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20. 10T1/2 fibroblasts or C2C12 myoblasts grown in GM (20% fetal calf serum) were transfected with Lipofectamine (Gibco BRL) according to the manufacturer's directions. After 36 hours, cells were fed DM [2% horse serum plus insulin (12 μg/ml)]; 48 hours later, cells were harvested for CAT assay, normalized to protein content, essentially as described [C. M. Gorman *et al.*, *Mol. Cell. Biol.* **2**, 1044 (1982)]. All experiments for CAT expression were repeated at least three times with similar results. Except where indicated, CAT activity is expressed as percent of CAT expression observed in the absence of ectopic cyclins. Plasmids for transfection contained the CMV promoter driving expression of the following: MyoD (CMV-MyoD) (provided by B. Novitch); cyclins A, B1, B2, D1, D2, D3, and E (provided by S. Dowdy and P. Hinds) (6); $p21^{CIP1}$ (provided by S. Elledge); nuclear β-galactosidase (CMV–β-GAL) (pCS2+nβ-GAL) (provided by D. Turner, R. Rupp, and H. Weintraub); and $p16^{INK4}$, which was made by subcloning p16 (provided by D. Beach) into the Not I and Apa I sites in pRC/CMV (Invitrogen). Reporter plasmids contained the CAT gene driven by 3300 base pairs of the muscle creatine kinase promoter-enhancer (MCK-CAT) [J. Jaynes *et al.*, *Mol. Cell. Biol.* **8**, 62 (1988)] (provided by S. Hauschka), four reiterated MEF1 sites driving the tk promoter (4Rtk-CAT) [H. Weintraub *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5623 (1990)], or the CMV-promoter (CMV-CAT) from the pCSA plasmid (provided by T. Roberts).
21. For immunoprecipitations, 10T1/2 cells were transfected and cultured as described (*20*). For MyoD immunoprecipitation, cells were lysed in NP-40 lysis buffer [50 mM tris (pH 7.4), 150 mM NaCl, 0.5% NP-40, and 20% glycerol with 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin and pepstatin (10 μg/ml each), aprotinin (100 μg/ml), and phosphatase inhibitors (10 mM $NaPPO_4$, 1 mM NaF, 0.1 mM Na_3VO_4)]. Cell extracts were sonicated and precleared with Zysorbin (Zymed Laboratories) and centrifugation. Extracts, normalized to DNA content by fluorometry (Hoefer Scientific), were immunoprecipitated with a rabbit polyclonal antiserum to the COOH-terminus of MyoD (*13*) and protein A–Sepharose beads. After immunoprecipitation, beads were washed with NP-40 buffer without phosphatase inhibitors, split, and resuspended in SDS gel loading buffer or in CIP buffer [10 mM tris (pH 8.0), 1 mM $MgCl_2$, 1 mM PMSF, aprotinin (1 μg/ml)] for phosphatase treatment with 50 U of CIP (New England Biolabs) at 50°C for 60 min. Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE), immunoblotted with a monoclonal antibody to MyoD (5.8A) (provided by P. Houghton), and analyzed by enhanced chemiluminescence (Amersham). For detection of hemagglutinin (HA)-tagged D-cyclins (provided by P. Hinds), cells were labeled for 3 hours with ^{35}S-methionine (TransLabel, ICN), lysed in RIPA buffer [10 mM tris (pH 7.4), 200 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and protease and phosphatase inhibitors as described (*21*)], sonicated, and precleared as described (*21*). Extracts, with equivalent ^{35}S-methionine incorporation, were immunoprecipitated with a monoclonal antibody to the HA epitope (12CA5) [J. Field *et al.*, *Mol. Cell. Biol.* **8**, 2159 (1988)] and analyzed by SDS-PAGE and fluorography.
22. Whole-cell extracts from C2C12 myoblasts (MB) (subconfluent and growing in GM) or differentiated myotubes (MT) (in DM for 72 hours) were made in NP-40 lysis buffer as described (*21*). Proteins were separated by SDS-PAGE and analyzed by immunoblotting and enhanced chemiluminescence (Amersham). Cyclin D1 was detected with mAb D1-72-13G, D3 was detected with mAb D3-18B6-10B, and Cdk4 was detected with rabbit antiserum Rz (*15*) (provided by C. Sherr).

