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Characterization of *Bcor* Expression in Mouse Development

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Abstract

Mutation of the gene encoding the transcriptional corepressor BCOR results in the X-linked disorder Oculofaciocardiodental Syndrome (OFCD or MCOPS2). Female OFCD patients suffer from severe ocular, craniofacial, cardiac and digital developmental defects and males do not survive through gestation. BCOR can mediate transcriptional repression by the oncoprotein BCL6 and has the ability to reduce transcriptional activation by AF9, a known mixed-lineage leukemia (MLL) fusion partner. The essential role of BCOR in development and its ability to modulate activity of known oncogenic proteins prompted us to determine the expression profile of *Bcor* during mouse development. Identification of independently transcribed exons in the 5′ untranslated region of *Bcor* suggests that three independent promoters control the expression of *Bcor* in mice. Although *Bcor* is widely expressed in adult mouse tissues, analysis of known spliced isoforms in the coding region of *Bcor* reveals differential isoform usage. Whole mount in situ hybridization of mouse embryos shows that *Bcor* is strongly expressed in the extraembryonic tissue during gastrulation and expression significantly increases throughout the embryo after embryonic turning. During organogenesis and fetal stages *Bcor* is differentially expressed in multiple tissue lineages, with a notable presence in the developing nervous system. Strikingly, we observed that *Bcor* expression in the eye, brain, neural tube and branchial arches correlates with tissues affected in OFCD patients.

Keywords

Bcor; Oculofaciocardiodental syndrome; MCOPS2; extraembryonic

Results and Discussion

BCOR (BCL6 corepressor) is a transcriptional co-repressor that was identified based on its ability to interact with the POZ domain of the oncoprotein BCL6 (Huynh et al., 2000). Chromosomal translocations in the promoter and 5′ untranslated region of the human BCL6 gene are a common genomic alteration in non-Hodgkin's B cell lymphomas (Baron et al., 1993;Dalla-Favera et al., 1999;Kerckaert et al., 1993;Miki et al., 1994;Ye et al., 1993). These translocations result in aberrant expression of BCL6 (Chen et al., 1998;Ye et al., 1995). Mice engineered to model one of these translocations develop B cell lymphomas, demonstrating that BCL6 is a bona fide oncogene (Cattoretti et al., 2005). BCOR potentiates BCL6 mediated transcriptional repression of reporter constructs in transiently transfected cells (Huynh et al.,

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2000). In germinal center B cells, BCOR is found with BCL6 at several known BCL6 target genes, including regulators of cell proliferation and apoptosis (Gearhart et al., 2006). BCOR co-purifies with an 800 kDa complex comprised of Polycomb group transcriptional repressor proteins and SCF ubiquitin E3 ligase components (Gearhart et al., 2006). Epigenetic modification of BCL6 target gene chromatin by the BCOR repression complex is likely to play a role in mediating their repression.

In addition to its role in B-cells, BCOR aids in the control of gene expression in multiple tissues and organ systems during development and into adulthood as mutations in human *BCOR* result in X-linked Oculofaciocardiodental syndrome (OFCD) (Ng et al., 2004). OFCD is the primary subtype of OMIM #300166 microphthalmia, syndromic 2 (MCOPS2) and is characterized by ocular, dental, cardiac and digital anomalies in heterozygous females (Ng et al., 2004;Schulze et al., 1999). Males with OFCD do not survive due to presumed embryonic lethality (Ng et al., 2004). Since *BCOR* lies on the X chromosome in both mice and humans, random X-inactivation results in mosaic expression of the mutant allele contributing to varying disease severity in females (Ng et al., 2004;Schulze et al., 1999). Additionally, peripheral leukocytes of female patients show preferential survival of cells in which the mutant allele of *BCOR* lies on the inactivated X chromosome, indicating a strong requirement for *BCOR* in hematopoiesis (Hedera and Gorski, 2003;Ng et al., 2004). Another form of MCOPS2, which is distinct from OFCD, occurs in males with a single missense mutation (p.P85L) in the fourth coding exon of *BCOR* (Horn et al., 2005;Ng et al., 2004). In the described family, this syndrome is inherited in an X-linked recessive pattern and comprises microphthalmia/anophthalmia, mental retardation, and skeletal and other anomalies (Ng et al., 2002). RNAi knock-down of *Bcor* in zebrafish results in eye, skeletal and nervous system abnormalities consistent with those found in MCOPS2 patients (Ng et al., 2004). BCOR has also been shown to interact with and repress transcriptional activation of the MLL fusion protein AF9 (Srinivasan et al., 2003), a known regulator of Hox gene expression (Collins et al., 2002) and skeletal development, further implicating BCOR as a key developmental regulator. The pleiotropic effects induced by the loss of functional BCOR in humans and zebrafish (Ng et al., 2004) clearly illustrate the essential role of *BCOR* during embryogenesis and emphasizes the importance of determining the spatial and temporal expression of *BCOR* during development. Herein, we provide a detailed analysis of *Bcor* mRNA expression during mouse development and in the adult mouse.

