Int. J. Dev. Biol. 53: 69-79 (2009)
doi: 10.1387/ijdb.082682hn

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Transcriptional regulation by Pax3 and TGFβ2 signaling: a potential gene regulatory network in neural crest development

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ABSTRACT Pax3 regulates neural crest cell migration and is critical during neural crest development. TGFβs modify neural crest cell migration and differentiation. *TGFβ2* nullizygous embryos *(TGFβ2 $^{-/-}$Pax3$^{+/+}$)* display open neural tube and bifid spine, whereas in wild type embryos the neural tube is closed. In previous work, we have demonstrated that Pax3 regulates *TGFβ2* by directly binding to *cis*-regulatory elements on its promoter. In this study, we found that the *TGFβ2* nullizygous phenotype can be reversed to the wild type phenotype by down-regulating one allele of *Pax3,* as in *TGFβ2 $^{-/-}$Pax3 $^{+/-}$* embryos obtained through breeding *TGFβ2 $^{+/-}$Pax3 $^{+/-}$* mice. The data in this paper suggest that Pax3 and TGFβ2 interact in a coordinated gene regulatory network, linked by common downstream effector genes, to bring about this phenotypic reversal. Downstream effectors may include *Hes1, Ngn2* and *Sox9,* as well as other genes involved in neuronal differentiation.

KEY WORDS: *Hes1, Ngn2, Sox9, epithelial-to-mesenchymal transformation*

Introduction

In 1954, Auerbach observed that developmental defects in homozygous *Splotch* embryos are associated with neural crest cell deficiency. In the homozygous condition the *Splotch* (*Sp*) mutation is lethal by embryonic day 13.5. These embryos display defects in neural tissue, neural crest derivatives, and limb musculature. *Splotch* heterozygotes exhibit defects in neural crest derivatives, which result in white patches on their bellies caused by defective development of neural crest derived melanocytes (Foy *et al.,* 1990). Chromosome localization in conjunction with the spatio-temporal tissue distribution of *Pax3* transcripts and tissues affected in *Sp* mutants led to direct experimental analysis demonstrating that *Splotch* and *Pax3* are the same gene (Epstein *et al.,* 1991; Goulding *et al.,* 1993) and the homozygous *Splotch* condition is *Pax3*$^{-/-}$. These studies show that Pax3 has a regulatory role in neural crest development.

Other studies also indicate that Pax3 has a role in regulating neurogenesis in neural crest derived precursor cells. Goulding *et al.* (1991) demonstrated *Pax3* expression in a population of migrating neural crest cells (NCCs) that contribute to the dorsal root ganglia and cephalic mesenchyme. In a separate study Li *et al.* (1999) found that *Pax3* replacement rescued neural tube closure and other neural crest related anomalies in *Sp* mutants. This group produced transgenic mice that over expressed *Pax3* in neural tube and neural crest. They then took *Pax3* overexpressing transgenic lines and crossed them with heterozygous *Splotch* mice. Transgenic heterozygous *Splotch* mice were identified, backcrossed to *Splotch* heterozygotes and examined at E13.5. All transgenic *Splotch* homozygous embryos had normal closed neural tubes in comparison to non-transgenic *Splotch* homozygous embryos, all of which displayed neural tube defects in the lumbrosacral region and in some cases exencephaly. More

Abbreviations used in this paper: NCC, neural crest cell; ngn, neurogenin.

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Accepted: 5 June 2008. *Published online:* 5 December 2008. *Edited by:* Angie Rizzino.

ISSN: Online 1696-3547, Print 0214-6282

recently, our lab (Nakazaki *et al.*, 2008) investigated the mechanism by which Pax3 regulates neurogenesis in neural crest derived precursor cells. We found that Pax3 regulates two bHLH transcription factor genes, *Hairy and enhancer of split homolog-1 (Hes1)* and *Neurogenin2* (*Ngn2*). *Hes1* and other *Hes* genes prevent premature neurogenesis (Hirata *et al.*, 2001) and *Ngn2* plays a critical role in sensory neurogenesis (Lo *et al.*, 2002).

TGFβs are essential for normal neural crest development (Nie *et al.*, 2008). Loss of TGFβ signaling causes decreased chondrocyte proliferation and premature differentiation of cartilage to bone (Hosokawa *et al.,* 2007). Expression of *Msx2*, a critical factor in the formation of the dorso-ventral axis, is diminished in *Tgfbr2* mutants (Hosokawa *et al.*, 2007). The TGFβ intracellular effector Smad3 regulates neuronal differentiation and cell fate specification in the developing spinal cord. Additionally, Smad3 –mediated TGFβ activity promotes neurogenesis, cell-cycle exit and lateral migration of neuroepithelial progenitor cells (García-Campmany and Martí, 2007). Nie *et al.* (2008) investigated the role of Smad4, a TGFβ/BMP signaling intermediate, in the development of NCCs. A Cre/loxP system was used to specifically disrupt Smad4 in NCCs. Expression of multiple genes, including *Msx1, 2*, *Ap-2α*, *Pax3*, and *Sox9*, which play critical roles in NCC development, was down regulated by Smad4 disruption. Mutant mice died at mid-gestation due to severe molecular defects.

TGFβ2-null embryos show open caudal neural tube with unfused spine (Sanford *et al.,* 1997), similar to *Pax3*$^{-/-}$ embryos. Work from our lab established a link between Pax3 and TGFβ2 (Mayanil *et al.,* 2006). Pax3 regulates the *TGFβ2* promoter by binding to its *cis*-regulatory elements, and *TGFβ2* expression is diminished in *Pax3*$^{-/-}$ embryos. Thus a regulatory relationship exists between Pax3 and TGFβ2 (Mayanil *et al.,* 2006).

In the present study we further examined the relationship between Pax3 and TGFβ2 in neural crest development. We found that the open neural tube and bifid spine observed in *TGFβ*$^{-/-}$ *Pax3*$^{+/+}$ embryos was significantly reversed by down-regulating one allele of *Pax3* as in *TGFβ2*$^{-/-}$*Pax3*$^{+/-}$ embryos. This reversal may be due to Pax3 and TGFβ2 working together in a regulated fashion to affect the actions of downstream target genes important in neural crest development.

Results

Incidence of neural tube defects in embryos bred from TGFβ2$^{+/-}$*Pax3*$^{+/-}$ *double heterozygous mice: phenotypic reversal in TGFβ2 null embryos by Pax3 down-regulation*

In order to investigate how Pax3 and TGFβ interact in neural crest development we first bred *TGFβ2*$^{+/-}$*Pax3*$^{+/-}$ double heterozygous mice and examined the phenotypes of resulting offspring. Table 1 compares Mendelian projections for breeding *TGFβ2*$^{+/-}$ *Pax3*$^{+/-}$ double heterozygotes, with live births at post natal day 0 (P0). Live births were seen in all genotypes where there was at least one copy of *TGFβ2* and *Pax3*. The table also shows the observed frequency of the open neural tube phenotype in E10.0 embryos bred from *TGFβ2*$^{+/-}$*Pax3*$^{+/-}$ double heterozygotes. Approximately 4% (10/254 embryos) of the wild type *TGFβ2*$^{+/+}$*Pax3*$^{+/+}$ embryos showed neural tube defects (NTDs), which was not statistically significant. No NTDs were observed in *TGFβ2*$^{+/-}$ *Pax3*$^{+/+}$ embryos. Open NTDs were seen in 85% (24 out of 28) of the *TGFβ2*$^{/-}$*Pax3*$^{+/+}$ embryos, however only 36% (9 out of 25) of the *TGFβ2*$^{/-}$*Pax3*$^{+/-}$ embryos exhibited open NTDs. The difference seen between the latter two groups is statistically significant (p=0.0002) as analyzed by Fisher's Exact test (Prism v.4.0 software). The closed neural tube phenotype in *TGFβ2*$^{-/-}$*Pax3*$^{+/-}$ embryos can be seen in Fig. 1. E10.0 *TGFβ2*$^{/-}$*Pax3*$^{+/+}$ and *TGFβ2*$^{/-}$*Pax3*$^{+/-}$ embryos did not exhibit additional external abnormalities. However Sanford *et al.,*1997) have shown that *TGFβ2* null mice exhibit perinatal mortality and a wide range of developmental defects. These include cardiac, lung, craniofacial, limb, spinal column, eye, inner ear and urogenital defects. The developmental processes commonly involved in the affected tissues include epithelial-mesenchymal interactions, cell growth, extracellular matrix production and tissue remodeling. In addition, many affected tissues have neural crest-derived components and simulate neural crest deficiencies (Sanford *et al.*, 1997).

