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HMGA2, MicroRNAs, and Stem Cell Aging

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Abstract

Mammalian aging results from a replicative decline in the function of somatic stem cells and other self-renewing cells. Recent studies (Monzen et al., 2008; Nishino et al., 2008; Sanna et al., 2008; Weedon et al., 2008) link a chromatin-associated protein, HMGA2, to development, height, and mouse stem cell aging during late fetal development and young adulthood.

Aging: A Disease of Declining Replicative Function

It appears that we get old in part because our somatic stem cells get old. In several types of tissues, the replicative function of somatic stem cells declines with age, and this in turn is associated with many common phenotypes characteristic of aging. This decline in replicative function clearly has multiple causes, including both intrinsic changes within self-renewing cells and extrinsic changes to the supporting milieu (Sharpless and DePinho, 2007). Some aspects of stem cell aging, however, seem to result from the activation of important anticancer mechanisms. Continuously proliferating, self-renewing cells make mistakes during DNA replication and accumulate DNA damage with age (Rossi et al., 2007). Therefore, the activation of tumor suppressor mechanisms in aged, damaged cells is desirable, as this prevents their malignant transformation. Unfortunately, these mechanisms also contribute to the functional decline of self-renewing compartments with aging, and thus we grow old.

The evidence for this model of tumor suppressor-driven aging is perhaps strongest for the $p16^{INK4a}$ tumor suppressor gene, encoded by the *INK4/ARF* (or *CDKN2a/b*) locus. This protein is a potent effector of cell-cycle arrest and plays a significant role in the in vitro senescence of numerous cell types (Gil and Peters, 2006). Expression of $p16^{INK4a}$ increases sharply with age in the majority of mammalian tissues (Sharpless and DePinho, 2007). In stem cell populations in the brain and bone marrow, $p16^{INK4a}$ expression mediates a heritable decline in replicative function due to either a deficiency in BMI-1 (a repressor of *INK4a/ARF* expression) or noxious insults such as ionizing radiation. Mice lacking $p16^{INK4a}$ maintain the replicative function of neural stem cells, hematopoietic stem cells, and pancreatic β cells as they age (Sharpless and DePinho, 2007). Most recently, loss of $p16^{INK4a}$ has been shown to attenuate many complex age-related phenotypes in a progeroid mouse strain that ages prematurely (Baker et al., 2008). Therefore, $p16^{INK4a}$ expression not only correlates with aging in these tissues but also in part causes aspects of their aging.

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Support for this model in humans has come from recent candidate and genome-wide association studies independently linking single-nucleotide polymorphisms (SNPs) near the *INK4/ARF* locus to several human age-associated conditions, including type 2 diabetes, atherosclerosis, and the geriatric wasting disorder termed frailty syndrome (Sharpless and DePinho, 2007).

The *INK4/ARF* Locus: Handle with Care

Several different labs have generated over 15 different mouse strains harboring disruptions of the murine *Ink4/Arf* locus, which encodes $p16^{INK4a}$ and two other tumor suppressor genes, $p15^{INK4b}$ and *Arf* (Gil and Peters, 2006). A consistent finding is that these animals are surprisingly normal through young adulthood. Mice lacking $p16^{INK4a}$, *Arf*, and $p15^{INK4b}$, either alone or in combination, are viable, fertile, and not easily distinguished from their wild-type littermates, until they develop tumors. This observation suggests that the *INK4/ARF* locus, and in particular the $p16^{INK4a}$ gene, is dispensable in the development of most tissues but plays a life-long role in tumor suppression. This potent tumor suppressor locus appears to be activated at an early stage of neoplastic progression by common features present in would-be cancer cells. However, as cells activate *INK4/ARF* expression, they also lose their capacity for further proliferation, perhaps permanently. Therefore, this beneficial anticancer mechanism also contributes to the attrition of functional stem cells with aging.

