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A Role for Runx2 in Normal Mammary Gland and Breast Cancer Bone Metastasis

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Abstract The transcription factor Runx2 is essential for the formation of the skeleton. It has therefore primarily been considered as a specific regulator of bone genes. However, mice containing a LacZ insertion at the Runx2 locus also revealed expression in the nascent mammary epithelium. Reports of Runx2 expression in breast cancer cell lines, combined with the fact that breast cancers preferentially metastasise to bone, have also hinted at a potential role for Runx2 in the formation of bone metastasese. These initial observations have prompted further analysis of Runx2 function in mammary epithelial cells and recent findings have demonstrated that Runx2 does indeed contribute to the ability of metastatic breast cancer cell lines to form osteolytic bone lesions. In addition, evidence is accumulating that Runx2 has a role in the regulation of normal mammary gland gene expression and recent data demonstrate that it regulates transcription of the mammary gland-specific gene, β-casein. In this article I discuss recent advances that link Runx2 with normal mammary epithelial cell function and the development of bone metastasese in breast cancer. J. Cell. Biochem. 96: 484–489, 2005.

Key words: Runx2; transcription; mammary gland; bone; metastasis

An increasing number of studies have revealed molecular connections between breast and bone. For example, genes important in the control of bone remodelling are expressed in breast cancer and lactating breast, such as RANK, RANKL, vitamin D, bone sialoprotein (BSP), osteopontin (OPN), and calcitonin [Martin and Gillespie, 2001]. In addition, breast cancers preferentially metastasise to the bone [Yin et al., 2005; Yoneda and Hiraga, 2005]. Breast cancer cells that establish themselves in the bone are thought to modulate, either directly or indirectly, the activity of osteogenic cells, resulting in local bone degradation. What causes breast cancer cells to preferentially colonize the bone and influence osteogenic cell activity is a key question in breast cancer research and identification of the genes involved in this metastatic process should provide potential targets for therapeutic intervention.

The transcription factor Runx2 is a master-regulator of bone development, and mutations in Runx2 are found in patients suffering from the skeletal disorder cleidocranial dysplasia [reviewed in Karsenty, 2000; Komori, 2002; Otto et al., 2002; Stein et al., 2004]. Targeted deletion of the *Runx2* gene in mice has demonstrated that Runx2 is required for osteoblast differentiation and chondrocyte hypertrophy. However, accumulating evidence also suggests that Runx2 has a role in normal mammary epithelial cells and in the formation of bone metastases in breast cancers. Here I review the evidence that Runx2 has a role in normal breast and breast cancer metastasis. The implications and future prospects for this newly emerging area of Runx2 research are also discussed.

RUNX2 EXPRESSION IN MAMMARY EPITHELIAL CELLS

The first indication that Runx2 was expressed in human breast cell lines was the observation by Selvamuragan and Partridge that Runx2 transcripts were present in MDA-MB-231 and

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MCF-7 cells [Selvamurugan and Partridge, 2000]. Runx2 protein has since been observed in these latter cell lines and in HC11, MTSV1-7, and LCC-15MB cells, as determined by Western analysis and DNA-binding activity [Barnes et al., 2003; Inman and Shore, 2003; Selvamurugan et al., 2004; Inman et al., 2005]. Runx2 DNA-binding activity has also been detected in primary mammary epithelial cells from pregnant mice [Inman and Shore, 2003]. In isolation these findings are not particularly surprising, since cultured cells often aberrantly express genes. However, Otto et al. have also documented expression of the Runx2 gene in the nascent mammary epithelium of mice in vivo, suggesting that Runx2 has a role in the mammary gland [Otto et al., 1997]. Furthermore, it is well established that breast cancers preferentially metastasise to the bone and that they express "bone genes" [Martin and Gillespie, 2001; Yin et al., 2005; Yoneda and Hiraga, 2005]. From the above observations it is reasonable to hypothesize that Runx2 regulates gene expression in the mammary epithelium and that the Runx2 genetic program somehow contributes to the ability of cancerous mammary epithelial cells to metastasise to the bone. Unfortunately, at this stage the data on Runx2 expression are limited, but further important evidence for Runx2 function in normal mammary gland and breast cancer cells has been provided from molecular studies of mammary gland-specific gene transcription and from work demonstrating that Runx2 contributes to the metastatic potential of breast cancer cell lines.