In the type of study done here there is a possibility of a partial rescue of the *TGFβ2* null phenotype due to a mixed genetic

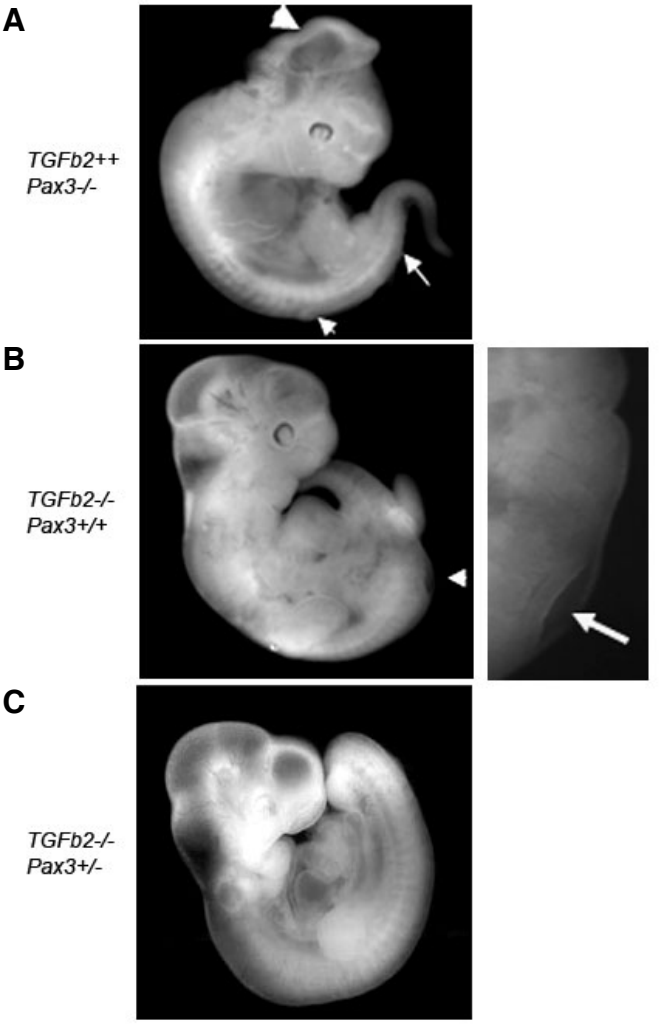


Fig. 1. Phenotypic recovery in *TGFβ2* nullizygous embryos by *Pax3* down-regulation. (A) *TGFβ2*$^{+/+}$*Pax3*$^{-/-}$ *embryo with open cranial and caudal neural tube (arrows).* **(B)** *TGFβ2*$^{-/-}$*Pax3*$^{+/+}$ *embryo with open caudal neural tube (arrow). Enlarged version of the open neural tube in (B) on the right.* **(C)** *TGFβ2*$^{-/-}$*Pax3*$^{+/-}$ *embryo with no neural tube defect; the phenotype here is comparable to wild type (TGFβ*$^{+/+}$*Pax3*$^{+/+}$*).*

background. To rule out possible contributions of background effects on the partial rescue of the *TGFβ2* null phenotype, we took *TGFβ2*$^{+/-}$ *Pax3*$^{+/-}$ double heterozygous females from the F1 generation of the original cross and back-crossed these with black Swiss3T3 males for 6 generations. The results demonstrated that even in a mixed background, *TGFβ2*$^{-/-}$ *Pax3*$^{+/+}$ embryos showed open neural tube phenotype which was reversed in *TGFβ2*$^{-/-}$ *Pax3*$^{+/-}$ embryos. These results show that: (a) *Pax3* down regulation decreases the incidence of open neural tube in *TGFβ2* null embryos; (b) Down-regulation of *Pax3* in *TGFβ2* null embryos does not totally rescue these embryos, as no live *TGFβ2*$^{-/-}$ *Pax3*$^{+/-}$ births were observed. The lack of live births is not surprising as *TGFβ2* null genotype produces a wide range of developmental defects, in addition to open caudal neural tube with unfused spine (Sanford *et al.*, 1997); (c) Down-regulating *TGFβ2* does not the rescue the open neural tube phenotype in *Pax3*$^{-/-}$ embryos.

Hes1, Ngn2 *and* Sox9 *expression levels* in vivo

Based on the above observations and earlier work from our laboratory showing Pax3 regulation of T *GFβ2* in migrating NCCs (Mayanil *et al.*, 2006) we surmised that Pax3 and TGFβ2 regulated common effecter genes involved in neural crest development. The significant reversal from the open neural tube phenotype seen in *TGFβ2*$^{-/-}$ *Pax3*$^{+/+}$ embryos to closed neural tubes seen in *TGFβ2*$^{-/-}$ *Pax3*$^{+/-}$ embryos suggested a possible interplay between Pax3 and TGFβ2, where these two may act in a reciprocal fashion to maintain levels of some common downstream genes in proper balance.

To begin elucidating the relationship between Pax3 and TGFβ2 we investigated *Hes1*, *Ngn2* and *Sox9* expression levels in embryos with the following 6 genotypes: *TGFβ2*$^{+/+}$ *Pax3*$^{+/+}$, *TGFβ2*$^{-/-}$ *Pax3*$^{+/+}$, *TGFβ2*$^{+/+}$ *Pax3*$^{-/-}$, *TGFβ2*$^{+/+}$ *Pax3*$^{+/-}$, *TGFβ2*$^{+/-}$ *Pax3*$^{+/-}$ and *TGFβ2*$^{-/-}$Pax3$^{+/-}$. We choose *Hes1* and *Ngn2* because these molecules are critical in neurogenesis and Pax3 regulates *Hes1* and *Ngn2* by directly binding to *cis*-regulatory elements on their promoters (Nakazaki *et al.*, 2008). Moreover, *Hes1* is down-regulated by TGFβ (Zavadil *et al.*, 2001; Blokzijl *et al.*, 2003) and its expression oscillates with 2 hour periodicity during development (Hirata *et al.*, 2002). *Ngn2* expression is coincident with *Pax3* during neural tube closure and *Ngn2* knockout embryos display neural tube defects (Fode *et al.*, 1998). Additionally, down-regulation of *Hes1* expression, which induces neural progenitor differentiation, leads to sustained up-regulation of *Ngn2* (Shimojo *et al.*, 2008). *Sox9* was chosen because it regulates neural crest development (Cheung and Briscoe, 2003), and it is up-regulated by TGFβ (Zavadil *et al.*, 2001) and down-regulated by Pax3 over-expression (Mayanil *et al.*, 2001).

Whole mount *in situ* hybridization was done on E10.0 (30 somite stage) embryos, using digoxigenin labeled *Hes1* (Fig. 2A), *Ngn2* (Fig. 2B) and *Sox9* riboprobes (Fig. 2C). W*ild type* (*TGFβ2*$^{+/+}$ *Pax3*$^{+/+}$) embryos expressed *Hes1* in dorsal neural tube (red arrow) and in migrating neural crest cells (yellow arrows) (Fig. 2Ai). Semi-quantification (Fig. 3) of our *in situ* hybridization results with densitometry (total density 8 bit gray) showed a slight but insignificant increase in *Hes1* levels in *TGFβ2*$^{-/-}$ *Pax3*$^{+/+}$ embryos in both the dorsal neural tube and migrating neural crest cells (Fig. 2A-ii) as compared with *wild type* embryos (Fig. 2A-i). *Hes1* expression was down-regulated in both expression domains in *TGFβ2*$^{+/+}$ *Pax3*$^{-/-}$ (* p<0.001) (Fig. 2A-iii) and in *TGFβ2*$^{++}$ *Pax3*$^{+/-}$ (** p<0.0005) embryos (Fig. 2A-iv). Expression was close to *wild-type* in *TGFβ2*$^{+/-}$ *Pax3*$^{+/-}$ (Fig. 2A-v), and *TGFβ2*$^{-/-}$ *Pax3*$^{+/-}$ embryos (Fig. 2A-vi). These studies suggest that both Pax3 and TGFβ2 play a role in regulating *Hes1* expression in the dorsal neural tube and migrating NCCs during embryonic development.