In adult mice and humans, *Bcor* is widely expressed (Huynh et al., 2000;Nagase et al., 2000). However, these studies are limited in scope, relying on RNA dot blot analysis of human tissues and reverse transcription PCR (RT-PCR) on a limited number of mouse tissues. Expanding upon these results, we have dissected the pattern of *Bcor* expression by Northern blot analysis, more extensive RT-PCR, and whole mount and section in situ hybridization experiments. To determine the expression pattern of *Bcor* in adult mouse tissues, we harvested total RNA from 14 different organs and conducted Northern blot analysis on 4 organs and RT-PCR on all 14 organs using a probe that recognizes all mRNA isoforms (Fig. 1F). Three transcripts migrating at approximately 7 kb are found in all four organs analyzed however the stoichiometry of the different transcripts is not identical (Fig. 1A). *Bcor* mRNA is detected by RT-PCR in all tissues tested (Fig. 1B). The ubiquitous expression of *Bcor* in adult mouse tissue is consistent with expression of *BCOR* in human adult tissue (Huynh et al., 2000).

The identification of three *Bcor* transcripts by Northern blot analysis prompted us to consider that the *Bcor* gene may utilize multiple promoters, alternative splicing and/or polyadenylation sites. Although alternative splicing in the coding region has been reported (Fig. 1F and (Srinivasan et al., 2003)) this can only affect transcript size by up to 156 bp. In silico analysis of spliced expressed sequence tags (EST) databases supported use of alternative promoters and polyadenylation sites at the mouse *Bcor* genomic locus. Two alternative polyadenylation sites separated by 550 bp are present. CpG islands are also found in close proximity upstream of

exons 1a, 1b and 1c, further supporting the presence of three independent promoters (Fig. 1F). To determine whether all three promoters suggested by the EST database are actively used in most tissues, we generated an exon 2 specific reverse primer and three forward primers in putative *Bcor* exons 1a, 1b and 1c that correspond with sequences specifically driven by putative promoters P1, P2 and P3 (Fig. 1F). RT-PCR and sequencing of the products demonstrates that *Bcor* uses all three putative independent promoters (Fig. 1B and data not shown). In the panel of adult mouse tissues tested, each promoter appears to be used at similar levels in all tissues tested, with the exception of whole blood, which appears to preferentially use promoter 3 relative to other tissues. Interestingly, amplification from promoter 2 results in two specific amplicons (Fig. 1B) due to the use of an alternative splice donor sites in exon 1b (data not shown). We also examined promoter use during embryonic development (E11.5 - E18.5). Although all promoters are used at these stages the distal splice donor of exon 1b is not used at E15.5 and E16.5 (Fig. 1D).

Splicing bypassing exon 5 and/or alternative splice acceptor usage at exon 8 results in the previously identified *Bcor* isoforms a-d (Fig. 1F) (Srinivasan et al., 2003). Only isoforms a and b contain sequences required for the interaction with the transcriptional regulator AF9 (Ng et al., 2004;Srinivasan et al., 2003). To determine which isoforms are differentially expressed in the embryo and adult mouse tissues, we conducted RT-PCR using primers that span exon 4 to exon 10 on samples used in Figure 1B and 1D. *Bcor* isoforms a, c and d can be amplified from the samples tested (Fig. 1C and 1E). Isoform c shows the most ubiquitous expression in the sample set and isoform a shows the most tissue-specific expression. Relative to the other tissues, isoform a is more strongly represented in the brain and testis. Isoform b is only barely detected in ovary, eye, spleen and kidney at this level of amplification but not at embryonic stages.

The clinical presentation of OFCD in female patients and presumed embryonic lethality in males suggested that *Bcor* might have a unique expression pattern during embryogenesis that might give insight into future studies on *Bcor* function. To determine the spatial and temporal expression of *Bcor* during gastrulation and early organogenesis we conducted whole mount and section in situ hybridization on embryonic day 7.5 - 15.5 CD-1 outbred mouse embyros. Using the same probe sequence as was used for northern analysis we generated digoxigenin labeled RNA antisense and sense (- control) probes that would detect all known *Bcor* transcripts. These probes were used in all subsequent whole mount and section in situ expression images.

