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Twist-ing Cell Fate: Mechanistic Insights Into the Role of Twist in Lineage Specification/Differentiation and Tumorigenesis

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ABSTRACT

Bone marrow-derived mesenchymal stem cells (MSCs), are multipotent cells that give rise to multiple lineages including osteoblasts, adipocytes, muscle, and fibroblasts. MSCs are useful for clinical applications such as cell therapy because they can be isolated from an individual and expanded for use in tissue repair, as well as other therapeutic applications, without immune rejection. However, one of the key problems in the use of MSCs for these applications is the efficiency of these cells to engraft and fully regenerate damaged tissues. Therefore, to optimize this process, a comprehensive understanding of the key regulators of MSCs self-renewal and maintenance are critical to the success of future cell therapy as well as other clinical applications. The basic helix loop helix transcription factor, Twist, plays a master regulatory role in all of these processes and, therefore, a thorough understanding of the mechanistic insights in the role of Twist in lineage specification/differentiation and tumorigenesis is vital to the success of future clinical applications for the therapeutic use of MSCs. In this article, we highlight the basic mechanisms and signaling pathways that are important to MSC fate, maintenance, and differentiation, as well as the critical role that Twist plays in these processes. In addition, we review the known literature suggesting a critical role for Twist in the generation of cancer stem cells, as this information may contribute to a broader understanding of stem cell biology and stem-cell-based therapeutics. J. Cell. Biochem. 110: 1288–1298, 2010.

KEY WORDS: TWIST; WNT; BMP; TGF-β; SMAD; MSC; OSTEOBLAST; CHONDROCYTE; ADIPOCYTES; CANCER STEM CELLS

The basic helix loop helix (bHLH) family of transcription factors can be categorized into three classes. Class A bHLH proteins, also known as E proteins, are ubiquitously expressed and form heterodimers with other tissue-specific bHLH proteins. Class B bHLH proteins are tissue specific and include members of the MyoD protein family and Twist. Class C bHLH proteins contain a leucine zipper motif C-terminal to the bHLH motif. In addition, members of the Id family of proteins lacking a DNA-binding basic region N-terminal to the bHLH motif act as negative regulators of Class A and B bHLH factors by sequestering these proteins and preventing them from forming functional complexes [Massari and Murre, 2000].

In mammals there are six Twist orthologs: Twist1, Twist2, Hand1, Hand 2, Paraxis, and Scleraxis. The bHLH domain is an evolutionary conserved motif consisting of a short stretch of basic amino acids followed by two amphipathic α-helices separated by a loop [Massari and Murre, 2000]. The α-helices participate in protein–protein dimerization with other bHLH proteins, which results in a juxtaposition of the basic domain and creates a DNA-binding motif that binds to the E-Box consensus sequence (CANNTG) [Massari and Murre, 2000].

Twist was originally discovered in *Drosophila* as essential for the onset of gastrulation and mesoderm formation [Simpson, 1983; Leptin, 1991]. Twist mutant *Drosophila* embryos fail to develop mesodermally derived organs, have abnormal head involution, and the embryo is twisted in the egg. When misexpressed in other tissues, Twist induced mesoderm-specific formation of muscle in inappropriate locations [Castanon et al., 2001]. In mice, Twist is expressed in the mesoderm, somites, cranial mesenchyme, limb bud mesenchyme, tooth bud, and sutural tissues of the skull [Wolf et al., 1991]. Twist null mice die at embryonic day 11.5 due to failure of neural tube closure and defects in the head mesenchyme, somites, and limb buds [Chen et al., 1995]. Heterozygous mice are viable but

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display abnormal craniofacial structures, poor suture growth, asymmetrical facial skeleton, and preaxial polydactyly of the hind limb [Bourgeois et al., 1998]. These observations suggest that Twist-1, and possibly Twist-2, inhibit osteoblast differentiation.

In humans, loss of function heterozygous mutations in the *twist* gene are associated with an autosomal-dominant craniosynostosis disorder known as Saethre–Chotzen Syndrome (SCS) [Reardon and Winter, 1994; el Ghouzzi et al., 1997; Johnson et al., 1998]. Over 75 mutations have been identified in SCS patients, involving large deletions and mostly point mutations that cluster near the DNA-binding domain [Gripp et al., 2000; Corsi et al., 2002; Jabs, 2004]. SCS is characterized by premature fusion of cranial sutures, low frontal hairline, facial asymmetry, eyelid ptosis, and limb defects such as polydactyly, brachydactyly, and syndactyly [Howard et al., 1997]. It has been proposed that these effects are due to accelerated bone growth and increased differentiation, as Twist mutant cells show an increased ability to form bone-like nodular structures with increased expression of osteoblastic markers, alkaline phosphatase (ALP), and type 1 collagen [Yousfi et al., 2001]. However, the mechanisms by which Twist regulates the processes of osteogenic development and postnatal bone homeostasis remain to be determined.

