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A BAC transgenic Hes1-EGFP reporter reveals novel expression domains in mouse embryos

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Abstract

Expression of the basic helix-loop-helix factor Hairy and Enhancer of Split-1 (Hes1) is required for normal development of a number of tissues during embryonic development. Depending on context, Hes1 may act as a Notch signalling effector which promotes the undifferentiated and proliferative state of progenitor cells, but increasing evidence also points to Notch independent regulation of Hes1 expression. Here we use high resolution confocal scanning of EGFP in a novel BAC transgenic mouse reporter line, Tg(Hes1-EGFP)^{1Hri}, to analyse *Hes1* expression from embryonic day 7.0 (e7.0). Our data recapitulates some previous observations on *Hes1* expression and suggests new, hitherto unrecognised expression domains including expression in the definitive endoderm at early somite stages before gut tube closure and thus preceding organogenesis. This mouse line will be a valuable tool for studies addressing the role of Hes1 in a number of different research areas including organ specification, development and regeneration.

1. Introduction

Hes1 is a basic helix-loop-helix (bHLH) transcriptional repressor which is required for normal development of several tissues and organs including the nervous system (Ishibashi et al., 1995), the eyes (Tomita et al., 1996), the pancreas (Jensen et al., 2000), the thymus (Tomita et al., 1999) and the lungs (Ito et al., 2000). Hes1 is also expressed in the developing kidneys (Chen and Al-Awqati, 2005; Piscione et al., 2004), the intestine (Jensen et al., 2000) and the stomach (Nyeng et al., 2007). Loss of Hes1 in the developing nervous system leads to up-regulation of the neural differentiation factor Mash1 and subsequently premature neural differentiation (Ishibashi et al., 1995). Also, the loss of Hes1 results in premature endocrine differentiation in the developing mouse gut tube as well as in the pancreas, resulting in pancreatic hypoplasia (Apelqvist et al., 1999; Esni et al., 2004; Fujikura et al., 2006; Jensen et al., 2000). Cell culture studies have shown that Hes1 is a downstream target of Notch signalling in some contexts (Jarriault et al., 1995; Jarriault et al., 1998; Ohtsuka et al., 1999), and loss-of-function studies often show similar phenotypes

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when comparing *Hes1* and other Notch pathway null mutants which all result in premature differentiation (Apelqvist et al., 1999; Esni et al., 2004; Fujikura et al., 2006).

In the pancreas, *Hes1* regulates endocrine differentiation (Jensen et al., 2000) by inhibiting the expression of the pro-endocrine differentiation factor *Neurog3* (Apelqvist et al., 1999; Fukuda et al., 2006), and studies in cell culture suggest that exocrine differentiation may be regulated by direct interactions between *Hes1* and Pancreatic transcription factor 1a (*Ptf1a*) (Esni et al., 2004; Ghosh and Leach, 2006). In the adult pancreas, *Hes1* is restricted to the centroacinar cells but becomes activated by pancreatic injury, e.g. during chemically induced pancreatitis (Jensen et al., 2005), indicating a possible role in regeneration. *Hes1* is also upregulated during induction of pancreatic cancer (Miyamoto et al., 2003; Pasca di Magliano et al., 2006).

Hence, a key function of *Hes1* is to prevent differentiation and keep a pool of progenitor cells in a proliferative state to ensure appropriate growth of the developing tissue. However, more recent studies point to several Notch independent ways of activating *Hes1* expression. During somitogenesis, segmentation is controlled by a molecular clock (Pourquie, 2003) where FGF has been shown to induce the oscillations of *Hes1* expression (Nakayama et al., 2008), while in retina explants, Sonic hedgehog (*Shh*) has been demonstrated to regulate *Hes1* activity in a *Gli2* dependent manner (Ingram et al., 2008; Wall et al., 2009).

Previous investigations of *Hes1* mutant mice revealed formation of ectopic pancreatic tissue in the common bile duct (Sumazaki et al., 2004), stomach, and duodenum, along with gall bladder agenesis (Fukuda et al., 2006). It has recently been proposed that *Hes1* acts in conjunction with the SRY-box containing HMG transcription factor *Sox17* to define the pancreato-biliary boundary in the ventral posterior foregut (Spence et al., 2009).

Here, we have used a publicly available BAC-clone from the GENSAT project (Gong et al., 2003), where the coding sequence of Enhanced Green Fluorescent Protein (EGFP) is inserted in the 5'UTR of the *Hes1* gene to generate a transgenic mouse line, Tg(*Hes1*-EGFP)^{1Hri}. We present the analysis of *Hes1*-EGFP expression in the developing mouse embryo from late streak stage around e7.0 with focus on the pancreas, the intestine, the liver, the kidneys, and the lungs. Our results confirm previous reports on *Hes1* expression and reveal novel *Hes1* expression domains.

2. Results

To investigate the temporal and spatial patterns of *Hes1* expression, we performed pronuclear injections to generate a transgenic mouse line expressing EGFP under control of *Hes1* regulatory sequences. We used a BAC clone containing more than 224 kb of chromosome 16 including the *Hes1* gene (Fig. 1A). The insertion of the whole 224 kb fragment was verified by Southern blots with probes that recognise the ends (Fig. 1B, C). Immunofluorescent stainings for *Hes1* and EGFP on adjacent sections confirm co-expression of EGFP with *Hes1* in the inner neural layer and the outer pigment layer of the future retina in the e10.5 developing eye (Fig. 1D, E), in e10.5 developing pancreas epithelium (but not in the mesenchymal cells) (Fig. 1F, G), in roof plate (but not in the ependymal cells) in e14.5 neural tube (Fig. 1I, J), and in e14.5 oesophagus (Fig. 1J, K). We have used the Tg(*Hes1*-EGFP)^{1Hri} mouse line to analyse for EGFP expression at different stages of development in selected tissues.