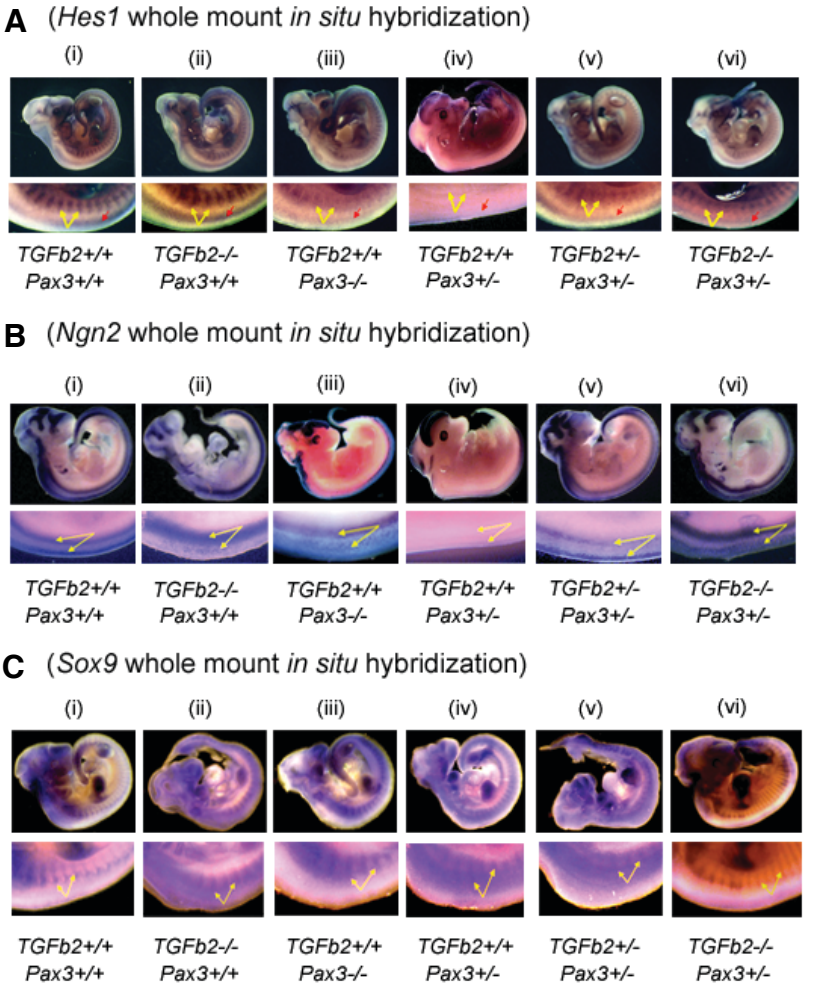


Fig. 2. Whole mount *in situ* hybridization. *E10.0 (30 somite) embryos with the following genotypes: (i) TGFβ2*$^{+/+}$*Pax3*$^{+/+}$*, (ii) TGFβ2*$^{-/-}$*Pax3*$^{+/+}$*, (iii)TGFβ2*$^{+/+}$*Pax3*$^{-/-}$*, (iv) TGFβ2*$^{+/+}$*Pax3*$^{+/-}$*, (v) TGFβ2*$^{+/-}$*Pax3*$^{+/-}$ *and (vi) TGFβ2*$^{-/-}$*Pax3*$^{+/-}$*, were used for* in situ *hybridization with digoxigenin-labeled murine Hes1, Ngn2 and Sox9 riboprobes.* **(A)** Hes1 *mRNA expression in embryonic NCCs. Yellow and red arrows represent* Hes1 *positive staining in migrating NCCs and neural tube respectively.* **(B)** Ngn2 *mRNA expression showing dorsal and ventral neural tube staining of* Ngn2 *transcripts. Yellow arrows point to dorsal and ventral neural tube regions.* **(C)** Sox9 *mRNA expression in embryonic NCCs. Yellow arrows indicate* Sox9 *positive migrating NCCs.* In situ *hybridization experiments for each genotype were done in quadruplicate, representative data is shown here.* In situ *hybridization using digoxigenin-labeled sense* Hes1, Ngn2 *and* Sox9 *riboprobes did not show staining (data not shown).*

TABLE 1

PHENOTYPIC RECOVERY OF *TGFβ2* NULLIZYGOUS EMBRYOS BY DOWN-REGULATING ONE ALLELE OF *PAX3*

Genotypes	*TGF*β*2+/+ Pax3+/+*	*TGF*β*2+/- Pax3+/+*	*TGF*β*2-/- Pax3+/+*	*TGF*β*2+/+ Pax3+/-*	*TGF*β*2+/- Pax3+/-*	*TGF*β*2-/- Pax3+/-*	*TGF*β*2+/+ Pax3-/-*	*TGF*β*2+/- Pax3-/-*	*TGF*β*2-/- Pax3-/-*
Mendelian Projection	6.25%	12.50%	6.25%	12.50%	25%	12.50%	6.25%	12.50%	6.25%
Number of Live BIRTHS	235 live births	110 live births	No live births	88 live births	125 live births	No live births	No live births	No live births	No live births
Frequency of open neural tube in E10.0 embryos	10 out of 245 embryos had NTD (4%)	4 out of 190 embryos had NTD (2%)	24 out of 28 embryos had NTD (85%)	6 out of 188 embryos had NTD (3%)	10 out of 55 embryos had NTD (18%)	9 out of 25 embryos had NTD (36%) (p=0.0002)	28 out of 28 embryos had NTD (100%)	32 out of 32 embryos had NTD (100%)	5 out of 5 embryos were resorped (100%)
			p=0.0002						

This table compares Mendelian projections and live births in populations bred from double heterozygous *TGFβ2*$^{+/-}$*Pax3*$^{+/-}$ mice. Live births were seen for the following genotypes *TGFβ*$^{+/+}$*Pax3*$^{+/+}$, *TGFβ*$^{+/-}$*Pax3*$^{+/+}$, *TGFβ*$^{+/+}$*Pax3*$^{+/-}$, *TGFβ*$^{+/-}$*Pax3*$^{+/-}$. There were no live births for *TGFβ*$^{-/-}$*Pax3*$^{+/+}$, *TGFβ*$^{-/-}$*Pax3*$^{+/-}$, *TGFβ*$^{+/+}$*Pax3*$^{-/-}$, *TGFβ*$^{+/-}$*Pax3*$^{-/-}$, and *TGFβ*$^{-/-}$*Pax3*$^{-/-}$ due to major developmental problems. The table also depicts the frequency of neural tube defects observed at E10.0 (30 somite stage). Neural tube defects were not present in *TGFβ*$^{+/-}$*Pax3*$^{+/+}$ and *TGFβ*$^{+/+}$*Pax3*$^{+/-}$ embryos. The number of *TGFβ*$^{+/+}$*Pax3*$^{+/+}$ embryos with neural tube defects was not significant. Two different populations of mice were used to obtain these results, one in which the embryos went full term and one in which they were removed from the dam at E 10.0. The data for E10.0 was analyzed with Fisher's Exact test (Prism v. 4.0 software), the difference in neural tube defect, 85% in *TGFβ2*$^{-/-}$*Pax3*$^{+/+}$ and 36% in *TGFβ2*$^{-/-}$*Pax3*$^{+/-}$ is statistically significant (p=0.0002).

In situ hybridization (Fig. 2B) using digoxigenin labeled *Ngn2* riboprobe in *TGFβ2*$^{+/+}$*Pax3*$^{+/+}$ embryos (Fig. 2B-i) showed dorsal and ventral expression patterns consistent with earlier reports that"*Ngn2* expression in dorsal and ventral spinal neural tube progenitor cells is regulated by distinct enhancers (Simmons *et al.*, 2001). Semi-quantification (Fig. 3) of the *in situ* hybridization results with densitometry (total density 8 bit gray) showed a significant decrease (§p<0.0001) in *Ngn2*expression in the dorsal neural tube, but a significant increase in *Ngn2* expression (§§p<0.002) in the ventral neural tube of *TGFβ2*$^{-/-}$*Pax3*$^{+/+}$ embryos (Fig. 2B-ii) compared to wild type. *TGFβ2*$^{+/+}$*Pax3*$^{-/-}$ embryos showed reduced *Ngn2* expression (Fig. 2B-iii) in the dorsal region. *Ngn2*expression was significantly reduced (§p<0.0001) in *TGFβ2*$^{+/+}$*Pax3*$^{+/-}$ embryos as compared to *wild type* in both expression domains (Fig. 2B-iv). In double heterozygous embryos (*TGFβ2*$^{+/-}$*Pax3*$^{+/-}$) *Ngn2* expression was reduced (§§§p<0.005) in the dorsal region (Fig. 2B-v) as compared with *wild type* embryos. In *TGFβ2*$^{-/-}$*Pax3*$^{+/-}$ embryos (Fig. 2B-vi) *Ngn2* levels in the ventral region were similar to those seen in *TGFβ2*$^{-/-}$*Pax3*$^{+/+}$ embryos, the dorsal region however showed a tendency to recover toward levels seen in w*ild type*. These observations indicate that Pax3 and TGFβ2 both play a role in regulating *Ngn2* expression.