Following initiation of the osteogenic pathway via the osteogenic master regulatory transcription factor, Runx2 [Ducy et al., 1997] a number of complementary genes, such as the zinc-finger transcription factors osterix and Krox-20, the homeobox-containing transcription factors, Msx2 and Dlx5, and various members of the Fos family of transcription factors (c-Fos, FosB, δ-FosB, Fra-1, and Fra-2) help maintain and regulate osteoblast differentiation [Shalhoub et al., 1989, 1992; McCabe et al., 1996; Inoue et al., 1999; Fang et al., 2001; Nakashima et al., 2002; Balint et al., 2003; Harris et al., 2003; Shah et al., 2004]. In contrast, little is known about the molecular mechanisms that determine the fate of the musculoskeletal precursors (Fig. 1) known as mesenchymal stem cells (MSC) [Pittenger et al., 1999; Gronthos et al., 2003b] during asymmetrical cell division. In particular, whether MSCs progress either towards a particular cell lineage or remain as a multi-potential stromal stem cell. Moreover, it is not clear which critical genes regulate the maintenance of multi-potential MSC populations throughout the life of an individual, or following ex vivo expansion.

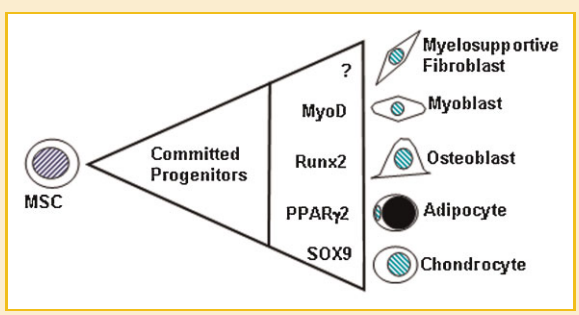

Fig. 1. Proposed stromal hierarchy of cellular differentiation. Mesenchymal stem cells (MSCs) undergo clonal expansion and differentiation via the upregulation of master regulatory genes associated with various stromal lineages.

TWIST: MECHANISMS GOVERNING MUSCULOSKELETAL DIFFERENTIATION

An important mechanistic insight into the action of Twist was discovered in 2004 by examination of skull development [Bialek et al., 2004]. In this report, it was noted that the master osteogenic regulator, Runx2, was expressed, together with Twist, at E12 in cells of the future temporal and parietal bones; however, no bone differentiation had occurred at this stage. By E14, however, the levels of Twist had decreased, Twist expression exhibited a mutually exclusive pattern with Runx2, and osteogenic differentiation was evident. This gave birth to the idea that Twist might function to antagonize the action of Runx2. Genetic interactions were then investigated and demonstrated that mice heterozygous for Twist and Runx2 have a normally shaped skull and intraparietal bones and no premature fusion of the coronal sutures. Although Twist homozygous and heterozygous knockout mice showed no difference in expression of Runx2, Twist inhibited Runx2-mediated transactivation of a Runx2-driven reporter, and this was mediated by the c-terminal region of Twist, known as the Twist box. Furthermore, this domain interacted with the Runt domain of Runx2 and reduced the ability of Runx2 to bind DNA (Fig. 2). This mechanism was recently also described in dentin-forming odontoblasts involved in tooth development, which are closely akin to osteoblasts, and is involved in tooth development. Mice heterozygous for Twist showed an earlier onset of dentin matrix, increased ALP activity, and pulp stones within the pulp. When crossed with $Runx2^{+/-}$ mice to produce Twist/Runx2 heterozygotes, this phenotype was found to be completely rescued [Galler et al., 2007]. Furthermore, in vivo evidence for the role of Twist on Runx2 function in regulating osteoblast differentiation is illustrated in the Charlie Chaplin (CC/+) mouse strain, which has a single amino acid substitution in the Twist box. These mice exhibit craniosynostosis with irregular lamboidal and coronal sutures. CC/CC mice have short limbs and polydacytly of the hind limbs but no neural tube defect. This suggests that although the above-described mechanism of Twist action is an essential part of regulating osteogenesis, it does not account for the complete story and does not rule out the possibility that Twist may interact with other proteins involved in osteogenesis. This idea is supported by genome-wide ChiP studies of *Drosophila* Twist showing that Twist can bind to more than 500 regions of the genome.