2.1 *Hes1* promoter-driven EGFP expression at late streak stage and early somite stages

We first analysed e7.0 and e8.0-e8.5 stage embryos using whole mount immunohistochemistry. The e7.0 late streak stage embryos show uniform EGFP expression

in the endodermal cell layer but is excluded from the mesoderm and ectoderm (Fig. 2A, B). A day later at e8.0-e8.5 just before embryo turning, we find a distinct area of EGFP expression in the posterior part, and another expression area in the anterior part of the embryo (Fig. 2C). In the tail region, we observe strong expression of EGFP in the neuroepithelium posterior to the forming somites (Fig. 2C, D). In addition, we find EGFP in the posterior definitive endoderm with an anterior border at the 4th somite pair (Fig. 2E-I), and in the presomitic paraxial mesoderm (Fig. 2C and J). The latter correlates well with previous data showing that *Hes1* is a part of the segmental clock in the presomitic mesoderm (Jouve et al., 2000). Anteriorly, we find EGFP expression in the endoderm of the foregut and in the anterior intestinal portal (AIP) (Fig. 2K-M).

2.2 *Hes1* promoter-driven EGFP expression at e9.0 and e10.5

Analysis of e9.0 embryos by whole mount immunohistochemistry shows continued expression of EGFP in the endodermal epithelium of the primitive gut tube. The dorsal Pdx1-positive pancreatic primordium shows strong EGFP expression and marks the anterior border of the posterior, dorsal endodermal expression domain at the level of the 4th somite pair (Fig. 3A). Only the dorsal part of the gut tube from the pancreas bud to the posterior part of the embryo is positive for EGFP, and at the most posterior end we also detect EGFP expression in the notochord (Fig. 3B). The ventral pancreatic progenitor cells marked by Pdx1 expression as well as the ventral part of the primitive gut tube posterior to the Pdx1 domain do not express EGFP at this stage (Fig. 3A). However, there is EGFP expression in the ventral foregut endoderm anterior to the ventral pancreas, but this does not appear to be in liver progenitor cells as there is no overlap between EGFP and the expression of *Prox1* (Fig. 6A-C) (see section 2.3.3). At e9.0, we also observe *Hes1*-EGFP expression in the mesonephric ridge and the presomitic mesoderm (Fig. 3A). In the head region of the embryo, EGFP expression is detected in the otic pit epithelium (Fig. 3A) demonstrating *Hes1* expression before the development of the primordial cochlea where expression of *Hes1* mRNA has been reported previously (Murata et al., 2009). Additionally, the optic vesicles express EGFP (Fig. 3A), a prelude to the *Hes1*-EGFP expression previously described in the mouse e13.5 retina (Ohtsuka et al., 2006). We also find EGFP expression in the frontonasal process and in the pharyngeal region (Fig. 3A) corresponding to *Hes1* mRNA expression described by Rochais et al. (Rochais et al., 2009). Moreover, there is EGFP expression in the neuroepithelium of forebrain, midbrain, and hindbrain (Fig. 3A).

Slightly later at e9.5, endodermal EGFP expression becomes confined to the dorsal pancreas anlage (Fig. 3C, D), whereas the more posterior dorsal gut tube epithelium as well as the ventral foregut epithelium both have ceased EGFP expression (Fig. 3C, D). EGFP expression in the mesonephric ridge becomes more pronounced (Fig. 3C).

At e10.5, the formation of many organs is more defined, and *Hes1*-EGFP expression has now become organ specific as seen by expression in the mesonephric tubules and in both pancreatic buds (Fig. 3E, F, F'). The EGFP expression is less intense in the dorsal pancreas bud compared to the mesonephros and even lower in the ventral pancreas bud (Fig. 3F', inset). A strong EGFP signal remains in the tail region (Fig. 3E), where continued *Hes1* expression is required in the presomitic mesoderm for normal somite segmentation (Jouve et al., 2000), but also the notochord and the posterior hindgut epithelium show EGFP expression in the most posterior part of the tail bud (Fig. 3G). Additionally, we see EGFP expression in the epidermal epithelium of the budding forelimbs (data not shown) corresponding with previous observations for *Hes1* mRNA (Rochais et al., 2009). In the head region of the e10.5 embryo, EGFP expression in the developing eye is now discernable to be in the inner neural layer of the optic cup (Fig. 3E). The neural EGFP expression is most pronounced in the telencephalic vesicle and the hindbrain, but also the neuroepithelium of the mesencephalon and the dorsal neural tube show significant EGFP expression (Fig.

3E). Overall, our findings of neural EGFP expression in e10.5 embryos reflect previously described neural expression of *Hes1* promoter activated EGFP (Ohtsuka et al., 2006).

2.3 Organ specific analysis of *Hes1* promoter-driven EGFP expression

Hes1 driven EGFP expression in the brain is well described by the GENSAT project (www.GENSAT.org) and in Ohtsuka et al. 2006 (Ohtsuka et al., 2006). We have therefore focused on the spatiotemporal expression pattern in different organs of the trunk, where the *Hes1* expression pattern is poorly characterized.

2.3.1 *Hes1*-EGFP expression in the developing pancreas—At e10.5, the pancreatic structures are well defined and distinct from the stomach, bile duct, and duodenum. Here, clear EGFP expression is seen in the dorsal pancreatic bud epithelium (Fig. 3F, F', 4A, D). The ventral pancreatic bud also displays EGFP expression although at a lower level (Fig. 3F inset, 4D). Others have reported *Hes1* immunoreactivity in the pancreatic mesenchyme at e11.5 (Seymour et al., 2007) and we see that as well when performing *Hes1* immunostainings (Fig. 1F), but we do not detect *Hes1*-EGFP expression in the pancreatic mesenchyme neither at e10.5 nor at e12.5 (Fig. 4A, B, D, E). These data correlate well with previous *in situ* hybridisation data (Lammert et al., 2000) demonstrating *Hes1* mRNA specifically in the e10.5 pancreas epithelium. However, we do see scattered EGFP expressing cells in the mesenchyme surrounding the duodenum (Fig. 4A, D, arrow heads).

