a single TVA+ cell (Holland et al., 1998a; Holland et al., 1998b; Holland and Varmus, 1998). Importantly, successful ALV infection has been possible only in replicating cells until the recent development of lentiviral counterparts that overcome the barrier of infection to low replicative target cells (Naldini et al., 1996; Lewis et al., 2001). Alternatively, direct introduction of the avian packaging cell line, DF-1, into the desired site of infection can be utilized as the DF-1 cells produce fresh virus for a short period of time (3-4 days) and then die off. This method has been used successfully for infecting cells in the brain and bone marrow in the past (Hughes and Crittenden, 1991). Many RCAS viral vectors have been developed to express exogenous genes in ALV-A infected mammalian cells (Federspiel and Hughes, 1997). These include the RCASBP(A) (RCAS, Bryan-RSV pol, subgroup A) vector that can accommodate an insert up to 2.5 kb and the RCASBP(B) (RCAS, Bryan-RSV polymerase, subgroup B) vector, which can accommodate a much larger insert. Virions produced from RCASBP(A) and RCASBP(B) can recognize only TVA and TVB expressing cells, respectively. Since an exogenous promoter is utilized to drive expression of genes of interest in the RCAS system, levels of expression can be enhanced using promoters such as CMV. Alternatively, a promoter of higher tissue specificity can be used to drive the gene of interest. For a hypothetical example, a TVA transgenic mouse driven off the FSP1 promoter can be infected with an RCASBP(A) virus with a gene of interest driven by the Collagen 1a1 promoter. This was only hypothetical, since no stromally directed TVA/TVB have been reported.

A number of laboratories have produced mouse lines expressing *TVA* under a variety of tissue-specific promoters, making it possible to direct the expression of an RCAS vector to a specific cell or tissue type. The beta-actin *TVA* mouse strain, which expresses TVA in all tissues, can enable preliminary experiments to test infectivity of the RCAS virus, expression of the gene of interest, and any promising phenotypes (Federspiel et al., 1996). The beta-actin *TVA* line is also a good source of primary cells/cell lines that can be efficiently infected in culture. Notably, the beta-actin *TVA* transgenic mice are in the C57/Bl6 background (of interest to those wanting to cross into their mouse line of interest or those wanting to perform adoptive transfer experiments). Additional promoter driven *TVA* mouse lines and the tissues they target include: albumin (hepatocytes) (Lewis et al., 2005), alpha-actin

(skeletal muscle) (Federspiel et al., 1994), BSP (bone) (Li et al., 2005), DCT (melanoblasts) (Dunn et al., 2000), elastase (pancreas) (Lewis et al., 2003), GFAP (astrocytes) (Holland and Varmus, 1998), GP-Iba (megakaryocyte lineage) (Murphy and Leavitt, 1999), keratin 5 (keratin 5 positive cells such as ovarian surface epithelial cells) (Orsulic et al., 2002), MMTV (mammary gland) (Du et al., 2006), and nestin promoters (glial progenitors) (Holland et al., 1998a; Holland et al., 1998b). Utility of this technology can be further enhanced through the creation of additional TVA expressing mice driven by additional epithelial or stromal compartment specific gene promoters.

Tissue Recombination Grafting – Transgenic Tissue Rescue & Compartment-Specific Gene Modulation

Manipulation of gene expression or signaling activity in transgenic mice often impairs viability and lifespan. Therefore, cells and tissues from these mice needed for experiments may not be available at the desired adult stage of development. A tissue of interest can be isolated from the host prior to death, for example the prostate from an adolescent male mouse, and rescued by surgical implantation under the renal capsule of a 7-10 weeks old normal adult male syngenic athymic nude (lacking T cells), or SCID mouse (lacking B cells and T cells) (Hayward et al., 1997; Day et al., 2002; Hayward, 2002). [An overview of the renal capsule grafting procedure is available online (<http://mammary.nih.gov/tools/mousework/cunha001/index.html>).] The kidney inherently has little stroma to interfere with implanted tissues and a rich vasculature that ensures the growth and survival of the implants. These characteristics make the kidney a suitable environment for the rescue of important tissues. To take advantage of both stromal conditional knockout mice, renal capsule allografting, as well as an intact immune system, we have successfully performed tissue rescues of prostate from *Tgfr2^{fsPKO}* mice in syngenic male C57/B16 mice enabling survival of the rescued prostate for up to a year from mice that would otherwise die by six to seven weeks of age.