23. We thank O. Halevy, P. Hinds, A. Münsterberg, B. Novitch, T. Schultheiss, and L. Webster for helpful discussions; S. Kohtz for sharing unpublished results; and D. Beach, S. Dowdy, S. Elledge, S. Hauschka, P. Hinds, P. Houghton, C. Sherr, D. Turner, T. Roberts, R. Rupp, and H. Weintraub for reagents. Supported by grants to A.B.L. from the National Science Foundation, the Lucille P. Markey Charitable Trust, the Muscular Dystrophy Association, The Council for Tobacco Research U.S.A., and a Basil O'Connor Award (1FY93-0848) from the March of Dimes Birth Defects Foundation. During the course of this study, A.B.L. was a Lucille P. Markey Scholar; D.B.S. is supported by NIH postdoctoral fellowship 1F32AR08214-01A1.

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p53-Independent Expression of $p21^{Cip1}$ in Muscle and Other Terminally Differentiating Cells

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Terminal differentiation is coupled to withdrawal from the cell cycle. The cyclin-dependent kinase inhibitor (CKI) $p21^{Cip1}$ is transcriptionally regulated by p53 and can induce growth arrest. CKIs are therefore potential mediators of developmental control of cell proliferation. The expression pattern of mouse p21 correlated with terminal differentiation of multiple cell lineages including skeletal muscle, cartilage, skin, and nasal epithelium in a p53-independent manner. Although the muscle-specific transcription factor MyoD is sufficient to activate p21 expression in 10T1/2 cells, p21 was expressed in myogenic cells of mice lacking the genes encoding MyoD and myogenin, demonstrating that p21 expression does not require these transcription factors. The p21 protein may function during development as an inducible growth inhibitor that contributes to cell cycle exit and differentiation.

Proper development of a multicellular organism is complex and requires precise spatial and temporal control of cell proliferation. A large network of regulatory genes has evolved to specify when and where in the embryo cells divide. This control is superimposed upon the basic cell cycle regulatory machinery.

Cell proliferation requires the action of cyclins, which serve as activators of their cognate cyclin-dependent kinases (Cdks). (*1*) D- and E-type cyclins have been implicated in controling passage through the "restriction" point (*2*), after which cells become committed to a round of cell division (*3*). G_1 cyclin accumulation is required for cell cycle entry and members of this family, particularly D-cyclins, have been identified as targets of growth factors (*1*).

Equally important in the execution of developmental programs is the arrest of growth once the program is complete. Whereas the control of terminal differentiation may be mediated by multiple, possibly redundant, mechanisms, cell cycle arrest through inactivation of Cdks is likely to be a central feature. Possible mediators of such negative control are two classes of Cdk inhibitory (CKI) proteins typified by $p21^{CIP1}$ (*4*) and $p16^{INK4/MTS1}$ (*5*). The p21 protein inhibits G_1 cyclin complexes containing Cdk2, Cdk3, Cdk4, and Cdk6 and is transcriptionally induced by overexpression of the tumor suppressor protein p53 (*6*) or by activation of p53 after DNA damage, consistent with a role for p21 in the p53-dependent G_1 checkpoint (*7*).

The ability of p21 to function as an inducible cell cycle inhibitor suggests that it

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might also function to mediate cell cycle arrest during development. Thus, knowledge of the timing and location of p21 expression in the embryo could provide evidence that this CKI participates in terminal differentiation in a developing organism. Because p53 regulates transcription of p21 in vitro (*6*, *7*), we also tested whether p21 expression in vivo was dependent upon p53.

We used in situ hybridization (ISH) to probe for p21 expression during mouse embryogenesis (*8*). Embryos of day 7.5 post coitum (p.c.) (0 to 5 somites) showed no expression of p21. By day 8.5 p.c., we detected hybridization along the midline of the neural tube and in the hindgut. Presomitic paraxial mesoderm did not express p21, but there was hybridization in the dermamyotome (Fig. 1A), where the first determined myocytes are localized. By day 10 p.c., there was strong expression of p21 in the muscle fibers extending from the anterior to the posterior margin of the myotome (Fig. 1B). Hybridization of adjacent sections with a probe for the muscle-specific basic-loop-helix protein myogenin (*9*) exhibited signals in the same regions in which p21 was detected (Fig. 1, C and G). Muscle cells in the myotome are post-mitotic (*10*). A transverse section through a day-10 p.c. embryo at the position of the forelimb revealed expression of p21 in the developing limb (Fig. 1D). In the section shown, there is a zone of hybridization in the dorsal region of the limb mesenchyme, representing the emerging dorsal muscle mass. Muscle primordia in the limb expressed the homeobox gene Pax3 (*11*) in the same region where p21 was expressed (Fig. 1, D and E). Later in development, limb and intercostal muscle strongly expressed p21 (Fig. 1, H through J).