At e12.5, EGFP is broadly expressed in the developing pancreas in both Pdx1 positive and Pdx1 negative cells (Fig. 4B, C, E) and the expression have reached similar levels in the dorsal and the ventral pancreas (not shown). This is in line with previous data showing co-expression of *Hes1* and Pdx1 in mouse e13.5 pancreas (Esni et al., 2004). It has also previously been demonstrated that *Hes1* expression is down regulated in glucagon positive cells in the e12.5 pancreas (Jensen et al., 2000), and we can confirm that many glucagon positive as well as insulin positive cells are negative for EGFP (Fig. 4B, E, arrow heads), but we also see examples of cells double positive for EGFP and glucagon (Fig. 4E, arrow) which can be explained by the longer half life of EGFP compared to endogenous *Hes1* mRNA and protein (Corish and Tyler-Smith, 1999; Hirata et al., 2002; Jouve et al., 2000). In the central epithelium, there is a quite uniform expression of EGFP whereas only a subset of cells in the tips of the forming branches are EGFP positive (Fig. 4C). We find that Carboxypeptidase A (*Cpa1*) is only expressed in tip cells with very low or no EGFP-expression (Fig. 4F). At e14.5, *Hes1*-EGFP expression is entirely restricted to the trunk epithelium and absent from the forming acini determined to become exocrine cells (Fig. 4G, K, O).

In the e17.5 pancreas, the EGFP expression is primarily observed in the central trunk epithelium, adjacent to insulin and glucagon expressing cells. EGFP is also seen in the exocrine tissue as scattered single cells or small clusters of 2-3 cells (Fig. 4H, L, P). These EGFP positive cell clusters all co-express the ductal marker *Sox9* (Fig. 4P inset) (Seymour et al., 2007).

At birth (P0), we find most of the EGFP expression located in proximity to the islets of Langerhans and the pancreatic ducts, but never in the differentiated insulin or glucagon positive cells (Fig. 4I, M). EGFP positive single cells can be observed in the central part of many acini (Fig. 4I, M, U, arrow heads). Eight days after birth (P8), EGFP expression is lost in the ducts and around the islets (Fig. 4J, N) but the single, scattered EGFP positive cells are still seen at the base of the acini (Fig. 4J, V, arrow heads) and they co-express *Sox9* (Fig. 4V inset) corresponding to the centroacinar cells in agreement with previous reports (Furuyama et al., 2010; Miyamoto et al., 2003). These cells can still be found in adult pancreas although they are very rare (Fig. 4X, arrow head and inset).

2.3.2 Hes1-EGFP expression in the developing duodenum—Immediately after closure of the gut endoderm at e9.0, we observe a Hes1-EGFP positive streak along the dorsal part of the prospective duodenum, posterior to the dorsal pancreas area (Fig. 3A). However, this expression disappears already at e9.5 (Fig. 3C, D), and at e10.5 there is only a few EGFP expressing cells left in the dorsal duodenal epithelium close to the pancreas (Fig. 5B), but many dispersed EGFP positive cells in the mesenchyme surrounding the developing duodenum and gut tube (Fig. 5A, B, arrow heads). At e12.5, there is a layer of EGFP expressing cells peripheral to the duodenal and midgut epithelium (Fig. 5C-E). These cells are located more peripherally than the smooth musculature marked by the expression of smooth muscle actin (Fig. 5C, E). Consistent with an absence of these cells from the e12.5 hindgut (data not shown), we observe *Hes1* expression to be overlapping with beta-III-tubulin expression, which marks enteric neurons (Fig. 5D).

At e14.5, EGFP expression appears in some but not all cells in the duodenal epithelium, and in the periphery we find a rim of cells with a strong EGFP signal together with a weaker EGFP signal in cells closer to the epithelium (Fig. 5G). We find EGFP expression in the e17.5 epithelium (Fig. 5H) confirming previously published data showing Hes1 immunoreactivity in the intestinal villi (Jensen et al., 2000). However, we do not find EGFP expression restricted to the intervillus regions (future crypts) and within the villus mesenchyme as others have previously detected by *in situ* hybridisation at e18.5 (Schroder and Gossler, 2002). Also at birth, we observe EGFP expression in cells distributed throughout the villus epithelium (Fig. 5I), whereas it becomes more intense near the crypts at postnatal day 8 (Fig. 5J). In adult tissue, we find an irregular distribution of EGFP expression in the duodenum. Occasionally, there is a single crypt which shows profound EGFP expression (Fig. 5K), and in other regions we find areas with EGFP expression along the villi (Fig. 5L), but parts of the duodenal tissue are negative for EGFP.

2.3.3 Hes1-EGFP expression in the developing liver—To determine if the EGFP expression observed in e9.0 ventral foregut endoderm anterior to the ventral pancreas is in the developing liver bud, we have made co-immunostainings for the liver and pancreas progenitor cells marker Prox1 (Burke and Oliver, 2002). Although there is nice co-expression of EGFP and Prox1 in the developing dorsal pancreas, we do not detect any EGFP expression in the Prox1 positive cells in the ventral part of the gut tube (Fig. 6A-C). Also at e10.5, we do not detect any EGFP expression in the developing liver bud epithelium or hepatoblasts (Fig. 6D, E), but at e12.5 and e14.5 we find EGFP expression in the Sox9 positive common bile duct epithelium (Fig. 6F, G) and in Sox9 positive primitive ductal cells along the portal veins (Fig. 6H, I). At e17.5, almost all the biliary epithelium along the portal veins express EGFP (Fig. 6J), and in newborn liver, the Sox9 positive bile ducts co-express EGFP (Fig. 6K). At P8, we still find EGFP positive cells along the portal veins and some bile ducts appear to be asymmetrical with EGFP expression in half of the epithelium whereas other bile ducts are completely negative for EGFP (Fig. 6L, M). These results correlate well with recent lineage tracing studies on Hes1 expression (Kopinke et al., 2011) and previous work showing asymmetrical Hes1 expression at e15.5 and symmetrical Hes1 expression at e18.5 in the developing bile ducts (Antoniou et al., 2009). We do not find EGFP expression in the adult liver (Fig. 6N) or in the common bile duct at e17.5 and older (data not shown).