Reinforcing the notion that Twist inhibits the action of transcription factors controlling tissue specificity is the fact that murine Twist inhibits MyoD transactivation by sequestering E-proteins and preventing the formation of E-protein–MyoD complexes and by inhibiting the action of MEF2, which results in strong inhibition of skeletal muscle differentiation. Murine Twist directly interacts with the myogenic bHLH protein, MyoD, via its basic DNA-binding domain and inhibits MyoD-driven transcription and muscle differentiation [Hamamori et al., 1997]. In addition, Twist can interact with other myogenic bHLH proteins, such as myf5, myogenin, and MRF4 [Hamamori et al., 1997], and these

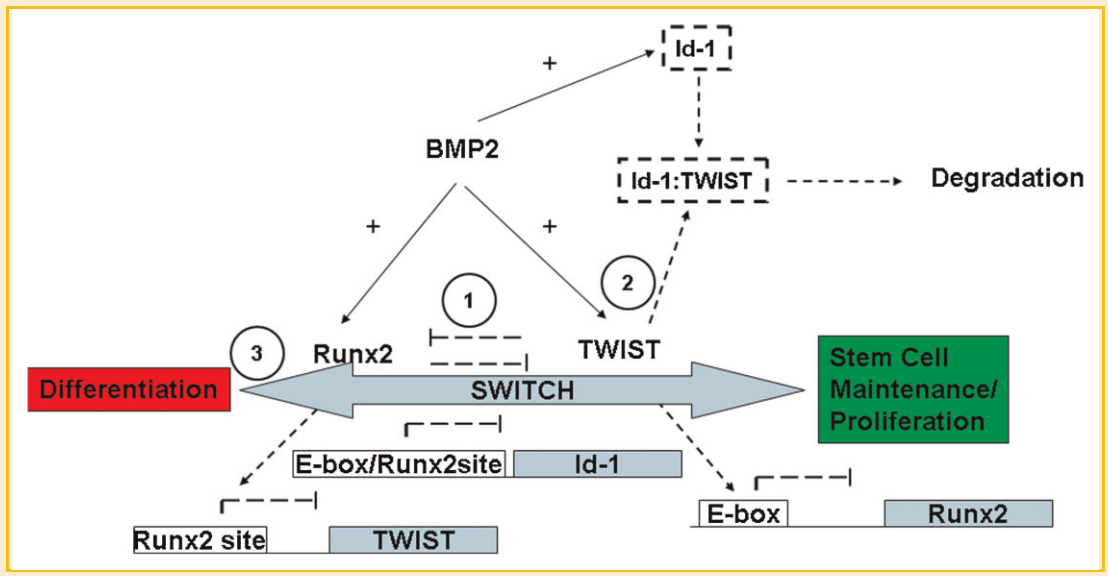

Fig. 2. Model depicting possible molecular mechanisms responsible for potentiating the osteoblastic lineage. This model postulates that Runx2 and Twist negatively regulate each other by protein–protein and protein–DNA interactions resulting in the observed shift from proliferation to differentiation (dotted arrows). We further postulate that degradation of Twist occurs through Id/Twist heterodimerization, which targets Twist for degradation, instead of allowing it to bind DNA. The absence of Twist then allows for differentiation to proceed. Additionally, both Runx2 and Twist regulate Id-1 by protein–DNA interactions and both processes are regulated by BMP2 signaling.

interactions are vital to other MSC lineages such as skeletal muscle. However, the mechanism by which Twist inhibits MyoD-mediated transcription has not been defined, although Twist has been shown to inhibit the histone acetylase activity of the MyoD coactivators, pCAF and CBP [Spicer et al., 1996]. In agreement with this, Dermo, a bHLH protein very closely related to Twist, has been shown to interact with MyoD via the HLH and C-terminal domains, thereby inhibiting transcription [Gong and Li, 2002]. This repression was partially alleviated by the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA). Moreover, the same effect was evident with Twist, suggesting that Twist and Dermo repress transcription by recruiting HDAC to the complex.

Twist can also function in parallel with the transcription factor Msx2, which is required for calvarial bone growth. In humans, Msx2 loss of function mutations exhibit skull vault ossification defects that are similar to the defects seen in individuals with Twist haploinsufficiency. In agreement with these observations, Msx2 and Twist double knockout mice have fewer neural crest cells expressing osteoblast markers, demonstrating cooperativity in differentiation and proliferation of the skeletogenic mesenchyme [Ishii et al., 2003]. This reduction in neural crest cells expressing osteoblast markers coincides with a reduction in the level of Runx2/ALP-expressing cells. However, the molecular mechanism by which this cooperation takes place remains unclear. Both Msx2 and Twist can inhibit transcription and interact in living cells. Therefore, they could potentially cooperate by repressing a common gene whose downregulation is required for differentiation of frontal bone skeletogenic mesenchyme [Ishii et al., 2003].