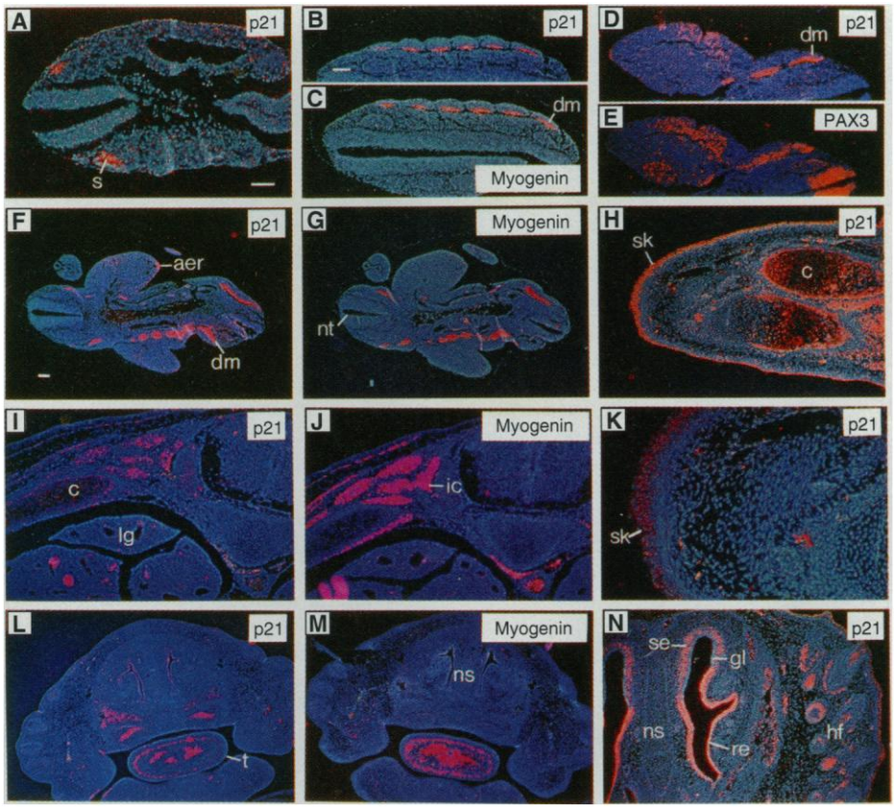

Fig. 1. Expression of p21, myogenin, and Pax3 during mouse embryogenesis. Sections are from C57 black embryos and were subjected to in situ hybridization with the indicated ^{35}S-labeled riboprobes (*8*). (**A**) Transverse section through an 8.5-day p.c. embryo: p21 probe. (**B** and **C**) Adjacent transverse sections through a 10-day p.c. embryo: p21 probe (B), myogenin probe (C). (**D** and **E**) Adjacent transverse sections through the limb bud and the body wall of a 10-day p.c. embryo: p21 probe (D), Pax3 probe (E). (**F** and **G**) Adjacent transverse section through a 10-day p.c. embryo: p21 probe (F), myogenin probe (G). (**H** and **K**) Cross-section through a 15.5-day p.c. forelimb: p21 probe. (K) Higher magnification of H. (**I** and **J**) Nearby transverse sections through the body wall and spinal cord of a 12.5-day p.c. embryo: p21 probe (I), myogenin probe (J). (**L** and **M**) Adjacent coronal sections illustrating p21 expression (L) in the nasal cavity and tongue muscles of a 12.5-day p.c. embryo, (M) was hybridized with a myogenin probe. (**N**) Coronal section through the nasal cavity of a 15.5-day p.c. embryo: p21 probe. Abbreviations: aer, apical ectodermal ridge; c, cartilage; dm, dermamyotome; gl, supporting cells; hf, hair follicle; lg, lung; ic, intercostal muscle; ns, nasal septum; nt, neural tube; re, respiratory epithelium; s, somite; se, olfactory sensory epithelium; sk, skin; and t, tongue. A, D, E, and K are the same magnification; scale bar in A is 50 μm. B, C, H, I, J, and N are the same magnification; scale bar in B is 100 μm. F, G, L, and M are the same magnification; scale bar in F is 100 μm. Hybridization signal is in red.