If the major function of $p16^{INK4a}$, and perhaps other members of the *INK4/ARF* locus, is to quell the hyperproliferation of once-normal cells that have stochastically "gone bad," one can clearly see that the expression of such a locus must be carefully controlled. In particular, regulation of *INK4/ARF* expression during embryonic development would be crucial. Many developing tissues demonstrate incredible rates of proliferation that are coupled with cell migration and rapid changes in the extracellular milieu. How would a cell know that these developmentally programmed events, which share many traits with aberrant malignant growth, are normal and not a cause for *INK4/ARF* activation? Evidence suggests that this problem is addressed in adult mammals by potent mechanisms that silence the *INK4/ARF* locus until it is derepressed by an age-induced activation of $p16^{INK4a}$. But how the *INK4a/ARF* locus is regulated during embryogenesis and early postnatal life is unclear.

HMGA2 Regulates *INK4a/ARF* in Stem Cells

Against this backdrop, a recent study in *Cell* by Nishino et al. (2008) identifies the chromatin-associated protein HMGA2 as a developmental regulator of stem cell self-renewal and *Ink4a/Arf* expression in mice. The authors performed an unbiased screen for transcripts that are highly expressed in fetal stem cells but decrease in expression postnatally and with aging. They identified one transcript, *Hmga2*, that satisfies these criteria and exhibits decreased expression in hematopoietic stem cells as well as in two types of neural stem cells.

There are four high mobility group A (HMGA) family proteins: three isoforms of HMGA1 and HMGA2. These chromatin-associated proteins appear to lack intrinsic transcriptional activity but instead bind to AT-rich DNA sequences and potentiate the effects of transcription factors by altering local chromatin structure (Fusco and Fedele, 2007). For example, a recent *Nature Cell Biology* paper by Monzen et al. (2008) reports that knockdown of *HMGA2* blocks cardiomyocyte differentiation of an embryonal carcinoma cell line and completely abrogates in vivo cardiogenesis in embryos of the frog *Xenopus laevis*. The authors demonstrate that HMGA2 cooperates with Smad transcription factors to induce expression of *Nkx2.5*, which encodes an important early transcription factor for cardiac development. This is accomplished through HMGA2 binding to a conserved AT-rich region in the *Nkx2.5* promoter. HMGA2 has also been strongly associated with

neoplasia, inducing diverse oncogenic effects on the cell cycle, apoptosis, and differentiation (Fusco and Fedele, 2007). Mice overexpressing *Hmga2* develop lymphoid, lipoid, and pituitary tumors. In humans, genetic amplifications or translocations of *HMGA2* that augment its expression are associated with a variety of common benign mesenchymal tumors as well as rare aggressive cancers (Fusco and Fedele, 2007).

Nishino et al. (2008) show that HMGA2 plays an age-associated role in the self-renewal of mouse neural stem cells (NSCs). Although HMGA2 does not appear to be required for the generation of NSCs during fetal development, NSCs from *Hmga2*-deficient mice have defects in proliferation and self-renewal. Differentiating neural progenitors from these mice do not exhibit proliferative defects, suggesting that *Hmga2* loss does not lead to a global decrease in cellular replication but specifically affects stem cell self-renewal. Importantly, in accordance with the observed expression pattern of *Hmga2*, the negative effects of HMGA2 on proliferation are most pronounced in NSCs derived from late embryos or very young mice and decline with aging. In fact, NSC number and function are similar in old *Hmga2*-deficient mice and wild-type littermates, indicating that physiological aging of normal mice reduces NSC function to a level comparable to that established in *Hmga2*-deficient mice during development. The alterations in stem cell function in *Hmga2*-deficient young mice are associated with neuroanatomical changes, including decreased cellular proliferation in the subventricular zone (where NSCs reside) and fewer neurons in the central and peripheral nervous systems.