The diverse mechanisms that Twist employs to alter transcription are further evident with the finding that dimer choice and posttranslational modifications can regulate Twist function. Twist can form homodimers and heterodimers with E-type proteins. Moreover, the ratio of Twist to Id proteins can influence dimerization. The use of cranial sutures as a model revealed that Twist homodimers are predominant at the osteogenic fronts and influence genes such as FGFR2 and periostin, whereas Twist/E protein heterodimers are predominant in the mid-sutures and inhibit osteogenesis. Dimer preference is altered in Twist heterozygous mice, favoring an increase in homodimers and expansion of the osteogenic fronts [Connerney et al., 2006]. In support of this, the premature suture fusion exhibited in Twist heterozygous mice was inhibited by increasing the expression of E2A or decreasing Id expression and favoring Twist/E2A heterodimer formation. Recently, it was discovered that a serine–threonine residue on the carboxyl end of the basic DNA-binding domain in the *twist* gene is highly conserved from *Drosophila* to humans. This residue is present in the Twist orthologs Hand1 and 2 and is phosphorylated by PKA/PKC and dephosphorylated by PP2A [Firulli and Conway, 2008]. Moreover, mutation of this site in the *twist* gene results in SCS and the hypophosphorylated form displays altered dimer preferences compared to wild-type Twist [Firulli et al., 2005]. These findings suggest that Twist phosphorylation may be crucial for dimerization and regulation of gene transcription. This also suggests that Twist:Hand2 heterodimers may regulate the neural crest lineages that give rise to intramembraneous calverial bone in the skull and mandible.

SIGNALING PATHWAYS IN LINEAGE DETERMINATION

Given the complexity and plethora of information regarding signaling networks in MSC lineage specification, we will briefly

mention the key players. A more comprehensive outline is given elsewhere [Karsenty and Wagner, 2002]. The Wnt family, which consists of 19 and 18 Wnt genes in the human and mouse genomes, respectively [Akiyama, 2000], is essential in embryonic bone development and formation [Westendorf et al., 2004]. Wnt signaling is required for limb bud initiation, patterning, and limb morphogenesis [Kengaku et al., 1998]. Wnt proteins bind to Frizzleds (Fzs), which are seven-span transmembrane receptor proteins, and lipoprotein receptor-related proteins 5 and 6 (LRP-5/6), and activate at least four distinct intracellular cascades: the Wnt/β-catenin, Wnt/Ca^{2+}, Wnt planar cell polarity, and Wnt/PKA pathways [Moon et al., 2002].

The Wnt/β-catenin pathway is the canonical pathway and Wnt-1, 2, 3, 3b, 4, 8, and 10b activate this pathway. In the absence of Wnt, β-catenin is phosphorylated at its amino terminal end, polyubiquitinated by βTRCP1/2, and degraded by a multi-protein complex consisting of glycogen synthase kinase (GSK-3β), adenomatous polyposis coli, and axin [Ikeda et al., 1998; Eastman and Grosschedl, 1999; Behrens, 2000]. In the presence of Wnt, the intracellular protein Dishevelled (Dvl) is activated. Dvl inhibits GSK-3β and causes dissociation of the multi-protein complex [Seto and Bellen, 2004]. Because β-catenin cannot then be targeted for degradation, it translocates to the nucleus and together with TCF/LEF family proteins activates transcription.

In addition to influencing cartilage development (by Wnt genes like Wnt-3a, 4, and 5a) [Hartmann and Tabin, 2001], Wnt-mediated regulation in bone mass was intensely studied after the initial description of mutations in one of the Wnt receptors, LRP-5, in patients with osteoporosis pseudoglioma syndrome [Gong et al., 2001]. Mutations in LRP-5 cause severe osteoporosis and recurrent fractures [Hartikka et al., 2005]. Wnt-3a promotes proliferation, suppresses osteoblast differentiation of adult MSCs [Boland et al., 2004], and promotes bone morphogenetic protein-2 (BMP-2)-mediated chondrogenesis in a murine mesenchymal cell line [Fischer et al., 2002a]. Wnt-10b, however, can promote osteoblastogenesis by inducing expression of the osteogenic-associated transcription factors, Cbfa1/Runx2, Dlx-5, and Osterix. A study implanting mature chick-derived chondrocytes by intramuscular injection found that gain of function of the downstream Wnt effector molecule, β-catenin, which mediates TCF/LEF signaling, accelerated chondrocyte maturation and bone formation while inactivation resulted in suppression [Kitagaki et al., 2003]. An essential role for β-catenin in BMP-2-induced endochondral ossification was established by implanting recombinant BMP-2 intramuscularly into mice, which induced endochondral bone formation. This activated β-catenin-mediated TCF/LEF transcription, and the use of conditional null alleles of β-catenin to inactivate the Wnt/β-catenin pathway inhibited chondrogenesis and bone formation [Chen et al., 2007].

Several BMPs (BMP-2, -4, and -7) potently induce osteogenesis, chondrogenesis, and adipogenesis, and inhibit myogenesis of MSCs. BMP, TGF-β, and Wnt pathway can promote osteogenesis while simultaneously suppressing adipogenesis by downregulating adipogenic transcription factors such as C/EBPα and PPARγ [Kang et al., 2007; Salazar et al., 2008; Locklin et al., 1998]. Overexpression of BMPs can induce ectopic bone formation [Wozney and Rosen, 1998]. BMPs function through type I and II receptors and activation of these receptors induces phosphorylation and heterodimerization of intracellular Smad1/5/8 (R-Smads) with Smad4 and other transcription factors, leading to gene expression [Massague, 1998].