2.3.4 Hes1-EGFP expression in the developing kidneys—We detect an incipient EGFP signal in the mesonephric ridge of e9.0-e9.5 embryos (Fig. 3A, C) that develops into strong EGFP expression in the mesonephric tubules at e10.5 (Fig. 3E, F and 7A) and e12.5 (Fig. 7B). At e14.5, Hes1-EGFP expression is observed in the tubules, the comma-shaped and the s-shaped bodies and the capsules of the maturing glomeruli (Fig. 7C). This confirms the *in situ* hybridisation results reported by others at e13.5 (Chen and Al-Awqati, 2005;

Piscione et al., 2004), except that we do not detect any EGFP expression in the condensed mesenchyme. At e17.5 and P0 we find EGFP expression primarily in stage I-III nephrons in the cortical region and in the collecting ducts, but also among the undifferentiated mesenchymal cells in the medullary region (Fig. 7D, D', E, E').

A shift in *Hes1* promoter activity can be observed between P0 and P8 where the EGFP expression is lost in the nephritic epithelia within the nephrogenic zone as the kidneys become close to fully developed. Only the EGFP expressing cells in the medullary interstitium remain (Fig 7F, F'). Kidney tissue from adult is completely devoid of EGFP expression (data not shown).

2.3.5 Hes1-EGFP expression in the developing lungs—*Hes1* mRNA expression has previously been detected by northern blot in foetal lungs from e12.5 to e18.5 (Ito et al., 2000). We find that the lung buds at e12.5 are mainly negative for EGFP expression except for a weak signal in the main bronchi epithelium (Fig. 8A). However at e14.5 and e17.5, we detect EGFP in the epithelial cell layer lining the bronchioles (Fig. 8B, C) correlating well with reported immunohistochemical detection of Hes1 protein in the bronchiolar epithelium at e16.5 (Collins et al., 2004). This expression pattern continues through birth (Fig. 8D) and into adulthood (Fig. 8E, F).

3. Discussion

Hes1 is expressed in multiple tissues during development, including organs derived from all three germ layers such as the brain, kidneys and pancreas (Chen and Al-Awqati, 2005; Jensen et al., 2000; Ohtsuka et al., 2006). In our hands, *Hes1* expression is difficult to detect reliably by *in situ* hybridisation and immunohistochemical techniques, and the reports on *Hes1* expression are often sparse and incomplete. Here we report a detailed analysis of the Tg(*Hes1*-EGFP)^{lHri} mouse strain that allows for easy investigation of *Hes1* expression. We assume that most if not all *Hes1* regulatory sequences are included in the BAC as it contains approximately 178 kb genomic sequence upstream and 43 kb downstream of the *Hes1* coding region. There are small discrepancies between EGFP expression and the immunohistochemical detection of Hes1 protein, and this can be due to the polyclonal antiserum not being absolutely specific to Hes1, or that it may take longer time for EGFP than Hes1 to reach detection levels, or the fact that EGFP has a longer half life than the tightly regulated Hes1 transcription factor, or that certain distant regulatory sequences is not included in the transgenic mouse. But in general, the results confirm previously described expression patterns of *Hes1* mRNA and Hes1 protein, indicating that the transgene has integrated in a region accessible for transcriptional regulation throughout development. There is no evidence for insertional mutagenesis as the mice breed well and develop normally both as heterozygotes and homozygotes.

In several tissues, *Hes1* mRNA and protein levels are tightly regulated and oscillates in a two hour cycle where Hes1 itself plays a role as a repressor of its own expression (Hirata et al., 2002; Kobayashi et al., 2009). This requires a very short half life of Hes1 compared to the relatively stable EGFP which has a half life of approximately 26 hours (Corish and Tyler-Smith, 1999). Therefore, the EGFP expression in this transgenic mouse line will reflect *Hes1* expression sites but not the post-transcriptional regulation.

Our analyses describe previously uncharacterised *Hes1* expression in the endoderm of late streak stage (e7.0) and in 5-6 somite (e8.0) embryos as well as in e9.0 dorsal gut tube epithelium. There is an anterior border of the caudal expression domain around the 4th somite pair that seems to stay fixed during growth of the embryo until e9.0 where it correlates with the anterior border of Pdx1 expression outlining the foregut/midgut boundary

(Zorn and Wells, 2009). The ventral foregut also shows *Hes1* expression bordering the liver diverticulum determined by *Prox1* expression and thus not including the prospective liver and the ventral pancreas. This suggests that *Hes1* may have a role in patterning of endodermal cells even before gut tube closure. The broad *Hes1* expression in the dorsal endoderm then becomes restricted to the dorsal pancreas epithelium at e9.5. *Sox17* has been shown to regulate ventral foregut patterning in conjunction with *Hes1*, where the timely appearance of *Hes1* is essential in order to repress *Sox17*, thereby defining the pancreato-biliary border with a *Sox17* positive biliary part and a *Hes1* positive pancreatic part (Spence et al., 2009). This model would predict that *Hes1* mutants should display pancreas to bile duct conversion, but actually the opposite seems to occur (Sumazaki et al., 2004). The entire pancreatic epithelium shows *Hes1* expression at e10.5, but as the pancreas starts to grow by branching morphogenesis at e12.5, *Hes1* expression becomes restricted to the trunk epithelium and disappears from the *Cpa1* positive cells. At e14.5, the tip cells destined to become exocrine acini are completely devoid of *Hes1* expression, which corresponds well with *Hes1* keeping pancreatic cells in a progenitor state. After the burst of endocrine differentiation at the secondary transition and towards the end of gestation, *Hes1* expression declines and in general we do not detect *Hes1* expression in the differentiated insulin or glucagon positive cells. However, we do occasionally find a few cells that are double positive for EGFP and a hormone, which is likely due to the longer half life of EGFP relative to *Hes1* protein and mRNA. As expected we detect *Hes1* expression in the centroacinar cells (Miyamoto et al., 2003).