Tissue recombination grafting provides another alternative to the creation of transgenic mice for the *in vivo* examination of gene function and interrogation of signal transduction pathways. For instance, to study prostate stromal-epithelial interactions, epithelial cells or intact prostatic organoids (in which the stroma has been removed by digestion with collagenase and DNase-I) can be recombined with stromal cells within a matrix of rat-tail collagen (Hayward et al., 1999). These grafts can be implanted immediately or incubated over-night and implanted the next day under the renal capsule of host mice (as described above). Recently, a UGM cell line (UGSM-2) has been isolated from a E16 mouse UGS. These cells participate in prostate glandular morphogenesis when recombined with UGE and may be a useful tool for gain- and loss-of-function studies in prostate tissue recombination models (Shaw et al., 2006; Shaw & Bushman, this issue)). Compartment-specific gene modulation is achievable through use of lentiviruses, which can infect both proliferative and non-proliferative cells (Naldini et al., 1996), to express a gene of interest or to knockdown a gene of interest by RNAi prior to the creation of the graft (Williams et al., 2005; Ao et al., 2006; Ao et al., 2007; He et al., 2007). Ad-Cre or cell-permeable Cre may also be used in culture or when assembling the graft to knockout the expression of a floxed gene *in situ*.

Concluding Remarks

While use of conditional promoter systems to create transgenic knock-in and knock-out mouse models have been invaluable, the technology available to date has yet to overcome long-standing challenges whose solution is essential to the molecular dissection of stromal tissues (Schmidt-Supprian and Rajewsky, 2007). Currently available promoter-regulated Cre's still target the stroma of multiple tissues often with mosaicism. This adds further variability to stromal compartments that are frequently heterogeneous in nature within normal tissues and between different tissues and organs. Moreover, currently available promoter-regulated Cre's are generally not specific enough to hit just the desired stromal compartment or cell-lineage within a single specific tissue. The ability to dissect functionally the stromal microenvironment during development or to study carcinogenesis is a balance of the stromal specificity of the targeting mechanism and inherent stromal heterogeneity. One solution can be to develop conditional knockout mice using combinatorial promoters, which will specifically restrict temporal (developmental) and spatial (compartment or cell-lineage specific) expression with higher fidelity than endogenous promoters.

In summary, the development and use of conditional transgenic mouse models has increased our understanding of the epithelial and stromal compartments of tissues including the prostate. Refinement of currently available transgenic technology as well as further elucidation and delineation of cell and tissue specific differentiation markers in stromal tissues will improve our ability to create increasingly informative mouse models utilizing targeted gene expression to and within specific stromal compartments.

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Figure 1. Prostate epithelial interactions with prostate stromal compartments

Glandular epithelium (basal, luminal, and occasional neuroendocrine cells) of the normal human adult prostate is in close contact with the underlying stromal compartment consisting predominately of ECM (collagen, fibronectin, etc.), differentiated smooth muscle cells, as well as occasional fibroblasts, myofibroblasts, and blood vessels. In prostate cancer, de-differentiation of the prostatic smooth muscle cells into fibroblasts promotes de-differentiation, proliferation, and stromal invasion of the prostatic epithelium. This results in a vicious cycle of disease progression, which includes localized increases in angiogenesis to/from the tumor as well increased recruitment of myeloid-lineage hematopoietic cells. Differentiation markers for which there are available Cre mice targeting prostate stromal compartments are listed.

Figure 2. Prostate tumor cell interactions with bone stromal compartments

The activities of bone resorption by osteoclasts and bone formation by osteoblasts are tightly coordinated. In bone, osteoclasts and osteoblasts are frequently localized in close proximity to each other and interact via paracrine signaling. Infiltration of metastatic prostate tumor cells begins a vicious cycle, mediated by cross-talk of paracrine signaling intermediates, such as TGF- β , Wnt and DKK1, Sonic Hedgehog (SHH), Endothelin-1 (ET-1), and Rank/RankL. Pro-osteolytic cancers stimulate osteoclast differentiation and activity resulting in bone destruction. Pro-osteoblastic cancers stimulate osteoblast differentiation and activity resulting in bone formation of lesions of weaker woven bone within the trabecular bone. Differentiation markers for which there are available Cre mice targeting bone stromal compartments are listed.

Figure 3. Conditional gene knockout in mice

(A) Integration of a gene targeting construct into a genomic locus by homologous recombination and recombinase-mediated excision. A typical gene knockout targeting construct has the following features: a region of DNA from a gene locus of interest containing one or more exons and the start codon(s) flanked by loxP sites (referred to as being “floxed”), a negative selection marker for integration such as the thymidine kinase (TK) cassette, and a positive selection marker for integration such as the Neomycin resistance gene (NEO) flanked by FRT sites (referred to as being “flrtd”). The linearized targeting construct is introduced into mouse embryonic stem cells by electroporation or microinjection. The construct integrates into the genome by homologous recombination resulting in loss of the negative selection marker. The positive selection marker is excised by transfection of FLPe recombinase, which specifically recognizes FRT sites. Subsequently, the ES cells are placed in blastocysts and implanted into a pseudo-pregnant female mouse resulting in the birth of mice bearing the desired floxed allele. Introduction of Cre recombinase, which specifically recognizes loxP sites, will result in excision of the floxed allele. (B) Generation of conditional knockout mice by selective breeding. Conditional knockout of gene expression in mice is accomplished through the mating of a mouse bearing two copies of the floxed allele with a mouse expressing Cre recombinase driven by a gene

promoter expressed in a developmental stage specific, tissue specific, and/or temporally restricted manner. For example, the mating of a FSP-1-Cre mouse with a floxed mouse will result in a proportion of the offspring having the desired FSP-1 directed knockout of the gene of interest (fspKO) in FSP-1 expressing stromal cells.

