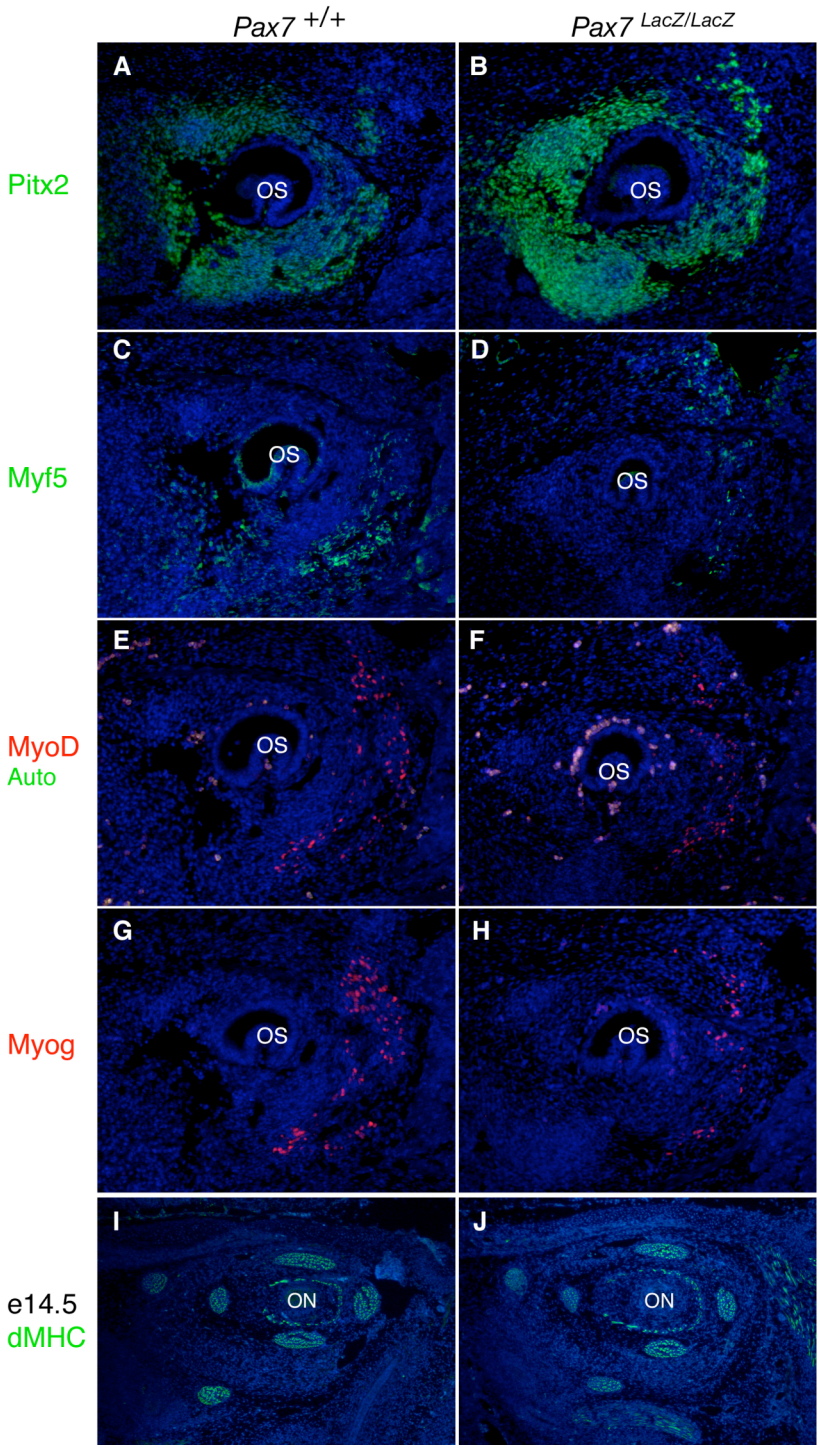


**Figure 4.1: *Pax7* is not required for extraocular muscle formation.** Sagittal sections of wildtype (**A**, **C**, **E**, **G**, **I**) and $Pax7^{LacZ/LacZ}$ (**B**, **D**, **F**, **H**, **J**) embryonic extraocular muscles. At e12.0, $Pax7^{LacZ/LacZ}$ embryos have normal expression of PITX2 (**A**, **B**) , MYF5 (**C**, **D**), MYOD (**E**, **F**, autofluorescent red blood cells shown in green), and MYOG (**G**, **H**), as compared to their wildtype littermates. By e14.5, these mice also have normal differentiation of all seven extraocular muscles as indicated by expression of developmental myosin heavy chain (dMHC) (**I**, **J**). OS, optic stalk; ON, optic nerve.

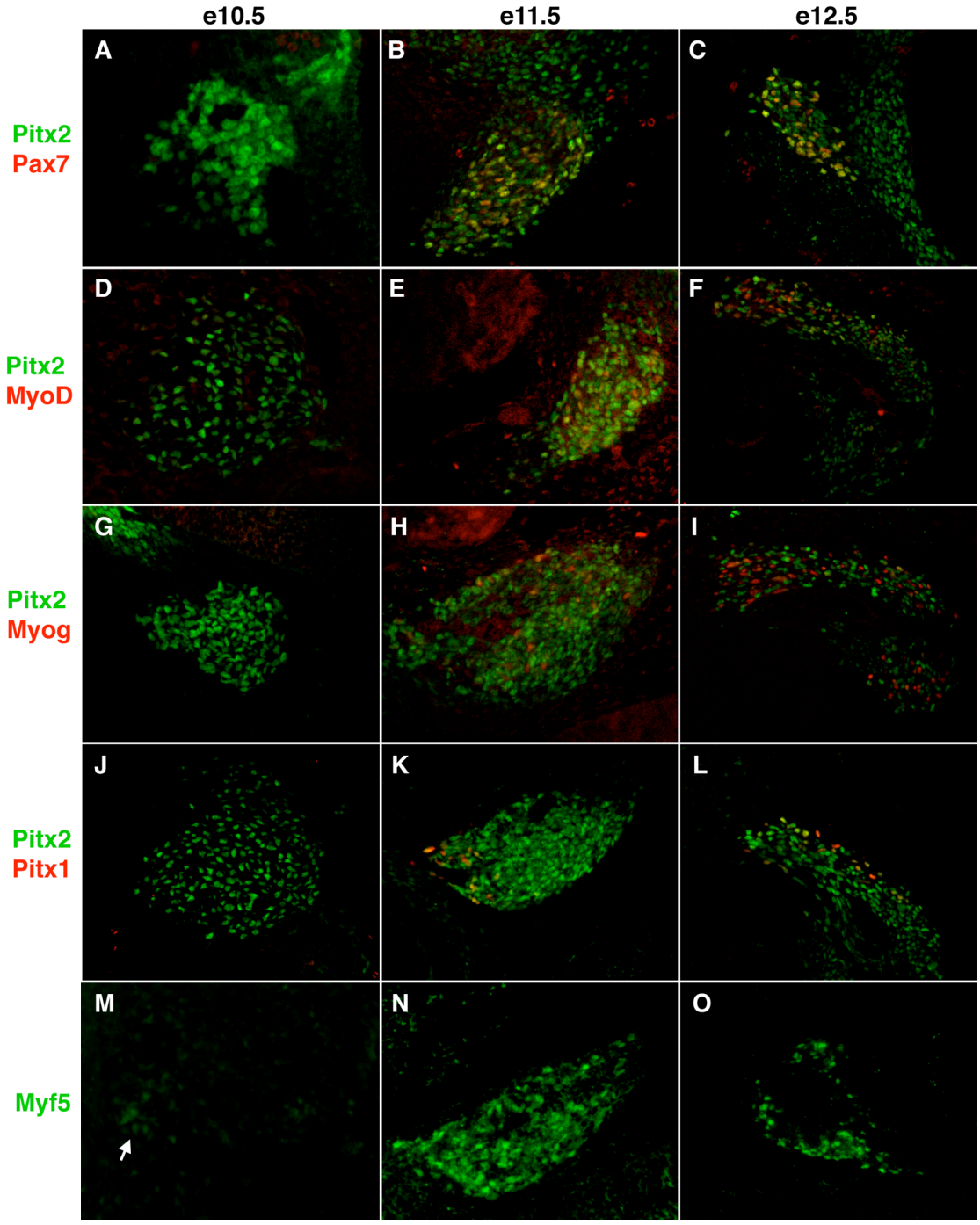


**Figure 4.2: PITX2 is expressed prior to markers of muscle specification.** Transverse sections of e10.5 (**A**, **D**, **G**, **J**, **M**), e11.5 (**B**, **E**, **H**, **K**, **N**) and e12.5 (**C**, **F**, **I**, **L**, **O**) wildtype extraocular muscle primordia. At e10.5, robust PITX2 expression is seen in the EOM primordia (**A**, **C**, **G**, **J**). MYF5 expression is seen only in a small patch of cells at e10.5 (**M**, arrow), while wide expression is seen later (**N**,**O**). Expression of the other MRFs, MYOD and MYOG does not begin until e11.5 (**E**, **H**). PAX7 and PITX1 expression are first seen at e11.5 (**B**, **K**), but PITX1 is only expressed in a small subset of cells (**K**, **L**). At e12.5, cells with both PITX2 and MYOD or MYOG expression are seen, as well as cells that express only one of the proteins (**F**, **I**).