Looking at other selected organs during embryonic development, we detect *Hes1* expression in neuronal class III beta-tubulin positive enteric neurons in the duodenal epithelium. Similarly, we observe an equivalent distribution of EGFP positive cells in the stomach mesenchyme at all stages examined (data not shown). In the duodenal/intestinal epithelium, the *Hes1* gene becomes active between e12.5 and e14.5 and is later found to be largely restricted to the proliferating crypt cells in neonatal and young animals. During liver development, we find the earliest *Hes1* activity at e12.5 in the common bile duct epithelium and at the first stages of intrahepatic bile duct differentiation. Here, *Sox9* has shown to be critical for bile duct formation (Antoniou et al., 2009) and it also marks progenitor cells in adult liver (Furuyama et al., 2010). In agreement with the work by Antoniou et al. (Antoniou et al., 2009), we find that *Hes1* activity in the biliary epithelium co-localise with *Sox9* expression during first the asymmetrical phase and later during the symmetrical phase of tubulogenesis, whereas mature bile ducts do not show *Hes1* expression. The kidneys show *Hes1* gene activity from the very early time points in the mesonephric ridge at e9.0, then in the mesonephric tubules, and later widely distributed in the first developmental stages of the nephrons. This correlates well with the more detailed descriptions of *Hes1* mRNA detection in the nephrons by Chen et al. and Piscione et al. (Chen and Al-Awqati, 2005; Piscione et al., 2004). However, there are some small discrepancies since we do not see *Hes1* activity in the condensed mesenchyme in the outer cortex as it has been reported at e13.5 by both groups (Chen and Al-Awqati, 2005; Piscione et al., 2004). This may reflect the delay in detection of EGFP expression compared to endogenous *Hes1*. In addition, the postnatal *Hes1* gene activity that we find in the medullary interstitium has not been described before. We expected to find *Hes1* gene activity in the developing lung buds at e12.5 as has been reported by *Hes1* northern blot analysis (Ito et al., 2000), but we could only detect a faint signal in the main bronchi and nothing in the segmental bronchi at that stage. Subsequently, we detect *Hes1* expression in the bronchiolar epithelia from e14.5 and onwards.

The results in this study support the concept of *Hes1* as a key component in progenitor cells as it is observed in the trunk epithelium in the developing pancreas. This agrees with the reports on pancreas hypoplasia in *Hes1* mutant mice (Jensen et al., 2000). *Hes1* expression in the developing common bile duct is in agreement with incorrect development of the

common bile duct in *Hes1* mutant mice (Sumazaki et al., 2004). Also in intrahepatic bile duct development, *Hes1* seems to play a role, but it has not been established whether it is in a Notch dependent manner (Antoniou et al., 2009). Along the same lines in the kidneys, it appears that *Hes1* primarily is active during development of the tubules, but it is not clear which role *Hes1* plays here.

The Tg(*Hes1*-EGFP)^{1Hri} mouse reported on here will be a useful tool to study the role of *Hes1* in a number of organ systems also allowing for prospective cell sorting.

4. Experimental procedures

Generation of Transgenic Mice

From the BACPAC Resources Center (<http://bacpac.chori.org/>) we obtained a BAC clone, GENSAT1-BX38, deposited by the GENSAT project (<http://gensat.org/>). The BAC contains 224,313 bp of chromosome 16, including 178 kb upstream of the transcriptional start site of the *Hes1* gene and 43 kb downstream of the *Hes1* gene. It also contains a 1.1 kb EGFP-PolyA fragment inserted into the 5'UTR of the *Hes1* gene, 27 bp upstream from the *Hes1* translation start codon. DNA from the BAC clone was prepared from *E. coli* cells using the NucleoBond® Xtra midi kit (MACHEREY-NAGEL), and purified according to (Nagy, 2003). The purified BAC DNA was resuspended in sterile filtered injection buffer (Moreira et al., 2004) to a final concentration of 1.5 ng/μl, kept on ice until injection, which was performed the same day. The DNA was injected into the male pronucleus of B6D2F2 zygotes which were then introduced into pseudopregnant NMRI female mice. Tail biopsies from 13 male offspring were positive for EGFP by PCR analysis, and all were mated with C57BL/6 female mice. Germ line transmission of EGFP expression in the embryos was analyzed by PCR (Primers: *Hes1* 5'-UTR: 5'-CGAGCGGTGCCGCGTGTCTCTTCCTCCC-3'; EGFP antisense: 5'-CGGCGAGCTGCACGCTGCCGTCCTC-3') and visually by fluorescence microscopy. Only one male founder gave rise to EGFP positive offspring. C57BL/6 female mice were time mated with heterozygous mutant males and EGFP positive embryos were identified by PCR using the primer pair described above. All mouse work conforms to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123).

Southern Blots

The two probes for verification of insertion of the BAC ends were amplified by PCR from purified BAC *Hes1*-EGFP DNA. Primers: Probe1: 5'-GGA TTG TTT GTC TAG GAT TTG AGG-3' and 5'-GGA AAG GAC ACT CTG ACC TGT T-3' (approx. 500 bp). Probe2: 5'-CTT TTT TGA TTT AGT GCA ATG CAC C-3' and 5'-GGT AGT GCA AGA CAA ACA AAC AAG-3' (approx. 500 bp). 3μg genomic DNA samples from a wild-type, a heterozygous, and a homozygous mouse were digested with BglII and EcoRV and subjected to standard Southern blot analysis with 100 ng radio labelled Probe1. Likewise 3μg genomic DNA samples were digested with NdeI and subjected to standard Southern blot analysis with 100 ng radio labelled Probe2.

Whole Mount Immunofluorescent Stainings

Whole mount immunofluorescent stainings were performed according to (Ahnfelt-Ronne et al., 2007) using the antibodies described below.

Immunohistochemical Stainings

Embryos were dissected in cold PBS and fixed O/N at 4°C in Lilly's formalin buffer, pH 7.4 (Bie & Berntsen, Denmark). The embryos were cryo protected O/N at 4°C in 30% sucrose in

PBS and then embedded in Tissue-Tek (Sakura, Denmark). The tissue was cryo sectioned at 10µm. Immunostainings were performed according to (Klinck et al., 2008) using the antibodies described below.