Interestingly, Smad4, a downstream component of BMP-2 signaling, has been found to interact with β-catenin and TCF/LEF, providing a point of convergence for these two pathways [Fischer et al., 2002b]. Using a rat fracture model, microarray studies have revealed Wnt genes including β-catenin, Dvl, and LRP-5 to be upregulated after injury [Hadjiargyrou et al., 2002; Zhong et al., 2006]. This parallels the stabilized tibia fracture mouse model in which TCF-dependant transcription of Wnt signaling genes including Wnt-4, 5b, 10b, 11, and 13, and LRP-6 is activated, and illustrates the importance of the Wnt pathway in fracture repair [Chen et al., 2007]. This model also illustrates the complexity of Wnt signaling, as bone healing was repressed in mice that conditionally expressed both null and stabilized β-catenin alleles. In contrast, a dramatic enhancement in bone healing was apparent in mice that conditionally expressed an active form of β-catenin [Chen et al., 2007].

Wnt/β-catenin promotes osteogenesis only when progenitor cells have been committed to the osteoblast lineage, and its role is dependent on the cell's differentiation state. β-Catenin is also essential in determining whether mesenchymal progenitor cells will become osteoblasts or chondrocytes, as genetic inactivation of β-catenin causes ectopic chondrogenesis at the expense of osteogenesis during intramembraneous and endochondral ossification [Day et al., 2005]. Along similar lines, another study found that constitutive Wnt signaling at an injury site promotes progenitor cell proliferation and reduces osteoblast differentiation, whereas inhibiting the Wnt pathway also resulted in a decrease in osteoblast differentiation [Kim et al., 2007]. These observations reveal that the Wnt pathway and the strength of the signal may have an initial role in proliferation of progenitor cells and that once the cells are committed to the osteoblast lineage, Wnt becomes crucial for osteoblast differentiation.

The transforming growth factor-beta (TGF-β) family of cytokines, which includes the BMP family, is crucial for embryogenesis, tissue homeostasis, proliferation, differentiation, apoptosis, migration, ECM remodeling, immune functions, and tumor invasion [Biere and Moses, 2006; Li et al., 2008; Massague, 2008]. TGF-β belongs to the same family as BMPs and also binds to type I and II receptors, resulting in the phosphorylation of Smad2 and 3, which associate with Smad4 and enter the nucleus to regulate gene expression [Verrecchia and Mauviel, 2002]. BMP-2 induces expression of the transcription factor Dlx5, an immediate early response gene [Lee et al., 2003]. Dlx5 then induces expression of Runx2 and promotes osteogenesis [Ryoo et al., 2006; Holleville et al., 2007]. TGF-β stimulates chondrogenesis in vitro and in vivo and inhibits adipogenesis in human bone marrow stromal fibroblasts [Locklin et al., 1998]. A microarray approach revealed that TGF-β can stimulate expression of Wnt2, 4, 5a, 7a, 10a, and the coreceptor LRP5 and β-catenin to promote osteo/chondrogenesis while inhibiting adipogenesis [Zhou et al., 2004]. Therefore, Wnt may synergize with TGF-β to inhibit adipogenesis of hMSCs, supporting

the notion that Wnt signaling is one of the mediators of TGF-β-induced osteo/chondrogenic differentiation.

Another important osteo/chondrogenic signaling cascade involves the Hedgehog family of proteins, including Indian hedgehog (Ihh), which exert their effects through Patched (PTCH) and Smoothened (Smo) [Denef et al., 2000]. When Ihh binds PTCH, Smo is activated and transduces the signal into the cytoplasm, thereby activating the Gli transcription factor family [Koebernick and Pieler, 2002], which is important in skeletogenesis [Hui and Joyner, 1993; Mo, 1997]. Mice deficient for *Ihh* show failure of osteoblast development in endochondral bones [Hilton et al., 2005]. In addition, Gli2 upregulates Runx2 expression and enhances the osteogenic action of Runx2 via direct physical interaction [Shimoyama et al., 2007]. These studies suggest that Ihh regulates osteoblast differentiation of mesenchymal cells through upregulation of the expression and function of Runx2 by Gli2.