Whole mount in situ hybridization on embryonic day 7.5 and 8.5 shows that *Bcor* is strongly expressed in the extraembryonic tissue during gastrulation (Fig. 2A, 2C). Anti-sense probe specificity is validated by absence of signal in embryo hybridized with the sense strand control (Fig. 2B, 2D, 2F). Figure 2E and 2G show that expression of *Bcor* is restricted to the ectoplacental cone and trophectoderm but absent from extraembryonic visceral and parietal endoderm. Consistent with this, trophoblastic stem cells express *Bcor* (data not shown). Although *Bcor* is weakly expressed in the neuroectoderm of the embryo proper (Fig. 2E and 2H), the level is significantly lower in comparison to extraembryonic tissues (Fig. 2E and 2G).

After completion of embryonic turning at approximately E9.0, *Bcor* transcript levels in the embryo proper increase dramatically. Whole mount in situ hybridization of E9.0 embryos (Figure 3A and B) reveals the initial increase in *Bcor* expression throughout the embryo proper, led by striking increases in the ectodermal tissue of the tail bud. By E9.5 *Bcor* transcripts are differentially expressed showing strong expression in the limb buds and branchial arches (Fig. 3C, 3D, 3E; sense control is 3F), while maintaining less robust expression throughout the rest of the embryo. Strong expression of *Bcor* also extends from the tail bud (shown in detail in Fig. 3G and 3H) down the ridge of the closing neural tube along the dorsal side of the embryo.

Strong expression of *Bcor* in tissues that will give rise to future craniofacial structures correlates well with craniofacial abnormalities present in female OFCD patients.

After E9.5, *Bcor* is differentially expressed in multiple tissue and organ systems during the fetal period of mouse development. Section in situ hybridization of a mid-body cross section at E11.5 reveals strong expression of *Bcor* in the neural tube and flanking dorsal root ganglion (Fig. 4A and 4D, sense control in 4B). *Bcor* is also expressed in the notochord and cervical sclerotome and myotome in addition to more subtle and general expression in the forelimb region (Fig. 4A and 4D). *Bcor* expression can also be found surrounding the esophagus, right and left bronchi and connected lung buds and in the sympathetic chains (Fig. 4A and 4C). A cross section through the head of an E13.5 fetal mouse displays significant expression in the eye, neural tube, the olfactory epithelium and the teeth primordium (Fig. 4E, 4G and 4H, sense control in 4F). *Bcor* expression in the eye is interestingly found in the retina, lens tissues and in the boundary of the eyelid (Fig. 4E and 4G).

At E14.5, a sagittal section near the midline of a fetus reveals expression of *Bcor* in the trigeminal (V) ganglion, the neopallial cortex, the corpus striatum and the olfactory epithelium (Fig. 5A, 5C and 5D, sense control in 5B). In more lateral sagittal sections through an E15.5 fetus, *Bcor* expression is retained in the eye but is localized to the retina and the dorsal side of the lens (Fig. 5E and 5G, sense control in 5F). *Bcor* is also expressed in the lung and gut epithelium and muscle tissue in the fetus e.g. in the temporalis and masseter muscles (Fig. 5E and 5H).

Here we have characterized the unique expression pattern of the transcriptional corepressor *Bcor*. We have shown that *Bcor* is widely expressed in adult tissue, is expressed in ES cells and displays a widespread but specific expression during embryonic development. There is very strong expression of *Bcor* in extraembryonic tissue. Expression of *Bcor* in the developing eye, tooth primordium, limb buds, branchial arches and multiple nervous system tissues also distinctly correlates with many of the tissues adversely affected in OFCD and LM patients (Ng et al., 2004;Schulze et al., 1999). This analysis provides important information for the analysis of future mouse models of OFCD.

Experimental Procedures

Mice

CD-1 outbred mice from Jackson Laboratory were kept on a normal light dark cycle and maintained by Research Animal Resources at the University of Minnesota.