Antibodies

Primary antibodies: Rabbit anti-GFP “Living Colors” (Clontech) diluted 1:2000. Goat anti-GFP (Abcam) diluted 1:1000. For the whole mount GFP stainings a Tyramide Signal Amplification (TSA) step was applied (PerkinElmer, USA). Rabbit anti-Hes1 1:800 with TSA (kind gift from Dr. T. Sudo), Goat anti-Sox17 (R&D systems) diluted 1:1000. Guinea Pig anti-Insulin (Abcam) diluted 1:500. Goat anti-Pdx1 (kind gift from Chris Wright), diluted 1:10.000. Mouse anti-E-cadherin (BD Biosciences) diluted 1:1000. Rat anti-E-cadherin (R&D Systems) diluted 1:1000. Guinea pig anti-Glucagon (Millipore) diluted 1:10000. Goat anti-Carboxypeptidase A (R&D Systems) diluted 1:1000. Mouse anti smooth muscle actin (DAKO) diluted 1:500. Rabbit anti-beta-III-tubulin (TuJ1) (Bio Site) diluted 1:1000. Rabbit anti-Sox9 (Millipore) diluted 1:500. Goat anti-HNF4a (Santa Cruz) diluted 1:100. Secondary Antibodies: Whole IgG biotin anti-rabbit (cat. #711-065-152; for TSA amplification), Cy2-anti-goat (cat. #705-225-147), Cy3-anti-rabbit (cat. #711-165-152), Cy3-anti-rat (Cat. No. 712-165-153), Cy5-anti-mouse (cat. #715-175-151), all from Jackson ImmunoResearch Laboratories raised in donkey. Diluted 1:500.

Confocal Microscopy

Images were obtained using a Zeiss LSM510 META Axio Imager connected to a LSM 510 laser module with the following lasers: Ar laser (488 nm; 50% laser power), He/Ne laser (543 nm), and He/Ne laser (633 nm) (Carl Zeiss; Germany). Embryos stained as whole mount were cleared in BABB (benzyl alcohol:benzyl benzoate 1:2) before scanning. Alignment of images to produce the composite e10.5 image was done manually with Canvas 9 (ACD systems). List of Objectives: Plan-Neofluar 10x/0.3, WD 5.5 mm; Achromplan 20x/0.5 W Ph2, WD 7.9 mm; Plan-Neofluar 25x/0.8, WD 0,21; EC Plan-Neofluar 40x/1,30 Oil DIC, WD 0.21 mm.

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Research highlights

- We have generated a transgenic *Hes1*-EGFP reporter mouse line, Tg(*Hes1*-EGFP)^{1Hri}.
- We present a characterization of *Hes1* expression in whole embryos from e7.0-e10.5.
- Detection of *Hes1* expression in the definitive endoderm before gut tube closure.
- We provide an analysis of *Hes1* expression in selected organs during development.

Figure 1.

The whole BAC Hes1-EGFP sequence is inserted in the genome of the $Tg(Hes1-EGFP)^{1Hri}$ mouse strain.

(A) The BAC Hes1-EGFP construct encompasses more than 224 kb of chromosome 16 from base pair number 29887283 to 30111596. This fragment includes the *Hes1* gene with the four exons shown as black boxes. EGFP shown in green is inserted in the 5'UTR. Restriction enzymes and the two probes for Southern blot verification of insertion of the BAC Hes1-EGFP ends are indicated (B) Southern blot on genomic DNA from wild-type, heterozygous, and homozygous Hes1-EGFP mice as indicated on the lanes, digested with BglII and EcoRV and hybridised with Probe1. The blot reveals the expected 6023 bp wild-type band in all three mice and a BAC Hes1-EGFP specific band around 4 kb in the transgenic mice. (C) Southern blot on genomic DNA from wild-type, heterozygous, and homozygous Hes1-EGFP mice as indicated on the lanes, digested with NdeI and hybridised with Probe2. The blot reveals the expected 4222 bp wild-type band in all three mice and BAC Hes1-EGFP specific bands of approximately 9 kb and 11 kb in the transgenic mice. (D) Hes1 immunofluorescence in the inner and outer layer of the optic cup at e10.5. (E) EGFP immunofluorescence on the adjacent section to D. (F) Hes1 immunofluorescence in the dorsal pancreas tissue at e10.5. (G) EGFP immunofluorescence on the adjacent section to G. (H) Hes1 immunofluorescence in the neural tube at e14.5. (I) EGFP immunofluorescence on the adjacent section to H. (J) Hes1 immunofluorescence in the oesophagus at e14.5. (K) EGFP immunofluorescence on the adjacent section to J. il, inner layer of optic cup; ol, outer layer of optic cup; dp, dorsal pancreas; d, duodenum; cc, central canal; rp, roof plate; el, ependymal layer; oe, oesophagus. Dorsal is towards the top. Scale bars are 50µm.

Figure 2.

Hes1 is expressed in the endoderm at late primitive streak stage and in the endoderm, presomitic mesoderm, and neuroepithelium in e8.0 mouse embryos.

(A) Whole mount immunostaining for EGFP in an e7.0 embryo of the Hes1-EGFP mouse strain. The endodermal cell layer is positive for EGFP expression. (B) An optical section of the embryo in A showing that it is only the endodermal cells and not the mesoderm nor the ectoderm that express EGFP. (C) Whole mount immunostaining of a 6 somite (e8.0) embryo of the Hes1-EGFP mouse strain. EGFP is in green and the endoderm marker Sox17 is in red. Posterior is to the left and anterior is to the right. It shows the area of strong EGFP expression in the posterior part of the embryo as well as the anterior expression area at the

