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Musashi1: A Stem Cell Marker No Longer in Search of a Function

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One of the earliest genes identified with stem and early progenitor cells is the RNA-binding protein, Musashi1 (Msi1). Through gene profiling of mammary epithelial cells transduced with Msi1, a unique autocrine signaling pathway was identified that activates both the Wnt and Notch pathways [1]. This process was associated with increased secretion of the growth factor, PLF1 and inhibition of the secreted Wnt pathway inhibitor, DKK3. Identification of PLF1 as an effector of these pathways in the absence of the DKK3 tumor suppressor provides a new avenue for investigating differences between normal and malignant tissues, and potentially targeting tumor stem cells.

Introduction

Msi1 was first identified in *Drosophila* as a determinant of sensory organ development [2], and later as a cell fate determinant of neuroglial stem cells [3]. Msi1 blocks translation of Numb, a negative regulator of Notch [4], as well as Ttk69, a transcriptional repressor downstream of Notch [5], and each gene is inherited asymmetrically and separately by the daughter cells. There are no studies of Ttk69 orthologs in mammalian cells, but it has been shown to alter signal transduction downstream of growth factor receptor activation in insect cells [6]. Msi1 expression is also impacted by a second family of RNA-binding proteins related to *Drosophila* Elav that are involved in the development and maintenance of the nervous system in the fly and mouse [7, 8]. Mammalian Elav orthologs HuB, HuC and HuD promote mRNA stabilization by binding to AU-rich elements in the 3'-UTR of several target mRNAs, including Msi1[9]. Their activity has been linked to PKCα[10], a protein kinase involved in multiple signaling pathways [11], and are localized to the nucleus of neuronal stem cells [12] similarly to Msi1 [13].

Most studies of Msi1 have focused on regulation of the Notch pathway. Notch is activated by sequential proteolytic cleavage of its membrane-associated form to a constitutively active coactivator [14], whose expression is regulated by Msi1 and Numb [15]. Numb promotes ubiquitination of intracellular Notch [16] and interferes with its nuclear translocation [17]. Msi1 associates with the *cis*-acting repressor motif, $GU_{3–5}$(G/AG), in the 3'-UTR of Numb and other targets to block translation [4].

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Musashi1 and mammary progenitor cells

Msi1 maintains the proliferation of multipotential neural stem/progenitor cells[15], and is rapidly downregulated in post-mitotic neurons[3] and upregulated in central nervous system tumors originating from neural stem cells [18, 19]. Mammary stem cells, like other stem cells, exhibit the ubiquitous feature of either remaining quiescent or undergoing self-renewal in response to their microenvironment [20], and retain the ability to pass on newly labeled DNA to their progeny by asymmetric cell division [21] (Fig. 1). “Label-retaining cells” are enriched in the “side population”, which express higher levels of ABC transporter proteins, Msi1, Notch1, CK19, ERα and a progenitor cell morphology[22], as well as the $CD24^{hi}/CD133^{+}$ phenotype[23]. Human breast stem cells are enriched in Notch3[24], and Notch ligands promote the proliferation of epithelial and myoepithelial progenitor cells[24]. Since the mammary gland contains a population of multipotent stem/progenitor cells throughout development [25], it is an ideal tissue in which to assess the pathways regulating stem/progenitor cell proliferation, as well as those leading to malignant transformation [26].

We recently discovered that Msi1 regulated a unique autocrine signaling pathway in mammary epithelial cells[1]. Msi1-transduced cells expressed the $CD24^{hi}/Sca\text{-}1^{+}$ and $CD24^{hi}/CD29^{+}$ phenotypes. It remains controversial whether Sca-1 is a stem cell or progenitor cell marker since $CD24^{hi}/Sca\text{-}1^{+}$ cells had no mammary gland repopulating activity compared to $CD24^{hi}/Sca\text{-}1^{-}$ cells [23, 27], whereas $Sca\text{-}1^{+}$, but not $Sca\text{-}1^{-}$ cells, exhibited mammary outgrowth activity [28, 29]. On the other hand, $CD24^{+}/CD29^{hi}$ mammary cells were found to be multipotent self-renewing stem cells capable of reconstituting the mammary gland from a single cell[30]. Preneoplastic tissue from MMTV-Wnt1 mice exhibited an increased percentage of $CD24^{+}/CD29^{+}$ cells [30], and mammary outgrowth capacity segregated with $CD24^{lo}$ rather than $CD24^{hi}$ cells [27]. The upregulation of $CD24^{hi}/CD29^{+}$ cells by Msi1 therefore appears to be more consistent with its ability to drive expansion of multipotent progenitor cells rather than pluripotent stem cells.

