Understanding urothelial stem cell biology and differentiation has been limited by the lack of methods for their unlimited propagation. Here, we establish mouse urothelial organoids that can be maintained uninterruptedly for >1 year. Organoid growth is dependent on EGF and Wnt activators. High CD49f/ITGA6 expression features a subpopulation of organoid-forming cells expressing basal markers. Upon differentiation, multilayered organoids undergo reduced proliferation, decreased cell layer number, urothelial program activation, and acquisition of barrier function. Pharmacological modulation of PPARγ and EGFR promotes differentiation. RNA sequencing highlighted genesets enriched in proliferative organoids (i.e. ribosome) and transcriptional networks involved in differentiation, including expression of Wnt ligands and Notch components. Single-cell RNA sequencing (scRNA-Seq) analysis of the organoids revealed five clusters with distinct gene expression profiles. Together with the use of γ-secretase inhibitors, scRNA-Seq confirms that Notch signaling is required for differentiation. Urothelial organoids provide a powerful tool to study cell regeneration and differentiation.
Urinary bladder diseases, most notably cystitis and bladder cancer, are important medical problems that generate high costs to the health systems worldwide. The bladder, ureters, renal pelvis, and part of the urethra are lined by a multilayered epithelium with some features reminiscent of the skin epidermis. The urothelium consists of three cell types (basal, intermediate, and umbrella) organized in 3–7 layers, with considerable species-related variation (Fig. 1a). In the mouse, basal cells are small, cuboidal, and express CD44 and KRT5; a fraction thereof express KRT14 and have stem cell properties. Intermediate cells are larger, express KRT5, KRT8, KRT18, and uroplakins UPK1a, 1b, 2, 3a and 3b. Luminal umbrella cells are...
largest, multicellular, highly specialized cells expressing high levels of uroplakins and KRT20–24. Umbrella cells constitute the physiological barrier to the passage of water, electrolytes, and urea through tight junctions, responsible for the high resistance paracellular pathway; a role for other urothelial cell types in barrier function has not been demonstrated8. Unlike the skin epidermis, the urothelium has a very slow turnover yet it preserves a robust capacity to restore epithelial integrity upon damage1,8.

Several key transcription factor networks involved in urothelial proliferation/differentiation have been identified using 2D cultures and genetic mouse models. PPARγ is expressed in the urothelium throughout embryonic development and in the adult9 and it has been shown to participate in proliferation and differentiation, cooperating with FOXA1, KLF5, and EGFR signaling10–12. The Southgate laboratory has shown that EGFR inhibition can potentiate the activity of PPARγ agonists and upregulate the expression of urothelial differentiation markers13,14. Retinoic acid signaling also plays an important role in the differentiation of urothelial cells during development and in tissue regeneration upon damage in the adult bladder15. However, the role of these pathways in urothelial differentiation is incompletely understood, in part due to the lack of methods to continually propagate normal cells, and improved cellular models are critically required.

In recent years, three-dimensional (3D) organoids have become a powerful tool to study the molecular and cellular basis of epithelial differentiation, allowing consistent culture and perpetuation16. Organoids are derived from cells capable of self-renewal and self-organization through cell sorting and lineage commitment in an in vivo-like manner17. The Cleverson laboratory has pioneered the establishment of organoids from a wide variety of epithelia, including mouse small intestine16, liver18, prostate19, and pancreas20. Organoids facilitate studying tissue biology, modeling disease, drug screening, and establishing a solid basis for regenerative medicine and gene therapy21. The majority of published studies have focused on organoids derived from simple epithelia. Recently, Lee et al.22 have reported the establishment of organoids from human bladder tumors and Mullenders et al.23 have described the features of normal mouse basal organoids and human bladder organoids. However, these reports have not explored in depth the potential of urothelial organoids to understand urothelial biology.

Here, we establish and characterize healthy tissue-derived mouse urothelial organoids and show that high CD49f (integrin α6, ITGA6) expression (CD49fhigh) characterizes a urothelial cell population containing stem cells able to self-perpetuate as organoids. We define their requirements for growth and differentiation and demonstrate their functional properties including barrier formation. Using bulk transcriptomics we identify a role for the Notch pathway in urothelial differentiation. scRNA-Seq allowed us to unveil gene expression signatures featuring Basal, Basal-Proliferative, Intermediate, and Luminal urothelial cells. This analysis further supports that expression of Notch target genes is transiently activated during urothelial differentiation. The organoids described should facilitate and accelerate the study of the molecular pathophysiology of bladder diseases, including the interaction of epithelial cells with pathogens and the mechanisms involved in malignant transformation of the urothelium.

Results

Urothelial organoids can be established and perpetuated. To establish organoids, we isolated cells from digest of urothelial scrapings. This unselected cell population allowed establishing urothelial organoids that could be consistently passaged. To characterize the cell populations present therein, we used flow cytometry analysis with specific antibodies detecting leukocytes (CD45), fibroblasts (CD140a), endothelial cells (CD31), and erythrocytes (Ter119); approximately 20% of single cells lacked these markers. The majority of the cells lacking these markers were EpCAM+ (>60%), indicating their epithelial nature (Supplementary Fig. 1a).

Unselected cells from urothelial digest, or EpCAM+ cells sorted from them, cultivated in Matrigel with complete medium (including EGF, LY2157299, Noggin, WNT3A, RSPO1)21,24 led to growth of multilayered organoids over 1 week. Under these conditions, lumen-containing organoids were very rare (Fig. 1b) and lumen-containing organoids were very rare (Fig. 1b). Organoids could be consistently passaged and maintained in culture uninterrupted for >1 year with stable morphology but a tendency for organoids to become enriched in cells with basal features over time was noted (Supplementary Fig. 1b–d). Unless otherwise indicated, the experiments reported used unsorted urothelial cells and >2 independent organoid cultures at passage <10.

To identify critical growth factors required for organoid formation, we performed leave-one-out experiments where we removed each complete medium component individually or in combination. At day 7, we observed a statistically significant reduction of organoid number upon omission of EGF, WNT3A, RSPO1, or WNT3A + RSPO1 (Fig. 1b, c). As reported for other tissues, Noggin was not required to establish organoids but it was essential for long-term perpetuation25. Despite their high proliferative potential, organoids did not form tumors upon xenotransplantation under the skin (Supplementary Fig. 1e) or in the kidney capsule.