anterior intestinal portal (AIP). **(D)** An optical section of another e8.0 embryo showing a ventral view of the strong EGFP fluorescence in the neuroepithelium posterior to the forming somites. **(E, E', E'')** An optical cross section of the embryo in C showing the EGFP fluorescence in the endodermal cell layer (E), marked by nuclear Sox17 expression in red (E'), and a merged image (E''). The epithelial marker E-cadherin is in blue. Posterior endodermal EGFP expression is detected from the 4th somite pair to the tip of the tail. **(F)** A whole mount image stack of an 8 somite embryo showing a more explicit EGFP expression in the posterior endoderm marked by Sox17 in red (arrowheads). **(G)** A higher magnification subset stack of the embryo in F showing more details of the posterior EGFP expression area. **(H)** An optical section from the image stack in G showing the EGFP fluorescence in the endodermal cell layer with an anterior border at the 4th somite pair (arrow). Also the expression in the neuroepithelium posterior to the forming somites is shown. **(I, I', I'')** An optical section of the embryo in F showing the EGFP fluorescence in the endodermal cell layer (I), marked by nuclear Sox17 expression in red (I'), and a merged image (I''). The epithelial marker E-cadherin is in blue. **(J, J', J'')** Another optical section of the embryo in F showing the EGFP fluorescence in the presomitic paraxial mesoderm (J), adjacent to the endodermal Sox17 expression in red (J'), and a merged image (J''). The epithelial marker E-cadherin is in blue. **(K)** A high magnification image stack showing the anterior expression area in the foregut endoderm and the AIP of the embryo in F. **(L)** A high magnification image stack showing the anterior expression area in the foregut endoderm and the AIP of the embryo in C. **(M)** An optical cross section from the image stack in L showing the EGFP expression in the foregut epithelium. The epithelial marker E-cadherin is in blue. nt: neural tube; nf: neural fold; fg: foregut; h: primitive heart. Scale bars are 100µm.

Figure 3.

Whole embryo overview of the Hes1-EGFP expression from e9.0 to e10.5. (A) Whole mount immunostaining of an e9.0 embryo (17 somite pairs) showing EGFP expression in green and the pancreatic progenitor cell marker Pdx1 in red. EGFP expression is seen in the dorsal pancreatic primordium (dp) with a clear anterior border of expression indicated by the dashed line. There is also EGFP expression in the ventral foregut endoderm (fg) but not in the ventral pancreatic primordium (vp). EGFP expression is also seen in the dorsal part of the gut tube posterior to the pancreas area, in the mesonephric ridge (mr), and in the presomitic mesoderm (psm). Moreover, EGFP expression is observed in the branchial arches (b), the pharyngeal region (ph), the otic pit (o), and optic vesicle (op). EGFP expression is also detected in the frontonasal process (fp) and the neuroepithelium of forebrain, midbrain,

and hindbrain. Scale bar is 100 μ m. **(B)** Cross sectional view of the tail bud at e9.0 (17 somite pairs) showing EGFP expression in neural tube (nt) and psm, but also in the notochord (n) and the dorsal endoderm of the posterior hindgut (hg). Co-immunostaining for the epithelial cell marker E-cadherin is in blue. Scale bar is 100 μ m. **(C, D)** Whole mount immunostaining at e9.5 (20 somite pairs in C, 26 somite pairs in D) showing EGFP expression in the dorsal pancreas. Scale bars are 200 μ m. **(E)** Whole mount immunostaining at e10.5 showing EGFP expression in green and the epithelial cell marker E-cadherin in blue. EGFP expression is seen in the mesonephric tubules (m), dorsal pancreatic bud (dp), retina (r), the tail bud, the telencephalic vesicle (t), and the neuroepithelium of midbrain (m), hindbrain (h), ventricular zone (vz), and dorsal neural tube. Scale bar is 500 μ m. **(F, F')** Higher magnification images of the pancreas and mesonephros of the e10.5 embryo shown in E. Co-staining for Pdx1 is shown in red. **(F', inset)** The ventral bud of the pancreas (vp) is also positive for EGFP expression when analysed at a higher magnification. Scale bars are 200 μ m and 50 μ m in inset. **(G)** Cross sectional view of the tail bud at e10.5 showing EGFP expression in the notochord (n) and posterior hindgut (hg), but also in the mesoderm and the neural tube (nt). Scale bar is 100 μ m.

Figure 4.

Hes1 promoter activity at different stages in the developing pancreas.

Cross sections of mouse pancreas at embryonic days e10.5 (**A, D**), e12.5 (**B, C, E, F**), e14.5 (**G, K, O**), e17.5 (**H, L, P**), neonatal P0 (**I, M, U**), postnatal P8 (**J, N, V**), and one year old adult (**X**) all stained by immunofluorescence for EGFP expression shown in green. (**A, B, G-J**) Co-stainings for Pdx1 expression shown in red and insulin expression shown in blue. (**D, E, K-N**) Co-stainings for Pdx1 expression shown in red and glucagon expression shown in blue. (**C**) Co-staining for Pdx1 expression shown in red. (**F**) Co-staining for Carboxypeptidase A (Cpa1) expression shown in red. (**A, D**) EGFP is detected throughout both the dorsal (dp) and the ventral (vp) pancreatic epithelium at e10.5. There is no EGFP in

the surrounding pancreatic mesenchyme, while the mesenchyme around the duodenum (d) contains scattered EGFP positive cells (arrow heads). **(B, E)** EGFP-expression is seen in the majority of the e12.5 pancreatic epithelium, but also Pdx1-negative cells can be found that express EGFP. Cells expressing insulin and glucagon are often negative for EGFP (arrow heads), but we also find EGFP to be co-expressed with the hormones (arrow). **(C)** In the e12.5 tips of the branching structures, EGFP is only expressed in some of the cells. **(F)** Cpa1 is expressed in the branching tip cells which have very low amounts or no EGFP (small arrow heads). **(G, K, O)** At e14.5, the entire central epithelium shows strong EGFP expression whereas the branching tips are completely negative for EGFP. Insulin and glucagon positive cells are also negative for EGFP. **(H, L, P)** At e17.5, EGFP expression is found as large clusters in connection with regions of developing endocrine cells in the central areas of the tissue. In the developing exocrine part, small clusters of 2-3 cells or single cells that co-express EGFP and Sox9 are observed. **(I, M, U)** In neonatal pancreas tissue (P0), EGFP is detected in and around the ducts and the islets of Langerhans but never overlapping with neither insulin nor glucagon. Single EGFP positive cells are often seen in the centre of the acini (arrow heads). **(J, N, V)** Eight days after birth (P8), the islets are completely devoid of EGFP expression. However, the scattered EGFP positive single cells among the exocrine cells remain (arrow heads) and they co-express Sox9 (inset). **(X)** Very rarely, EGFP positive single cells among the exocrine cells can be found in adult pancreas tissue (arrow heads). They co-express Sox9 (inset). Scale bars are 50 μ m.