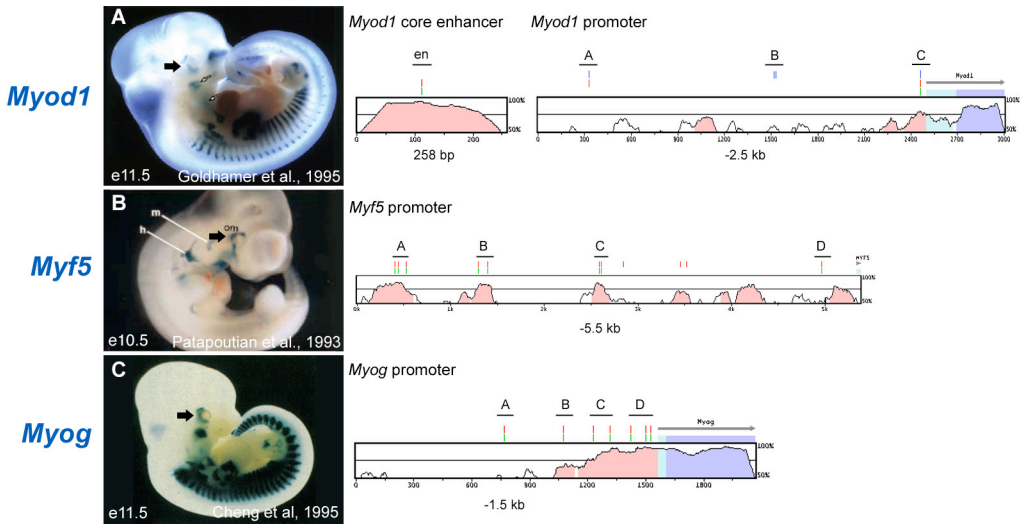


**Figure 4.3: MRF promoters drive expression in mouse EOM primordia and contain predicted PITX2 binding sites.** The previously described *Myod1* 258bp core enhancer, which is 20 kb upstream of its -2.5 kb promoter drive *LacZ* expression in the extraocular muscle primordia at e11.5 (**A**, arrow, image from Goldhamer et al., 1995). The *Myod1* enhancer and promoter contain conserved (green tick marks), aligned (conserved in location but not sequence, red tick marks) and non-conserved (blue tick marks) predicted PITX2 binding sites identified by rVISTA (Loots et al., 2002). The VISTA plots show conservation between mouse and human along the length of the promoter. Locations amplified in ChIP are shown above the VISTA plot. The *Myf5* -5.5 kb promoter drives *LacZ* expression in the extraocular muscle primordia at e10.5 (**B**, arrow, image from Patapoutian et al., 1993) and it contains predicted PITX2 sites identified by rVISTA. The *Myog* -1.5 kb promoter drives expression in the EOM primordia at e11.5 (**C**, arrow, image from Cheng et al., 1995) and contains predicted PITX2 binding sites identified by rVISTA.

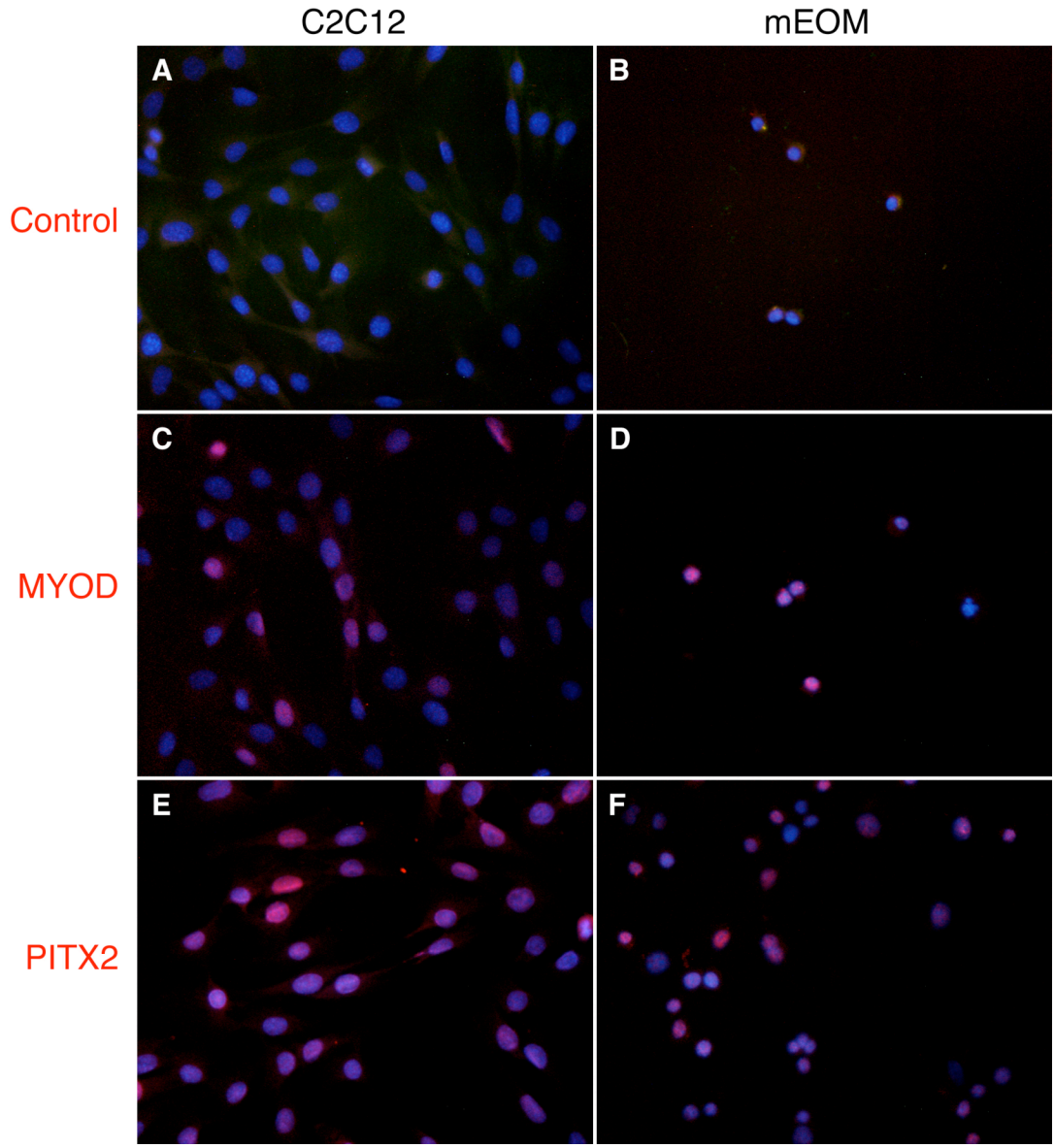


**Figure 4.4: PITX2 is expressed in muscle cell lines.** Immunocytochemistry shows that MYOD (**C**, **D**), as well as PITX2 protein (**E**, **F**) are expressed in both the C2C12 limb muscle precursor cell line and the mEOM extraocular muscle precursor primary cell line. Omission of the primary antibody in the staining process results in mild background staining in the cytoplasm (**A**, **B**). Note that the mEOM cells have smaller nuclei and less extensive cytoplasm than the C2C12 cells.