Probe Generation

An amplicon containing nucleotides 280 to 1294 of the *Bcor* open reading frame was generated by PCR from a bacterial artificial chromosome containing the mouse *Bcor* gene using the following primers: Forward: 5′-TACGGGTACCATCGATGGCCTGGCAGCACT-3′ and Reverse: 5′-CATGTCTAGATTGGACAGCCTGGATGTAGAG-3′. The PCR amplicon was gel purified (Qiagen, PCR clean up kit) and directly cloned into the Kpn and Xba restriction sites of a pBluescript KS+ vector (Strategene). Radiolabeled DNA probes for Northern blot analysis were generated using a Promega Prime-A-Gene kit and ^{32}P labeled dCTP. Digoxigenin labeled RNA antisense and sense in situ hybridization probes were *in vitro* transcribed from the T7/T3 promoter containing pKS+ plasmid with *Bcor* insert using an Ambion Maxiscript kit and Digoxigenin labeleld dUTP from Roche.

Northern Blot Analysis

Total RNA was harvested from embryonic mice and adult mouse organs using TriZol (Invitrogen). Messenger RNA was then extracted using a Miltenyi mRNA isolation kit. Northern blot analysis using one ug per lane was carried out using standard procedures (Ausubel, 1988).

RT-PCR / qRT-PCR

Total RNA was isolated from mouse embryos and tissues using TriZol and cDNA was generated using SuperScript III cDNA sythesis kit (Invitrogen). *Bcor* promoter RT-PCR was performed using the following primers: *Bcor* exon 1a forward: 5′-TGCTGGGTTTGAACGGAATG-3′, *Bcor* exon 1b forward: 5′-GAGAGATAAATGGCCTCTGACAGC-3′, *Bcor* exon 1c forward: 5′-GAAAACTTCAAAGCGCCGGATC-3′ and *Bcor* exon 2 reverse: 5′-GACCCTCTCGCTGTTCATCCAG-3′. *Bcor* isoform a-d RT-PCR was performed using the following primers: *Bcor* isoform a-d forward: 5′-CTAGCCGGGCAGCCCGCTGCAGGAG-3′ and *Bcor* isoform a-d reverse: 5′-GTGGGCGGTGCCCCTCCAAACATGGA-3′. HPRT expression was used as a loading control for *Bcor* promoter and isoform a-d RT-PCR analysis. These HPRT primers are as follows: HPRT forward: 5′-AGCTACTGTAATGATCAGTCAACG-3′ and HPRT reverse: 5′-AGAGGTCCTTTTCACCAGCA-3′.

Whole mount and Section In Situ Hybridization

Whole mount in situ hybridization was performed as described (Nagy, 2003). Section in situ hybridization was performed as described (http://genetics.med.harvard.edu/~cepko/protocol/insituprotocol.pdf).

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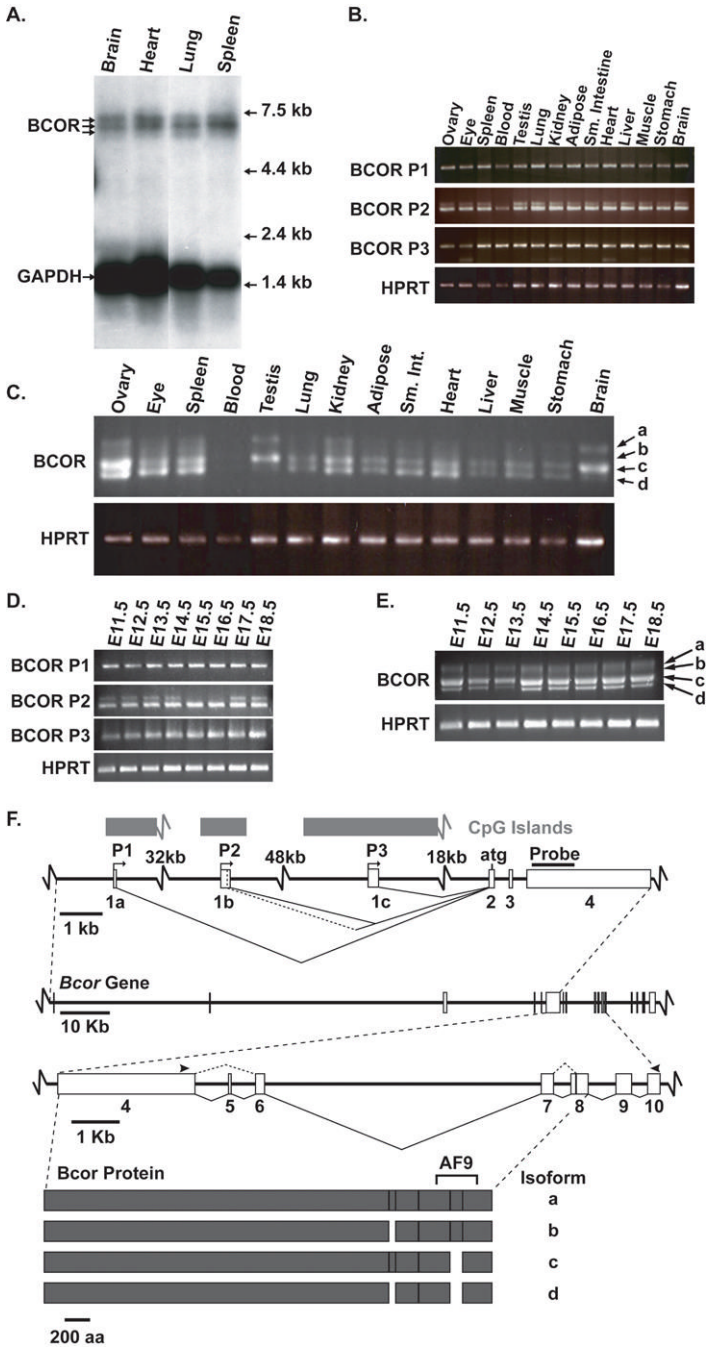