At days 13 to 15 p.c. we detected expression of p21 in nasal epithelium, tongue muscles, hair follicles, the outer most layer of embryonic epidermis, and cartilage (Fig. 1, H through M). With the exception of cartilage, which has been studied in less detail, each of these sites of expression contain post-mitotic, differentiated cells (*10*). This is also true of the apical ectodermal ridge (AER) where expression of p21 was observed as early as day 10 p.c. (Fig. 1F). During limb outgrowth, the AER maintains the underlying mesenchymal tissue in an undifferentiated state, but the cells of the AER itself do not divide. Selective expression of p21 in differentiated epithelium was evident in the nasal region. At day 15.5 p.c., p21 was expressed throughout the post-mitotic respiratory epithelium, whereas the adjacent olfactory epithelium had a delimited pattern of expression (Fig. 1N). We detected no p21 mRNA in the mitotic germinal layer of the olfactory epithelium, but p21 was expressed in the layer containing differentiating olfactory neurons (Fig. 1N).

The mRNA encoding p53 is widely expressed in the day-12 p.c. embryo (Fig. 2A) (*12*), suggesting that normal amounts of p53 are not sufficient for p21 induction in many cell types. To determine whether expression of p21 was dependent upon p53, we examined expression of p21 in sections of mice lacking the gene encoding p53 [p53(−/−)] (*13*). Mice that lack p53 develop normally but incur tumors much more rapidly than do wild-type animals (*13*, *14*). As judged by ISH, expression of p21 during early embryogenesis was independent of expression of p53 (Fig. 2A). Sagittal sections through day-11.5 p.c. embryos lacking p53 stained strongly for p21 in somites (Fig. 2A). A survey of the major sites of p21 expression, including cartilage, nasal epithelium, intercostal tongue, and limb muscle from day-12 to -14.5 p.c. embryos lacking p53 revealed that p21 expression in these tissues was independent of p53 expression (Fig. 2A).

We determined expression of p21 in adult mouse tissues (Fig. 2C). Because adult tissues are a primary target for tumorigenesis, it was conceivable that p21 expression in certain adult tissues is p53-dependent. Analysis of small intestine and stomach revealed that p21 is expressed in a highly selective manner and is found in large amounts only in the fully differentiated columnar epithelium (Fig. 2B). The p21 mRNA was absent from the embryonic brain and spinal cord, but large amounts of p21 mRNA were detected in the adult brain (Fig. 2C), especially in the olfactory bulbs (Fig. 2B). Uniform expression of p21 was observed in adult lungs, heart, and skeletal muscle (Fig. 2B). In all of