CD49fhigh defines cells with organoid-forming capacity. To define the cell type of origin of the organoids, we isolated urothelial cell subpopulations based on marker expression and size.
CD49f expression identifies a cancer cell population with basal features\(^2\), suggesting that it might serve as a stem cell marker. Immunofluorescence analysis of normal mouse bladder showed that CD49f is expressed both in the epithelium and in the lamina propria; in the urothelium, CD49f selectively labels basal cells (Fig. 1a)\(^2\). Compared to CD49f\(^{low}\) cells, FACS-purified CD49f\(^{high}\) cells were enriched in basal markers whereas CD49f\(^{low}\) cells were enriched in luminal markers (Supplementary Fig. 1f). Lectins have also been used effectively as cell-type specific markers. Therefore, we screened a panel of lectins and found that the cytoplasm and plasma membrane of umbrella cells is strongly labeled by wheat germ agglutinin (WGA), while the remaining urothelial cells are weakly labeled (Fig. 1a), FACS-sorting of freshly isolated urothelial EpCAM\(^{+}\) cells on the basis of CD49f, WGA-binding, and cell size—which augments towards the lumen—showed that CD49f\(^{high}\)/WGA\(^{+}\) cells (basal) have the highest organoid-forming capacity. By contrast, CD49f\(^{low}\) cells (intermediate and luminal) were essentially unable to form organoids, regardless of WGA labeling or size (small vs. large) (Fig. 1d). CD44 has also been proposed as an urothelial stem/basal cell marker\(^3\), most CD44\(^{+}\) cells were CD49f\(^{+}\) and both CD49f\(^{high}\)/CD44\(^{high}\) and CD49f\(^{high}\)/CD44\(^{low}\) cells were able to form organoids displaying a similar phenotype (Fig. 1e, Supplementary Fig. 1g, h). However, the number of organoids was highest in the CD49f\(^{high}\)/CD44\(^{high}\) population (\(P = 0.029\)) (Fig. 1f). Low-density seeding of freshly isolated urothelial cells (1–100 cells/drop) showed that CD49f\(^{high}\) cells have a markedly increased clonal growth capacity and that organoids can be established from single cells only from this cell population (Fig. 1g). GFP- and Tomato-labeled sorted cells derived from organoids were mixed prior to seeding in Matrigel at 1:1 ratios and only single-colored organoids were identified, supporting the notion that organoids are monoclonal in origin (Fig. 1h). Altogether, these results indicate that CD49f\(^{high}\) and CD44\(^{high}\) label the urothelial cell population with highest organoid-forming potential.

### Organoids recapitulate urothelial differentiation and function

To induce differentiation, organoids that had been maintained for 7 days in proliferation conditions (P) were cultured in medium without EGF, LY2157299, Noggin, WNT3A, and RSPO1 (differentiation medium, D) for an additional 7 days: a dramatic morphological change was observed, including an increase in diameter and organoid lumen formation, and reduced cell layer thickness (Fig. 2a–d). In proliferation conditions, organoids expressed high levels of Ccn1 transcripts and Ki67 and resemble basal cells expressing Cd49f, Cd44, Tp63, Krt14, and Krt5 and low levels of uroplakins (Fig. 2e–g). By contrast, upon differentiation, organoids showed marked downregulation of cell cycle mRNAs and proteins, a modestly decreased expression of basal markers, and upregulation of mRNA expression of Foxa1 and Ppary, intermediate cell markers (Krt8 and Krt18), uroplakins (Upk2 and Upk3a), and Krt20 (Fig. 2e–g). The corresponding proteins displayed the canonical distribution observed in the urothelium: TP63 and CD49f were found in the outer layer of proliferative organoids while PPAR\(^{y}\) and UPK3a displayed heterogeneous expression in cells lining the lumen of differentiated organoids (Fig. 2f, g). Expression of KRT14 and KRT5 persisted in differentiated organoids, possibly reflecting the half-life of these proteins and the slow differentiation dynamics of urothelial cells in tissues. KRT20 was generally undetectable at the protein level, as were multicellular umbrella cells.

Functional competence of organoids was assessed using urothelial barrier assays based on paracellular diffusion of FITC-labeled low molecular weight dextran (FITC-dextran) and fluorescence recovery after photobleaching (FRAP) (Fig. 3a–d). Urothelial organoids were cultured with medium containing FITC-dextran during both proliferation and differentiation stages. Prior to photobleaching, the lumen of D organoids showed a significantly higher normalized FITC intensity than the lumen of P organoids, suggesting epithelial layer tightness (Fig. 3b, c). After photobleaching, and during a recovery period of up to 14 h, differentiated organoids proved to be impermeable to FITC-dextran whereas proliferative cultures were heterogeneous and contained a mixture of impermeable and permeable organoids (Fig. 3b, d, Supplementary Movie 1). The differences in barrier function acquisition were statistically significant and increased over time of recovery. These findings confirm the ability of organoids to acquire features of differentiated urothelium.

**PPAR\(^{y}\) activation and EGFR inhibition enhance differentiation.** We used the PPAR\(^{y}\) agonist Roziglitazone (Rz) and the EGFR inhibitor Erlotinib to determine if they could further induce urothelial differentiation. P organoids cultured for an additional 7 days in the presence of Rz + Erlotinib acquired larger lumina and showed significantly lower Ki67 labeling, when compared to untreated samples (Fig. 4a, b). Cd49f and Cd44 mRNAs were down-regulated while uroplakin transcripts and proteins were up-regulated (Fig. 4a–c). In D organoids, Rz or Erlotinib alone caused reduced expression of Cd49f and Cd44 mRNAs (Supplementary Fig. 2a). When combined, they led to highest Foxa1 and uroplakin mRNA expression and to a significant reduction of lumen formation. UPK expression and lumen formation were often, but not always, correlated. There were no major changes in K67 and cleaved-caspase-3 labeling upon culture of differentiated organoids with Rz + Erlotinib (Fig. 4a, b). Treatment of organoids with the PPAR\(^{y}\) inverse agonist T0070907 at the initiation of the differentiation protocol had minor effects on lumen formation, K67, and UPK3a expression (Fig. 4a–c, Supplementary Fig. 2b, c), suggesting that pathways other than PPAR\(^{y}\) activation contribute to differentiation. KRT20 was not detected in any of the conditions analyzed. These results indicate that PPAR\(^{y}\) activation, together with EGFR inhibition, effectively promote urothelial organoid differentiation.