When digoxigenin labeled *Sox9* riboprobe (Fig. 2C) was used for whole mount *in situ* hybridization, *Sox9* positive staining was seen in migrating NCCs in *TGFβ2*$^{+/+}$*Pax3*$^{+/+}$ embryos (Fig. 2C-i). Semi-quantification (Fig. 3) of the *in situ* hybridization results with densitometry (total density 8 bit gray) showed a significant decrease ($^{¥}$ p<0.001) in *Sox9* expression in *TGFβ2*$^{-/-}$*Pax3*$^{+/+}$ (Fig. 2C-ii) embryos as compared to *wild type* controls. *TGFβ2*$^{+/+}$*Pax3*$^{-/-}$ embryos showed an increase ($^{¥¥}$ p<0.005) in *Sox9* expression (Fig. 2C-iii) compared with *wild type*. *TGFβ2*$^{+/+}$*Pax3*$^{+/-}$ embryos showed a small insignificant increase in *Sox9* expression (Fig. 2C-iv). *Sox9* expression was significantly decreased in *TGFβ2*$^{+/-}$*Pax3*$^{+/-}$ embryos ($^{¥¥¥}$ p<0.05) (2C-v). Staining in *TGFβ2*$^{-/-}$*Pax3*$^{+/-}$ embryos (Fig. 2C-vi) was similar to the pattern seen in *wild-type* (*TGFβ2*$^{+/+}$*Pax3*$^{+/+}$). These data suggest that *Sox9* expression is

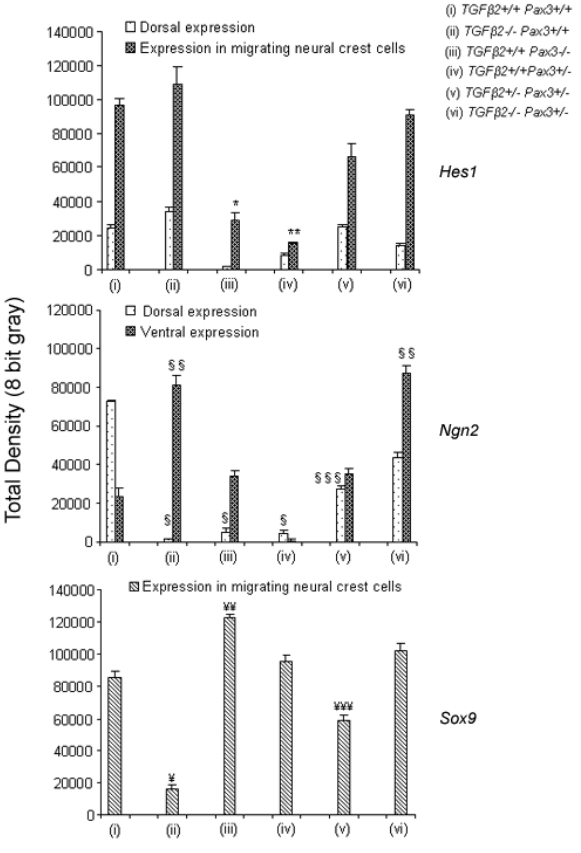


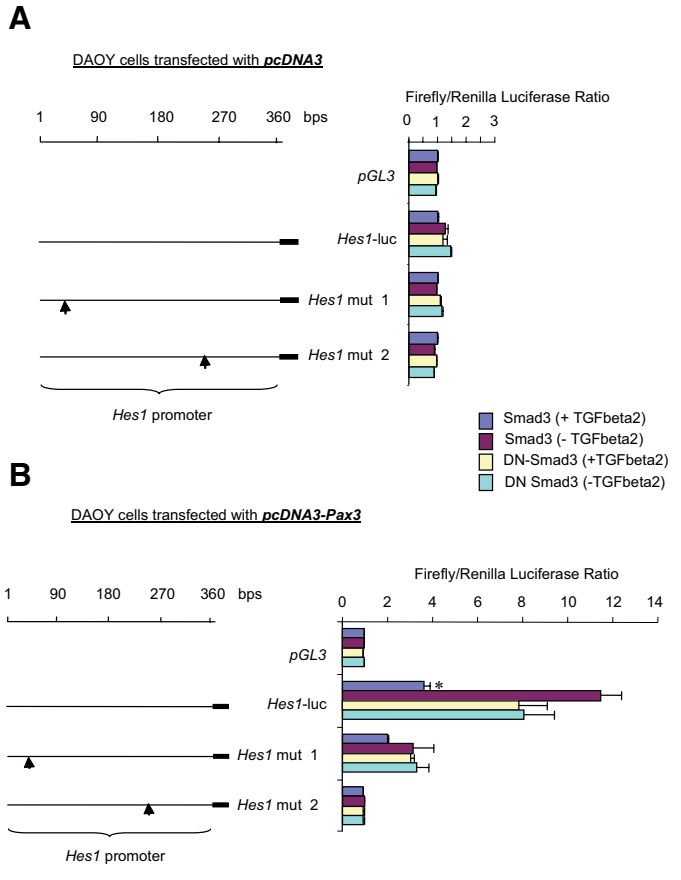
Fig. 3. Semi-quantification of digoxigenin-labeled *Hes1, Ngn2* and *Sox9* riboprobe staining using densitometry scanning (Open Lab program -Leica). *Total density was determined from images scanned in 256 gray scale. The data is represented as the total density (8 bit gray), which is defined as the area multiplied by the mean of the relative densitometry units in the embryonic region under investigation. For* Hes1, *the data is represented as total density (8 bit gray) of* Hes1 *positive staining in dorsal neural tube and in migrating NCCs; (* p<0.001; ** p<0.0005, Student T test). For* Ngn2, *the data is represented as total density (8 bit gray) of the dorsal and ventral* Ngn2 *positive staining; (§ p<0.0001;§§ p<0.002, §§§ p<0.005, Student T test). For* Sox9, *the data is represented as total density (8 bit gray) of the* Sox9 *positive staining in migrating NCCs; ($^{¥}$p<0.001;$^{¥¥}$p<0.005;$^{¥¥¥}$p<0.05, Student T test). The values represent the average ± SEM of 4 embryos for each genotype.*

regulated by Pax3 and TGFβ2 during embryonic development.

TGFβ2 effect on Pax3 regulation of Hes1, Ngn2 and Sox9 promoter activity

The TGFβ2 intracellular effector phospho-Smad3 regulates neuronal differentiation and cell fate specification in the developing spinal cord (García-Campmany and Martí, 2007). To further examine the relationship between Pax3 and TGFβ2 we co-transfected DAOY cells with *pcDNA3* or *pcDNA3-Pax3* expression constructs, *Hes1-luciferase* (Fig. 4 A,B), *Ngn2-luciferase* (Fig. 5 A,B) or *Sox9-luciferase* (Fig. 6 A,B) promoter constructs, and *Flag-Smad3* or *Flag-DN-Smad3* (dominant negative Smad3) expression vectors. Transfected cells were then untreated or treated with 10ng/ml human recombinant TGFβ2 (10 ng/ml) for 6 hours. Luciferase activity was measured 48 hours after transfection.