SIGNALING PATHWAYS REGULATED BY TWIST

The mechanisms by which Twist influences MSC differentiation are not fully understood. That Twist uses multiple mechanisms to influence MSC differentiation is apparent; however, its mode of action is still not fully understood. Apart from inhibiting lineage-specific transcription factors, Twist can interact with and alter many signaling pathways. For example, it is well established that increases in Twist expression can inhibit osteoblast differentiation in vitro, and craniosynostosis is associated with activating mutations in the fibroblast growth factor receptor FGFR genes and haploinsufficiency of Twist-1 [Cunningham et al., 2007]. FGFR2 is detected in the osteogenic fronts of cranial sutures and this expression is extended to the mid-suture in Twist heterozygous mice [Connerney et al., 2006], suggesting enhanced FGF signaling in the sutures of these mice. Because BMP signaling is active in the osteogenic fronts [Warren et al., 2003], it was predicted that Twist heterozygous mice would exhibit enhanced BMP signaling. In agreement with this, in Twist heterozygous mice, which favor formation of Twist/Twist homodimers (T/T) as opposed to Twist/bHLH E protein heterodimers (T/E), the levels of noggin, a repressor of BMP signaling, were decreased [Connerney et al., 2008]. BMPs signal through Smad proteins, resulting in the initial phosphorylation of Smad1, 5, and 8, followed by the formation of complexes with Smad4, which activate transcription. In Twist heterozygous mice, phospho-Smads 1/5/8 were found in the osteogenic fronts and the mid-sutures, suggesting an increase in BMP signaling.

To definitively show that the craniosynostosis phenotype in Twist heterozygous mice is mediated by activation of FGF signaling, a transgenic mouse conditionally expressing the receptor tyrosine kinase inhibitor, Sprouty1 (Spry1), was used. Conditional expression of Spry1 was achieved by cre-recombinase-induced recombination in which activation of CRE-ERT2 protein was inducible by tamoxifen. Tamoxifen was directly injected into the sutures of mice to activate conditional expression of Spry1 by CRE-ERT2. Activation of Spry1 in Twist heterozygous mice resulted in almost complete inhibition of suture fusion in these mice. Therefore, inhibition of FGF signaling prevented craniosynostosis in Twist heterozygous mice [Connerney et al., 2008]. Further studies revealed that ectopic expression of T/E heterodimers dramatically reduced osteoblast differentiation and FGFR2 expression, whereas T/T expression enhanced differentiation and FGFR2 expression. Furthermore, overexpression of T/T homodimers in a wild-type background resulted in a similar phenotype as Twist-1 haploinsufficiency, supporting the idea that the ratio of Twist heterodimers can integrate signaling pathways, such as the FGF and BMP pathways, and deregulate osteogenesis and cranial suture growth [Connerney et al., 2008]. Further evidence that Twist influences BMP signaling was illustrated in a study using an osteoblastic cell line, MC3T3E-1, in which overexpression of Twist-1 suppressed BMP-induced differentiation, whereas downregulation of Twist had the opposite effect [Hayashi et al., 2007]. Twist-1 formed a complex with Smad4 and the corepressor HDAC1, suggesting that Twist-1 inhibits expression of differentiation genes by recruiting HDAC1 to Smad4. These effects were overcome by overexpressing Id1, which promoted degradation of Twist-1 and positively regulated BMP signaling [Hayashi et al., 2007].

Another signaling pathway that is important for regulating Twist is the Wnt signaling pathway. Wnt signaling is extremely complex and is essential for both chondrogenesis and osteogenesis. Wnt3a can inhibit chondrogenesis and chondrogenic gene expression. Use of a stable form of β-catenin revealed that this repression is through the canonical Wnt pathway. Twist-1 is expressed in the limb bud mesenchyme and overlaps sites of Wnt signaling [O'Rourke and Tam, 2002; Reinhold et al., 2006]. Furthermore, in limb bud mesenchymal cells, Twist-1 expression was induced by canonical Wnt signaling and overexpression of Twist could inhibit chondrogenesis via reduced expression of aggrecan and collagen II, whereas knockdown of Twist had the opposite effect. The same effect was also evident when BMP2 was used to stimulate chondrogenesis, as overexpression of Twist-1 inhibited BMP2-mediated chondrogenesis. Therefore, Twist-1 is involved in Wnt-mediated inhibition of chondrogenesis, although its mechanism of action in this process remains unresolved.

Recent accumulative data illustrate that although Twist inhibits osteogenesis, it enhances adipogenesis. A recent investigation of brown fat metabolism in adipose tissue found that Twist is highly expressed in this tissue. The transcription coactivator PGC-1a is a central regulator in brown fat thermogenesis [Lin et al., 2005]. PGC-1a expression is highly sensitive to nutrient status. Overexpression of Twist in a white fat preadipocyte cell line (3T3-L1) did not affect adipocyte differentiation, which led to the conclusion that Twist has no role in adipogenesis. It should be noted, however, that in the same article, Twist transgenic mice displayed obesity and increased weight gain when placed on a high fat diet as compared to matched controls [Pan et al., 2009]. Twist heterozygous mice were obesity-resistant and showed less lipid accumulation in the brown fat. Moreover, expression of oxidation genes was higher in brown fat with no effect on white fat. The effect of Twist on energy dissipation was examined and revealed that Twist inhibited PGC-1a-mediated transcription by directly interacting with PGC-1a and suppressed expression of PGC-1a

target genes and mitochondrial biogenesis. Furthermore, Twist inhibited PGC-1a-mediated histone H3 acetylation by recruiting the histone deacetylase HDAC5. PPARδ was associated with the Twist promoter and directed Twist-1 expression in brown fat cells. Because PPARδ is a nutrient sensor, this network would allow brown fat to respond to the body's nutritional status [Pan et al., 2009]. When overexpressed in human bone marrow MSCs, Twist inhibited osteogenesis and chondrogenic differentiation, while promoting adipogenesis [Isenmann et al., 2009]. This correlated with an increase in the PPARγ2 transcription factor and the adipocyte-related markers leptin and adipsin. Therefore, it appears that Twist expression is a positive regulator of adipose development.