**Cells in differentiated organoids can re-enter the cell cycle.** To determine whether cells could re-enter the cell cycle after inducing differentiation, organoids cultured in differentiation medium, or in differentiation medium supplemented with Rz and Erlotinib, were maintained for an additional 7 days in complete medium (Fig. 5a–c, Supplementary Fig. 3). In both cases, differentiated organoids (D→P and D + Rz + Erlo→P, Fig. 5a) acquired a multilayer organization similar to that of proliferative organoids and basal cells showed uniform expression of KI67 and TP63 (Fig. 5b). However, the differences in marker gene expression at the mRNA level were modest, possibly due to pre-existing transcripts (Fig. 5b). The wide re-expression of KI67 in the basal cell layer strongly suggests that incompletely differentiated urothelial cells re-enter the cell cycle rather than overgrowth of a low-frequency stem cell population (Fig. 5c). Accordingly, 88.9% of cells in differentiated organoids were CD49f\(^{high}\) (Supplementary Fig. 3c). Organoids maintained in differentiation medium (D→D) showed highest mRNA expression of Foxa1, Ppary, and uroplakins; however, smaller lumina and higher cell death were noted (Supplementary Fig. 3b, c). These results indicate that organoids acquire a cyto-organization and differentiation characteristic of the urothelium and retain proliferative potential.

**Transcriptome analysis unveils urothelial differentiation pathways.** To interrogate the transcriptomic programs governing organoid differentiation we performed RNAseq of three
independent paired P and D organoid cultures (Supplementary Fig. 4a). Principal component analysis (PCA) showed that proliferative organoids clustered closely whereas their differentiated counterparts showed greater transcriptome divergence (Supplementary Fig. 4b); 4100 genes were differentially expressed (FDR < 0.05; Supplementary Data 1). Among the top upregulated transcripts in P conditions were those involved in cell cycle (i.e. Cdk1, Aurkb, Ccnb2, and Ki67), inhibition of apoptosis (Birc5), epidermal differentiation (i.e. Sprr2f, Crnn, Stfa3), cytoskeletal regulation (Kif15, Kif4), and stemness (Cd34). By contrast, transcripts significantly up-regulated upon differentiation included those involved in urothelial cell functions (i.e. Upk1a, Upk2, Upk3a), glycosylation (i.e. Wbscr17, Ugt2b34, Galnt14), and TGF-β signaling (i.e. Fstl1, Ltbp1, Fstl4) (Supplementary Data 1). Of note, several genes involved in xenobiotic metabolism (i.e. Cyp2f2, Adh7, Gstm1) are among those differentially expressed, underscoring the relevance of this pathway to bladder carcinogenesis29. Manual curation revealed the downregulation of canonical basal...
growth factor-depleted organoids recapitulate the urothelial differentiation program. 

**Fig. 2** Growth factor-depleted organoids recapitulate the urothelial differentiation program. a Experimental design applied to induce urothelial organoid differentiation: organoids cultured until day 7 in complete medium were maintained for seven additional days in differentiation medium. b Image of organoids displaying the features quantified in panel c: d, diameter; l, lumen; t, thickness of the epithelial layer. The signal distribution was measured across the organoids as indicated by the arrow in both cases (scale bar, 100 μm). c Signal distribution (in microns) acquired by confocal microscopy displaying the quantification of organoid features (diameter) of individual proliferative (P) (n = 57) and differentiated (D) (n = 71) organoids; color code indicates the intensity of the signal: green, low; yellow, intermediate; red, high. d Violin plots representing organoid features. e RT–qPCR analysis of expression of genes regulated during differentiation. Data are normalized to Hprt expression (Mann–Whitney test, error bars indicate SD). f Western blot (WB) analysis showing expression of TP63 (basal marker), UPK3a, and UPK1b (luminal markers) in P and D organoids in three independent experiments. Urothelial bladder cancer cell lines (ScaBER, RT112, VMUCUB1, and RT4) were used as controls. g Immunofluorescence analysis of urothelial markers in P and D organoids. Normal urothelium is shown for comparison. DAPI staining is shown in blue (scale bar, 1000 μm). Source data are provided as a Source Data file.

**Fig. 3** Organoids cultured in differentiated conditions are functionally competent and acquire barrier function. a Experimental design to assess barrier function in organoid cultures using FITC-dextran and fluorescence recovery after photobleaching (FRAP). b Example of P and D organoids during the FRAP assay (pre-bleaching, post-bleaching and recovery—3.5 and 14 h) (scale bar, 1000 μm). c Quantification of FITC-dextran intensity of P (n = 5) and D (n = 7) organoids in the pre-bleaching phase showing specific FITC-dextran retention in differentiated organoids (Mann–Whitney test, error bars indicate SD). d Fluorescence recovery in P (n = 5) (left) and D (n = 7) organoids (middle); mean fluorescence recovery in P vs D organoids (Mann–Whitney test, error bars indicate SD). ‘p ≤ 0.05, **p ≤ 0.01. Source data are provided as a Source Data file.

urothelial markers (i.e. Krt14, Krt15, Cd44, Cd49f, and Trp63) and the upregulation of suprabasal markers (i.e. Krt19, Krt8, Foxa1, Ppary, Upk1a, Upk1b, Upk2, and Upk3a) in D conditions (Fig. 6a, Supplementary Fig. 4c), as well as the robust regulation of additional keratin species (Supplementary Fig. 4d).

Manual curation also revealed dynamic changes in expression of cell–cell adhesion genes. Transcripts coding for tight junction components showed two distinct expression patterns: Cldn1, Cldn8, Cldn12, and Cldn25 were down-regulated upon differentiation whereas Cldn3, Cldn4, Cldn7, Cldn23, Ocnn, Zo-1, Zo-2, and Zo-3 were up-regulated (Fig. 6b), suggesting distinct functions and cellular distribution for the corresponding proteins. Selected mRNA expression changes were confirmed in independent organoid samples and in normal urothelium (Supplementary Fig. 4e, f). Apical expression of ZO-1 and CLDN4 was confirmed in differentiated organoids (Fig. 6c). The predicted expression pattern of CLDN1, CLDN3, and CLDN4 was validated using information from the Human Protein Atlas (https://www.proteinatlas.org/). Epithelial impermeability and endocytic traffic were significantly up-regulated in D organoids, in agreement with results of the FITC-dextran assays described above.