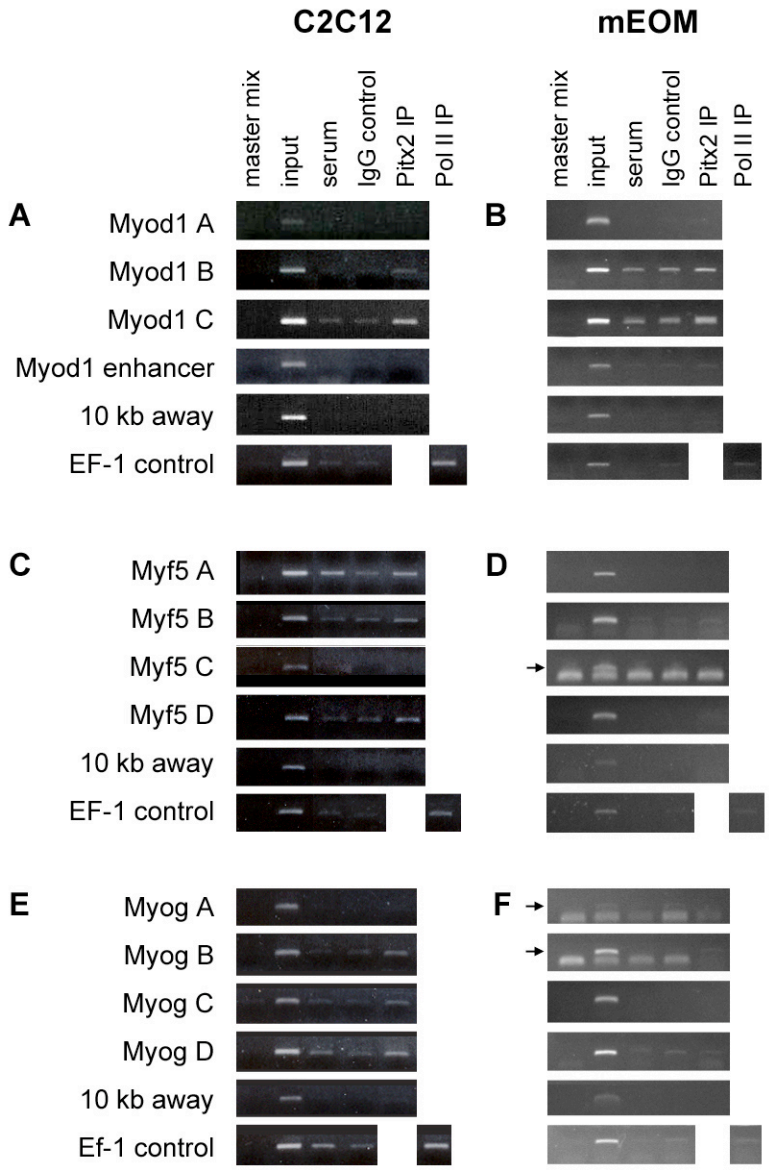


**Figure 4.5: PITX2 binds specific sites in MRF promoters.** Chromatin immuno-precipitation of sheared chromatin from C2C12 (**A**, **C**, **E**) and mEOM (**B**, **D**, **F**) was immunoprecipitated with anti-PITX2 or control (serum, IgG control) antibodies. Regions 10 kb upstream of each promoter with no identifiable PITX2 binding sites were used as negative-controls. Chromatin bound to PITX2 was enriched for sequences containing the *Myod1* B and C sites over control in both C2C12 and mEOM cells (**A**, **B**). The *Myod1* enhancer and site A were not enriched over the controls (**A**, **B**). The *Myf5* promoter showed enrichment of the B and D sites but not the A and C sites in the PITX2 IP over the control IPs in both cell lines (a faint band is visible in the Pitx2 IP lane for site D in the mEOM cells) (**C**, **D**). The B, C and D sites in the *Myog* promoter all showed PITX2 enrichment over controls in the C2C12 cell line (**E**), but only the B site is enriched in the mEOM cells (**F**). When primer dimer is visible, arrows indicate the specific PCR product.

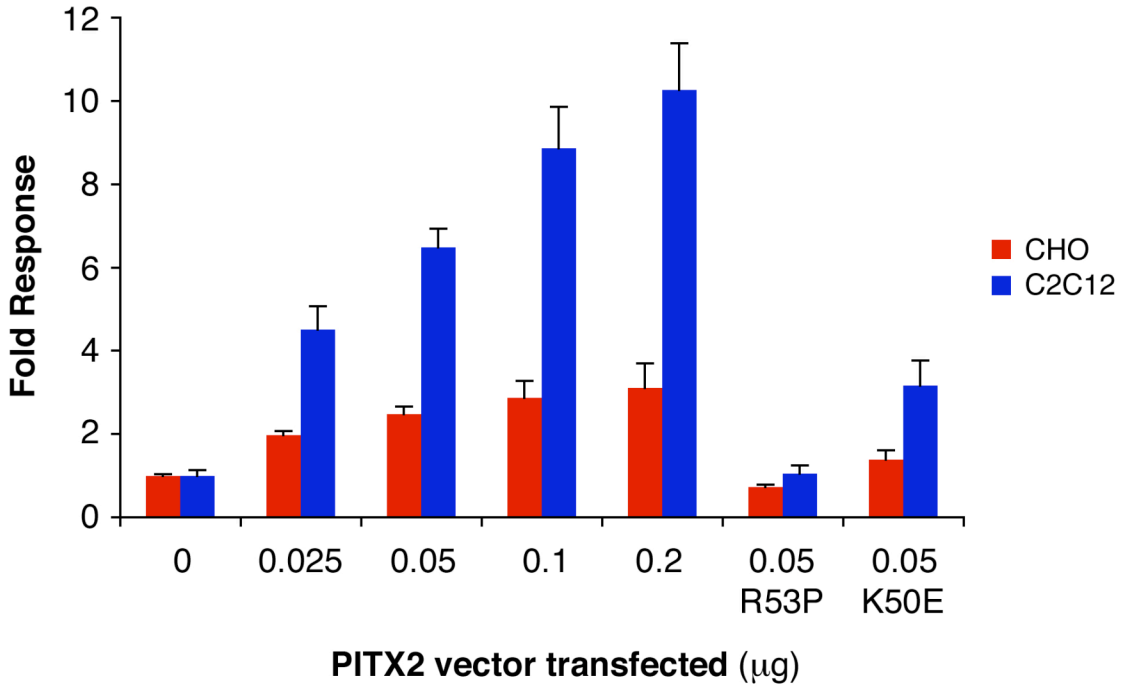


**Figure 4.6: PITX2 activates the human *MYOD1* promoter in muscle and non-muscle cell lines.** Increasing doses of PITX2-expression vector transfected into CHO and C2C12 cells results in an increasing response of the human *MYOD1* promoter in luciferase reporter assays. Mutations in PITX2 shown to be deficient in promoter activation (R53P and K50E) activate the *MYOD1* promoter significantly less than the equivalent wildtype dose. The *MYOD1* promoter responds at significantly higher levels over baseline in the C2C12 muscle precursor cell line than in the CHO Chinese hamster ovary cell line.