A ROLE FOR RUNX2 IN NORMAL MAMMARY GLAND

Whilst Runx2 is expressed in several mammary cell lines, not all of them exhibit a preference to metastasise to the bone, suggesting that its expression might not just be associated with a metastatic phenotype. The expression of Runx2 in HC11 cells is particularly interesting because these cells do not originate from a tumor but are derived from the mammary gland of a pregnant mouse [Ball et al., 1988]. In contrast to the cancer cell lines they retain the ability to produce milk proteins in response to lactogenic hormones and have been used extensively as a model to investigate mammary gland-specific gene regulation. Work in my own laboratory has used this model cell line to investigate whether Runx2 contributes to the expression of genes known to be expressed in the mammary gland. Our studies have focused on two genes, the milk protein gene, β-casein, and OPN, a gene known to be normally expressed in both osteoblasts and mammary epithelial cells [Inman and Shore, 2003; Inman et al., 2005].

β-casein is a milk protein whose expression is specifically induced in mammary gland at late pregnancy and during lactation [reviewed in Groner, 2002]. It is an established marker of mammary epithelial cell differentiation and a paradigm for hormonal regulation of gene transcription. The mouse β-casein gene promoter contains a Runx binding site which is essential for its lactogenic hormone stimulation [Saito and Oka, 1996; Inman et al., 2005]. A combination of RNAi, chromatin immunoprecipitation (ChIP) assays, and luciferase reporter assays demonstrated that Runx2 contributes to the activity of the β-casein promoter [Inman et al., 2005]. In addition, it was noted that the Runx site in the β-casein promoter is adjacent to an octamer element and that this configuration is highly conserved in casein promoters from a wide range of mammals. This Runx/octamer element enables the formation of a novel Runx2/Oct-1 complex, analysis of which revealed that Oct-1 stimulates recruitment of Runx2 to the β-casein promoter by interacting with the C-terminal region of Runx2.

At this time it is not clear what the role of this Runx2/Oct-1 complex is in mediating lactogenic hormone signals to activate β-casein transcription. It is well established that the primary mechanism of activation of the β-casein gene is via STAT5 and the Glucocorticoid Receptor (GR) [Groner, 2002] and it is possible that the Runx2/Oct-1 complex acts as a "landing pad" for these transcription factors. Indeed, both Runx2 and Oct-1 have been shown to interact with GR [Ning and Robins, 1999; Prefontaine et al., 1999], suggesting that the Runx2/Oct-1 complex may recruit GR. Furthermore, since Runx2 has been shown to interact with STAT1 [Kim et al., 2003], it might also be able to interact with STAT5, thus providing a link between prolactin signaling and Runx2 activity. As Runx2 is a nuclear matrix-binding protein it is also possible to envisage a role in recruiting the β-casein promoter to transcriptionally active nuclear sub-domains via its interaction with Oct-1. Finally, it is intriguing to note that osteocalcin,

a Runx2 target in osteoblasts, and β-casein are both calcium-phosphate binding proteins, which may reflect a conserved functional role for Runx2 in the physiological regulation of calcium [Hauschka, 1986; Vegarud et al., 2000].

Another gene that we have investigated is OPN [Inman and Shore, 2003]. OPN has long been established as a Runx2 target gene in osteoblasts, and analysis of its regulation in HC11 cells using a combination of RNAi, promoter reporter assays and RT-PCR demonstrated that Runx2 also contributes to the expression of the OPN gene in these cells. OPN is a secreted integrin-binding extracellular matrix protein, which has several functions including stimulation of cell adhesion, cell signaling, cell migration, and protection against apoptosis [reviewed in Weber, 2001]. It is potentially a very important Runx2 target gene since it is expressed in normal mammary gland and its overexpression is strongly associated with metastasis. Further analysis of OPN transcriptional regulation by Runx2 in normal and metastatic cells may reveal important differences in Runx2 activity in these two cell types.

Whilst the complete picture is still far from clear, the available data on Runx2 in mammary epithelial cells indicate that it contributes to the expression of mammary gland-specific casein genes and OPN. Interestingly both of these genes are expressed during lactation, which suggests that Runx2 is expressed in lactating breast. Moreover, given the established precedence for Runx transcription factors in tissue development [reviewed in Coffman, 2003], it is possible that Runx2 has a more extensive role in mammary gland. These questions will be best addressed in vivo by generating mice in which the Runx2 gene has been specifically deleted in the mammary gland.