Gene set enrichment analysis (GSEA) revealed a highly significant downregulation of the activity of pathways involved in cell cycle, DNA repair, RNA biology, protein synthesis, and cytokines in D organoids; this was accompanied by increased activity of pathways involved in epithelial differentiation/cell–cell adhesion, intracellular traffic (endocytosis, lysosome), and
Fig. 4 Pharmacological modulation of EGFR and PPARγ activity potentiates organoid differentiation. 

a. Representative phase contrast and immunofluorescence images of proliferative (P) and differentiated (D) organoids cultured in the presence of drugs modulating PPARγ (Roziglitazone, Rz) or EGFR activity (Erlotinib, Erlo) (scale bars: brightfield, 500 μm; immunofluorescence, 250 μm).

b. Quantification of lumen formation (Mann-Whitney test), Ki67, UPK3a, and cleaved-caspase-3 expression (Bonferroni test) in organoids cultured as described in panel a.

c. Heatmap representing RT-qPCR expression analysis of cell cycle and canonical urothelial differentiation markers in P or D organoids treated with Rz + Erlotinib, and with the PPARγ inverse agonist T0070907 (n = 2). *p ≤ 0.05, **p ≤ 0.01; ***p ≤ 0.001. Source data are provided as a Source Data file.
Fig. 5 Differentiated organoids are able to re-enter the cell cycle upon exposure to complete medium. 

a Experimental strategy: day 7 P organoids were maintained for 7 additional days either in complete medium (P), differentiation medium (D), or differentiation medium supplemented with Roziglitazone and Erlotinib (D + Rz + Erlo). D organoids were then switched to complete medium (D –> P; D + Rz + Erlo –> P) for 7 additional days. 

b RT-qPCR analysis of expression of genes regulated during differentiation. Data are normalized to Hprt expression (n = 1 biological replicate). 

c Representative phase contrast images of organoid cultures (scale bar, 500 μm), H&E staining, and immunohistochemistry for Ki67, TP63, and UPK3a (n = 1 biological replicate) (scale bar, 250 μm). Source data are provided as a Source Data file.
Fig. 6 Transcriptome analysis reveals organoid differentiation and identifies pathways involved therein. a Heatmap showing the expression (FPKM, RNAseq) of key urothelial differentiation genes in P and D organoids (n = 3/group; paired samples). b Heatmap showing the expression of genes related to tight junctions (claudins and tight junction/Zo proteins) (FPKM) in P and D organoids (n = 3/group). c Immuno fluorescence analysis of the expression of ZO-1 and KRT5; immunohistochemical analysis of CLDN4 in the same samples; L (Lumen) (scale bar, 250 μm). d Enrichment plots showing the upregulation of ribosome pathway genes and the downregulation of tight junction component genes in P organoids. e ISMARA analysis of top transcription factor motifs (ranked by z-scores) significantly enriched in the promoters of genes differentially expressed in P vs. D organoids; for the z-score of motifs enriched in D organoids. Source data are provided as a Source Data file.
signalization (phosphatidylinositol, Notch, Wnt, MAPK, mTOR) (Table 1).

Promoter motif analysis of differentially expressed genes using ISMARA revealed significant enrichment in motifs of TF involved in proliferation (E2F, MYC, MYB) and hypoxia regulation (HIF1A) in proliferative conditions. By contrast, the genes differentially expressed upon differentiation showed enrichment in motifs of TF involved in cell cycle genes (Fig. 8c, Supplementary Data 2). The Basal-Proliferative cluster was selectively enriched in cell cycle genes (Fig. 8c, Supplementary Data 2). The Basal-Proliferative cluster was identified as a marker of urothelial differentiation (Fig. 8a, b). The two Basal clusters identified in proliferative conditions shared expression of Krt14, Trp63, Igha6/Cd49f, and Ighb4 whereas the Basal-Proliferative was selectively enriched in cell cycle genes (Fig. 8c, Supplementary Data 2). The Intermediate-Low cluster was characterized by expression of basal and intermediate (i.e. Krt8) markers, transcripts of tight junction components (Tip1, Cldn4, Cldn7), and known (Klf5) as well as candidate (Foxq1) urethelial transcription factors. The Basal-Proliferative cluster was also found in organoids maintained in complete medium whereas the Luminal cluster was exclusive to the differentiated organoids (Fig. 8a, b). The two Basal clusters identified in proliferative conditions showed expression of Krt14, Trp63, Igha6/Cd49f, and Ighb4 whereas the Basal-Proliferative was selectively enriched in cell cycle genes (Fig. 8c, Supplementary Data 2). The Intermediate-Low cluster was characterized by expression of basal and intermediate (i.e. Krt8) markers, transcripts of tight junction components (Tip1, Cldn4, Cldn7), and known (Klf5) as well as candidate (Foxq1) urethelial transcription factors. The Basal-Proliferative cluster showed increased expression of genes up-regulated in the Intermediate-Low cluster and of Pscb, identified in this study as a marker of urothelial differentiation (Fig. 8c). The genenesets enriched in the Basal and Intermediate clusters were similar in proliferative and differentiated organoids. The Luminal cluster showed higher levels of expression of Intermediate cluster markers as well as of uroplakins (Fig. 8c).

Among the differentially expressed genes (DEGs) in the Basal clusters were those coding for several Wnt ligands (Wls, Wnt10a, Wnt4, and Wnt5a) (Fig. 8d). Importantly, components of the Notch pathway were also differentially expressed: Notch1 was enriched in Basal clusters whereas Jag1 and Hes1 were enriched in the Intermediate-Low and Intermediate clusters (P and D organoids, respectively) (Fig. 8d). These results suggest that the Notch pathway is transiently activated during urothelial differentiation.