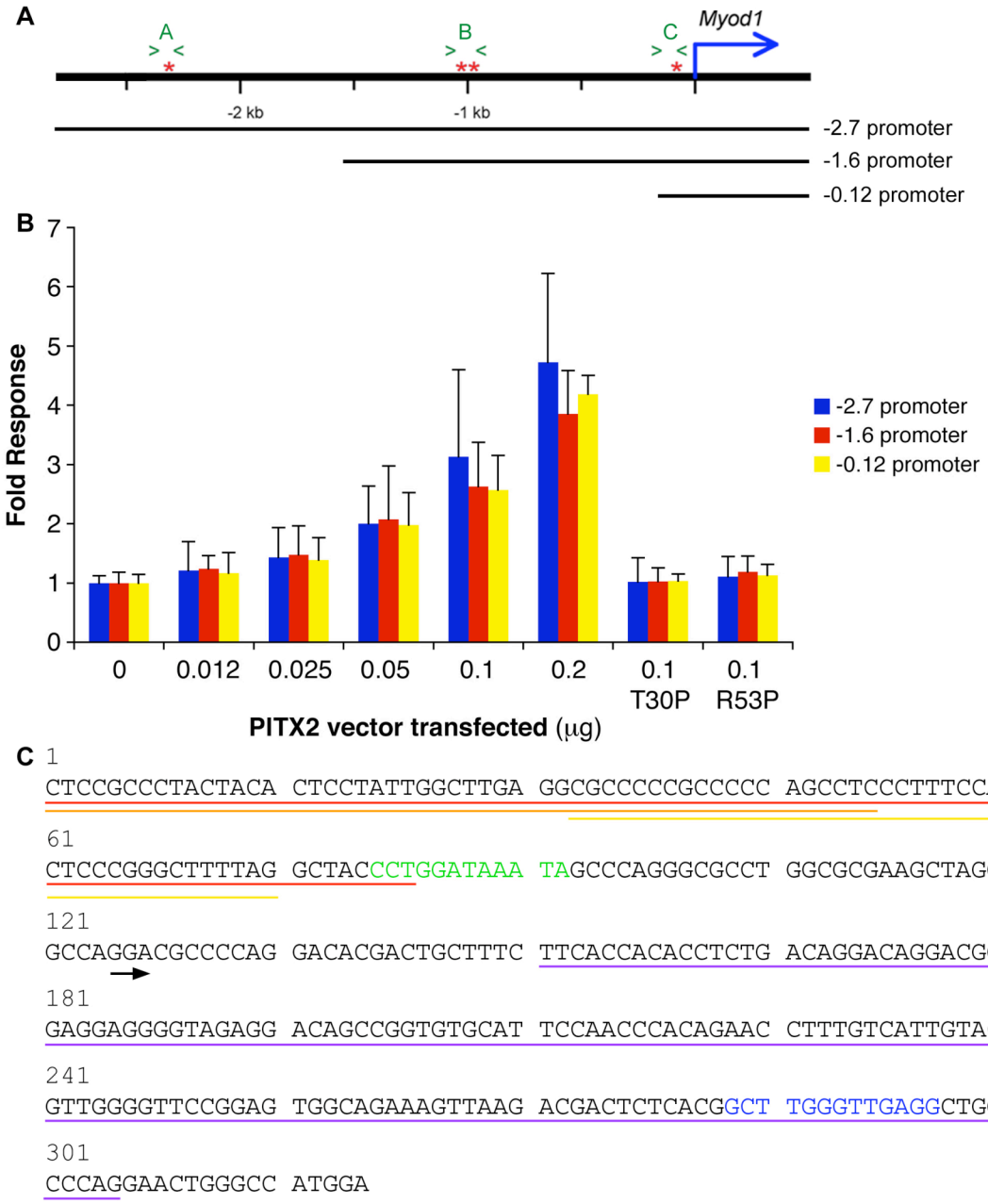


**Figure 4.7: A minimal fragment of the mouse *Myod1* promoter responds to PITX2.** A diagram shows the mouse *Myod1* promoter constructs, with the predicted PITX2 binding sites (red asterisks) and ChIP sites (green > <) indicated (**A**). The mouse *Myod1* promoter also responds to wildtype but not mutant forms of PITX2 in C2C12 cells (**B**). Deleting 1.1 kb at the 5' end of the promoter, which contains ChIP site A, does not significantly affect its ability to respond to PITX2, nor does deleting a further 1.5 kb, which contains ChIP site B. The minimal -124 bp *Myod1* promoter is sufficient to respond to PITX2 (**B**). The sequence of the -124 bp *Myod1* promoter fragment, which includes 196 bp of the 5' untranslated region (**C**). The transcriptional start site is indicated by a black arrow. Conserved (green) and non-conserved (blue) predicted PITX2 binding sites are shown. Underlined regions indicate sequences deleted in the promoter deletion (red), 5' UTR deletion (purple), deletion A (orange), and deletion B (yellow).

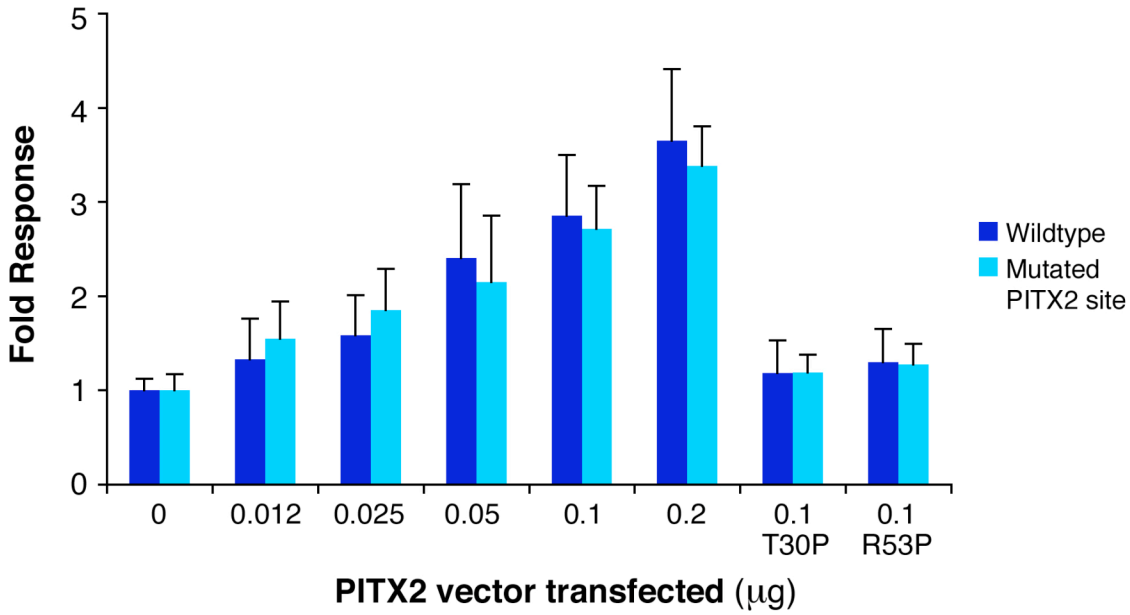


**Figure 4.8: A predicted PITX2 binding site is not required for activation of the *Myod1* minimal promoter.** Mutagenesis of the conserved predicted PITX2 binding site found in ChIP site C in the *Myod1* minimal promoter does not significantly affect its ability to respond to PITX2 in C2C12 cells. The location of the conserved binding site is indicated in Figure 4.7C and 4.9A.

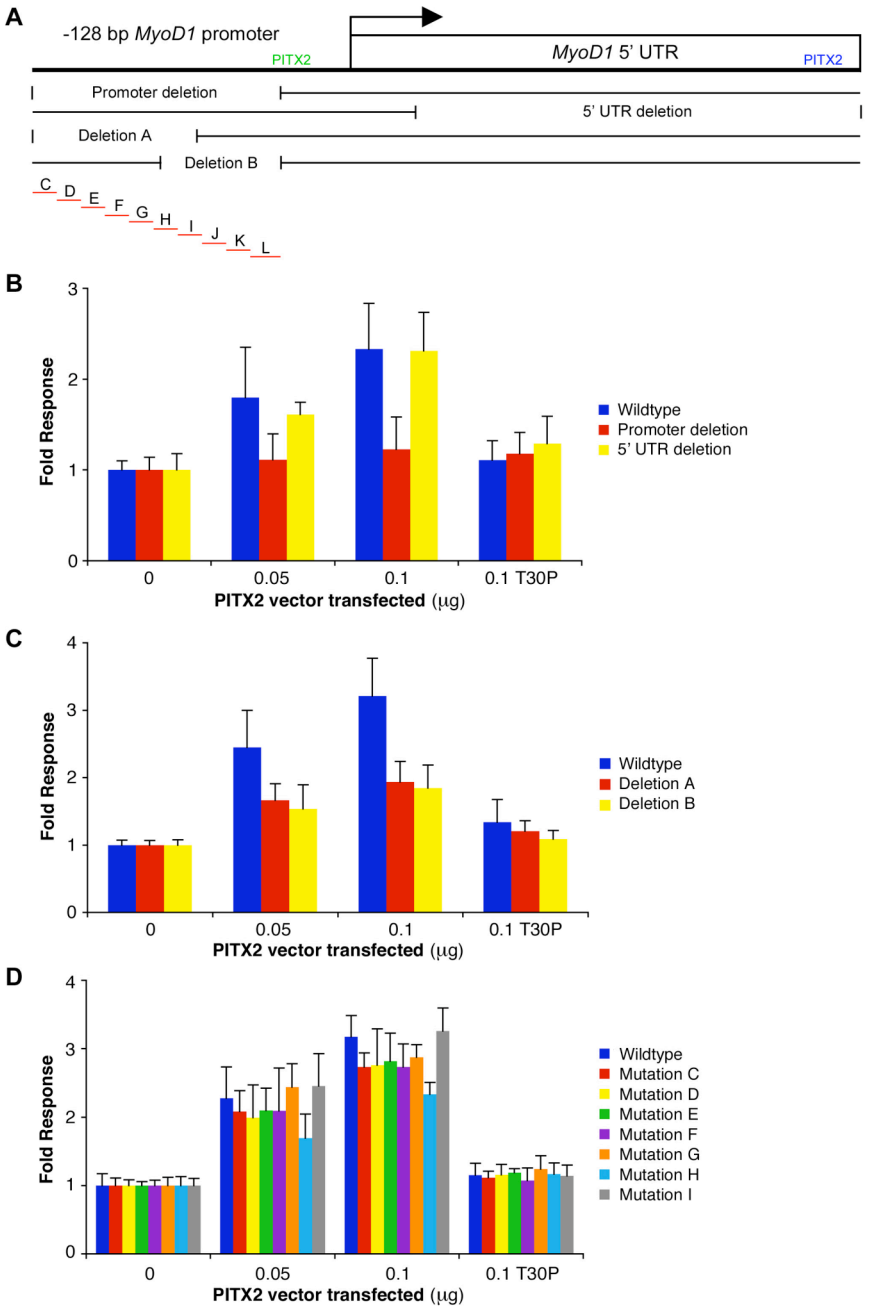


**Figure 4.9: A small region of the *Myod1* promoter responds to PITX2.** A diagram of the *Myod1* minimal promoter shows the conserved (green PITX2) and non-conserved (blue PITX2) predicted PITX2 binding sites (**A**). Regions removed by various deletion constructs are shown in black, and regions subjected to adenine mutagenesis are shown in red (A). The promoter region upstream of the *Myod1* start site is required for PITX2 responsiveness, while the 5'UTR is not in C2C12 cells (**B**). Two overlapping deletions of the *Myod1* promoter region both reduce PITX2 responsiveness in C2C12 cells (**C**). Short scanning adenine mutants of the *Myod1* minimal promoter were created to identify the precise location of the PITX2-responsive region (**D**). Only mutation H is significantly reduced from the wildtype response in C2C12 cells (**D**). Mutations J, K, and L remain to be studied.