When the authors analyzed $p16^{INK4a}$ and *Arf* expression in mouse NSCs lacking *Hmga2*, they found that stem cells from late-stage embryos and young mice markedly overexpress both genes of the *Ink4a/Arf* locus. Interestingly, the degree of *Ink4a/Arf* overexpression inversely correlates with declining *Hmga2* expression from late fetal life to old age, suggesting a direct link between this locus and HMGA2. Indeed, the self-renewal defects of *Hmga2*-deficient NSCs could be partially rescued by the loss of $p16^{INK4a}$ or *Arf* expression. Consistent with the proliferative defects seen in NSCs, the alterations in *Ink4a/Arf* expression are strongly correlated with age. Overexpression of $p16^{INK4a}$ and *Arf* is most pronounced in late-stage embryos and young-adult mice but decreases with age. As expression of *Ink4a/Arf* normally sharply increases in NSCs with age, wild-type mice appear to eventually “catch up” with *Hmga2*-deficient mice regarding *Ink4a/Arf* expression. The expression of $p16^{INK4a}$ and *Arf* is roughly comparable in NSCs from old (~2 years) mutant and wild-type mice, indicating that the effects of HMGA2 are most pronounced from late fetal development to young adulthood. Because no HMGA2 binding could be detected at the *Ink4a/Arf* locus, Nishino et al. suggest that HMGA2 may control expression from this locus by repressing the expression of *JunB*, an activator of *Ink4a/Arf* expression in stem cells.

But how is *Hmga2* expression regulated in NSCs? Recent work has defined a posttranscriptional mechanism for the regulation of HMGA2 protein production by the *let-7* microRNA (miRNA). The *HMGA2* mRNA contains in its 3′ untranslated region (UTR) seven target sites for *let-7* binding (Figure 1) (Fusco and Fedele, 2007). Expression of *HMGA2* is high in embryonic cells and very low in differentiated cells, a pattern exactly inverse to that of *let-7* miRNA family members, further suggesting a regulatory relationship. Nishino and colleagues analyzed the expression of the *let-7* family in aging NSCs. They show that the expression of a specific *let-7* family member, *let-7b*, inversely correlated with *Hmga2* expression, increasing during late fetal development and adult aging. Satisfyingly, overexpression of *let-7b* in NSCs from young mice decreases *Hmga2* expression and increases *INK4a/ARF* expression. Furthermore, miRNA overexpression decreases NSC self-renewal in vitro. Together, these observations suggest an appealing model for how regulation of stem cell self-renewal by controlling the *INK4a/ARF* tumor suppressor locus

can be achieved through modulating expression of the pleiotropic transcriptional regulator HMGA2 during late embryogenesis and young adulthood with *let-7b*, a developmentally regulated miRNA.

The MicroRNA Connection

Accumulating evidence links miRNAs to the biology of stem cells. Over 600 distinct miRNAs have been discovered in the human genome, and each is predicted to regulate several hundred target mRNAs. The enormous regulatory potential of miRNAs may even surpass that of transcription factor networks. Expression of miRNAs is crucial for embryonic stem cell (ESC) function. Indeed, loss of the essential miRNA-processing enzyme Dicer causes a defect in ESC differentiation in vitro and the death of mouse embryos early in development (Stadler and Ruohola-Baker, 2008).

The *let-7* miRNA family was among the first group of miRNAs suggested to regulate stem cells. This evolutionarily conserved family of miRNAs was first described in the worm *Caenorhabditis elegans* (Bussing et al., 2008). There are intriguing parallels between the effects of *let-7* in *C. elegans* and the observations of Nishino et al. in the mouse. In *C. elegans*, *let-7* is strongly induced in hypodermal stem cells (seam cells) coincident with their terminal differentiation, and loss of *let-7* function results in the continued proliferation of these cells. In mammals, *let-7* expression is similarly induced during embryonic development and has been suggested to negatively regulate stem cell function in a variety of tissues.