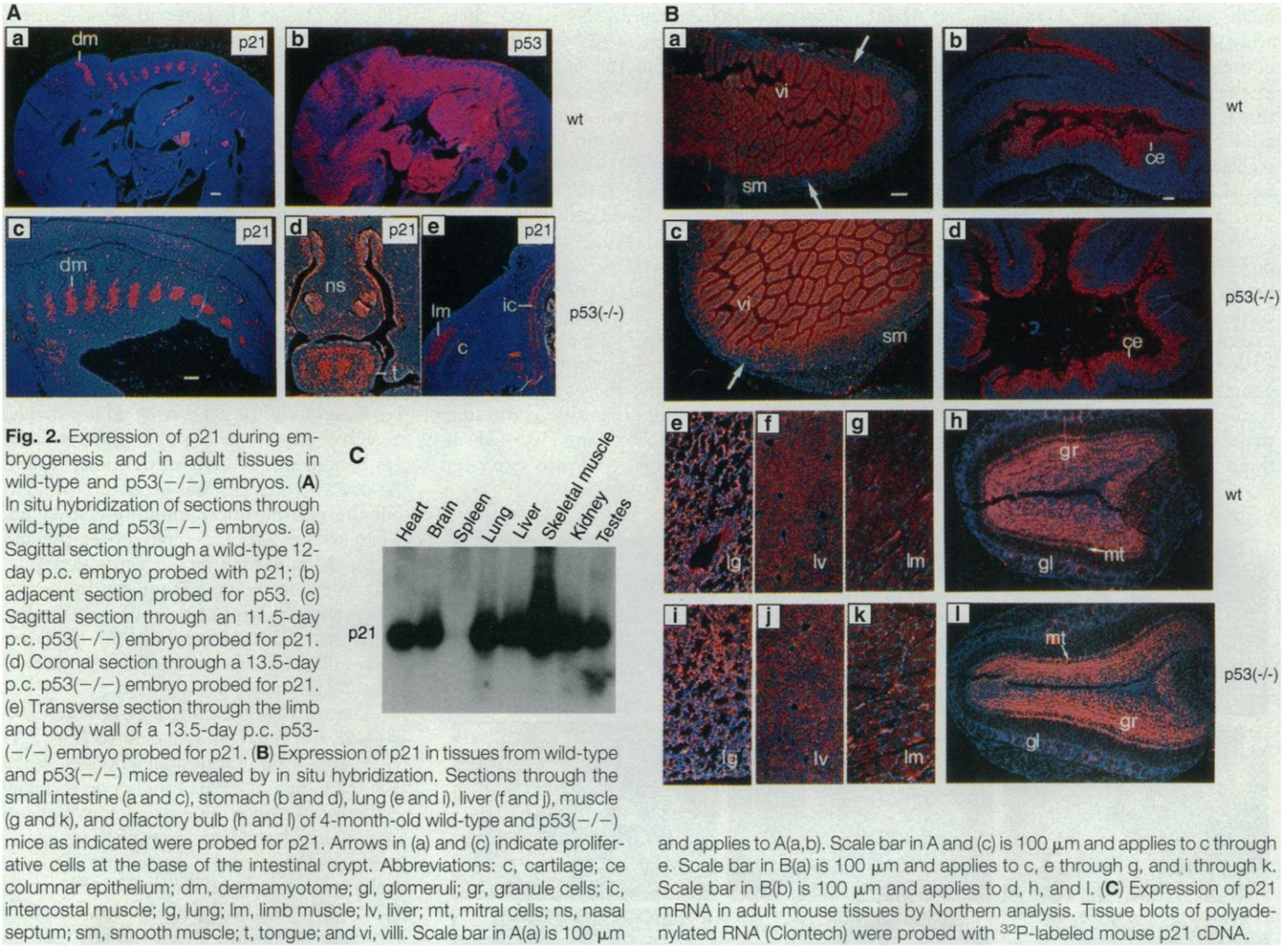

Fig. 2. Expression of p21 during embryogenesis and in adult tissues in wild-type and p53(−/−) embryos. (**A**) In situ hybridization of sections through wild-type and p53(−/−) embryos. (a) Sagittal section through a wild-type 12-day p.c. embryo probed with p21; (b) adjacent section probed for p53. (c) Sagittal section through an 11.5-day p.c. p53(−/−) embryo probed for p21. (d) Coronal section through a 13.5-day p.c. p53(−/−) embryo probed for p21. (e) Transverse section through the limb and body wall of a 13.5-day p.c. p53(−/−) embryo probed for p21. (**B**) Expression of p21 in tissues from wild-type and p53(−/−) mice revealed by in situ hybridization. Sections through the small intestine (a and c), stomach (b and d), lung (e and i), liver (f and j), muscle (g and k), and olfactory bulb (h and l) of 4-month-old wild-type and p53(−/−) mice as indicated were probed for p21. Arrows in (a) and (c) indicate proliferative cells at the base of the intestinal crypt. Abbreviations: c, cartilage; ce columnar epithelium; dm, dermamyotome; gl, glomeruli; gr, granule cells; ic, intercostal muscle; lg, lung; lm, limb muscle; lv, liver; mt, mitral cells; ns, nasal septum; sm, smooth muscle; t, tongue; and vi, villi. Scale bar in A(a) is 100 μm and applies to A(a,b). Scale bar in A and (c) is 100 μm and applies to c through e. Scale bar in B(a) is 100 μm and applies to c, e through g, and i through k. Scale bar in B(b) is 100 μm and applies to d, h, and l. (**C**) Expression of p21 mRNA in adult mouse tissues by Northern analysis. Tissue blots of polyadenylated RNA (Clontech) were probed with ^{32}P-labeled mouse p21 cDNA.