Msi1-transduced cells expressed a higher percentage of CK6, CK19 and double-positive CK14/CK18 cells, which are indicative of basal cells, a mixture of stem and progenitor cells [1]. CK6 is abundant in stem and basal cells [29, 31–33] and has been linked to proliferation of alveolar epithelium and activation of the Wnt pathway [34]. CK19 is expressed in luminal progenitor cells that give rise to $CK14^{+}$ basal cells [35, 36], and double-positive CK14/CK18 cells are bipotential progenitor cells[32]. The Wnt pathway drives alveolar proliferation, as shown in MMTV-Wnt1 [37] and MMTV-ΔN89β-catenin [38] transgenic mice. Activation of ΔN89β-catenin in mammary basal cells under the control of the CK5 promoter produced abundant end bud development [39]. β-Catenin also participates in establishing the mitotic spindle [40], suggesting an additional role in stem cell self-renewal and progenitor cell proliferation. Expansion of mammary basal cells with characteristic CK6/CK14 expression has also been noted in mice with increased Notch pathway activation [32]. These results are also consistent with Msi1 being a transducer of multipotential progenitor cell expansion rather than stem cell self-renewal.

An additional finding of our study was that $p21^{Cip1}$ was absent in Msi1-expressing cells, which is in agreement with the ability of Msi1 to block its translation[41]. $p21^{Cip1}$ is believed to function as a rheostat to maintain a balance between stem cell quiescence and stem cell exhaustion resulting from increased cell cycle entry[42, 43]. $p21^{Cip1}$ is also associated with chromosome segregation during mitosis [44], as well as the negative regulation of Wnt4 transcription [45]. Like β-catenin [40], $p21^{Cip1}$ may play a role in mitosis to regulate progenitor cell expansion.

Msi1 regulates the proliferin signaling pathway

Gene profiling of Msi1-expressing cells presented the initial clue that Msi1 regulated a unique autocrine pathway. Msi1 produced an increase in the growth factor, PLF1, and an equally large reduction of the secreted Wnt pathway inhibitor, DKK3 [1], and closely paralleled their respective changes in protein level in the conditioned medium of Msi1-transduced cells (Fig. 2). PLF1 is one of three highly homologous genes related to the prolactin gene family that map to a single locus on mouse chromosome 13 [46]. PLF1 is a ligand for the Gi-protein-coupled IGF2R [47, 48] that mediates prolactin-induced alveolar development in the mammary gland through activation of ERK and Jak2 [49, 50]. Receptor activation by PLF1 activates ERK [48, 51] and transcription factor AP-1 [52], and is blocked by pertussis toxin [48, 51], which catalyzes ADP-ribosylation of the Giα subunit to prevent its interaction with the receptor [53]. This mechanism was corroborated in Msi1-expressing cells by showing that pertussis toxin inhibited PLF1-mediated ERK activation by conditioned medium from Msi1-transduced cells, and that depletion of PLF1 from the medium or its downregulation by RNA interference inhibited ERK activation and Notch and Wnt signaling[1]. IGF2R activation is known to increase β-catenin nuclear localization and EMT [54], which are associated with growth and invasion [55]. IGF2 increases the number of Msi1-positive intestinal stem/progenitor cells and their susceptibility to tumorigenesis[56]. Also pertinent to our findings is the identification of PLF2 and PLF3 as Wnt-1 target genes[57]. Since the three PLF1 genes are transcribed from a single locus, it is likely that they are all regulated in a similar manner. We previously found that PLF1 and PLF3 expression increased in primary mouse mammary tumors, particularly in those with basal cell characteristics[58, 59]. Interestingly, PLF2 has been shown to increase expansion of mouse hematopoietic stem cells *ex vivo* [60]. These results therefore support a role for PLF1 in Msi1-mediated activation of the Wnt and Notch pathways and in mammary progenitor cell expansion.