Although miRNA expression is well characterized in ESCs, the picture is less clear in adult stem cells. This is partly due to the imprecise definition of most adult stem cell populations, as well as the inability to purify these cells to homogeneity without contamination from committed progenitor cells. Nonetheless, even though comprehensive expression analyses have not been performed, it is clear that specific miRNAs control the differentiation state of somatic cells. Certain miRNAs have been identified that promote proliferation of epithelial progenitors, whereas other miRNAs promote differentiation of progenitors (Stadler and Ruohola-Baker, 2008). Interestingly, the *let-7* family has made an appearance in this latter class. Progenitor cells isolated from normal mammary epithelial cells have reduced *let-7* expression, and enforced *let-7* expression reduces the progenitor cell population (Ibarra et al., 2007). Similarly, tumor-initiating cells from primary breast carcinoma have reduced levels of *let-7* compared to the bulk population. Enforced *let-7* expression in these cells reduces their proliferation and self-renewal, in part through posttranscriptional regulation of *HMGA2* (Yu et al., 2007a). These data present an overall theme of *let-7* as a mediator of differentiation state and as an inhibitor of self-renewal.

A Lin-28-*let-7*-HMGA2 Axis?

Given these extensive changes in miRNA expression during mammalian development, how are these miRNAs regulated? Most miRNAs are products of RNA polymerase II and thus are controlled by the same transcriptional machinery as protein coding genes. In fact, the core promoters of many miRNA genes have been shown to be regulated by established transcription factors such as c-Myc, E2F, and serum response factor. Therefore, it came as a surprise that *let-7* is not transcriptionally induced during mammalian development but is instead regulated posttranscriptionally (Bussing et al., 2008). All miRNAs are initially generated as a primary transcript several thousand nucleotides in length. These long transcripts undergo two processing steps during maturation (Figure 1). The first step, catalyzed by the Drosha/DGCR8 heterodimer, liberates the stem-loop precursor for further processing by Dicer to yield the mature 22 nucleotide miRNA. Biogenesis of *let-7* is specifically inhibited at both processing steps in embryonic cells (Bussing et al., 2008). It is

the release of this block that allows mature *let-7* production during differentiation, with all of the attendant effects on development and differentiation. Quite surprisingly, the processing block of *let-7* miRNAs is achieved by interaction with the protein Lin-28 (Bussing et al., 2008; Heo et al., 2008). The Lin-28 protein is highly expressed in ESCs and is also able to promote reprogramming of differentiated somatic cells into pluripotent stem cells (Yu et al., 2007b). It should be noted that Lin-28 does not appear to be a general inhibitor of miRNA processing but instead specifically inhibits *let-7* production.

Thus, a linear pathway from Lin-28 to *let-7* to HMGA2 to *INK4/ARF* expression can be constructed (Figure 1). The expression of Lin-28 is high in ESCs, thus repressing the production of *let-7*. Given that *let-7* inhibits translation of *HMGA2* mRNA by the miRNA pathway, low levels of *let-7* miRNA are predicted to allow HMGA2 protein production in ESCs. This is precisely what is observed in ESCs, but could such a relationship also exist in somatic stem cells in adult mammals? Although the new work from Nishino and colleagues describes *let-7* regulation of *Hmga2* and *Ink4a/Arf* in mouse NSCs, Lin-28 expression has not been observed in nonembryonic tissues. However, a functionally related protein, Lin-28B, has been detected in somatic tumor cells (Guo et al., 2006), raising the possibility that Lin-28B substitutes for Lin-28 in NSCs. Alternatively, *let-7* in NSCs could be regulated at the level of transcription.

Unresolved Questions

Although a pathway linking Lin-28 to *INK4a/ARF* expression through the actions of *let-7* and HMGA2 is appealing (Figure 1), it is likely to be too simplistic. HMGA2 undoubtedly regulates the expression of many genes other than *JunB*, $p16^{INK4a}$, and *ARF*. Because the effects of HMGA2 on NSC self-renewal are only partly rescued by *Ink4a/Arf* loss, HMGA2 must have *Ink4a/Arf*-independent effects on self-renewal. Likewise, the *let-7* family of miRNAs has many targets other than *HMGA2*. For example, *let-7* mediates the repression of *RAS* oncogenes in the self-renewal of tumor-initiating cells (Yu et al., 2007a). It also remains unclear whether the cancer-relevant activities of *let-7* or HMGA2 include influencing the expression of the *INK4a/ARF* tumor suppressor locus. Therefore, elucidating other mediators of *let-7* and HMGA2 function is essential to understanding their roles in somatic stem cells.