Figure 1.

Three independent promoters drive widespread expression of Bcor mRNA in embryonic and adult mouse tissue (A-F). Northern blot analysis of adult mouse organs reveals three major Bcor transcripts. Gapdh was used as a loading control (A). Promoter 1, 2 and 3 (P1, P2 and P3) specific RT-PCR analysis on panel of adult mouse organs (B). RT-PCR product sizes are as follows P1 166 bp, P2 shorter 164 bp, P2 longer 187 bp, P3 159 bp. Bcor isoform a-d specific RT-PCR on mouse organs panel (C). RT-PCR product sizes are as follows a 1642 bp, b 1588 bp, c 1540 bp, and d 1486 bp. P1, P2 and P3 specific RT-PCR analysis of embryonic stages 11.5-18.5 (D). Bcor isoform a-d specific RT-PCR on stages E11.5-E18.5 (E). Hprt was used as a loading control, product 200 bp (B-E). Illustration depicting promoter usage and splicing

of the mouse *Bcor* gene and resulting protein (F). Arrowheads indicate primer binding sites used in C and E. Exon 1b distal splice donor site adds an additional 23 bp. Exon 5 is 54 bp and alternative proximal splice acceptor for exon 8 adds 102 bp. NCBI sequences supporting exons 1a-1c; size given for first exon: exon 1a (AK129398, 67bp), exon 1b (CJ073674, 159 bp; BY207441, 148 bp; CD554764, 99bp; CN677848, 132 bp) exon 1c (AY161171, 51bp; AK086385, 241 bp; BB662958, 242 bp; BC058656, 237 bp; BU056818, 237 bp; AK053309, 736 bp)