| ChIP Site | Forward primer | Reverse Primer | Product size |
|---|---|---|---|
| Myod1 A | TTTGCCTCCCAATGCTAAAC | ATCTCGCTGCTCTCAGCTTT | 189 |
| Myod1 B | TACCCCCTGGACATTGTCAT | GCTATGGGTTTGTGCCATCT | 194 |
| Myod1 C | CAAGCTCCGCCCTACTACAC | TGAAGAAAGCAGTCGTGTCC | 158 |
| Myod1 enh | GGGCATTTATGGGTCTTCCT | CCAACTGGCTGTGTTGTGAG | 152 |
| Myod1 -10 kb | CACAGTGCCTGCACATAAGG | ACCAGAGGGTGTCATTCCTG | 157 |
| Myf5 A | CCAATGAAATCCTTGGTGTG | GGTCCTGCTATGGTGATGAA | 297 |
| Myf5 B | CCTCTCCAGGCTGCTAAATG | CTCTGGAAGCTGGGCACAC | 164 |
| Myf5 C | CCCTGCGTCTTTAGTTCCAC | ACTGGGAAGCTGCTGTCACT | 136 |
| Myf5 D | AATGTCTTGCTACCGTGCTG | GGTCCCTTTGACGCTAATGA | 157 |
| Myf5 -10 kb | TCCTTCTCCCACTCTTTCTGA | GACATGGCAACTGTGGAATG | 169 |
| Myog A | AGAAACCCAGAAGGGCAAAT | GAAGGCAATGTAGAGTAGTCTGTGA | 198 |
| Myog B | CTCTCTCCTCCATGGTCCAA | GGGTCTCATGGGACTGACAT | 160 |
| Myog C | TCCCCTTCCCTCTCCTTTT | CTTGGACCATGGAGGAGAGA | 146 |
| Myog D | AAGGCTTGTTCCTGCCACT | GAGAGGGAAGGGGAATCACA | 196 |
| Myog -10 kb | TCCAGACAGGGTCTGAGGAC | AGCCAGGGCTACACAGAGAA | 202 |

**Table 1: Chromatin immunoprecipitation primers.** Primers used in the chromatin immunoprecipitation experiment in Figure 4.5. The approximate location of the regions the primers amplify within the gene promoters are indicated in Figure 4.3.

| Mm *Myod1* cloning primers | Forward Primer | Reverse Primer |
|---|---|---|
| -2.7 Mm *Myod1* | **TTCTCGAG**ATGTCCCTCTTGTCCCTGTG | **TTACTAGT**TCGTCTGCTGTCTCAAAGGA |
| -1.6 Mm *Myod1* | **TTCTCGAG**CCATGGTGAATGCTGAATGA | **TTACTAGT**TCGTCTGCTGTCTCAAAGGA |
| Sequencing 1 | GGAGCCATTAAGAAGAATGGTG | |
| Sequencing 2 | GAGAGGGCTTTCCAGTTTGTAA | |

**Table 2. Cloning primers for the Mus musculus *Myod1* promoters.** Bold sequences indicate XhoI and SpeI sites added for cloning purposes. Two *Myod1* sequencing primers were also needed to sequence the entire promoter.

| *Myod1* minimal promoter construct | Forward & Reverse Primers |
|---|---|
| Conserved site mutagenesis | cgggcttttaggctacccggtagacagagcccagggcgcctggcg |
| TGGATAAAT to GGTAGACAG | cgccaggcgccctgggctctgtctaccgggtagcctaaaagcccg |
| Non-conserved site mutagenesis | gaaagttaagacgactctcacggatggtggttatgctggacccaggaactgggccat |
| CTTGGGTTGAG to ATGGTGGTTAT | atggcccagttcctgggtccagcataaccaccatccgtgagagtcgtcttaactttc |
| Promoter deletion | GATCCGAGCTCGGTACCAGGATAAATAGCCCAGGGCGC |
| deletes 83 bp | GCGCCCTGGGCTATTTATCCTGGTACCGAGCTCGGATC |
| 5' UTR deletion | CCCAGGACACGACTGCTTTCGAACTGGGCCATGGAAGACG |
| deletes 155 bp | CGTCTTCCATGGCCCAGTTCGAAAGCAGTCGTGTCCTGGG |
| Deletion A | GCGGCCGTTACTAGTGGATCCTTTCCAGCTCCCGG |
| deletes 51 bp | CCGGGAGCTGGAAAGGATCCACTAGTAACGGCCGC |
| Deletion B | ACACTCCTATTGGCTTGAGGGCTACCCTGGATAAATAGCCC |
| deletes 43 bp | GGGCTATTTATCCAGGGTAGCCCTCAAGCCAATAGGAGTGT |
| Mutagenesis C | tggatccgagctcggtaccaagcttagaaaaaaactactacactcctattggcttgaggcg |
| CTCCGCC to AAAAAAA | cgcctcaagccaataggagtgtagtagttttttctaagcttggtaccgagctcggatcca |
| Mutagenesis D | ctcggtaccaagcttagctccgccaaaaaaaaaacctattggcttgaggcgcccccgc |
| CTACTACACT to AAAAAAAAAA | gcgggggcgcctcaagccaataggttttttttttggcggagctaagcttggtaccgag |
| Mutagenesis E | gcttagctccgccctactacactaaaaaaaacttgaggcgcccccgcccccagc |
| CCTATTGG to AAAAAAAA | gctgggggcgggggcgcctcaagttttttttagtgtagtagggcggagctaagc |
| Mutagenesis F | ttagctccgccctactacactcctattggaaaaaaaagcccccgcccccagcctc |
| CTTGAGGC to AAAAAAAA | gaggctgggggcgggggctttttttttccaataggagtgtagtagggcggagctaa |
| Mutagenesis G | actacactcctattggcttgaggcaaaaaaaccccccagcctccctttccagctcc |
| GCCCCCG to AAAAAAA | ggagctggaaagggaggctgggggttttttgcctcaagccaataggagtgtagt |
| Mutagenesis H | cctattggcttgaggcgcccccgaaaaaaaactccctttccagctcccgggctt |
| CCCCCAGC to AAAAAAAA | aagcccgggagctggaaagggagttttttttcgggggcgcctcaagccaatagg |
| Mutagenesis I | gaggcgcccccgcccccagcaaaaaaatccagctcccgggcttttag |
| CTCCCTT to AAAAAAA | ctaaaagcccgggagctggatttttttgctgggggcgggggcgcctc |
| Mutagenesis J | gaggcgcccccgcccccagcctccctaaaaaaaaccgggcttttaggctac |
| TCCAGCTC to AAAAAAAA | gtagcctaaaagcccggttttttttaagggaggctgggggcgggggcgcctc |
| Mutagenesis K | cgcccccagcctccctttccagctcaaaaaaatttaggctaccctggataaatagcc |
| CCGGGCT to AAAAAAA | ggctatttatccagggtagcctaaatttttttgagctggaaagggaggctgggggcg |
| Mutagenesis L | cccagcctccctttccagctcccggaaaaaaaagctaccctggataaatagcccaggg |
| GCTTTTAG to AAAAAAAA | ccctgggctatttatccagggtagctttttttttccgggagctggaaagggaggctggg |

**Table 3. Primers for the identification of the PITX2 responsive region in the *Myod1* minimal promoter.**

# Chapter 5: Conclusions

Like most scientific endeavors, the research in this thesis has raised as many questions as it has answered. Prior to the initiation of my research, it was known that *Pitx2* was a critical gene in eye development that was expressed in two embryonic lineages, the neural crest and mesoderm (Gage et al., 2005). *Pitx2* knockout mice were described to have an eye phenotype that included hypercellular corneas, optic nerve dysplasia, and absence of the extraocular muscles (Gage et al., 1999; Kitamura et al., 1999; Lu et al., 1999). *Pitx2* was shown to be required in a dose-dependent manner for regulating extraocular muscle size and differentiation as well as the expression levels of muscle-related genes at e12.5, including the muscle regulatory factors (Diehl et al., 2006). The creation of lineage-specific knockouts of *Pitx2*, as described in this thesis, enabled the assignment of many aspects of the $Pitx2^{null/null}$ eye phenotype to a requirement for gene function in either the neural crest or mesoderm. It has also provided new insights into the underlying mechanisms of *Pitx2* functions, as well as the discovery of new functions in eye development.

The neural crest-specific knockout of *Pitx2* enabled the identification of new cell-autonomous and non-cell autonomous functions of *Pitx2*. We identified cell autonomous roles for *Pitx2* in sclera and ocular blood vessel formation. These defects are present in the global *Pitx2* knockout mice but were not previously recognized. The sclera is critical for eye shape and thus visual acuity, but scleral development is poorly understood (Dakubo et al., 2008; Sundin et al., 2005). The identification of *Pitx2* as a required developmental transcription factor is a critical finding for improving our understanding of the development of this important tissue. It remains to be determined what function *Pitx2* plays in scleral development; it could be required for cell proliferation, cell survival

and/or cell fate specification. Analysis of cell death and proliferation in the *Pitx2* mutant sclera can easily be achieved by staining for markers such as Ki67 and TUNEL, as well as a careful analysis of the expression of *Pitx2* mRNA. If the defect is in scleral cell fate specification, it will be important to determine if the function of *Pitx2* is to activate the expression of other transcription factors or if it is involved in directly regulating the deposition of extracellular matrix proteins that form the membranous sclera (Zhou et al., 2006). Recent work by others showing that *Pitx2* is indirectly downstream of *Indian hedgehog* signaling from the choroid vasculature may also help lead to new insights (Dakubo et al., 2008).