An integrated analysis of both organoid scRNA-Seq datasets yielded five clusters with differentially expressed genes featuring Basal, Basal-Proliferative, Intermediate, Intermediate-High, and

### Table 1 GSEA showing the top 25 pathways (Kyoto Encyclopedia of Genes) significantly enriched in proliferative and differentiated organoids

| Proliferative organoids | Differentiated organoids |
|-------------------------|--------------------------|
| Ribosome                | Lysosome                 |
| DNA replication         | Endocytosis              |
| Cell cycle              | Focal adhesion           |
| Parkinsons disease      | Neuroactive ligand receptor |
| Huntington's disease    | Regulation of actin cytoskeleton |
| Oxidative phosphorylation | Phosphatidylinositol signaling system |
| Spliceosome             | Melanogenesis            |
| Pyrimidine metabolism   | Pathways in cancer       |
| Alzheimers disease      | Tight Junction           |
| Purine metabolism       | B cell receptor signaling |
| Base excision repair    | Axon guidance            |
| Mismatch repair         | Inositol phosphate metabolism |
| Oocyte meiosis          | Other glycan degradation |
| Nucleotide excision repair | Leukocyte transendothelial migration |
| Homologous recombination| Notch signaling pathway  |
| RNA polymerase          | Fcy R-mediated phagocytosis |
| RNA degradation         | Endometrial cancer       |
| Systemic lupus erythematous| Neurotrophin signaling pathway |
| Proteasome              | Wnt signaling pathway    |
| N-glycan biosynthesis   | Mapk signaling pathway   |
| Protein export          | Apoptosis                |
| Amino sugar and nucleotide sugar metabolism | Adherens Junction |
| Steroid biosynthesis    | Acute myeloid leukemia   |
| Progesterone-mediated oocyte maturation | Insulin signaling pathway |
| Cardiac muscle contraction | mTOR signaling pathway |

*All pathways significant with FDR q-value <0.05*
Luminal cells (Fig. 8e, Supplementary Fig. 7c). The DEGs characteristic of each cluster highly overlap with the ones of the individual analysis (Supplementary Fig. 8) suggesting a robust clusterization. Indeed, the Basal and a single Intermediate cluster are largely shared by both organoid populations. The Basal-Proliferative and Luminal clusters were consistently preserved in the integrated analysis (Fig. 8e). Supplementary Fig. 9 shows the expression of selected cell layer markers. Altogether, these findings indicate that the transcriptomes of organoids in proliferation and differentiation conditions share similarities (Basal, Intermediate clusters) but also display unique features (Basal-Proliferative or Luminal clusters).
Fig. 8 Single-cell RNAseq reveals distinct cell populations within P and D organoids. 

a Transcriptomic clusters in P (n = 6826 cells) and D (n = 4896 cells) organoids visualized using uniform manifold approximation and projection (uMAP) plots, colored according to cluster.

b Proportion of cells from P and D organoids contributing to each of the clusters shown in panel a.

c Heatmap depicting expression of selected cluster markers identified by differential expression analysis (Wilcoxon rank-sum test).

d Dot plot depicting the expression of significantly differentially expressed genes from the Wnt and Notch signaling pathways in P and D organoids, according to cell clusters.

e UMAP plots visualizing integrated analysis of cells from P and D organoids as a joint plot (left, n = 11722) or separate plots (P, center; D, right). For P organoids: B (Basal), BP (Basal-Proliferative), IL (Intermediate-Low) and IH (Intermediate-High); for D organoids: B (Basal), I (Intermediate), and L (Luminal). Source data are provided as a Source Data file.

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Discussion

The establishment of conditions for the self-renewal and differentiation of urothelial stem cells and the identification of markers thereof are crucial for an improved understanding of homeostasis and dysregulation in disease. In this work, we show that the Cd49f^{high} basal urothelial population contains a subset of cells with stem cell properties and long-term growth potential. Upon depletion of growth factors, these cells largely recapitulate urothelial differentiation, allowing to uncover pathways involved therein and providing an important resource for cell biology studies and approaches to regenerative and cancer medicine.

Cd49f/as integrin labels a population of urothelial basal cells that is reminiscent of the a2β1 and a3β1 integrin-expressing basal skin epidermal stem cells^{30,31}, underscoring that similar hierarchies exist in stratified epithelia despite fundamental differences in proliferation dynamics. The integrated scRNA-Seq analysis revealed that α4 integrin is also significantly up-regulated in the Basal cell cluster (Supplementary Fig. 9). The population of Cd49f^{high} cells is heterogeneous and it will be important to identify subsets with highest self-renewal potential.

The growth factor dependencies of Cd49f^{high} cells and the growth pathways involved in bladder cancer reveals striking parallelisms. Cd49f and Cd44, highly expressed in proliferative cells with highest organoid-forming capacity, have been proposed as markers of the aggressive basal/squamous-like (BASQ) bladder cancer subtype^{27,31,32}. The urothelial organoids reported here display a predominant basal phenotype and are critically dependent on EGF for growth. Similarly, BASQ-type bladder tumors show EGFR pathway deregulation, mainly through EGFR amplification, and are highly sensitive to EGFR inhibitors both in vitro and in xenograft models^{33}. Wnt ligands are also required for optimal urothelial organoid growth, consistent with in vivo data indicating that stromal Wnt induced by epithelial Shh is required for urothelial recovery from damage impinged by infection or chemical injury^{34}. Addition of the GSK3β inhibitor, acting as a Wnt agonist^{15}, to organoids prevented the upregulation of urothelial markers in differentiation conditions. scRNA-Seq analysis showed that Wnt ligand mRNAs are selectively upregulated in cells with basal features and Wnt pathway inhibition reduced the number of lumen-containing organoids, suggesting that epithelial-autonomous Wnt production could contribute to the maintenance of urothelial progenitors. Indeed, inhibition of Wnt secretion led to reduced expression of proliferation markers without major effects on the differentiation signature genes. In bladder cancer, genes involved in Wnt signaling are rarely regulated in cells with basal features and Wnt pathway inhibition was conceivably that Claudins—and other tight junction proteins—contribute to other processes beyond cell–cell adhesion^{6}.