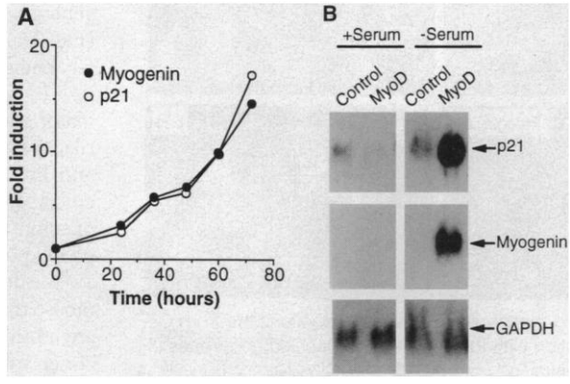

Fig. 3. Induction of p21 mRNA during myoblast differentiation in vitro. (**A**) Northern blot analysis of p21 and myogenin mRNA in C2 myoblasts deprived of serum for the indicated times in hours. Fold induction represents mRNA levels normalized to GAPDH relative to time 0. (**B**) 10T1/2 cells and 10T1/2 cells expressing MyoD (*23*) were grown in the presence of high or low concentrations of serum for 4 days; total RNA was isolated, and Northern blots were probed with p21, myogenin, and GAPDH.

the adult tissues analyzed, p21 expression was unaltered in p53(−/−) mice (Fig. 2B). Consistent with this, mRNA prepared from a typical tissue, the stomach, showed no p53-dependent changes in the amount of p21 mRNA (*15*).

Our expression studies suggest that p21 may function in muscle cell differentiation. The myogenic program is controlled by helix-loop-helix transcription factors of the MyoD family and expression of either MyoD, Myf5, or myogenin is sufficient to convert a number of cell types into muscle (*9*, *16*). Whereas Myf5 and MyoD can functionally replace each other in vivo (*17*), myogenin has a separate function and is required for the formation of differentiated muscle fibers (*18*). A critical step in this differentiation process is cell cycle arrest, and overexpression of MyoD in various cell types leads to a block in DNA replication (*20*). The process of muscle cell differentiation can be mimicked in vitro in C2 myoblasts, which form post-mitotic, multinucleated myotubes when grown in the absence of growth factors (*19*). Consistent with a role for p21 in the differentiation process, withdrawal of serum from C2 myoblasts results in induction of p21 mRNA as detected by Northern analysis (Fig. 3) (*21*). After 72 hours, at which time ~50% of the cells have been incorporated into myotubes, p21 mRNA levels had increased by 17-fold. The time course for myogenin mRNA induction was similar to that of p21 mRNA. Furthermore, MyoD expression in 10T1/2 cells in low serum was sufficient to induce both p21 (Fig. 3B) (*22*) and differentiation into myotubes (*23*).

To examine whether p21 expression is dependent upon myogenin, ISH analysis was done with mice lacking the gene encoding myogenin [myogenin(−/−)]. These animals produce muscle precursor cells expressing MyoD and Myf5 but lack fully differentiated muscle fibers and die shortly after birth (*18*). Expression of p21 was retained in myoblasts