Hes1-promoter-luciferase and mutant constructs are fully described in our previous paper (Nakazaki *et al.*, 2008). When *pGL3* control vector, *Hes-1*-luc, *Hes1* mut #1 or *Hes1* mut #2 constructs were co-transfected with *pcDNA3* in DAOY cells, neither Smad3 nor TGFβ2 affected promoter activity (Fig. 4A). When *Hes1*-luc was co-transfected with *pcDNA3-Pax3*, with DN-Smad3 minus TGFβ2, there was a 7-8 fold increase in *Hes1* promoter activity in comparison to *pcDNA3* transfection (Fig. 4B). Promoter activity did not increase significantly when *Hes1* mut1 or *Hes1* mut2 were co-transfected with *pcDNA3-Pax3*. These results agree with our previous work (Nakazaki *et al.*, 2008). Pax3 mediated *Hes1* promoter activity (DN-Smad3 minus TGFβ2) decreased significantly ($p<0.05$) in the presence of Smad3 and TGFβ2. Smad3 alone or TGFβ2 and DN-Smad3 did not decrease the Pax3



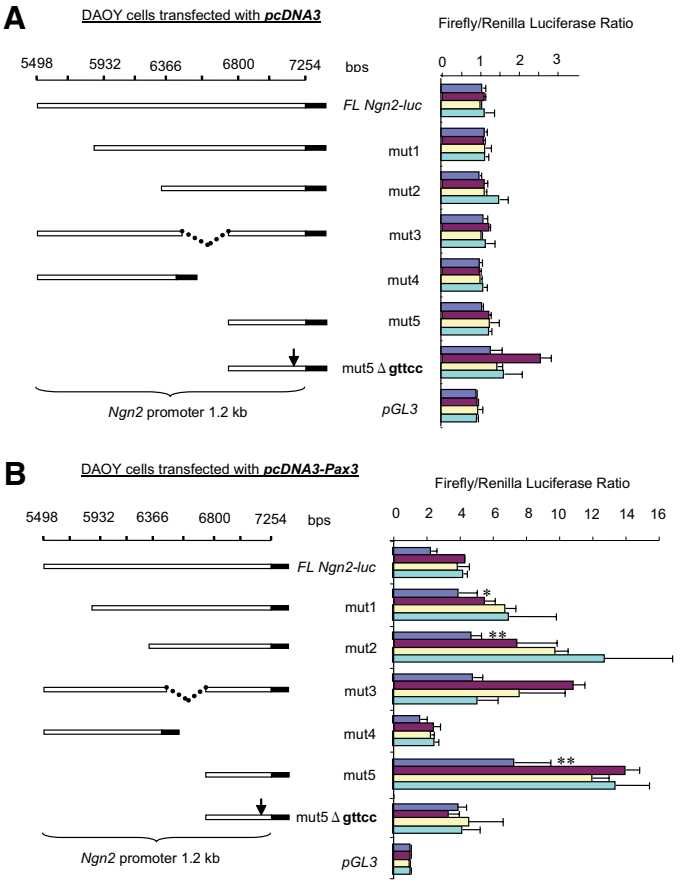


Fig. 4 (Left). Effect of Pax3 and TGFβ2 on *Hes1*-promoter luciferase activity. *DAOY cells were co-transfected with* **(A)** pcDNA3 *or* **(B)** pcDNA3-Pax3 *construct, Hes1-promoter-luciferase or mutant constructs and wild type Flag-Smad3 or dominant negative Flag DN-Smad3. pGL3 served as a control vector. Transfected cells were treated or not treated with TGFβ, and luciferase activity was measured. Renilla luciferase (pRL-null) was used as a transfection control and luciferase activities are expressed as a ratio of Firefly/Renilla luciferase. Each experiment was done in quadruplicate, with each data point in triplicate. Promoter activity in the presence of Smad3 plusTGFβ2 was compared with activity in the presence of DN-Smad3 minus TGFβ2, significance was determined with the Student T test (*p<0.05).*

Fig. 5 (Right). Effect of Pax3 and TGFβ2 on *Ngn2*-promoter luciferase activity. *DAOY cells were co-transfected with* **(A)** pcDNA3 *or* **(B)** pcDNA3-Pax3 *construct, full length (FL) Ngn2-luciferase or truncated mutant constructs and wild type Flag-Smad3 or dominant negative Flag DN-Smad3. pGL3 served as a control vector. Transfected cells were treated or not treated with TGFβ2 and luciferase activity was measured. Renilla luciferase (pRL-null) was used as a transfection control and luciferase activities are expressed as a ratio of Firefly/Renilla luciferase. Each experiment was done 4 times and each data point was in triplicate. Promoter activity in the presence of Smad3 plusTGFβ2 was compared with activity in the presence of DN-Smad3 minus TGFβ2, significance was determined with the Student T test (*p<0.05; **p<0.001). Key, same as for Fig. 4.*

mediated increase in *Hes1* promoter activity. These observations suggest that *Hes1* promoter activity is itself not affected by TGFβ2, but TGFβ2 decreases Pax3 induced *Hes1* activity.

Ngn2-promoter-luciferase and mutant constructs are fully described in our previous paper (Nakazaki *et al.*, 2008). When *pGL3* control vector, *FLNgn2*-luc (1.2 kb), mut1, mut2, mut4, mut5, mut5 Δ gttcc constructs were co-transfected with *pcDNA3* in DAOY cells, neither Smad3 nor TGFβ2 affected promoter activity (Fig. 5A). When *FLNgn2*-luc was co-transfected with *pcDNA3-Pax3*, with DN-Smad3 minus TGFβ2, there was a 2 fold increase in promoter activity (Fig. 5B). Pax3 mediated promoter activity was also observed with mut1 (5 fold increase), mut2 (11-12 fold increase) and mut5 (12-14 fold increase) in the presence of DN-Smad3 minus TGFβ2. These results agree with our previous work (Nakazaki *et al.*, 2008). In the presence of Smad3 plus TGFβ2, Pax3 mediated promoter activity was decreased in *FLNgn2*-luc, mut1 (*p<0.05), mut2 (**p<0.001) and mut5 (**p<0.001). Smad3 alone or TGFβ2 with DN-Smad3 did not decrease Pax3 mediated *Ngn2* promoter activity. These observations suggest that *Ngn2* promoter activity is itself not affected by TGFβ2, but TGFβ2 decreases Pax3 induced *Ngn2* activity.

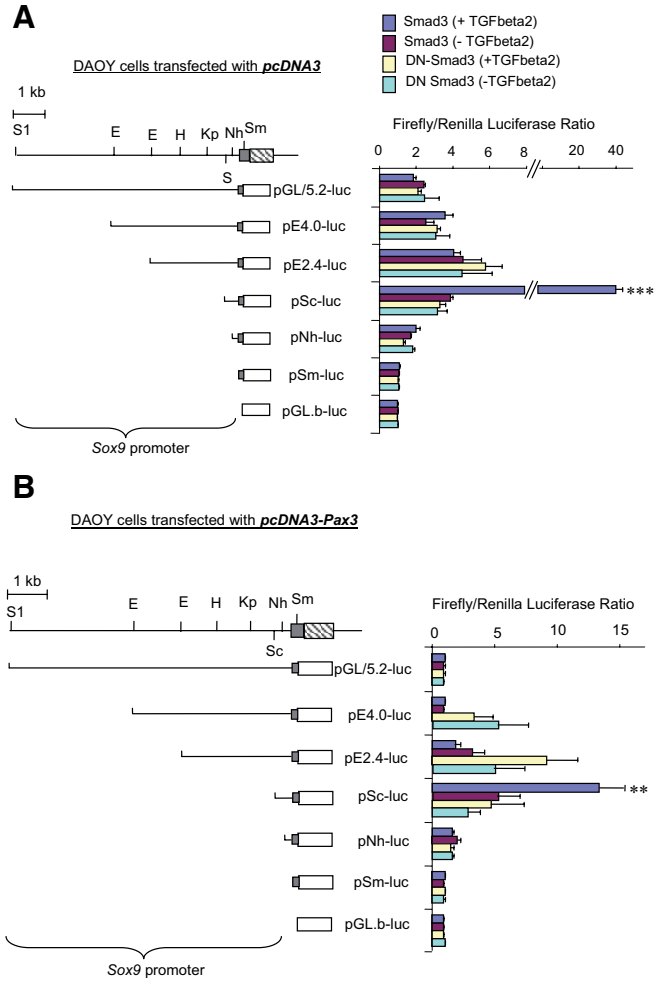


Fig. 6. Effect of Pax3 and TGFβ2 on *Sox9*-promoter luciferase activity. *DAOY cells were co-transfected with* **(A)** pcDNA3 *or* **(B)** pcDNA3-Pax3 *construct and Sox9-luciferase or truncated mutant constructs, along with wild type Flag-Smad3 or dominant negative Flag DN-Smad3. pGL.b-luc served as a vector. Transfected cells were treated or not treated with TGFβ2 and luciferase activity was measured. Renilla luciferase (pRL-null) was used as a transfection control and luciferase activities are expressed as a ratio of Firefly/Renilla luciferase. Each experiment was done in quadruplicate, with each data point in triplicate. Promoter activity in the presence of Smad3 plusTGFβ2 was compared with activity in the presence of DN-Smad3 minus TGFβ2, significance was determined with the Student T test (**p<0.001; ***p<0.0001).*