Conversely, earlier studies showed that when Twist-1 was overexpressed in osteoblast cell lines they remained in an undifferentiated state and their morphology changed from a cuboidal osteoblast phenotype to a spindle shape fibroblast that represented an osteoprogenitor cell [Lee et al., 1999]. In contrast, knockdown of Twist-1 resulted in a cuboidal, differentiated-like phenotype as compared to controls. These results correlated with a change in expression of bone marker genes such as alkaline phosphatase, type I collagen, and osteopontin. It is known that basic FGF (bFGF) acts as a potent mitogen of osteoblast cells, increasing the pre-osteoblast population and eventually bone formation [Nakamura et al., 1995]. Very early populations of pre-osteoblasts do not respond to bFGF, whereas more differentiated populations do respond [Long et al., 1995]. In support of Twist promoting an immature phenotype, it was discovered that Twist overexpressing cells do not respond to bFGF, in contrast to cells in which Twist expression was knocked down, which did respond to bFGF. The latter finding gave support to the idea that Twist promotes a premature osteoblast phenotype and can possibly de-differentiate osteoblasts to a more progenitor-like state. A later study using a novel subtraction hybridization cDNA library to characterize the gene expression profile of purified preparations of prospectively isolated human bone-marrow-derived MSCs revealed that Twist-1 and -2 were two of the highly expressed clones in this population [Gronthos et al., 2003a]. MSCs that overexpressed Twist-1 and -2 had a higher proliferation rate and greater life span. Moreover, FACS analysis revealed that both Twist-1 and -2 promoted higher expression levels of the MSC-associated antigen, STRO-1, and the early osteogenic transcription factors Runx2 and MSX2. This study suggests that Twist promotes an osteo-progenitor-like phenotype and maintains an immature mesenchymal precursor population [Isenmann et al., 2009].

An area of future interest is to determine if Twist indeed plays a role in stem cell maintenance. The putative role of Twist in stem cell maintenance is of major interest because of its potential use for optimizing the number of undifferentiated adult stem cells for therapeutic applications, as well as elucidating stem cell biology. Overall, it is clear that Twist can modulate signaling pathways such as BMP and FGF; however, its role and mechanism of action in regulating TGF-β, Wnt, and other pathways that control MSC differentiation and/or maintenance remain largely unexplored. Given the complexity of these signaling pathways related to cell types, ligand specificities, differentiation status and experimental conditions, this area will be a challenge for future studies.

TWIST: MASTER REGULATOR OF EMT AND ITS ROLE IN TUMORIGENESIS

The process of epithelial to mesenchymal transition (EMT) occurs during several stages of embryogenesis, wound healing, and mammary gland development [Dvorak, 1986; Savagner et al., 1994, 2005; Hay, 1995; Thiery and Chopin, 1999; Gammill and Bronner-Fraser, 2003; Shook and Keller, 2003; Thiery, 2003; Fata et al., 2004]. Moreover, an EMT-like process has been hypothesized to occur in pathological conditions such as tissue fibrosis and tumor progression of epithelial-based cancers [Savagner et al., 1994; Hay, 1995; Thiery and Chopin, 1999; Thiery, 2002; Kalluri and Neilson, 2003; Thiery, 2003; Agiostratidou et al., 2007; Kokkinos et al., 2007; Moustakas and Heldin, 2007; Baum et al., 2008; Gavert and Ben-Ze'ev, 2008; Turley et al., 2008; Yang and Weinberg, 2008]. EMT is characterized by a loss of epithelial cell characteristics, such as cell–cell and cell–substratum contacts; reorganization of the actin cytoskeleton; and gain of mesenchymal characteristics, such as loose organization and lack of contacts with neighboring cells. Thus, EMT allows cells to undergo changes in shape and polarity, which confer greater motility and the ability to penetrate through the basement membrane, away from the epithelial layer, and into the surrounding tissues (invasion). In cancer, the tumor cells in the periphery of the primary tumor mass are thought to undergo an EMT-like process that facilitates tumor cell invasion of the surrounding tissues (stroma). Invasion then facilitates the subsequent steps of metastasis (intravasation into the blood circulatory or lymphatic system, survival in the blood, extravasation into distant tissues, and colonization of a distant organ). Highly invasive and metastatic breast cancer cell lines possess characteristics of EMT passage [Sommers et al., 1992; Thompson et al., 1992; Gilles et al., 1999], and EMT passage-associated gene signatures have been linked to invasive breast carcinomas and poor breast cancer prognosis [Jechlinger et al., 2003; Zhang et al., 2005; Kleer et al., 2007; Lien et al., 2007; Gavert and Ben-Ze'ev, 2008; Sarrio et al., 2008; Yang and Weinberg, 2008; Weigelt et al., 2009]. Moreover, EMT confers resistance to various cancer therapies [Sabbah et al., 2008].