Downregulation of DKK3 was reciprocally related to PLF1 expression downstream of Msi1 signaling[1](Fig. 2). DKK3 (also known as REIC or Reduced Expression in Immortalized Cells) is one of four homologous secreted proteins [61] that function as tumor suppressors [62]. DKK1 and DKK2, but not DKK3, bind to the Wnt co-receptor, LRP5/6, to block Wnt pathway activation [63], but DKK3 similarly prevents nuclear localization of β-catenin through an as yet undefined mechanism[64]. Downregulation of DKK3 by RNA interference in control cells showed that it negatively regulated both β-catenin/TCF- and CBF1-dependent transcription, which resembled the phenotype resulting from Msi1 expression[1]. DKK3 expression in lung, prostate and liver tumor cells has been shown to induce apoptosis[65–67] and disrupt acinar morphogenesis and growth of prostate tumor cells [68]. In melanoma cells, reduction of DKK3 expression resulted in loss of cell adhesion, increased invasion, upregulation of the transcriptional repressor, Snail-1 [69], and reduction of E-cadherin [70], all of which are associated with EMT. However, gene profiling and western analysis of Msi1-tranduced cells did not reveal reduction in E-cadherin, suggesting that this mechanism is not operative. Reduction of DKK3 therefore appears to work in concert with PLF1 to promote increased progenitor cell expansion upstream of Wnt and Notch signaling, but not EMT *per se*.

Notch-mediated transformation has been reported downstream of Ras and ERK activation [71], which is also in agreement with the dependence of Msi1-induced Notch and Wnt signaling on ERK activation[1](Fig. 2). One mechanism common to activation of both pathways is inhibition of GSK3β by ERK, which is necessary to prime GSK3β for inactivation by other protein kinases [72](Fig. 2). Since GSK3β in its activated state phosphorylates and promotes ubiquitination and proteasomal degradation of β-catenin [73]

and intracellular Notch [74], this mechanism provides a link between Msi1 and Wnt and Notch pathway activation.

PLF1 induced ERK signaling correlated with the $CD24^{hi}/CD29^{+}$ progenitor cell phenotype of Msi1-transduced cells[1]. CD24 is highly expressed in invasive tumor cells[75], and mediates its effects through integrinβ1, the subunit expressed by CD29, and which itself is upregulated through the Ras/ERK pathway[76]. CD24 expression is linked to IGF2 signaling through IGF2R, the same receptor activated by PLF1. Importantly, deletion of the IGF2 gene reduced CD24 expression by 90% and suppressed invasion of glioblastoma cells[77]. Thus, increased PLF1 signaling through the IGF2R accounts for most, if not all, of the phenotypic changes occurring in Msi1-expressing cells. In summary, increased PLF1 secretion and reduced DKK3 expression by Msi1 leads to ERK activation and increased Notch and Wnt pathway activation. Inhibition of $p21^{Cip1}$ and Numb work cooperatively with Notch and Wnt to promote cell cycle transit and progenitor cell expansion, but not terminal differentiation. Still undefined in this process is the role of the Msi1 target, Ttk69, which may function in the regulation of noncoding RNA. We have recently found that Msi1 induces a microRNA signature that resembles a breast cancer phenotype[78] (X. Wang and R.I. Glazer, unpublished results). Thus, one role of Msi1 in mammary tissue is the expansion of an early multipotenital progenitor cell population during development. Whether this is a determinant of susceptibility to tumorigenesis will be the subject of future studies.

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Abbreviations

DKK3	Dickkopf3
EMT	epithelial to mesenchymal transition
Msi1	Musashi1
PLF	proliferin
Sca-1	stem cell antigen-1
TCF	T-cell factor
Ttk69	Tramtrack69
UTR	untranslated region