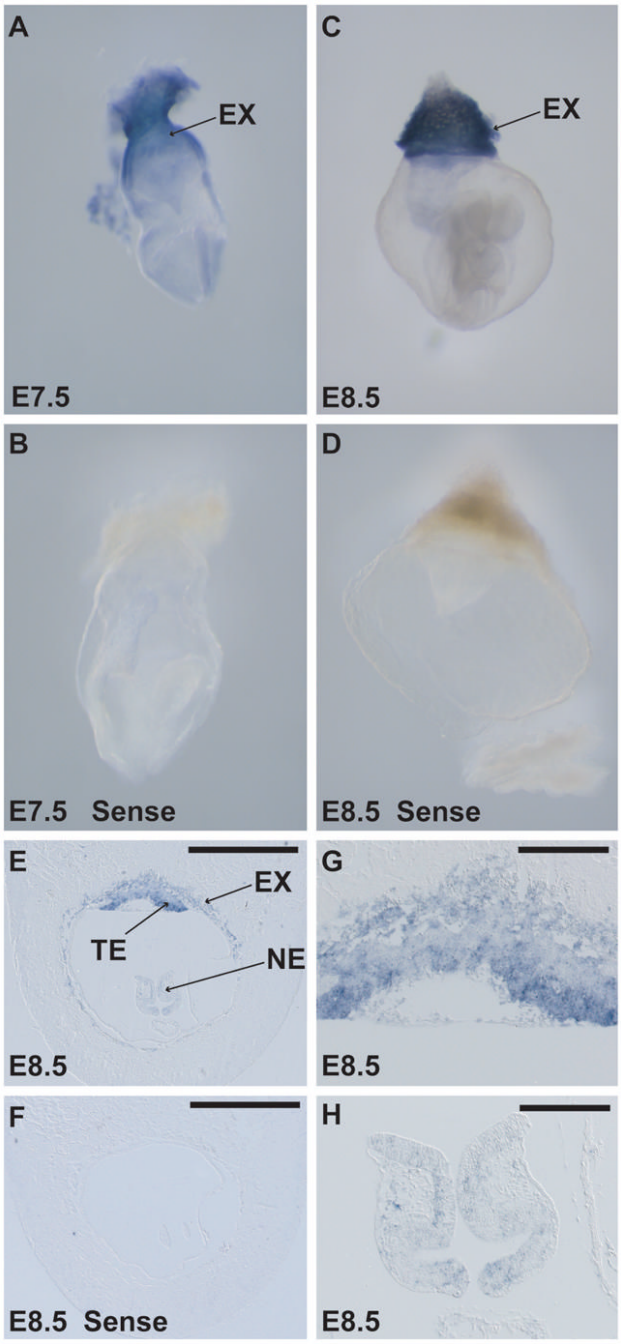

Figure 2.

Bcor mRNA is strongly expressed in extraembryonic tissue during early embryogenesis (A-G). Whole mount in situ hybridization of mouse embryos at day 7.5 and 8.5 shows strong expression of *Bcor* in the extraembryonic tissue (A and C). Sense strand control probes are negative on E7.5 and 8.5 embryos (B and D). Section in situ hybridization of a sagittal section of embryonic day 8.5 shows *Bcor* expression in the trophectoderm (TE), extraembryonic tissue (EX) and minimally in the neuroectoderm (NE) of the embryo proper (E, G and H). Sense strand control probe is negative on a parallel E8.5 sagittal section (F). (G and H) are insets from panel (E) at higher magnification. Scale bars represent 1 mm in panels (E and F) and 0.1 mm in panels (G and H).

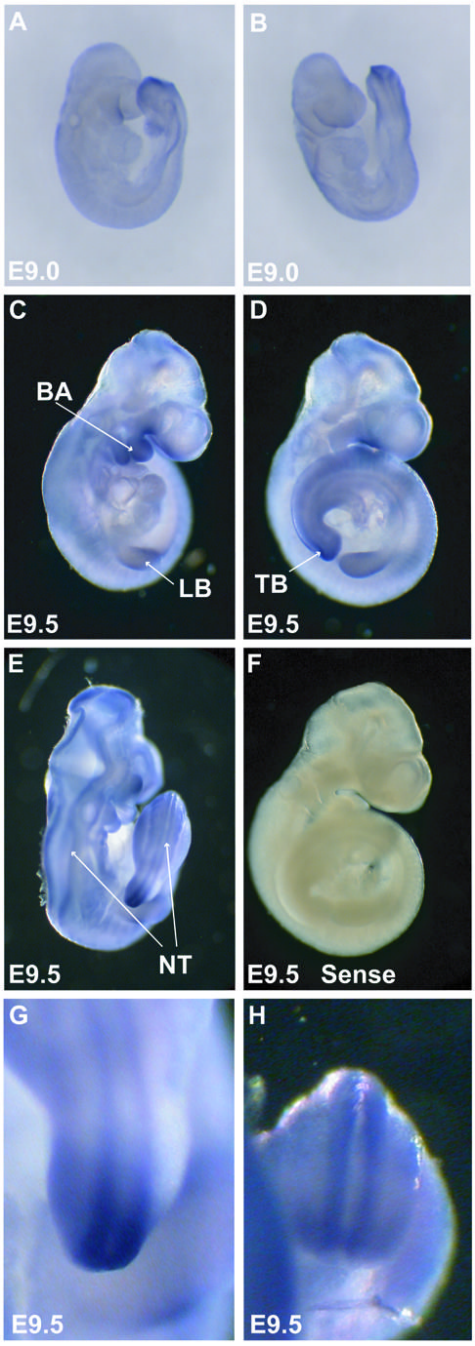

Figure 3.
Bcor mRNA expression increases throughout the embryo proper post embryonic turning. Whole mount in situ hybridization shows *Bcor* expression starting in the tail bud (TB) at embryonic day 9 (A and B) and abundant expression in the tail bud, branchial arches (BA), limb buds (LB) and neural tube (NT) (C, D and E). Sense strand control probes are negative on E9.5 embryos (F). E9.5 tail buds at higher magnification (G and H).