RUNX2 IN BREAST CANCER BONE METASTASIS

Breast cancer cells that have the potential to form bone metastasese have been shown to express several genes that are regulated by Runx2 including OPN, BSP, and collagenase-3 [Selvamurugan and Partridge, 2000; Barnes et al., 2003; Inman and Shore, 2003; Selvamurugan et al., 2004]. Expression of Runx2 target genes appears to confer features of an osteoblast-like phenotype upon breast cancer cells, a phenomenon termed osteomimicry [Barnes et al., 2003]. The first clue pointing to a role for Runx2 in bone metastases was the observation that the Runx2 target gene, collagenase-3, was constitutively expressed in the human breast cancer cells MDA-MB-231 [Selvamurugan and Partridge, 2000]; these cells are highly metastatic and form osteolytic lesions when injected into mice [Guise, 1997]. Collagenase-3 is a member of the family of matrix metalloproteinases (MMPs) which are often expressed by metastatic cancers and contribute to their ability to invade tissues [reviewed in Ala-aho and Kahari, 2005], it is a known in vivo target gene of Runx2 in bone and further analyses have demonstrated that Runx2 does indeed regulate collagenase-3 expression in MDA-MB-231 cells [Jimenez et al., 1999; Selvamurugan et al., 2004]. ChIP assays demonstrated that two Runx sites located within the endogenous collagenase-3 promoter are occupied by Runx2 in MDA-MB-231 cells [Selvamurugan et al., 2004]. Mutation of either of these Runx sites inhibited the activity of the collagenase-3 promoter in reporter assays, as did the expression of the Runx repressor protein AML/ETO. In separate studies, Barnes and colleagues investigated the role of Runx2 in the regulation of the BSP promoter in MDA-MB-231 and LCC15-MB cells [Barnes et al., 2003]. BSP is a major non-collagenous, extracellular matrix protein expressed in bone; it is expressed by terminally differentiated osteoblasts and is thought to function in the formation and remodelling of mineralized tissue. Increased levels of BSP expression are also found in malignant breast cancers and are associated with an increased incidence of bone metastases, implicating BSP in the development of bone metastases [Barnes et al., 2003]. EMSAs using nuclear extracts from MDA-MB-231 and LCC15-MB cells demonstrated that Runx2 in these cells can bind to the Runx element within the BSP promoter [Barnes et al., 2003]. Overexpression of the type I Runx2 isoform (MRIPV) was also shown to activate the BSP promoter in luciferase reporter assays. These findings provide compelling evidence that Runx2 is involved in the regulation of collagenase-3 and BSP expression in metastatic breast cancer cells and suggest that Runx2 has a role in the ability of these cell lines to form bone metastases.

Further elegantly designed experiments by Barnes et al used a mouse implantation model to

demonstrate that disruption of Runx2 activity in MDA-MB-231 cells does indeed abolish their ability to form osteolytic lesions in vivo [Barnes et al., 2004]. When injected into the intramedullary space in the tibia of immunocompromised mice MDA-MB-231 cells induced osteolytic lesions in more than 80% of recipients. In contrast, only 5% of mice developed detectable osteolytic lesions when injected with stably transfected MDA-MB-231 cells expressing a truncated version of Runx2 (Runx2Δ230). Runx2Δ230 acts as a dominant-negative to down-regulate Runx2 target genes and the stably transfected MDA-MB-231 cells used in these experiments displayed a 50% reduction in BSP promoter activity.

When examined in vitro, MDA-MB-231 cells inhibited differentiation of osteoblasts derived from bone marrow stromal cells (BMSCs) in co-culture, as determined by the reduced formation of osteoblast-associated mineralized nodules and a lack of expression of the osteoblast markers alkaline phosphatase, BSP, osteocalcin, and collagen type I [Barnes et al., 2004]. In contrast, osteoclasts in these cultures were induced to differentiate, as judged by the increase in cells expressing the osteoclast marker tartrate resistant acidic phosphatase. Strikingly, these effects on bone cell differentiation were not observed in analogous co-cultures in which the MDA-MB-231 cells expressed the dominant-negative protein Runx2Δ230. Since osteoblasts are the bone forming cells and osteoclasts the bone removing cells it is envisaged that perturbation of their differentiation by breast cancer cells contributes to localized degradation of the bone and that this process is dependent upon the expression of Runx2 in the cancer cells.

The correct localization of Runx2 to intranuclear subdomains has also been shown to be important for expression of target genes required for the osteolytic activity of metastatic breast cancer cells [Javed et al., 2005]. Runx2 contains a nuclear matrix-targeting signal (NMTS) in the C-terminal region which is responsible for its association with the nuclear matrix. Point mutations of the Runx2 nuclear matrix-targeting signal sequence (R398A and Y428A), that impair its association with the nuclear matrix, were used to investigate the effect of altered intranuclear trafficking of Runx2 on the ability of breast cancer cells to induce osteolysis. When the Runx2 NMTS mutant was expressed in MDA-MB-231 cells it was no-longer found to be associated with the nuclear matrix intermediate filament fraction. The effect of perturbing Runx2 nuclear matrix targeting on osteogenic differentiation was similar to that observed with the dominant-negative Runx2Δ230 protein [Barnes et al., 2004]. Importantly, the mutant Runx2 protein also inhibited the invasive and osteolytic properties of MDA-MB-231 cells as determined by their reduced migration ability on Matrigel and a reduction in the formation of osteolytic lesions in vivo [Javed et al., 2005]. These findings correlated with reduced expression of VEGF and collagenase-3, both factors known to be associated with cell invasion.