These observations also suggest a specific role for *let-7* and HMGA2 in cancer. The *let-7* miRNA appears to act as a tumor suppressor in part by repressing self-renewal, whereas HMGA2 seems to have oncogenic activities that include the enhancement of self-renewal. At first blush, this appears inconsistent with the finding that in cultured human fibroblasts, HMGA proteins cooperate with $p16^{INK4a}$ in the establishment of cellular senescence, which is a crucial tumor suppressor mechanism (Narita et al., 2006). However, one should consider that the effects of pleiotropic regulators like the HMGA-proteins are likely to be different between fibroblasts and stem cells. Furthermore, the senescence-promoting effects of HMGA2 in fibroblasts are far weaker than those of HMGA1. Thus, we do not believe these observations are in conflict. Rather, they illustrate that the functions of HMGA proteins are likely to be complex and to have cell-type and isoform-specific effects.

Like the documented effects of HMGA2 on cancer, its role in height determination is well established but remains mysterious. Two recent genome-wide association studies (GWAS) in *Nature Genetics* (Sanna et al., 2008; Weedon et al., 2008) confirm an association between common alleles of SNPs linked to *HMGA2* and human height. Although mechanistically understanding how a given SNP identified by GWAS is associated with a particular trait can be problematic, in this case, evidence supports a model in which these SNPs are associated with altered HMGA2 function. A spontaneous mutation of the *Hmga2* gene has been

previously shown to result in the murine *pygmy* phenotype, which includes reduced adult size (Zhou et al., 1995). Meanwhile, transgenic mice that overexpress a wild-type *Hmga2* gene or a truncated variant without the 3′ UTR exhibit gigantism (Battista et al., 1999). This latter *Hmga2* allele is of interest because the 3′ UTR truncation removes the *let-7* binding sites, thereby abrogating the repression of *Hmga2* by *let-7*. Furthermore, a germline chromosomal inversion that results in a similarly truncated human *HMGA2* gene was identified in a boy with severe overgrowth (Ligon et al., 2005). The *HMGA2*-linked SNPs that are most strongly associated with height are known to lie within the 3′ UTR of *HMGA2*, suggesting that the height-influencing genetic events linked to these SNPs may influence *let-7* binding. However, it remains to be determined how exactly increased *HMGA2* expression would alter human height and whether this is related to the effects of HMGA2 on stem cell self-renewal and tumorigenesis in mice.

In summary, control of the age-promoting expression of $p16^{INK4a}$ and possibly other *INK4/ARF*-associated transcripts in self-renewing cells appears to be a cradle-to-grave problem for mammals. During late fetal development, early postnatal life, and young adulthood, *Hmga2*, under the control of the *let-7* miRNA, seems to be a major regulator of *INK4a/ARF* expression. With the transition from young to old adulthood, the Polycomb group (PcG) chromatin remodeling complexes appear to play a principal role in the repression of the *INK4a/ARF* locus. With increasing age, a developmentally programmed increase in *let-7* expression and stochastic loss of PcG-mediated silencing results in the derepression of the *INK4a/ARF* locus and activation of $p16^{INK4a}$ expression in self-renewing cells. This more refined understanding of the regulation of somatic stem cell self-renewal can now be exploited in future studies to determine how healthy aging may be achieved without increasing the risk of malignant disease.