The organoid system described is validated by the response to established regulators of urothelial differentiation such as PPARγ and EGFR inhibitors. Previous studies using 2D cultures had shown that TERT-immortalized urothelial cells lost the ability to respond to PPARγ agonists^{40}. By contrast, our organoids undergo differentiation upon culture with PPARγ activators and EGFR inhibitors in complete medium after several in vitro passages. Organoids retained differentiated features in the absence of complete medium components and upon inhibition of PPARγ with an inverse agonist, suggesting either residual PPARγ activity or the participation of additional signaling pathways. In this regard, bulk organoid transcriptomics analysis pointed to a previously unknown role of Notch in normal urothelial differentiation that was confirmed using scRNA-Seq and two different γ-secretase inhibitors. The single-cell analyses revealed highest levels of the Hes1 in cells with intermediate differentiation features, suggesting transient pathway activation during differentiation. These findings are consistent with genetic evidences indicating that somatic mutations in genes coding for Notch pathway components occur in urothelial tumors^{41–44} and downregulation of NOTCH1 and DLL1 transcript levels in bladder cancer cells^{45}. Notch activation can suppress bladder cancer cell proliferation by direct upregulation of dual-specificity phosphatases; accordingly, ERK1 and ERK2 phosphorylation was associated with NOTCH inactivation and tumor aggressiveness^{41}. The findings in human samples are supported by studies using genetic mouse models: ubiquitous or urothelium-specific inactivation of Nicastrin, a γ-secretase complex component, led to BASQ-like tumors and this phenotype was suppressed by inducible overexpression of Notch-IC^{41}. In addition, urothelial deletion of Presenilin-1/2 or Rbpj accelerated carcinogen-induced squamous tumors with of epithelial-to-mesenchymal features^{44}. These results indicate that Notch is not only involved in tumorigenesis but it also supports differentiation in the urothelium. Inactivation of this pathway is co-opted during carcinogenesis to promote the development of poorly differentiated, aggressive, tumors.

Methods for the expansion of human urothelial tumors as organoids have been reported recently^{22,23}. This work shows that the organoids largely reflect the genetic alterations present in the original tumors and they display the phenotype of the major bladder cancer subtypes. They also maintain substantial phenotypic stability while exhibiting plasticity upon in vivo expansion. Furthermore, preliminary evidence indicates that organoids may
predict in vivo drug responses. The most fundamental differentiation of the culture conditions used in the study of Lee et al. and ours refers to the use of fetal bovine serum. The growth medium used in this study has not allowed the expansion of normal human urothelium or bladder tumors from patients. Further work is required to identify species- and tissue-specific growth requirements, considering the recent reports on human bladder cancer organoids.

We describe a single-cell transcriptomic analysis of the organoids that not only substantiates the findings at the whole population level but it also provides clues as to the molecular mechanisms involved in urothelial differentiation and homeostasis. For example, cells in the Basal cluster are enriched for expression of several collagen transcripts, suggesting epithelial–mesenchymal features. Intermediate cells are enriched in transcripts for several transcription factors not previously known to be involved in urothelial differentiation, including HOX1 and FOXQI. Interestingly, FOXQI is expressed at highest levels in the bladder (https://gtexportal.org/home/); while rarely altered in tumors, the highest frequency of FOXQI mutations occurs in bladder cancer, with clustering in the vicinity of the forkhead domain (https://www.intogen.org/search). By contrast, Gata3, Foxa1, and Ppary are not differentially expressed among the clusters, pointing to the existence of differentiation regulatory networks with distinct structures. We also identify cell surface markers of these populations that will allow the isolation and characterization of urothelial cell subsets with greater precision. Among them is PSCA, the expression of which is influenced by genetic polymorphisms associated with bladder cancer risk.

The identification of a mouse urothelial stem cell population able to form organoids with high efficiency, while maintaining differentiation capacity, should facilitate the study of the molecular pathophysiology of bladder diseases, including the interaction of epithelial cells with pathogens. These tools will also allow dissecting the molecular mechanisms through which oncogenes and tumor suppressors contribute to bladder cancer.

Methods

Mice. C57BL/6 (Jackson Laboratories, 000664 RRID:IMSR:JAX:000664), Hsd: Athymic Nude-Foxn1nu (Jackson Laboratories, 002189), 129-Gt-Rosa26 SOR-CAG-EGFPfloxed (Jackson Laboratories, 006053), and ROSA26 mTmG mice (Jackson Laboratories, 007576) were housed in a specific pathogen-free environment according to institutional guidelines. 129-Gt-Rosa26 SOR-CAG-EGFPfloxed mice were kindly provided by Maria A. Blasco (CNIO). Mice were sacrificed with oxLDL (15 μM) in culture with complete medium [Advanced DMEM/F12, 1× penicillin/streptomycin, 1× HEPES, 1× Glutamax, 50% WNT3A conditioned medium, 5% human RSP01 conditioned medium, 1× N2 (Gibco, 17004089), 1× B27 (Gibco, 12508701), 50 ng/ml human recombinant EGF (Invitrogen, PHG031L), 1 mM N-acetyl-ylglycine (Sigma-Aldrich, 616-91-1), 50 μg/ml human Noggin (Peprotech, 120-19C) and 1 μM LY2157299 (Axon), TGFβ inhibitor Y-27632 (10 μM) (Sigma-Aldrich, 129830-38-2) was added during the first 3 days of culture. For differentiation experiments, organoids were cultured for the first 7 days in complete medium, reseeded (without disaggregation) in fresh Matrigel, and cultured either with complete or differentiation medium (lacking WNT3A and RSP01 conditioned medium, 1× N2, 1× B27, Y-27632 (10 μM) (Sigma-Aldrich, 129830-38-2) was added during the first 3 days of culture. For differentiation experiments, organoids were cultured for the first 7 days in complete medium, reseeded (without disaggregation) in fresh Matrigel, and cultured either with complete or differentiation medium (lacking WNT3A and RSP01 conditioned medium, 1× N2, 1× B27, Y-27632 (10 μM) (Sigma-Aldrich, 129830-38-2) was added during the first 3 days of culture. 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For all major experiments, at least two independent biological replicates—in addition to technical replicates—were performed.
FACS and dead cells were excluded from subsequent analyses. In the experiments with organoids, samples were disaggregated as previously described and single-cell suspensions were incubated with PE-labeled anti-mouse/human CD49f antibody. In the case of D < 70 μm and D > 70 μm, organoids cultured for 7 days in complete medium were filtered using a 70 μm filter and the fall through and the retained organoids were reseded in Matrigel and cultured in differentiation medium. All samples were analyzed using a FACS Influx or AriaII (BD Biosciences) flow cytometer and at least 10,000 events were acquired. Analyses were performed using FlowJo version 10.2 flow cytometry analysis software.