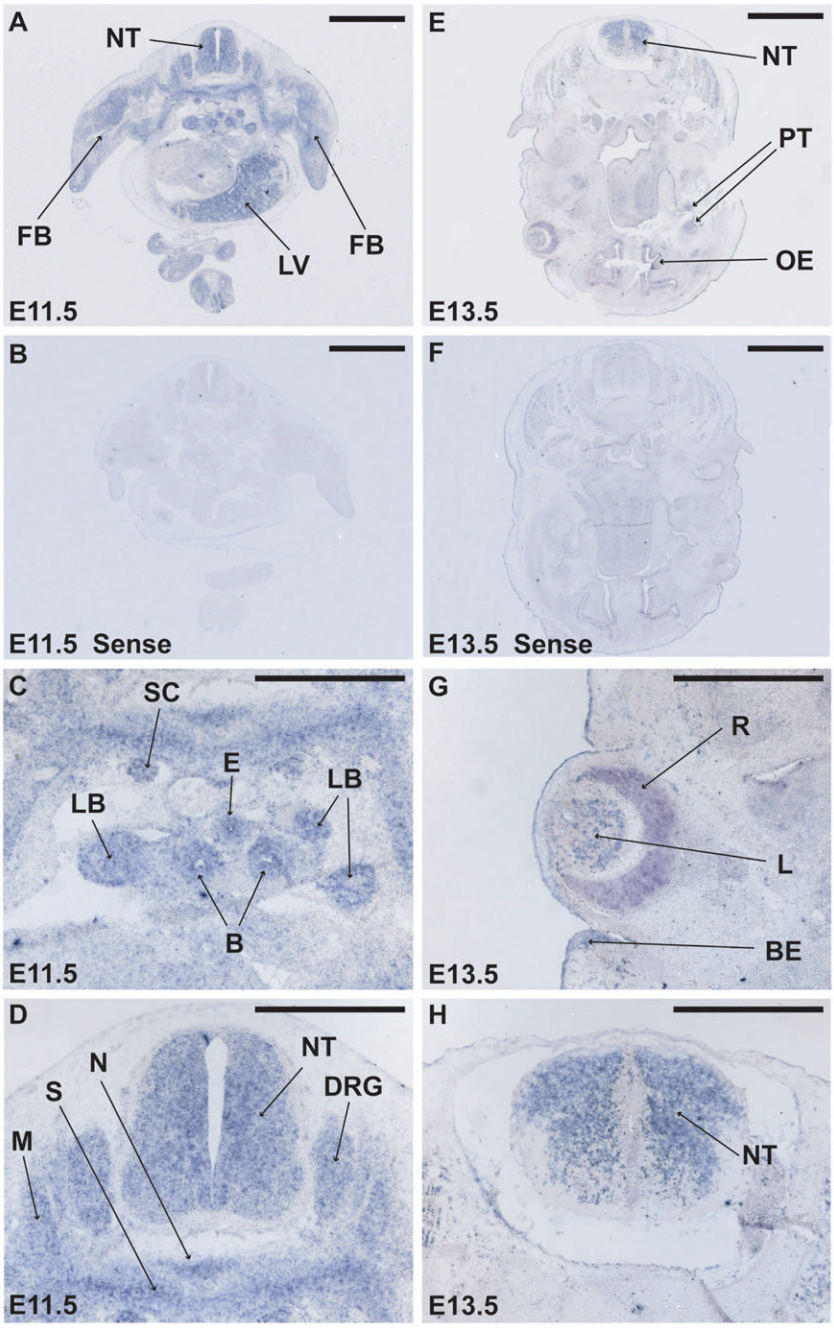

Figure 4.

Early fetal stage mouse embryos show widespread but differential expression of *Bcor* mRNA (A-H). Section in situ hybridization on horizontal sections of an E11.5 embryo shows *Bcor* expression in multiple tissues including the right and left forelimb buds (FB), liver (LV), left and right lung buds (LB), sympathetic chains (SC), esophagus (E), bronchi (B), myotome (M), sclerotome (S), notochord (N), neural tube (NT), and dorsal root ganglion (DRG) (A, B, C and D). C and D are enlargements from A. Near horizontal section of E13.5 embryo shows expression in the tooth primordium (PT), olfactory epithelium (OE), lens (L) retina (R), boundary of the eyelid (BE), and neural tube (NT), (D, E and F). Sense strand control probes are negative on E11.5 and E13.5 equivalent horizontal sections (B and F). G and H are

enlargements form E. Scale bars represent 1 mm in panels (A, B, E and F) and 0.5 mm in panels (C, D, G and H).