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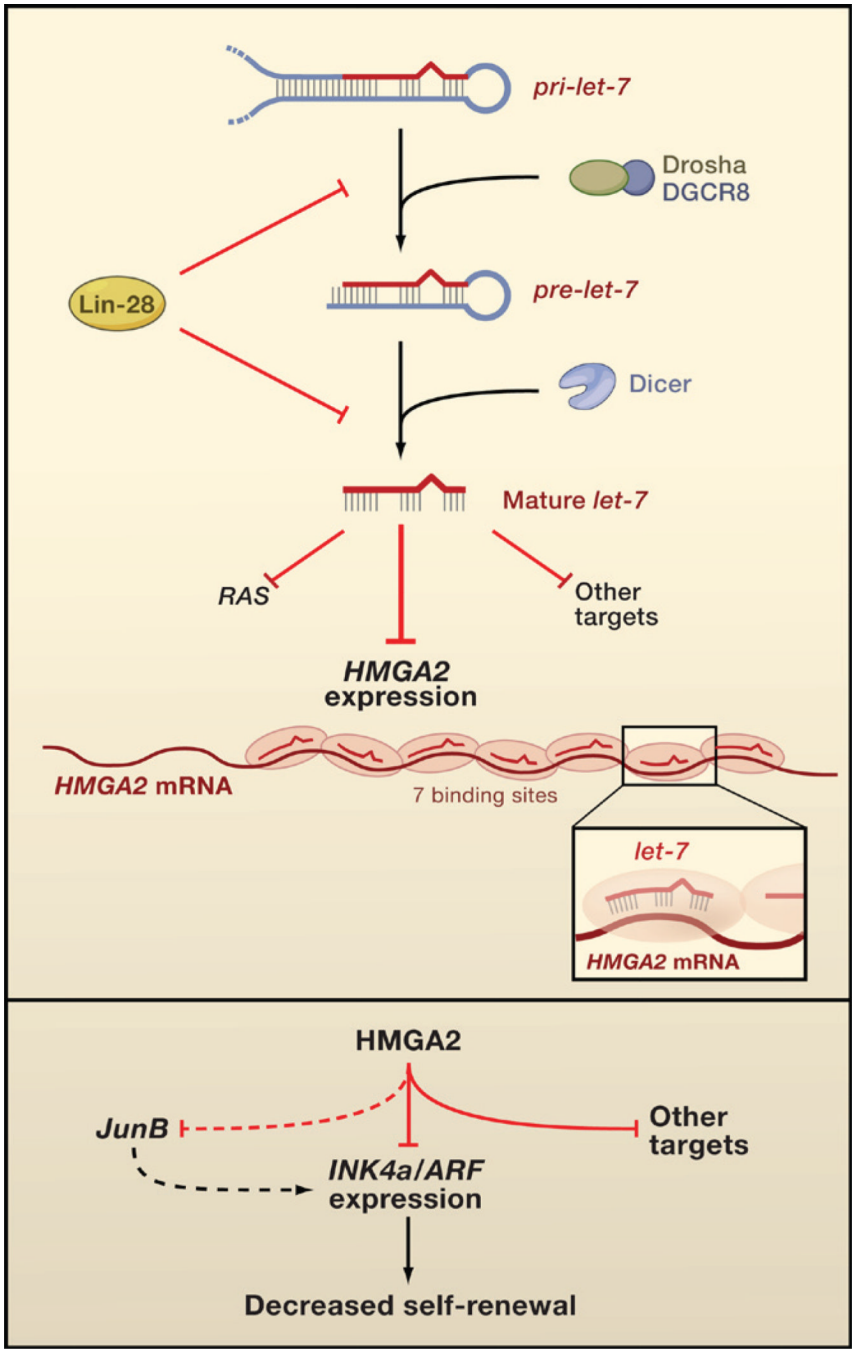

Figure 1. HMGA2 and *let-7* in Mammalian Development

MicroRNAs (miRNAs) are generated as long primary transcripts (pri-miRNAs) that are processed by the Drosha/DGC8 complex into pre-miRNAs. Further processing by the endoribonuclease Dicer produces the 22 nucleotide mature miRNAs. Production of the *let-7* miRNA during development is regulated by the RNA binding protein Lin-28. Mature *let-7* targets multiple genes, including *HMGA2*. This architectural transcription factor represses *INK4a/ARF* expression, possibly through the repression of *JunB*.