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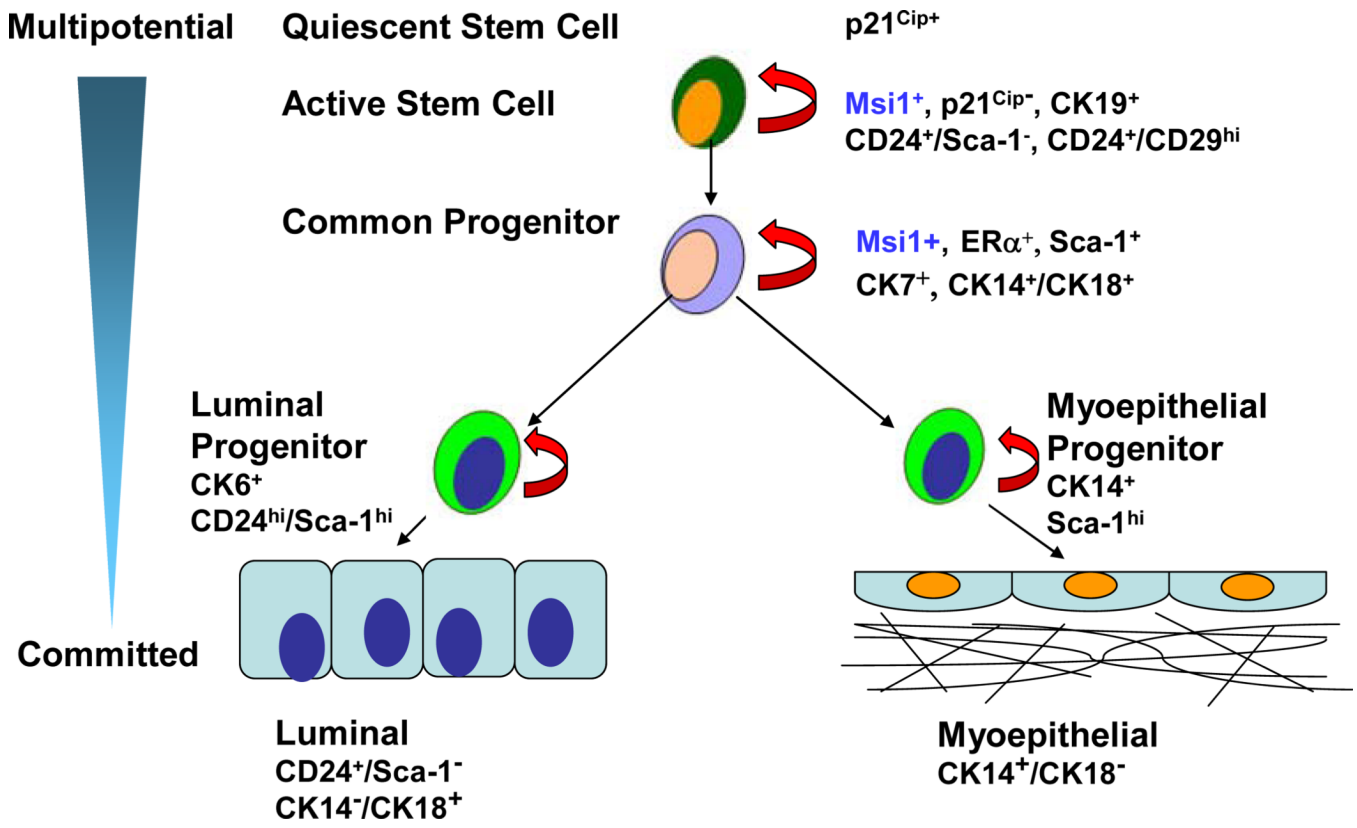

Figure 1.

Mammary gland differentiation. The degree of differentiation increases from top to bottom as cells become more committed. Stem cells are distinguished by their long label retention that reflects quiescence and nuclear expression of $p21^{Cip}$. Stem cells undergo self-renewal within the end bud niche through interactions by integrins on their cell surface, eg. CD29 (integrinβ1), with the extracellular matrix. In response to various stimuli, stem cells exit the niche and actively divide into early progenitors that express Musashi1 (Msi1), CK19, $CD24^{+}/Sca\text{-}1^{-}$ and $CK24^{+}/CD29^{hi}$. As cells become further committed, they differentiate into bipotential luminal and myoepithelial progenitor cells that express ERα, CK7, $CK14^{+}/CK18^{+}$ and Sca-1. These cells give rise to $CD24^{hi}/Sca\text{-}1^{hi}/CK6^{+}$ luminal progenitor cells, and $Sca\text{-}1^{+}/CK14^{+}$ myoepithelial progenitor cells. Committed luminal cells are $CD24^{+}/Sca\text{-}1^{-}/CK18^{+}/CK14^{-}$, whereas myoepithelial cells are $CK14^{+}/CK18^{-}$.