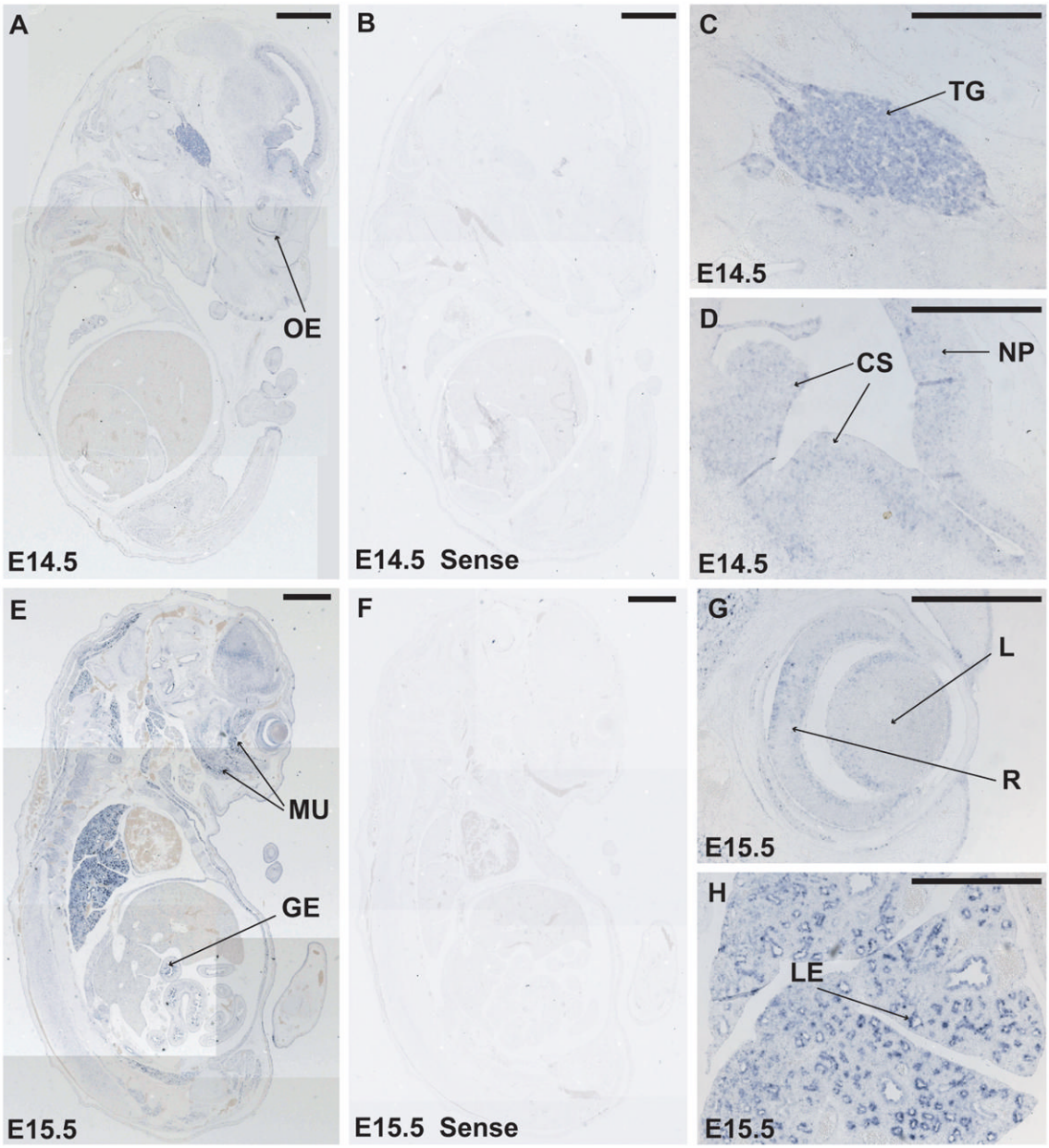

Figure 5.

Later fetal stage mouse embryos continue to express *Bcor* mRNA in a widespread yet tissue specific manner (A-H). Section in situ hybridization on a sagittal section of an E14.5 embyro (A-D) shows *Bcor* expression in trigeminal ganglion (TG), neopallial cortex (NP), the corpus striatum (CS) and the olfactory epithelium OE). At E15.5 *Bcor* continues to be expressed in the lens (L) and retina (R) of the eye and in the lung epithelium (LE), gut epithelium (GE), and temporalis and masseter muscle (MU) (E-H). Sense strand control probes are negative on E14.5 and E15.5 equivalent sagittal sections (B and E). Scale bars represent 1 mm in panels (A, B, E and F) and 0.5 mm in panels (C, D, G and H).