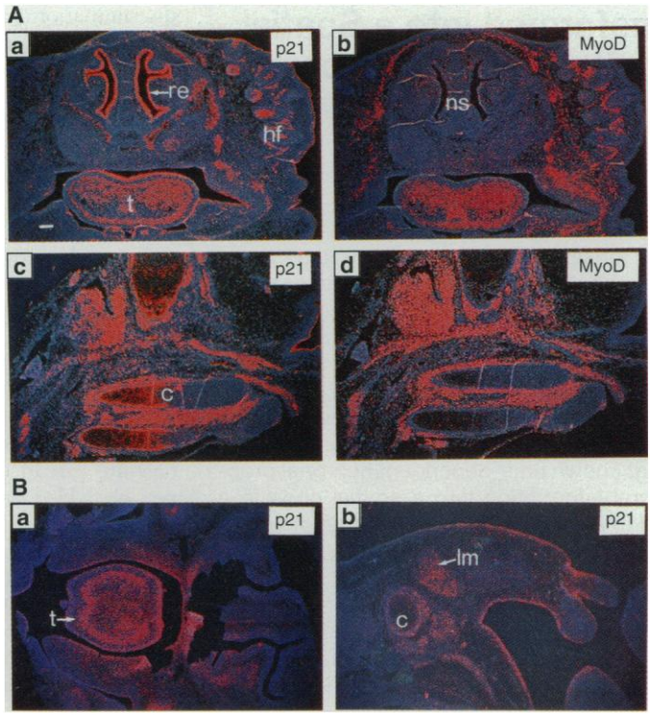

Fig. 4. Expression of p21 in myogenin(−/−) embryos and embryos lacking both MyoD and myogenin. **A**(a, b) Adjacent coronal sections, illustrating nasal cavity, tongue muscle, and hair follicles of 15.5-day p.c. myogenin(−/−) embryo sections, were probed with p21 (a) or MyoD (b) to identify muscle precursor cells. (c and d) Cross-sections through the forelimb of a 15.5-day p.c. myogenin(−/−) embryo probed with p21 (c) or MyoD (d). **B**(a, b) Sections through the tongue (a) and forelimb (b) of a 14-day p.c. MyoD(−/−); myogenin(−/−) embryo probed with p21. Abbreviations: c, cartilage; hf, hair follicle; lm, limb muscle; ns, nasal septum; re, respiratory epithelium; and t, tongue. Scale bar in A(a) is 100 μm and applies to all panels in Fig. 4.

in the forelimb and tongue of myogenin(−/−) embryos and in other muscle tissue (Fig. 4A), indicating that myogenin is not required for p21 induction during myogenesis. Although there is a block to myoblast differentiation in myogenin(−/−) mice, BrdU (bromodeoxyuridine)-labeling experiments indicate that the undifferentiated myoblasts that populate the presumptive muscle-forming regions withdraw from the cell cycle normally (*24*).

Although MyoD expression is sufficient to induce p21 (Fig. 3B) (*22*), p21 is expressed in somites at day 8.5 p.c. (Fig. 1A) before expression of MyoD at day 10.5 p.c. (*9*), consistent with the possibility that other transcription factors may control p21 induction during muscle cell differentiation in the embryo. One caveat is that at day 8.5, MyoD may be expressed in amounts sufficient to activate p21 but below the limit of detection by ISH. However, at day 14 p.c., p21 was expressed in muscle precursor cells from forelimb and tongue in MyoD(−/−); myogenin(−/−) mice in amounts comparable to that found in wild-type animals (Fig. 4B).

Our results revealed a strong correlation between arrest of cell proliferation and p21 expression in vivo. This correlation was particularly evident in the skeletal muscle lineage where p21 expression was similar to that of myogenin in vivo and in vitro. Although MyoD is sufficient to arrest the cell cycle and induce muscle differentiation, neither MyoD nor myogenin is required for p21 regulation in vivo, suggesting a possibly redundant role for Myf5. Other CKIs and negative cell cycle regulators, such as the retinoblastoma gene product Rb (*28*), may also contribute to differentiation of muscle and other cell lineages. Although the basal amount of p21 in fibroblast cell lines in vitro is p53-dependent (*26*), p21 expression in the embryo and adult does not require p53. Taken together, our results indicate that p21 functions as an inducible growth inhibitor both during development and in G_1 checkpoint control, and that p53's role in p21 expression is likely to be limited to the checkpoint function.

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8. The cDNA for mouse p21 in pBluescript was used for riboprobe synthesis. Embryo collection, sectioning, and in situ hybridization were done essentially as described (*27*). In those cases in which mice harboring null alleles of p53, myogenin, or both MyoD and myogenin were used, genotypes were determined by Southern blot analysis with yolk sac DNA as described for tail DNA (*13*). Plasmids were linearized with the appropriate enzymes, and [α-^{35}S]uridine triphosphate–labeled sense and anti-sense transcripts were generated by run-off transcription with either T7 or T3 polymerase. For p21, the 707-bp cDNA fragment was used as a probe. For p53, a 1.7-kb transcript derived from p53 cDNA pBluescript linearized with Eco RI was used as probe. Plasmids used to generate riboprobes for Pax3 (*11*), myogenin (*9*), and MyoD (*9*) have been described. Specimens were photographed by double exposure using darkfield illumination with a red filter and Hoechst epifluorescence optics (*27*).
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