Clonality experiments. FACS-sorted freshly isolated urothelial cells were embedded in 5 μL of Matrigel in a 96-well format at 1, 10, or 100 cells/well for the clonal growth experiments. For the monolclonality experiment, organoids derived from mice of the 129-Gt.ROSA26 sOR-CAG-GFPLuc and ROSA26 tmt/McUbcCreERT2 strains were separately established; after dissociation to single-cell suspensions they were mixed at a 1:1 ratio and allowed to grow as organoids in 20 μL of Matrigel/drop in 48-well plates at 1000 cells/well. Organoids containing cells of the corresponding fluorescent color (EGFP, Tomato, and chimeras) were counted.

Cell cycle re-entry experiments. Urothelial cell suspensions were seeded in complete medium for 7 days. Afterwards, organs were resuspended in Matrigel and cultured for the following 7 days either in complete medium, differentiation medium, or in differentiation medium supplemented with Rosiglitazone (1 μM) and Ertotinib (0.5 μM). On day 14, the medium was replaced with either complete or differentiation medium and cultures were allowed to grow until day 21.

Organoid quantification. For the leave-one-out experiments, images were acquired with ×40 resolution with CCD-microscope using a brightfield filter. Three pictures in the Z-axis were taken in order to collect the majority of the organoids. Then, a Z-stack was done using ImageJ software. For immunofluorescence and growth assays, quantification was performed with tailored routines programmed in Definiens XD v2.5 software. Data on organoid features (layer thickness, lumen, and diameter) were derived from the signal distribution upon Hoechst staining. Organoid growth and apoptosis videos were acquired using a DM4000B brightfield microscope from Leica Microsystems with an HC PL APO ×10 0.4 NA dry objective. Cells were maintained in a temperature-controlled (37 °C), humidified environment in the presence of 5% CO₂ during imaging.

Barrier function assays. A derivative of FRAP-derived strategy was used whereby the influx of Fluorescein isothiocyanate–dextran (average mol wt 3000–5000) (Sigma-Aldrich, 60842-46-8) into the organoid was measured after bleaching the original fluorescence. Organoids were cultured in μ-slide eight-well ibidi plates (Ibidi, 80826). In the pre-bleaching step, the original signal was registered. After bleaching by pointing the laser into the lumen for 20 s (488 nm wavelength at maximum power), the signal decayed and a new register was established. In the recovery phase, the entrance of FITC-dextran into the lumen was measured. The recovery period spanned for a total of 14 h, with images acquired every 30 min. The assay was performed using a Leica TCS-SP5 (AOBS) Leica Microsystems laser scanning confocal microscope with a ×40 immersion oil objective (HCX PLAPO 1.2 NA). The normalized intensity of luminal FITC-dextran was calculated by dividing the measurements corresponding to the region of interest in the lumen by the background in the Matrigel.

Western blotting. Organoids were pelleted after removing Matrigel with Cell Recovery Solution and whole cell extracts were prepared using RIPA buffer [0.05 M Tris-HCl pH 7.5, 0.1% SDS, 0.15 M NaCl, 1% Triton X-100 (Sigma-Aldrich) and 1% sodium deoxycholate] containing protease and phosphatase inhibitors (leupeptin, 10 μg/mL; aprotinin, 10 μg/mL; PMSF, 1 mM; orthovanadate, 1 mM; NaF, 1 mM; all from Sigma-Aldrich). Cell lysates were fractionated by SDS-PAGE, transferred to PVDF membranes, and incubated with the following primary antibodies: mouse monoclonal anti-UPK3a (Santa Cruz, sc-166808), mouse monoclonal anti-UPK1b (kind gift from Dr. A. Garcia-España), goat polyclonal anti-Lamin (Santa Cruz, sc-6216), rabbit polyclonal monoclonal anti-HES1 (a gift from Dr. T. Hung, mouse monoclonal anti-TβR (Abcam, ab815151), and rat monoclonal anti-HIF1 (395/A7, C57BL/6). Antibody concentrations were provided in the Nature Protocol Exchange article and Reporting summary.

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Real-time quantitative PCR. Total RNA was extracted from organoids using the TRIzol reagent (Invitrogen) followed by the PureLinkTM RNA Mini Kit (Life Technologies, 12183020), according to the manufacturer’s instructions. For cells grown in 2D culture, RNA was extracted using ReliaPrepTM RNA Cell Miniprep System Kit (Promega, TM370). Samples were treated with Dnase before reverse transcription (Life Technologies, AM1906). cDNA was generated from 200 to 1000 ng of RNA using random hexamers and reverse transcriptase using the TaqMan® Reverse Transcription Reagents (Life Technologies, N8080234). Reaction mixes lacking RT were used to ensure the absence of genomic DNA contamination. PCR amplification and analyses were conducted using the 7900HT Real-Time PCR System (Applied Biosystems, Life Technologies) using GoTaq® qPCR Master Mix (Promega, M318). Gene-specific primers were designed using the Primer3 software (http://folding.en.manchester.ac.uk/Primer3/). Primer pairs were designed to achieve inter-exon products of 200–250 bp. Primer sequences are provided in Supplementary Table 1.

Bulk RNA-sequencing analysis. RNA quality was assayed by laboratory chip technology on an Agilent 2100 Bioanalyzer. PolyA+ RNA was isolated from total RNA (1 μg, RIN > 9), randomly fragmented, converted to double stranded cDNA, and processed through subsequent enzymatic treatments of end-repair, dA-tailing and ligation to adapters according to Illumina’s TruSeq RNA Sample Preparation v.2 Protocol. The adaptor-ligated library was completed by limited-cycle PCR with Illumina PE primers (8 cycles). The resulting purified cDNA library was applied to pHiP polymerase chain reaction cell for cluster generation generation kit v.2.5 and sequenced on a Genome Analyzer IIX with SRS TruSeq v.5 reagents, according to the manufacturer’s protocols. The sample sequencing was paired-end. Three paired samples of organoids cultured in proliferative and differentiation conditions were analyzed. The Nextpresso version 1.9.1 analysis pipeline (Bioinformatics Unit, CNIO, Madrid) was used to process the data and the version MGGC37536mm of the mouse genome promoter motif analysis of differentially expressed genes was performed using ISMARA version 1.21.

Principal component analysis. The Pearson correlation was calculated from the expression values (expressed as fragments per kilobase of transcript per million mapped reads) of each gene for each sample by using the “cor” command in R (https://cran.r-project.org). Principal component analysis was performed using the “prcomp” command in R, from the correlation value of each sample.