Sox9-luciferase constructs are described in Kanai and Koopman (1999). When pGL.b-luc (control vector), pGL/5.2-luc, pE4.0-luc, pE2.4-luc, pSc-luc, pNh-luc and pSm-luc *Sox9* promoter constructs were co-transfected with *pcDNA3* (Fig. 6A), there was a significant (p<0.0001) ~ 40 fold increase in *Sox9* promoter activity with pSc-luc, minimal essential promoter. This increase was not seen with the other three treatment conditions. When *Sox9* promoter constructs were co-transfected with *pcDNA3-Pax3* (Fig. 6B), with DN-Smad3 minus TGFβ2, pE2.4-luc, pSc-luc, and pNh-luc constructs showed an insignificant increase in promoter activity in comparison to the vector control, suggesting that Pax3 does not have a direct effect on *Sox9* promoter activity. S*ox9* promoter may be regulated by genes downstream to Pax3 or co-regulators may be required for *Sox9* promoter regulation. pSc-luc *Sox9* promoter activity increased ~12 fold (p<0.001) as opposed to ~40 fold when co-transfected with *pcDNA3* under the same experimental conditions (Smad3 plus TGFβ2). These studies suggest that although the minimum essential *Sox9* promoter is not directly regulated by Pax3, it is directly up-regulated by Smad3 plus TGFβ2. Pax3 may negatively affect this increase.

The data presented in this section suggest Pax3 and TGFβ2 may reciprocally regulate *Hes1*, *Ngn2* and *Sox9* promoters during development. These downstream effector genes together with Pax3 and TGFβ2 may be part of a coordinated gene regulatory network present during neural crest development.

Discussion

In summary, (a) In *TGFβ2* nullizygous embryos, down-regulating one allele of *Pax3* (as in *TGFβ2*$^{-/-}$ *Pax3*$^{+/-}$) can bring about phenotypic reversal of open neural tube. 85% of *TGFβ2*$^{-/-}$ *Pax3*$^{+/-}$ E10.0 embryos, but only 36% of *TGFβ2*$^{-/-}$ *Pax3*$^{+/-}$ E10.0 embryos exhibit open neural tube, representing an approximate 60% success rate in reversing the open neural tube defect. (b) Phenotypic reversal seen in 60% of *TGFβ2*$^{-/-}$ *Pax3*$^{+/-}$ embryos is most likely due to differential gene changes, with some genes, possibly including *Hes1*, *Ngn2* and *Sox9*, affected by Pax3 and TGFβ2 in an opposing fashion (Fig. 7). Not all genes will be regulated by these molecules in an opposing fashion, as we did not observe a 100% phenotypic recovery of the open neural tube defect in E10.0 *TGFβ2*$^{-/-}$ *Pax3*$^{+/-}$ embryos and no phenotypic recovery in *TGFβ2*$^{+/-}$ *Pax3*$^{-/-}$ embryos.

Previous data from our lab on Pax3 regulation of genes (Mayanil *et al.*, 2001) and TGFβ regulation of genes from Zavadil *et al.* (2001) supports the fact that these two developmental regulators may act on certain genes in an opposing fashion. Table 2 shows genes that are up-regulated by Pax3 and down-regulated by TGFβ2, or *vice versa*. Genes selected for this table from the Mayanil *et al.* (2001) paper showed at least a 2-fold change in response to Pax3 over-expression in DAOY cells as measured by

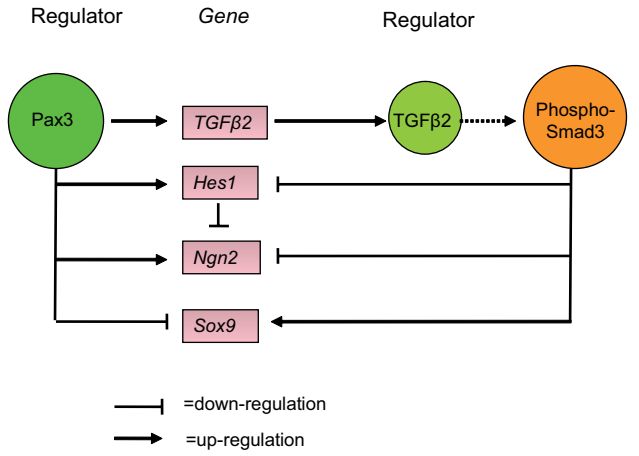


Fig. 7. A hypothetical gene regulatory network comprised of Pax3, TGFβ2 and downstream genes, *Hes1, Ngn2* and *Sox9*. *Some key neural crest genes such* Hes1, Ngn2 *and* Sox9, *may be regulated in an opposing fashion by Pax3 and TGFβ2. Additionally, Hes1 can negatively regulate* Ngn2 *expression (Shimojo et al., 2008).*

microarray analysis. In the paper by Zavadil *et al.* (2001) HaCaT keratinocytes were treated for 2 to 4 hours with TGFβ and gene levels were determined by microarray analysis. Only candidate genes that appear to be regulated in an opposing fashion are shown. Interestingly, *Sox4*, *Sox9*(Hong and Saint-Jeannet, 2005) and *type IV collagen* (Duband and Thiery, 1987), are involved in neural crest development and Rho GDP-dissociation inhibitor (*RhoGDI*) (Maddala *et al.*, 2008) and tissue inhibitor of matrix metalloprotease 3 (*TIMP3*) (DeBecker *et al.*, 2007) are involved in cell migration.

Role of a Pax3/TGFβ2 Gene Regulatory Network in neural tube development

During early embryonic development Pax3 ensures that NCCs do not execute a differentiation program inappropriate for their dorso-ventral position in the neural tube (Goulding and Lamar, 2000). Previous work from our lab (Nakazaki *et al.*, 2008) has shown that Pax3 regulates *Hes1* which regulates neural stem cell maintenance and *Ngn2* which is involved in neural crest cell neurogenesis (Theriault *et al.*, 2005). Regulation of these molecules may be part of the mechanism by which Pax3 initially maintains neural crest cells in an undifferentiated state prior to migration, and then ensures that positional information is integrated into the neurogenesis process and to the specification of progenitor-cell identity.

Pax3 also regulates *TGFβ2*(Mayanil *et al.,* 2006), which in turn decreases Pax3 increased *Hes1* and *Ngn2* promoter activity. These observations suggest that a coordinated gene regulatory network between Pax3 and TGFβ2 could play a role in proper timing of neural tube closure and NCC migration. For instance, in the early stages of epithelial to mesenchymal transition (EMT) it may be critical to inhibit *Hes1* and *Ngn2* without inhibiting Pax3. If *Ngn2* is not inhibited during early stages of EMT, migratory NCCs may undergo premature sensory neurogenesis and die prior to reaching their destination (Nakazaki *et al.*, 2008). Our data suggests that TGFβ2 may be an important player in down-regulating *Ngn2* in the early stages of EMT.

Recently, Shimojo *et al.* (2008) have shown a complex interaction between *Hes1* and *Ngn2*. This group found that *Hes1* expression dynamically oscillates in neural progenitors. They also found that *Hes1* oscillations regulate *Ngn2* oscillations. Down-regulation of *Hes1* expression, which induces neural progenitor differentiation, leads to sustained up-regulation of *Ngn2*. Our results also suggest a complex relation between *Hes1* and *Ngn2*, with Pax3 and TGFβ2 playing a role in appropriately timing the expression of these two molecules in NCCs.

Sox9 regulation by Pax3 and TGFβ2 was also examined in this paper. *Sox9* has a central role in neural crest formation and subsequent EMT and is required for trunk neural crest survival (Sakai *et al.*, 2006). Expression of multiple genes, including *Pax3* and *Sox9*, was downregulated by disrupting Smad4 in NCCs, thus providing evidence that Smad4-mediated activities of TGFβ signals are essential for appropriate NCC development (Nie *et al.*, 2008). Our results indicate that Pax3 does not directly affect *Sox9* activity. However, Pax3 may regulate TGFβ effects on *Sox9* promoter activity. This interaction may play a role in appropriately timing *Sox9* expression in NCCs.