The cell-autonomous role of *Pitx2* in ocular blood vessel development seems to be confined to the neural crest, because the mesoderm-specific *Pitx2* knockout mice have apparently normal vasculature. Neural crest-derived pericytes have been shown to play critical roles in the formation of the ocular blood vessels and *Pitx2* may be important for enabling these cells to enhance proliferation of the vascular endothelial cells, but apparently these cells do not require *Pitx2* to respond to these signals (Klinghoffer et al., 2001; Uemura et al., 2002). The role of Pitx2 in pericyte function remains unknown, but it may include enabling the cells to receive angiogenic signals or to signal the endothelial cells through the release of factors like VEGF (Vidro et al., 2008).

The neural crest-specific knockout of *Pitx2* also provided further insight into the non-cell autonomous functions of *Pitx2*. We found that the "dysmorphic optic nerves" examined only at relatively early timepoints (e12.5) in the global *Pitx2* knockout mice, were actually optic nerves that failed to extend, causing the optic cups to be pulled to the center of the head, where they are directly attached to the hypothalamus (Gage et al., 1999; Kitamura et al., 1999). This results in a complete disruption of cornea development because the optic cup is separated from the surface ectoderm, a phenotype which is quite distinct from the "hypercellular corneas" that were originally reported (Gage et al., 1999; Kitamura et al., 1999; Lu et al., 1999). The *Pax2/Pax6* boundary between the optic nerve and RPE was also disrupted in these mice, which we hypothesized was due to the

displacement of the eyes closer to the source of *Pax2*-activating *Sonic hedgehog* (Macdonald et al., 1995).

Our laboratory has recently examined neural crest-specific *β-catenin* knockout mice, which help shed some light on the non-cell autonomous neural crest-specific functions of *Pitx2* (Brault et al., 2001). These mice have normal activation of *Pitx2* in the neural crest at e10.5 (Figure 5.1A, B), but they lose almost all neural crest expression of *Pitx2* between e11.5 and e12.5 (Figure 5.1C, D). These mice do not have displacement of the optic cup, but do have severe optic nerve defects (Figure 5.1F, G) and disruption of the RPE/optic nerve boundary (Figure 5.1E) similar to the *Pitx2-NCKO* eyes. While we cannot rule out the fact that these defects are caused by other disrupted functions of *β-catenin* in the neural crest, the similarities to the *Pitx2-NCKO* phenotype indicate that they are primarily caused by the loss of *Pitx2* expression. These findings have caused us to modify our hypotheses about *Pitx2* function in the neural crest.

First, this indicates that while *Pitx2* expression in the neural crest is required for signaling to the optic nerve, it has additional functions in anchoring the optic cup adjacent to the surface ectoderm between e9.5 and e11.5. The mechanism may be that *Pitx2* expression in the mesenchyme activates the expression of cell adhesion molecules that adhere to the lens and RPE to prevent the movement of the optic cup. Several important cell adhesion molecules are downregulated in $Pitx2^{null/null}$ eyes and we are currently examining them to determine if they mediate this process and are direct targets of *Pitx2*. The nature of the signaling from the mesenchyme to the optic nerve that is dependent on *Pitx2* also remains to be determined. *Bmp3* has been suggested as a candidate because it is expressed in the mesenchyme surrounding the developing optic stalk and BMP receptors are found in the developing chick optic stalk (Belecky-Adams and Adler, 2001; Dudley and Robertson, 1997). *Bmp3* mutant mice have no reported eye phenotype, but other BMPs are expressed in the ocular mesenchyme so they could be redundant (Belecky-Adams and Adler, 2001; Daluiski et al., 2001). Zebrafish Fgf3/8 morphants have a fused optic stalks, which bears some resemblance to the *Pitx2* NCKO centrally placed eyes (Walshe and Mason, 2003). These genes should be investigated as

possible *Pitx2* targets. Besides a candidate gene approach, qRT-PCR profiling of signaling molecule expression could be done on microdissected eye mesenchyme to identify the signaling molecules expressed there.

Second, this indicates that disruption of the *Pax2/Pax6*-dependant RPE/optic nerve boundary may not secondary to the displacement of the eyes, but in fact a primary defect. It is easy to imagine that genes activated by a distant *Sonic hedgehog* signal might require refinement of their expression domains on a local level. It appears that *Pitx2* expression in the adjacent mesenchyme may be necessary to refine the expression domain of *Pax2* to prevent it from being inappropriately expressed in the posterior RPE. This could be mediated by an extracellular modifier of hedgehog signaling, such as *Gas1*, which is downregulated in $Pitx2^{null/null}$ eye primordia based on microarray analysis (Philip Gage, personal communication)(Allen et al., 2007; Martinelli and Fan, 2007). *Gas1* is expressed in the periocular mesenchyme as well as the RPE, and *Gas1* mutant mice do indeed have RPE specification defects, but their ventral RPE is converted to neural retina (Lee et al., 2001). It could be that *Gas1* expression in the mesenchyme is required to refine the RPE/optic nerve boundary, but *Gas1* expression in the RPE itself is required to maintain the RPE fate. This hypothesis requires much further investigation. *Pitx2* mutant eyes should be examined for the expression of *hedgehog* target genes such as *Patched* and *Gli1* in the RPE and optic nerve to determine if *hedgehog* signaling is actually disrupted. It may be that other mesenchyme-derived signals are required to repress *Pax2* expression in the RPE. Next, it remains to be proven that *Gas1* is actually a direct target in the mesenchyme. Finally, a neural crest-specific knockout of *Gas1* would be required to separate the neural crest and RPE functions of *Gas1*.

While the neural crest knockout of *Pitx2* uncovered many functions of this important gene, it did not enable us to determine the role of *Pitx2* in cornea development. Because the optic cup moves through the periocular mesenchyme as it is shifted away from the surface ectoderm, the signals from the lens and optic cup are unable to properly specify a population of corneal endothelium and stromal cells (Figure 2.4)(Coulombre and Coulombre, 1964; Genis-Galvez, 1966; Matt et al., 2005; Matt et al., 2008; Molotkov

et al., 2006). This makes it impossible to determine which aspects of the corneal phenotype are primary defects caused by the absence of *Pitx2*, and which are secondary to the displacement of the optic cup. *Pitx2* is likely to be required for normal corneal development, because human patients with *PITX2* mutations have corneal defects (Asai-Coakwell et al., 2006; Xia et al., 2004). The temporal knockouts of *Pitx2* that we have described in Chapter 3 may be a way to study *Pitx2* function in the cornea without the complication of eye displacement. Some mice treated with tamoxifen to induce global *Pitx2* knockout at e10.5 do not have eye displacement and mice treated even later would probably be spared eye displacement. The corneas of these mice can be examined for formation of the corneal endothelium, corneal stroma compaction, and expression of cytokeratins in the corneal epithelium and *keratocan* and *AP-2β* in the corneal stroma (Liu et al., 1998; Moser et al., 1997; West-Mays et al., 1999; Zieske, 2004). If the later temporal knockouts survive long enough, the role of *Pitx2* in the formation of the trabecular meshwork and Schlemm's canal can be examined. The success of these experiments will greatly enhance our understanding of the role of *Pitx2* in anterior segment development.

The creation of mesoderm specific *Pitx2* knockout mice also uncovered new functions for this gene. The requirement for *Pitx2* in eyelid closure was a new finding. The mechanism underlying this defect is unclear. Most genes identified in eyelid closure are expressed in the surface ectoderm and involved in formation of the periderm, cell migration and the fusion event (reviewed in Martin and Parkhurst, 2004; Xia and Karin, 2004). *Fgf10* is the one of the few genes required for eyelid closure that is expressed in the mesenchyme, besides *Foxc1* and *Foxc2*, which were found to have normal expression patterns in mutant eyelids. *Fgf10* mutant mice have short eyelids and some rudimentary periderm formation, similar to the *Pitx2-mko* mice (Tao et al., 2005). *Fgf10* expression should be examined in *Pitx2* mutant eyelids and evaluated as a potential target of *Pitx2*. Alternately, *Pitx2* could regulate proliferation in the eyelid mesenchyme or other signaling molecules that activate gene expression in the surface ectoderm.