Figure 4. Fluorescent detection of FSP-1 expression in the stromal compartments of multiple tissues of the bladder, prostate, and uterus

GFP expression driven by the FSP-1 promoter (green) is visible in these paraffin sections, nuclear counterstained with Hoechst 33258 (blue). Importantly, note that a fraction of the stromal compartment has FSP-1 expression. The epithelial (E) and stromal (S) compartments are indicated.

Table 1

Prostate epithelial and stromal differentiation markers

Differentiation Marker	Symbol(s)	Compartment	Reference
Androgen Receptor	AR	Epithelia	Cooke et al. (1991)
Cytokeratin 5	CK5	Basal epithelia	Wang et al. (2001)
Cytokeratin 8	CK8	Luminal epithelia	Wang et al. (2001)
Cytokeratin 14	CK14	Basal epithelia	Wang et al. (2001)
Cytokeratin 18	CK18	Luminal epithelia	Wang et al. (2001)
Desmin	DES	Myofibroblasts, smooth muscle	Hayward et al. (1996b)
Fibroblast Specific Protein-1	FSP-1, S100A4	Fibroblasts, myofibroblasts, and smooth muscle	Bhowmick et al. (2004a)
p63	p63	Basal epithelia	Wang et al. (2001)
Prostatic-Acid Phosphatase	PAP	Differentiated prostatic epithelium	Hayward et al. (1998)
Probasin	Pb	Epithelia	Zhang et al. (2004)
Prostate-Specific Antigen	PSA	Epithelia	Snoek et al. (1998)
SM22	SM22	Myofibroblasts, smooth muscle	Duband et al. (1993)
Smooth Muscle Actin (alpha)	α SMA	Myofibroblasts, smooth muscle	Tuxhorn et al. (2002a)
Smooth Muscle Myosin Heavy Chain	SMMHC	Myofibroblasts, smooth muscle	Regan et al. (2000)
Tenascin	TEN	Myofibroblasts, smooth muscle	Tuxhorn et al. (2002a)
Vimentin	Vim	Fibroblasts, myofibroblasts	Tuxhorn et al. (2002a)

Table 2

Cre mice available for targeting mesenchymal/stromal compartments of the prostate and bone

Mouse line	Targeted tissue for Cre expression	References
CD11b-Cre	Macrophages, monocytes, osteoclasts, granulocytes	Ferron and Vacher (2005)
Col1a1-Cre	Fibroblasts, myofibroblasts, smooth muscle, osteoblasts, chondrocytes	Dacquin et al. (2002), Liu et al. (2004), Kim et al. (2004)
Col1a2-Cre/Cre-ERT	Fibroblasts, myofibroblasts, smooth muscle, osteoblasts, chondrocytes	Zheng et al. (2002), Florin et al. (2004)
Col2a1-Cre/Cre-ERT	Chondrocytes	Long et al. (2001), Sakamoto et al. (2005), Nakamura et al. (2006)
CTSK-Cre	Osteoclasts	Chiu et al. (2004)
Dermo1-Cre	Chondrocytes, osteoblasts	Yu et al. (2003)
FSP1-Cre	Fibroblasts, myofibroblasts, smooth muscle, osteoblasts, chondrocytes, macrophages	Iwano et al. (2001, 2002), Bhowmick et al. (2004a); Inoue et al. (2005)
OC-Cre	Chondrocytes, osteoblasts	Zhang et al. (2002)
Prx1-Cre	Chondrocytes	Logan et al. (2002)
SMA-Cre	Myofibroblasts, smooth muscle	Miwa et al. (2000), Wu et al. (2007b)
SM22-Cre/Cre-ERT	Smooth muscle	Kuhbandner et al. (2000), Holtwick et al. (2002)
SMMHC-Cre	Smooth muscle	Regan et al. (2007)
Sox9-Cre	Chondrocytes, osteoblasts	Akiyama et al. (2005)
Tie1-Cre	Endothelial cells	Song et al. (2007)
Tie2-Cre	Endothelial cells, Macrophages	Kisanuki et al. (2001)
TRAP-Cre	Chondrocytes, osteoclasts	Chiu et al. (2004)

FSP, fibroblast specific protein; SMA, smooth muscle actin; SMMHC, smooth muscle myosin heavy chain.