Up-regulation of specific genes and delay in others is expected of a cell that is committed to migration. These events prepare the extracellular matrix (ECM) as well as intracellular cytoskeletal elements for efficient migration. Pax3 and TGFβ2 may also interact in altering levels of various ECM molecules. In a previous study Mayanil *et al.* (2000) found that Pax3 over-expression up-regulated *α-2, 8-polysialyltransferase* (*STX),* thereby increasing NCAM polysialylation (PSA-NCAM). PSA-NCAM prefers the migration permissive substrate heparin sulfate proteoglycan, whereas *Versican*, a large chondroitin sulfate proteoglycan (*CSPG2*), is a migration non-permissive substrate and promotes NCAM-NCAM-mediated homophilic adhesion (Mayanil *et al.*, 2000; Storms and Rutishauser, 1998). Migration of NCCs during early embryonic development would be facilitated if non-permissive substrates were down-regulated. Henderson *et al.* (1997) observed *Versican* overexpression in *Pax3*$^{-/-}$ mice, which exhibit defective NCC migration, and suggested that Pax3 may negatively regulate *Versican* expression. It is likely that extracellular matrix compo-

TABLE 2

REGULATION OF COMMON DOWNSTREAM GENES BY PAX3 AND TGFB2

Gene Name	Gene changes upon *Pax3* over-expression (Mayanil *et al.*, 2001)	Gene changes upon TGFβ2 treatment (Zavadil *et al.*, 2001)
SOX4	Decrease	Increase
SOX9 Also murine *Sox9* (present study)	Decrease	Increase
GDP-DISSOCIATION INHIBITOR	Decrease	Increase
TYPE IV COLLAGEN	Decrease	Increase
UBIQUITIN CONJUGATING ENZYME	Decrease	Increase
HES1 (Hairy and Enhancer of Split) Also murine *Hes1* (present study)	Increase	Decrease
PRAME *(preferentially expressed antigen of melanoma)*	Increase	Decrease
37KD LEUCINE RICH REPEAT PROTEIN	Increase	Decrease
TIMP3 *(Tissue inhibitor of matrix metalloproteases3)*	Increase	Decrease
Murine *Ngn2* (present study)	Increase	Decrease

Evaluation of data from Mayanil *et al.* (2001) and Zavadil *et al.* (2001), indicate that TGFβ negatively regulates certain Pax3 downstream genes.

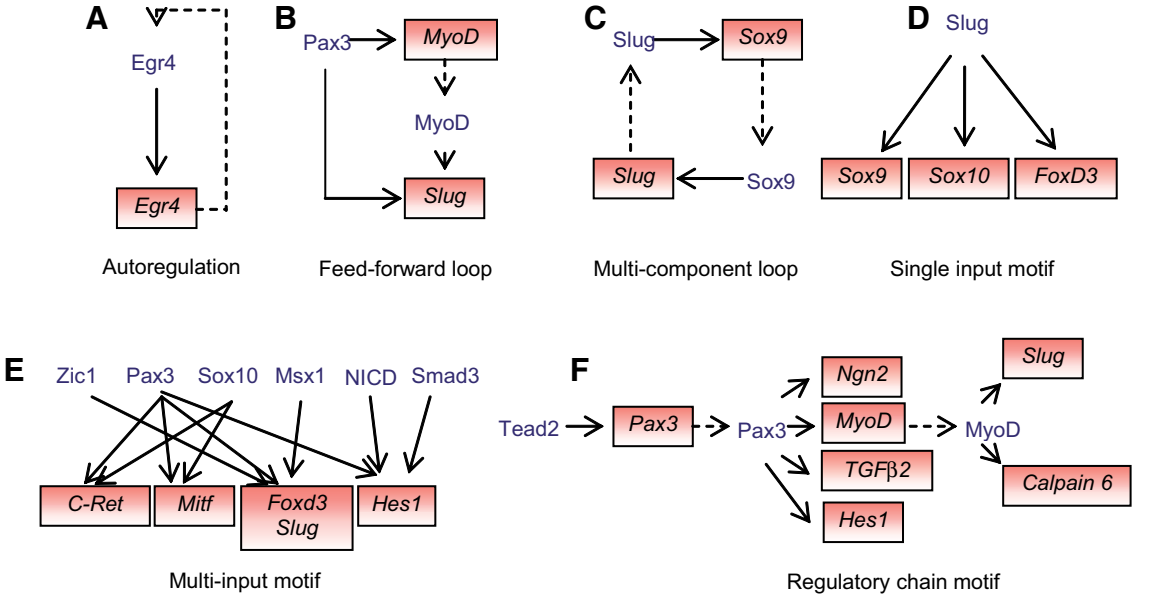


Fig. 8 . Gene regulatory networks *(network motif diagram adapted from Lee* et al., *2002, modified for neural crest development). Different network motifs may exist in developing neural crest.* **(A)** *Auto-regulation: transcription factor Egr4 acts as an auto-regulatory transcription factor (Zipfel* et al., *1997).* **(B)** *Feed forward loop: Pax3 regulating MyoD (Tajbakhsh* et al., *1997) and MyoD regulating Slug (Zhao* et al., *2002). Pax3 also regulating Slug (Monsoro-Burq* et al., *2005).* **(C)** *Multi-component loop: Slug regulating Sox9 and Sox9 regulating Slug (Meulemans and Bronner-Fraser, 2004).* **(D)** *Single input: Slug regulating expression levels of Sox9, Sox10 and FoxD3 (Meulemans and Bronner-Fraser, 2004).* **(E)** *Multi-input: seen in neural crest stem cell specifiers: Pax3 and Sox10 regulate c-ret (Lang and Epstein, 2003) and Mitf (Lang* et al., *2005). Zic1 and Pax3 regulate Foxd3 and Slug (Meulemans and Bronner-Fraser, 2004), and Smad3 and NICD regulate Hes1 (Blokzijl* et al., *2003).* **(F)** *Regulatory chain: Tead2 regulating Pax3 (Milewski* et al., *2004), Pax3 regulating MyoD among others (Tajbakhsh* et al., *1997); MyoD regulating Slug and Calpain 6 (Zhao* et al., *2002). Additionally, a regulatory network based on TGFβ2 regulation of Slug (Romano and Runyan, 2000) can be constructed by overlapping some of the motifs depicted here.*

nents such as Versican V3 are regulated by TGFβ2, given the fact that several extracellular components that affect cell-matrix interactions are remodeled by TGFβ for efficient EMT (Zavadil *et al.,* 2001). An unpublished observation from our lab shows that Pax3 down-regulation of *Versican V3* (Mayanil *et al.*, 2001) is partially reversed by TGFβ2 treatment.

A final point in relation to extracellular matrix components is that they could play a key role in making mature TGFβ available at an appropriate time to induce EMT by negatively regulating Pax3 downstream genes which keep NCCs in a non-migratory state. ECM components and membrane bound forms of TGFβ co-receptors determine bioavailability of TGFβ to NCCs (Dijke and Arthur, 2007). Thus TGFβ2 may be secreted by NCCs, but may not be accessible to interact with its receptors on the membrane because of covalent association with the ECM via latent TGFβ-binding protein (LTBP) (Mangasser-Stephan and Gressner, 1999). However at the critical time, ECM bound large latent TGFβ complex may be released by matrix metalloproteinases. The released complex may be targeted to the cell surface, and proteolytically activated. Interestingly the expression of *tissue inhibitor of matrix metalloprotease 3 (TIMP3)*, which assists in EMT, is increased by Pax3 over-expression and decreased by TGFβ treatment (Table 2).

In this paper we have begun to examine the regulatory interaction between Pax3 and TGFβ2. Pax3 and TGFβ2 may act as a gene regulatory network in neural tube development and NCC migration. The role of this type of network is to a specify sets of genes that must be expressed in specific spatio-temporal patterns (Meulemans and Bronner-Fraser, 2004). This regulatory network, in turn, is most likely a small part of a larger network involved in the highly complex behavior of neural crest cells. Fig. 8 is an adaptation of a diagram from Lee *et al.* (2002). We have modified this diagram based on published data to show different types of networks that may exist in developing NCCs. Each of these regulatory motifs in turn, may be building blocks that can be combined into larger network structures.

Further work needs to be done to elucidate the complex relationship between Pax3 and TGFβ2 in regulating gene expression levels in NCCs. Regulation of genes downstream of Pax3 and TGFβ2 will involve binding of transcription factors, histones, and other transcriptional regulators to the gene's regulatory region. The regulatory region may serve as an integration point, performing a logical computation of transcription factors to determine resulting gene expression levels (Siggia, 2005). Transcriptional regulatory proteins, which recruit and regulate chromatin modifying complexes and components of the transcription apparatus, will recognize specific promoter sequences and in turn affect gene expression programs. For instance, Blumenberg *et al.* (2007) reported that TGFβ-directed EMT is accompanied by chromatin structure regulation involving Polycomb group epigenetic silencers and histone-lysine methyl transferase EZH1 and EZH2. To add to the complexity, Zavadil *et al.* (2007) reported TGFβ-directed

microRNA: mRNA regulatory circuits in EMT. Future studies will examine epigenetic features on promoters of common Pax3 and TGFβ2 downstream targets, as well as gene expression.