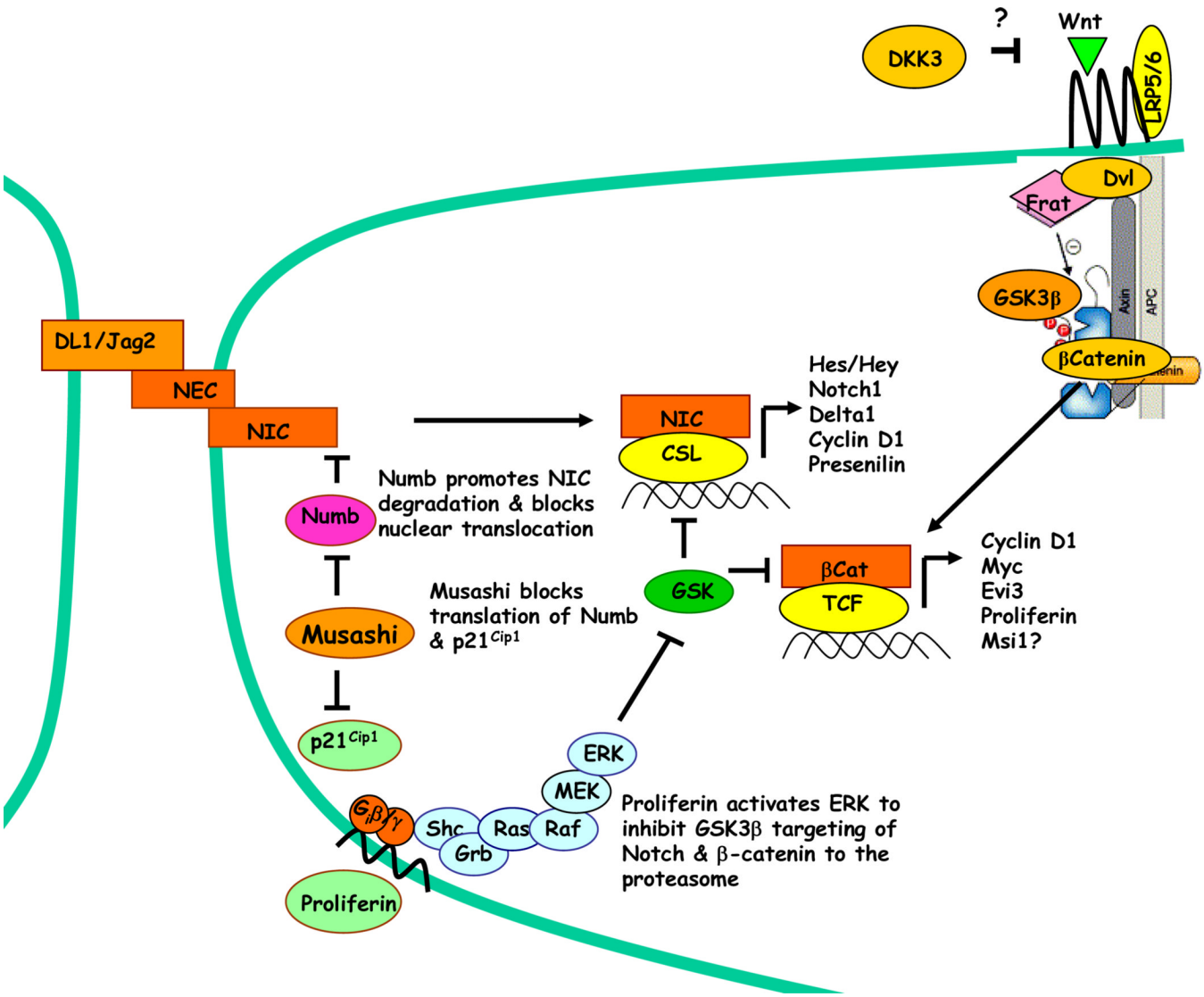

Figure 2.

Musashi signaling pathways associated with mammary progenitor cell expansion. Notch is processed proteolytically to an extracellular domain (NEC) and an intracellular domain (NIC). The Notch ligands Delta1 (DL1) and Jagged1 (Jag1) associate with NEC, and induce cleavage and release of membrane-bound NIC. NIC translocates to the nucleus, where it serves as a coactivator of CSL to activate transcription of Hes/Hey, Notch, DL1/Jag2 and cyclin D1. Musashi inhibits the translation of Numb and $p21^{Cip1}$ by binding to a motif in the 3-UTR. Inhibition of Numb prevents NIC degradation and nuclear translocation, whereas inhibition of p21Cip1 prevents inhibition of CDK's to promote G1/S transition. Preliminary studies indicate that Musashi increases secretion of the growth factor proliferin, which is known to mediate ERK activation through the Gi-coupled IGFII receptor and inhibit GSK3β activity. Msi1 also blocks expression of the Wnt pathway inhibitor, DKK3, to increase β-catenin/TCF activity by an unknown mechanism.