The analysis of the role of Runx2 in MDA-MB-231 cells clearly demonstrates that Runx2 confers upon breast cancer cells an ability to perturb osteogenic differentiation, suggesting that Runx2 target genes are instrumental in the formation of metastatic bone lesions in breast cancer. These findings suggest that considerable effort should be focused on identifying the Runx2 target genes which are responsible for the development of bone metastases.

FUTURE PERSPECTIVES

It is clear from the available data that Runx2 can contribute to the formation of bone metastases and the regulation of mammary-gland specific genes in experimental systems (Fig. 1). However, whilst this evidence is compelling it is still only a smoking gun, and much more needs to be done to establish Runx2 as a key contributor to gene expression in both normal mammary gland and bone metastases in vivo.

How Runx2 regulates gene expression in metastatic breast cancer cells could be important in the development of therapies to inhibit the metastasis of breast cancers to bone. In this regard it will also be critical to identify the Runx2 target genes which confer bone colonizing ability on the tumor cells. Such potential makes it a rather pressing priority to establish if Runx2 is indeed expressed in primary human breast tumors and their matched bone metastases. It is also possible that the metastatic role of Runx2 is not due to abnormal expression in breast cancer cells but rather a subversion of its normal role (Fig. 1). It will therefore be important to determine whether Runx2 expression is actually increased in breast cancers, as there is

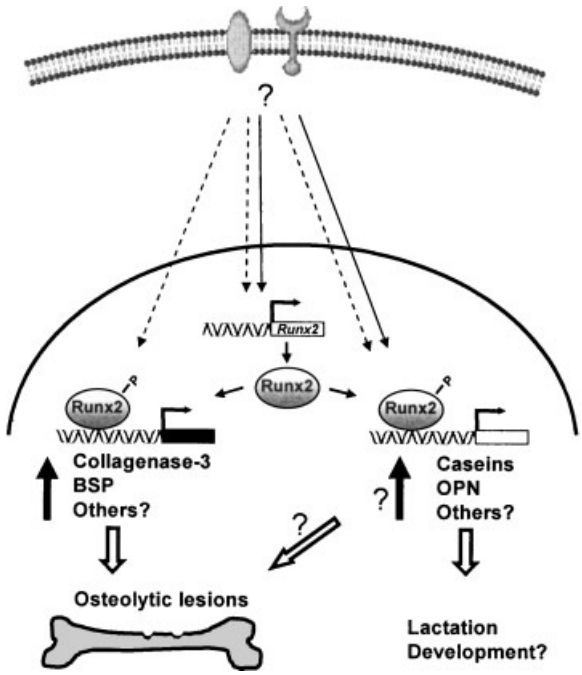

Fig. 1. Proposed scheme for normal and aberrant regulation of Runx2 target genes in mammary epithelial cells. Runx2 is depicted regulating both normal (**right**) and aberrant (**left**) target genes. The proposed normal Runx2 target genes include the casein genes and OPN. Collagenase-3 and BSP are known to be aberrantly up-regulated in metastatic cells and it is likely that other genes are too. OPN expression is also increased in metastatic cells. The bold arrow indicates increased expression in metastatic cells. Runx2 activity is known to be regulated by several signaling pathways in osteoblasts. These same pathways may regulate Runx2 in mammary epithelial cells but only hypothetical pathways are depicted to illustrate the principle that Runx2 could be regulated differently in normal and metastatic cells. Solid arrows indicate signaling in normal cells, broken arrows indicate aberrant signaling in metastatic cells. Runx2 expression and activity may also be increased by genetic mutation or other regulatory factors but these are not shown here for clarity.

still uncertainty as to whether Runx2 expression is up-regulated in metastatic cells compared to non-metastatic cells. Indeed, Runx2 is the target of a number of signal transduction pathways, some of which are known to be aberrantly activated in cancer cells [reviewed in Franceschi and Xiao, 2003; Campbell and Der, 2004]. In metastatic cells constitutive activation of signaling pathways may in turn lead to activation of Runx2, and thus increased expression of its target genes (Fig. 1). Alternatively, Runx2 activity could be altered by mutation, or the presence of collaborating factors that are not present, or active, in non-metastatic cells

In summary, studies to date suggest that Runx2 has a role in both normal mammary gland and in the development of breast cancer bone metastases. The stage is now set for further work to elucidate the precise expression pattern, target genes and mechanism of action of Runx2 in normal mammary gland and in breast cancer. In the future, this aspect of Runx2 research may prove to be very useful in the development of therapies to treat bone metastasese.

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