Materials and Methods

Double heterozygous mouse breeding and genotyping

The C57BL/6J-*Pax3*^{Sp}/J (stock = 2469) male and C57BL/6J breeding pairs were obtained from The Jackson Laboratory. Genotyping of *Pax3*$^{+/+}$*(wild-type), Pax3*$^{+/-}$heterozygous, and *Pax3*$^{-/-}$ homozygous embryos was performed on isolated genomic DNA from embryonic membranes using PCR with a *Pax3*$^{+/+}$ (*wild-type)* or (*Pax3*$^{-/-}$*)* homozygous specific reverse primer and a common forward primer in separate PCRs as described by Epstein *et al.* (1996). *TGFβ2* heterozygous and homozygous genotypes were detected with PCR using the exon 6 primers,
p5 (AATGTGCAGGATAATTGCTGC) and
p3 (AACTCCATAGATATGGGGATGC). The amplification conditions were 35 cycles at 94°C for 30 seconds, 57°C for 30 seconds and 72°C for 90 seconds (Sanford *et al.*, 1997). *TGFβ2* heterozygous breeding pairs were kindly provided by Dr. T. Doetschman (Sanford *et al.*, 1997) and were used to maintain the *TGFβ2* heterozygous colony and to obtain *TGFβ2* nullizygous embryos. Double heterozygous males and females were produced by mating *TGFβ2*$^{+/-}$ heterozygous females with *Pax3*$^{+/-}$ heterozygous males. The double heterozygous males and females were back-crossed with wild type Swiss black females and males respectively for 6 generations. The resulting double heterozygous males and females were than used to generate the *TGFβ2*$^{-/-}$ *Pax3*$^{+/-}$ genotypes and other genotypes as per Mendelian projection.

Whole mount* in situ *hybridization

Whole mount *in situ* hybridization was done as described earlier (Mayanil *et al.*, 2006). Washes were performed at 65°C. The antisense digoxigenin labeled mouse *Hes1* riboprobe was created by linearizing with EcoR1 and synthesized using T7 polymerase. The *Hes1* sense riboprobe was made by, linearizing with BamH1 and synthesized using T3 polymerase. The antisense digoxigenin labeled mouse *Ngn2* riboprobe was created by linearizing with BamH1 and synthesized using T7 polymerase, the *Ngn2* sense probe was made by linearizing with EcoR1 and synthesized using SP6 polymerase. The antisense digoxigenin labeled mouse *Sox9* riboprobe was created by linearizing with Hind III and synthesized using T7 polymerase. The *Sox9* sense riboprobe was made by linearizing with EcoR1 and synthesized using T3 polymerase. Embryos were stained and photographed with a Spot Camera (Diagnostic Instruments Inc.).

Densitometry was performed on appropriate stained areas, using the Open Lab program (Leica). Areas examined were the dorsal neural tube andÄmigratory neural crest cells in *Hes1* riboprobe stained embryos, dorsal and ventral tube regions in *Ngn2* riboprobe stained embryos and migratory NCCs in *Sox9* riboprobe stained embryos. Staining was quantified from the thoracic/lumbar area downward to include the entire caudal region. On an average, 14-15 somites were counted per embryo of a given genotype. Total density was determined from images scanned in 256 gray scale. The data is represented as the total density (8 bit gray), which is defined as the area multiplied by the mean of the relative densitometry units in the field under investigation. Total density of stained NCCs or dorsal or ventral regions of the neural tube were measured, and then the total density of each genotype was averaged. The data is expressed as average density of all embryos (+/- SEM) in each genotype category (n= 4 per genotype). Differences between *wild type embryos* and embryos with other genotypes were compared by subjecting the data to unpaired T-Test with two-tailed P value (Graph Pad PRISM 4). The data is expressed as the average density of all embryos (n=4 per genotype).

Analysis of Hes1, Ngn2 and Sox9 promoter activity in the presence and absence of TGFβ2

Constructs were kindly provided by: *Hes1- promoter luciferase construct,* Dr. R. Kageyama, *Ngn2*-promoter (AF303001) promoter luciferase constructs, Dr. J. Johnson, *Sox9*-promoter luciferase constructs Dr. P. Koopman. *Hes1*- and *Ngn2*-luciferase and their mutant constructs are fully described in our earlier work (Nakazaki *et al.*, 2008). *Sox9*-luciferase and its mutant constructs are described in Kanai and Koopman (1999). Each of these constructs (0.2 μg) were transiently co-transfected into DAOY cells with *Pax3-pcDNA3*or *pcDNA3* vector control and *Flag-Smad3* wild type (0.2 μg) or dominant negative *Flag-DN-Smad3* (0.2 μg) cloned in HSV-TK-pEXL vector (kindly provided by Dr. H. F. Lodish, MIT). DAOY cells were used to maintain consistency with previous experiments done in our laboratory (Mayanil *et al.*, 2000; 2001; 2006 and Nakazaki *et al.*, 2008). 24 hrs post-transfection, transfected DAOY cells were treated or not treated with 10ng/ml human recombinant TGFβ2 for 6 hrs. Luciferase activity was measured 48 hrs post-transfection using the Dual Luciferase Kit from Promega, as previously described (Mayanil *et al.*, 2006). Transfected cells not treated with TGFβ2 served as the negative control.

Comparison of common downstream genes regulated by Pax3 or TGFβ2

Microarray data from Mayanil *et al.* (2001) and Zavadil *et al.* (2001) were compared. Examination of 270 genes altered by Pax3, as examined in *Pax3* stably transfected DAOY cells (Mayanil *et al.*, 2001), and 728 genes, in HaCaT keratinocytes, altered by 2 to 4 hour treatment with TGFβ (Zavadil *et al.*, 2001), yielded 58 genes that were altered in both studies. To increase stringency 2 fold or higher increase or decrease in expression levels was used as a cut off. This resulted in a total of 22 genes altered by both Pax3 overexpression and TGFβ treatment. From these 22, 10 candidate genes, 5 up-regulated and 5 down-regulated by Pax3 (Mayanil *et al.*, 2001) and oppositely regulated by TGFβ treatment (Zavadil *et al.*, 2001) are shown in Table 2. Out of these 10 genes, we used three for our *in situ* hybridization and promoter-luciferase studies. Several genes not listed in Table 2 did show up-regulation or down-regulation in response to both Pax3 overexpression and TGFβ treatment.

Acknowledgements

The authors would like to thank Dr. P. Gruss Max Planck Institute, Gottingen, Germany, for the pBH3.2 plasmid containing Pax3; Dr. N. L Brown, Division of Developmental Biology, Departments of Pediatrics and Ophthalmology, University of Cincinnati College of Medicine, Cincinnati, OH, USA, for pBluescriptHes1 and pGEM3Ngn2 plasmids; Dr. de Crombrugghe, University of Texas, MD Anderson Cancer Center, and Department of Molecular Genetics, Houston Texas for murine Sox9 cDNA probe. TGFβ2$^{+/-}$ *heterozygous breeding pairs were provided by Dr. T. Doetschman, Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Ohio. The authors also wish to thank Dr. R. Kageyama, Institute for Virus Research, Kyoto University for Hes1-promoter luciferase constructs; Dr. J. Johnson, Department of Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX for Ngn2-promoter-luciferase constructs; and Dr. P. Koopman, Division of Molecular Genetics and Development, Institute for Molecular Bioscience, The University of Queensland, Brisbane, Australia for Sox9-promoter-luciferase constructs. The Flag-Smad3 and Flag-DN-Smad3 constructs were gifts from Dr H.F. Lodish, Whitehead Institute for Biomedical Research, Department of Biology, Massachusetts Institute of Technology. The authors also wish to thank Dr. J. A. Kessler, Feinberg School of Medicine, Northwestern University, and Dr. S. Ahlgren Developmental Biology Program, Childrens Memorial Research Center, Chicago for valuable discussions, Dr H. Chung for statistical assistance and Bill Goossens for microscope assistance. This work was supported by* the

State of Illinois Excellence in Academic Medicine award (CSKM) and a Grant from the Spastic Paralysis Research Foundation of Illinois-Eastern Iowa District of Kiwanis (CSKM and DGM). The earlier preliminary part of the work described in this paper was done in the Neurobiology Program at Childrens Memorial Research Center, Chicago, IL 60614.

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