The non-cell autonomous requirement of *Pitx2* for retinal fissure closure was also a newly identified function for *Pitx2*. Since the initial observation was made, we have observed rare retinal colobomas in $Pitx2^{+/null}$ and $Pitx2^{neo/neo}$ embryos, indicating it can occur due to reduced *Pitx2* levels (data not shown). Human patients with *PITX2* mutations also have coloboma in some cases (Ozeki et al., 1999). *Pitx2* function in either the mesoderm adjacent to the outside of the optic fissure or the hyaloid vasculature on the inside of the fissure is required for signaling to initiate fissure closure. In either case, the process is dependant on *Pitx2*, which is one of the few mesenchymal genes involved in optic fissure closure (Gregory-Evans et al., 2004).

Although there are cells of mesoderm origin that contribute to the anterior segment of the eye, we did not find that *Pitx2* function is required in these cells for the development of anterior segment structures. These cells are hypothesized to be immune surveillance cells, but we were unable to assess their specification and differentiation. Little is known about the embryonic development of these immune cells and the *Pitx2-mko* mice do not survive past late gestation (Gage et al., 2005). Markers are available to label dendritic and Langerhans cells in the adult cornea, so the function of *Pitx2* in the mesodermal cells of the anterior segment could be better assessed with a late *Pitx2* temporal knockout model (Hamrah et al., 2003a; Hamrah et al., 2003b; Hamrah et al., 2002).

The mesoderm-specific knockout of *Pitx2* also showed that the requirement for *Pitx2* in the extraocular muscles was cell-autonomous. We showed that *Pitx2* was required for extraocular muscle precursor survival in a dose dependant manner, which explains the previously described dependence of extraocular muscle number and size on *Pitx2* dose (Diehl et al., 2006). The requirement for *Pitx2* in extraocular muscle survival extends beyond a single developmental stage, which suggests that Pitx2 is playing a more active role in cell survival than simply permitting precursor cells to continue past a single checkpoint. The window in which *Pitx2* is required for survival can be determined using the *Pitx2* temporal knockout mice. It would be interesting if *Pitx2* is required for survival even after the EOM precursors are specified as muscle that expresses *Myf5*, *MyoD*, and

*Myog*, because this would suggest that the requirement for *Pitx2* is not a developmental check to dispose of unspecified or incorrectly specified cells.

The mechanism by which *Pitx2* prevents EOM precursor apoptosis remains to be determined, although we have ruled out a role for *p53*. This is an important area for future studies. It will be important to investigate the role of *Pitx2* in proliferation of the EOM precursors and how this might relate to the apoptosis phenotype. We were unable to find any proliferation changes in $Pitx2^{null/null}$ EOM primordia, and proliferation was generally low in the mesoderm at the timepoints we examined, although this may be due to the comparison with the highly proliferative optic cup. Expression of CyclinD1 and CyclinD2, which are direct Pitx2 targets, remain to be examined (Kioussi et al., 2002). If the apoptosis is not due to alterations of the cell cycle, the loss of *Pitx2* may result in apoptosis through other mechanisms such as activation of caspase-2 and caspase-3, and the function of *Bax*, *Bak*, and *AIF*, which are important for interdigital apoptosis in the limbs, and activation of caspase-9 and Apaf-1, which are important in the neuronal apoptosis in the brain (reviewed in Mirkes, 2008). The expression and activation of these factors can be examined in $Pitx2^{null/null}$ EOM primordia at e9.5.

The early requirement for *Pitx2* in extraocular muscle precursor survival has made it difficult to address the function of *Pitx2* in later extraocular muscle development. The potential functions of *Pitx2* include specification of the cells as myoblasts by activation of the muscle regulatory factors (MRFs), specification of the unique properties of extraocular muscle, and/or specification and regulation of satellite cells. We have addressed the ability of *Pitx2* to directly regulate the MRFs with chromatin immunoprecipitation (ChIP) and luciferase reporter assays using cultured cell lines. We found that PITX2 binds specific regions in the promoters of each of the MRFs, *Myf5*, *Myod1*, and *Myog*, in both limb and extraocular muscle precursor cell lines. We showed that PITX2 is able to activate the *Myod1* promoter in a dose dependant manner, and it does so more robustly in a muscle cell line than an unrelated cell line. The specific PITX2-responsive site in the *Myod1* promoter remains to be identified, but all typical PITX2 binding sites have been ruled out. This indicates that PITX2 binds a non-canonical

site or has a co-factor in activating the *Myod1* promoter that alters its DNA binding site. Identification of the PITX2-responsive site may help identify this co-factor, if it resembles the binding site for a known transcriptional co-factor, such as MEF2 (Phan et al., 2005; Toro et al., 2004). Otherwise, this co-factor could be identified by using mass-spectroscopy to characterize co-precipitants of a PITX2 immunoprecipitation of C2C12 lysate, or by screening a C2C12 cDNA library with a PITX2 yeast two-hybrid assay. These experiments could also identify the muscle specific co-factor that elevates the PITX2-dependant response of *Myod1* in muscle cells. It would also be useful to determine the effect of PITX2 on the *Myf5* and *Myog* promoters to verify the ChIP findings, determine if they activated or repressed, and if they also use muscle-specific co-factors for activation.

Unfortunately, the lineage-specific knockouts of *Pitx2* did not provide much insight into the interactions between the neural crest and mesoderm lineages in extraocular muscle development. We did find that *Pitx2* expression in the neural crest is not required for the specification and differentiation of extraocular muscles, but the displacement of the eyes made it difficult to assess whether all of the muscles were present and of normal size. It was also difficult to determine *Pitx2* function in the formation of tendons from the neural crest. We did not examine the expression of *Scleraxis*, which marks developing tendons in the head, but if expression is altered, it would not be possible to determine if it was primary or secondary to the loss of *Pitx2* in the tendon precursors because the attachment points at the orbit and the sclera are disrupted (Grenier et al., 2009; Pryce et al., 2007). The similarity between the fibrous connective tissue of the sclera, which requires *Pitx2* function, and the tendons suggests that *Pitx2* expression in the neural crest may be important for tendon formation. The mesoderm specific knockout mice did not provide any information on how extraocular muscle precursors lacking *Pitx2* interact with wildtype neural crest, because the precursors die prior to the initiation of these interactions. The *Pitx2* temporal knockout mice may prove useful for studying the interactions between neural crest and mesoderm during extraocular muscle development, if a timepoint can be identified when *Pitx2* is not

required for EOM precursor survival. Otherwise, it may be necessary to identify and inhibit the cause of EOM precursor apoptosis in *Pitx2*$^{null/null}$ embryos.

It is also important to determine if *Pitx2* is required to specify the unique properties of extraocular muscles. The extraocular muscles have fiber types and gene expression patterns that are atypical from other skeletal muscles and these unique properties must be specified during development, although the timing and mechanisms are unknown (reviewed in Porter, 2002; Spencer and Porter, 2006). Because *Pitx2* is the only transcription factor required for extraocular muscle development identified to date, it seems like a promising candidate in some respects (Diehl et al., 2006; Gage et al., 1999; Kitamura et al., 1999). It is known to have critical functions in EOM development, and it could directly activate the expression of some of the unique proteins that are only expressed in the EOMs. However, *Pitx2* is also required for the development of the muscles of mastication from the first branchial arch. While these muscles share a few properties of the EOMs, such as high resistance to fatigue and an increased proportion of satellite cells, they are largely different, suggesting that *Pitx2* alone does not specify the unique properties of EOMs (Noden and Francis-West, 2006). *Pitx2* may still be involved in the specification of the unique properties of EOMs, which could be assessed in later temporal knockouts of *Pitx2*. It has already been shown that post-natal deletion of *Pitx2* does not result in fiber type changes in the short term, although it does result in decreases of some EOM-specific proteins. It may be that *Pitx2* is important for specifying the unique fiber types at an earlier timepoint in development, or it may be that other factors are involved. There may be as yet unidentified extraocular muscle-specific transcription factors or microRNAs that specify many of the unique properties. Alternately, it may be the expression of transcription factors *Pitx2*, *Tbx1*, and *Musculin*, and absence of *Tcf21*, a combination distinct from expression patterns in both the branchial arches and somitic muscles, specifies the unique properties of extraocular muscles (Grenier et al., 2009; Kelly et al., 2004; Lu et al., 2002).