Figure 5.

Hes1 promoter activity in the developing duodenum.

(A) Whole mount immunostaining showing the duodenal region in a lateral view of an e10.5 embryo. E-cadherin (Cdh1) staining in blue outlines the epithelium, and staining for EGFP is in green. The dorsal (dp) and ventral (vp) pancreas epithelia are EGFP positive as well as scattered cells in the duodenal mesenchyme (arrow heads). d: duodenum; gb: gall bladder.

(B) Cross section of e10.5 duodenum (d) showing the scattered EGFP positive cells in the surrounding mesenchyme (arrow heads), but also some EGFP in the dorsal epithelium. (C-F) Cross sections of e12.5 duodenum. (C) Shows a layer of green EGFP positive cells at the periphery of the mesenchyme. Immunostaining for smooth muscle actin (SMA) in red labels

the smooth muscle cells and Cdh1 in blue outlines the epithelium. **(D)** Immunostaining for the neuronal marker β -III-tubulin in red on the adjacent section to **(C)** shows overlapping expression with EGFP in the enteric neurons. **(E, F)** Immunostaining for SMA in red and EGFP in green in duodenum in an e12.5 Hes1-EGFP transgenic embryo **(E)** and a wild-type littermate **(F)** showing that the EGFP signal in the enteric neurons is not background fluorescence. **(G)** At e14.5 there are EGFP positive cells appearing in the duodenal epithelium and in the mesenchyme there is strong EGFP expression in a cell layer in the outer periphery and a weaker EGFP signal in the mesenchyme closer to the epithelium. **(H)** In e17.5 duodenum, EGFP in green is detectable in the epithelium but no longer in the mesenchyme. **(I)** Staining for EGFP expression in green shows continued Hes1 promoter activity in the epithelium at P0. **(J)** At P8, the EGFP expression is less abundant but still found in the epithelium and primarily seen in the crypts. **(K, L)** In one year old adult tissue, a crypt is occasionally found to express EGFP **(K)**, or there are regions with EGFP expression along the villi **(L)**. In all panels **(G-K)**, EGFP expression is in green and the epithelium is outlined by Cdh1 staining in red. Panel **(L)** shows a co-staining of EGFP in green with Sox9 positive crypt cells in red. Scale bars are 50 μ m in A-F and 100 μ m in G-L.

Figure 6.

Hes1-promotor activity in the developing liver.

(A, A', A'') Optical section from a whole mount immunostaining of an e9.0 embryo (17 somite pairs) showing EGFP expression in the dorsal pancreas (dp) and the ventral foregut (fg) in (A), expression of Prox1 in the dp and the liver diverticulum (ld) in (A'), and the merged image in (A''). Scale bar is 100 μ m. (B, C) Cross sections of e9.0 primitive gut at (B) the most anterior part of the liver diverticulum (ld), and (C) through the middle of the ld. EGFP expression co-localise with Prox1 in the dp but there is no detectable EGFP in the Prox1 positive developing liver cells. Scale bars are 100 μ m. (D, E) Adjacent sections of e10.5 liver bud (lb) showing (D) Hnf4a expression in the hepatoblasts (hb) and (E) Sox9 in the liver bud epithelium. EGFP is expressed together with Sox9 in the dorsal pancreas (dp) but not in the developing liver tissue. (F, G) e12.5 and e14.5 common bile duct (cbd) epithelium co-express Sox9 and EGFP. (H) Two Sox9 positive neighbouring cells along the e12.5 portal vein (pv). One of the two cells is also expresses EGFP. (I) At e14.5, many cells along the portal veins co-express EGFP and Sox9. (J, K) At e17.5 and P0, most of the biliary epithelium co-express EGFP and Sox9. (L, M) There are many EGFP positive cells along the portal veins at P8, but regarding the bile ducts there are two different observations where (L) shows an asymmetrical bile duct with EGFP expression in half of the epithelium and (M) shows a bile duct with no EGFP expression. (N) In adult (one year old) liver, we have not found any EGFP positive cells. Scale bars are 50 μ m in (D-N). Asterisks mark the lumen of the bile ducts.

Figure 7.

Hes1-promotor activity in the developing kidneys.

(A) Whole mount immunostaining showing the mesonephros in a lateral view of an e10.5 embryo. Hes1 promoter-driven EGFP in green is seen in the mesonephric tubules (mt) but not in the mesonephric ducts (md). Staining for E-cadherin in red outlines the epithelium. (B) Cross section of e12.5 metanephros showing persistent EGFP expression in the tubules. Co-staining for E-cadherin is shown in red. (C) Cross section of e14.5 metanephros shows EGFP expression in all the epithelialized derivatives of the metanephric mesenchyme. Co-staining for E-cadherin is shown in red. (D, D', E, E') Cross sections at e17.5 and P0 show that there is EGFP expression in stage I-III nephrons in the cortical region and in collecting ducts and mesenchymal cells in the medullary region. (F, F') Cross section at P8 shows EGFP expression in cells only in the medullary interstitium. Scale bars in (A-C) are 50µm. Scale bars in (D-F) are 100 µm.

Figure 8.

Hes1-promotor activity in the developing lungs.

(A) Cross section showing a weak EGFP immunoreactivity (green) in the main bronchus (mb) epithelium of the e12.5 lung bud (arrow), but no EGFP expression in the segmental bronchus (sb) epithelium marked in red by Sox9 co-immunostaining. Cdh1 marks the epithelium in blue. oe, oesophagus. (B) Cross section of e14.5 developing lung tissue shows EGFP expression in green in the epithelium of the primary bronchus. (C) e17.5 cross section showing EGFP immunostaining in the epithelium of the bronchioles. (D, E, F) Cross sections of P0, P8, and adult lung tissue showing persistent EGFP immunoreactivity in the epithelia of the central bronchioles. Scale bars are 50µm.