Gene Set Enrichment Analysis. The list of genes was ranked by the “t-stat” statistical value from the cuffdiff output file. The list of pre-ranked genes was then analyzed with GSEA for Gene Ontology (GO) database. Significantly enriched GO terms were identified using a false discovery rate q value of less than 0.25. The GSEA tool is available online at http://www.broadinstitute.org/gSEA/GSEAUserGuideFrame.html?Interpreting_GSEA.

Droplet based single-cell mRNA sequencing. Organoids were enzymatically dissociated and cells were suspended in PBS with 0.04% BSA (Ambion AM2616). Cells (10,000 per condition, cell viability >70%) were loaded onto a 10x Chromium Single Cell Controller chip B (10x Genomics) as described in the manufacturer’s instructions: Chromium Single Cells Value kit II (10x Genomics, G06270075). Generation of gel beads in emulsion (GEMs), barcoding, GEM-RT clean up, cDNA amplification, and library construction were performed following the
manufacturer’s recommendations. Libraries were loaded at a concentration of 1.8 pM and sequenced in an asymmetrical pair-end format, with 28 bases for read 1 and 56 for read 2 in a NextSeq500 instrument (Illunina). Sequencing depth was 58 and 53 million paired reads for proliferative and differentiated organoids, respectively.

**Single-cell RNAseq data computational analysis.** Reads were locally processed with Bcl2fastq (bcl2fastq 2.19.0; Illumina). Cell Ranger version 3.0.2 software (10x Genomics) pipeline was then used to demultiplex and align reads to the GRCm38/mm10 genome. FastQC software was used to check sequencing read quality. Cell Ranger count generated the matrices used in the next analysis step.

Cell Ranger matrix data (barcodes, features, and count matrix) were loaded onto the Seurat R package (version 3.0.0.9000). In order to identify and exclude low-quality cells from downstream analyses pre-processing was performed for both datasets. The distribution of UMI’s (Unique Molecular Identifiers), genes, and the percentage of mitochondrial-encoded genes across cells were visualized using the ggplot2 R package. Cells with >800 genes detected were kept. Since a high percentage of mitochondrial genes is considered a characteristic of low-quality cells, cells expressing >12% of mitochondrial genes were excluded from downstream analysis (Supplementary Fig. 8). After the filtering steps outlined above, 6826 cells with a median gene count of 1151.5 were retained for downstream analysis of the proliferative organoids and 4896 cells with a median gene count of 1346.5 were retained for downstream analysis of the differentiated organoids.

Normalization of filtered cells was performed by dividing gene counts for each cell by the total number of counts for that cell multiplied by a scale factor (10,000–above, respectively). This method performs feature selection by computing the mean and variance of each gene using unnormalized data and applying log 10 transformation to both and then fitting a line to the relationship of log(variance) and log(mean) using local polynomial regression (loess). Standardized feature values are obtained with the observed mean and the expected variance, given by the fitted line. Feature variance is then calculated as the squared value of the line fitted to a maximum starting value (default parameter: square root of the number of cells). This variance is used to rank the features for each dataset. A subset of 2000 genes (Supplementary Fig. 7b, Supplementary Data 2) exhibiting high cell-to-cell variation was selected for downstream analysis. Lastly, genes were scaled and centered in each dataset by z-normalization to standardize the dynamic range across genes. This was done to ensure subsequent downstream analysis was not biased towards highly expressed genes.

Linear dimensional reduction was performed on the scaled data using principal component analysis (PCA). Elbow plots and JackStraw permutations test were applied to determine the dimensionality of each dataset as significant principal components (p value cut-off of less than 0.05) (25 PCs for proliferative and 21 PCs for differentiated organoids).

The minimal number of clusters was estimated by determining the robustness of the consensus matrix using SC3 R package. The identification of biologically relevant communities was performed by projecting a graph-based similarity matrix in Seurat through the construction of a KNN graph and posterior cell clustering using the Louvain algorithm (resolution 0.2). Cell clusters were visualized using uniform manifold approximation and projection (UMAP) plots with previously selected significant components as an input.

Cluster gene markers were detected with Seurat R package using a Wilcoxon rank sum test between each cluster and the rest of the cells in the dataset and p value adjustment was performed using Bonferroni correction based on the total number of genes in the dataset.

Both organoid datasets were integrated into a shared space through the identification of common shared features termed anchors between cells across P and D organoids. Both datasets were pre-processed and normalized separately as described above. Dimensional reduction was performed by means of canonical correlation analysis (CCA). We defined the dimensionality of the integrated dataset by using 25 canonical vectors CCA (maximum number of significant principal components) (27) as performed in individual analysis (27) that projected the two datasets into a correlated low dimensional space. After dimensional reduction, we identified the K nearest neighbors (KNNs) for each cell within both datasets, using the L2 normalized CCA. Lastly, we identified the “anchors” as pairwise correspondences by mutual nearest neighbors (MNN). These anchors were filtered and scored to reduce the effect of incorrect identification and weighted in a matrix that defines the strength of association of each cell and each anchor. Batch correction was then performed by matching MNN as described by Haghverdi et al. (28). These steps were implemented in the FindIntegrationAnchors and IntegrateData Seurat functions. Downstream analysis on the integrated dataset was performed as it was done for individual analysis (Clustering (resolution 0.17), Cluster markers, and differential expression analysis).

**Additional resources.** Claudin expression data were extracted from the Human Protein Atlas (www.proteinatlas.org). GenePattern was used to compute all heat-maps here presented. IDraw was used to generate the panels and figures.

**Quantification and statistical analyses.** All quantitative data are presented as mean ± s.e.m. from ≥2 experiments or samples per data point (n is mentioned in each figure legend). Non-parametric Mann-Whitney U test (two-tailed) was used to assess significance levels and ANOVA was used to compare more than two groups. Statistical analysis was performed using GraphPad Prism version 6 software. For further statistical details, refer to each figure legend. For in vitro experiments, sample size required was not determined a priori. The experiments were not randomized.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** The authors declare that all data supporting the findings of this study are available within the article and its supplementary information. The bulk RNAseq data generated in this study have been deposited in the GEO database under accession code: GSE109566. The single-cell RNA-seq data have been deposited in the GEO database under accession code: GSE119909. Materials, other data, and Excel files with significantly differentially regulated genes and pathways are available upon reasonable request to the authors.

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