A final aspect of *Pitx2* function in extraocular muscle cells that remains unknown is its function in satellite cells, the muscle stem cell population. Little is known about the

satellite cells in the muscles of the head, except that they exist in a greater proportion per fiber than in the somitic muscles (Karpati et al., 1988; Noden and Francis-West, 2006). It is unclear whether their specification or function is different than the satellite cells of the trunk. An easy first step in this area would be to examine the cranial muscles of *Pax7* mutant mice. *Pax7* knockout mice lose almost all of their satellite cells to apoptosis during development and the remaining satellite cells express *Pax3*, at least in the somite derived muscles (Relaix et al., 2006). The state of the satellite cell population in any *Pax7* mutant cranial muscles has not been described in the literature (Kuang et al., 2006; Oustanina et al., 2004; Relaix et al., 2006; Seale et al., 2000). These muscles would be expected to have more severe loss of satellite cells in the absence of compensatory *Pax3* expression, unless other factors participate in satellite cell specification in the head. *Pitx2* could be such a factor in satellite cell specification; it is expressed during cranial muscle development like *Pax3/Pax7* in the somites, and it is expressed in the satellite cells of adult extraocular muscles (Shih et al., 2007b; Zhou et al., 2009). A post-natal knockout of *Pitx2* in the extraocular muscles was recently described, but the presence and function of satellite cells was not examined (Zhou et al., 2009). It will be very important to determine if satellite cells can survive without *Pitx2* expression, and if they do, *Pitx2* mutant satellite cells may be extremely useful in determining the *in vivo* role of *Pitx2* in activating MRF expression. When a muscle is injured, satellite cells become activated from their quiescent state, proliferate, and initiate the expression of *Myf5*, *MyoD*, and *Myogenin* in a manner that roughly recapitulates development (Kuang et al., 2006; Relaix et al., 2006). If $Pitx2^{null/null}$ EOM-derived satellite cells could be generated with a temporal or tissue-specific knockout, they could be activated and assessed for their ability to initiate MRF expression. However, it is possible that *Pax7* is the primary activator of MRF expression in activated satellite cells in the cranial muscles, as it is in the somitic muscles. The formation and function of satellite cells in the extraocular muscles and other cranial muscles is an area that deserves much future study and the necessary genetic tools are readily available.

The critical functions of *Pitx2* in extraocular and branchiomeric muscle development raise interesting questions about what functions it might have in somitic

muscle development. *Pitx2* is expressed at some point in development in virtually all muscles, but its expression overlaps with its paralogs *Pitx1* in the limb muscles and *Pitx3* in the trunk muscle (L'Honore et al., 2007; Lanctot et al., 1997; Shang et al., 1997; Shih et al., 2007b). The expression of the *Pitx* genes is not seen in the somites until after myogenesis has already initiated, so they cannot be required for initial activation of the MRFs, although they might be responsible for MRF maintenance (L'Honore et al., 2007; Shih et al., 2007b). In the developing somites, *Pitx2* expression is downregulated in the later stages of myogenesis, while the expression of *Pitx3* is simultaneously upregulated. In *Pitx3* knockout mice, *Pitx2* fails to be downregulated, in an apparent compensation for the loss of *Pitx3* (L'Honore et al., 2007). The *Pitx* genes have similar homeodomains and DNA binding sites and activate some of the same targets in the pituitary (Charles et al., 2005; Quentien et al., 2002a). The overlapping expression patterns and compensatory ability of the *Pitx* genes suggest functional redundancy, but complicate the analysis of their functions during myogenesis. Double or triple knockouts of the *Pitx* genes are necessary to determine their functions during muscle development, but this has so far proved very challenging. *Pitx1;Pitx2* double knockout embryos were extremely difficult to generate; $Pitx1^{+/-}Pitx2^{+/-}$ mice display severely reduced viability, and only one $Pitx1^{-/-}$ $Pitx2^{-/-}$ embryo was ever found (Marcil et al., 2003). *Pitx2; Pitx3* double mutant mouse embryos have proved similarly difficult to generate, suggesting that the *Pitx* genes may have overlapping functions in early embryo viability (Jacques Drouin, personal communication). To overcome these difficulties, $UBC\text{-}CreER^{T2}+;\ Pitx2^{flox/flox};\ Pitx3^{flox/flox}$ embryos could be generated and treated with Tamoxifen prior to e9.75, when *Pitx2* expression is initiated in the somites. Since, *Pitx1* is only expressed in a few limb muscles, the functions of the *Pitx* genes in somitic muscle development could be uncovered with these mice (L'Honore et al., 2007; Lanctot et al., 1997; Shang et al., 1997). Identifying these functions in somitic muscle development could also provide new insights into the role of *Pitx* genes in extraocular and branchiomeric muscle development.

In conclusion, we have identified new cell autonomous and non-cell autonomous functions of *Pitx2* in eye development in both the neural crest and mesodermal lineages of the periocular mesenchyme. Many of the underlying mechanisms for these functions

remain to be identified, but these represent tractable problems. The ongoing requirement of *Pitx2* for cell survival in the extraocular muscles presents particular challenges for identifying its later functions there. While some creative genetic tricks may be able to overcome these challenges, the identification of the mechanism by which the absence of *Pitx2* leads to cell death and a method of inhibiting it would greatly aid the understanding of *Pitx2* functions in muscle development. The differences in the functions of *Pitx2* between the two lineages further underscores the multifunctional nature of this important transcription factor, and may ultimately enable the identification of cell type-specific *Pitx2* co-factors that modulate its function in the many cell types where it is expressed.

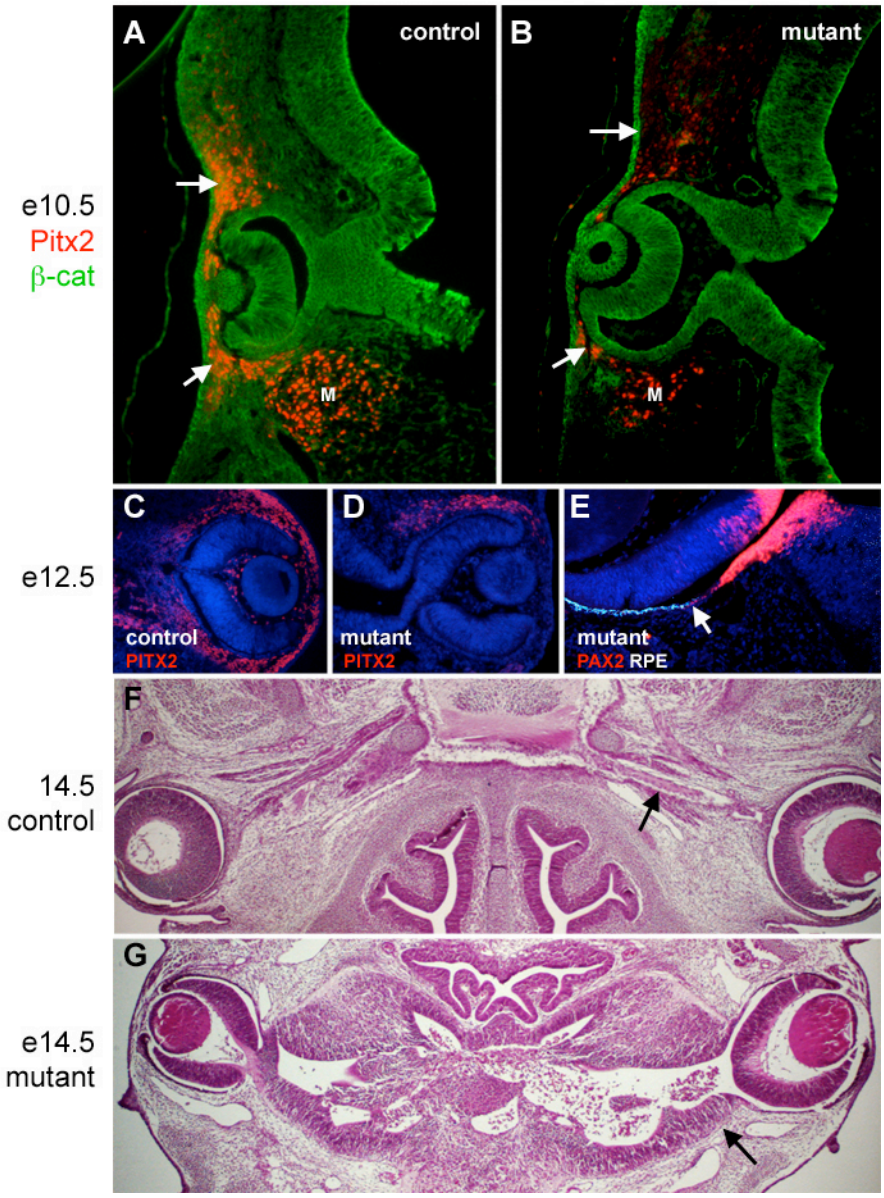


**Figure 5.1: β-catenin is required for PITX2 expression in the neural crest after e10.5.** β-catenin neural crest specific knockout control (**A**, **C**, **F**) and mutant (**B**, **D**, **E**, **G**) eyes. At e10.5, PITX2 is expressed in the neural crest in both the control and mutant embryos (**A**, **B**, arrows) even though the expression of β-catenin is lost. By e12.5, the expression of PITX2 in the neural crest surrounding the optic cup is severely reduced in the mutant, especially around the optic stalk (**C**, **D**). The mutant eyes also display expansion of PAX2 expression into the outer layer of the optic cup which forms the RPE, shown here in reverse contrast (**E**, arrow). By e14.5, the mutant optic nerves are severely dysmorphic and hyperblastic (**F**, **G**, arrows), but the eyes are not internally displaced. β-catenin expression is not affected in the mutant optic nerves (data not shown).

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