Twist regulates the expression of many genes involved in EMT, allowing cells to undergo EMT passage. Twist is highly expressed in invasive carcinoma and in highly invasive cell lines [Yang et al., 2004; Mironchik et al., 2005]. In addition to conferring migratory and invasive capabilities, Twist gives cells the ability to resist apoptosis upon treatment with paclitaxel [Cheng et al., 2007; Kajiyama et al., 2007] and cisplatin [Zhuo et al., 2008], as well as to inactivate premature senescence in cancer cells [Ansieau et al., 2008].

A vast amount of evidence points to the existence of cancer stem cells (CSCs) within tumors and cancer cell lines [Reya et al., 2001; Clarke et al., 2006; Li et al., 2007; Lobo et al., 2007; Cariati and Purushotham, 2008; Hurt and Farrar, 2008]. In addition to enabling metastasis, Twist-induced EMT passage can induce the generation of

breast CSCs from differentiated epithelial cancer cells [Mani et al., 2008; Morel et al., 2008; Santisteban et al., 2009]. Moreover, unlike the rest of the cancer cell population, breast CSCs display the mesenchymal morphologic and phenotypic characteristics of cells that have undergone EMT passage, including high expression of Twist and other genes involved in motility, invasion, resistance to apoptosis, and ECM remodeling [Al-Hajj et al., 2003; Sheridan et al., 2006; Stingl et al., 2006; Liao et al., 2007; Liu et al., 2007; Shipitsin et al., 2007; Stingl and Caldas, 2007; Mani et al., 2008; Hennessy et al., 2009]. Because breast CSCs possess EMT-passage characteristics and the self-renewing and differentiation capacities needed to seed new tumor growth and differentiate into a heterogeneous population of tumor cells [Al-Hajj et al., 2003; Mani et al., 2008], it is likely that they are responsible for breast tumor cell dissemination and distant tumor formation during breast cancer metastasis [Brabletz et al., 2005]. According to this model, normal mammary stem cells may become transformed, undergo EMT and generate breast CSCs, which may represent the disseminating population during cancer metastasis. Alternatively, differentiated, transformed breast epithelial cells may undergo EMT and, as a result, acquire stem-cell-like properties, disseminate to distant organs and seed secondary tumor growth.

In contrast to the reported role of Twist-mediated EMT in CSC generation, Twist has been shown to induce a breast CSC phenotype through downregulation of CD24 cell surface expression independently of EMT [Vesuna et al., 2009]. Therefore, Twist may enable stem cell function via EMT-dependent and -independent pathways. Altogether, Twist inhibition, as a therapeutic modality, may represent an ideal approach to directly target breast CSC function and, consequently, prevent tumor growth initiation (at primary and secondary sites) and tumor relapse due to therapeutic resistance.

FUTURE DIRECTIONS

Future studies will undoubtedly decipher the role of Twist in regulating the complex signaling cascades associated with MSC differentiation into various lineages. Of vital importance to understanding the functions of Twist, however, is determining to which regions of the genome Twist is recruited and if these regions are binding sites for Twist or binding sites for other factors essential for MSC differentiation. Furthermore, understanding the role of Twist in the regulation of CSC function will uncover novel factors involved in stem cell self-renewal and differentiation, as well as factors that could potentially be used as therapeutic targets. The most critical problem facing stem cell therapy today is the limited number of ex vivo expanded adult progenitor cells available for transplant studies. Therefore, identification of genes involved in self-renewal and differentiation will greatly benefit the field of stem cell therapy.

In tumor models, Twist inhibition should help prevent EMT, cell migration, and invasion, as well as sensitize tumor cells to chemotherapeutic agents such as paclitaxol. In addition, stem cells are currently being tested as vehicles to target therapeutics agents to tumor cells. Stem-cell-mediated delivery of Twist inhibitors may prove to be a useful approach to prevent metastasis of invasive tumors. A recent review cites 22 preclinical studies describing the use of neural or MSCs for cancer therapy [Aboody et al., 2008]. These studies document the remarkable tumor-tropism of normal human stem/progenitor cells and describe the use of neural or MSCs to deliver therapeutic gene products selectively to various types of solid tumors. If successful, the use of targeting Twist inhibitors with a stem cell delivery vehicle as a therapeutic strategy will create a new paradigm for cancer treatments by eliminating the toxic side effects commonly experienced by high-dose chemotherapeutic regimens. Overall, improved survival combined with fewer side effects from treatments will positively impact clinical cancer care.

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